Pyranose 2-oxidase: playground for enzyme evolution

Ph.D. thesis

DI Oiver Spadiut

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For Mam, Nik and Hörmsch
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Curriculum vitae
Abstract

The sugar oxidoreductase pyranose 2-oxidase (P2Ox) is a member of the glucose-methanol-choline (GMC) family of FAD-dependent oxidoreductases. Pyranose 2-oxidase is widespread in wood-degrading basidiomycetes, where it is localized in the hyphal periplasmic space. Presumably, P2Ox supplies lignin and manganese peroxidases with H$_2$O$_2$, an essential co-substrate for ligninolysis by wood-rotting fungi. The crystal structure, which was determined at 1.8 Å resolution, revealed that P2Ox from Trametes multicolor (TmP2Ox) is a homotetrameric enzyme with a molecular mass of 270 kDa with each of the four 68-kDa subunits carrying one flavin adenine dinucleotide (FAD) covalently bound. In accordance with other flavoprotein oxidoreductases the reaction mechanism of P2Ox is of the typical Ping Pong Bi Bi type. In a reductive half-reaction an aldopyranose is oxidized at position C-2 to yield a 2-ketoaldose, while FAD is reduced to FADH$_2$. During an ensuing oxidative half-reaction FADH$_2$ is re-oxidized by the second substrate oxygen, yielding the oxidized prosthetic group and H$_2$O$_2$. In addition, alternative electron acceptors, including benzoquinones and chelated metal ions, can be used efficiently by P2Ox instead of oxygen.

The monosaccharide D-glucose is the preferred sugar-substrate of P2Ox, whereas D-galactose is a rather poor substrate with only 5.7% relative activity. Oxidation of D-galactose at position C2, however, is interesting from an applied point of view, since 2-keto-D-galactose can be easily reduced at position C1 to yield D-tagatose, which is used as a non-cariogenic, low caloric sweetener in food industry. For effective biotechnological applications, the catalytic activity of P2Ox with D-galactose is too low, leading to either very long conversion times or disproportionate amounts of required enzyme. Thus, enzyme variants with improved catalytic activity towards D-galactose are required for industrial applications. Besides applications in food industry, P2Ox is increasingly getting attractive for bio-electrochemical applications, e.g. in enzymatic biosensors and biofuel cells. In these applications, the enzyme
communicates with an electrode through small redox-active compounds, so-called mediators, which collect the electrons from the prosthetic group of the enzymes and transfer them to a graphite electrode in a process referred to as mediated electron transfer (MET). Thus, biofuel cells convert sugars into electrical energy by employing oxidoreductases as an anodic biocomponent, and coupling this with a suitable enzyme on the cathode. To improve the performance of biofuel cells it is crucial to increase both the catalytic activity and the stability of the enzymes applied.

We used rational protein design, semi-rational protein design and different methods of directed evolution to generate variants of $TmP2Ox$, which are useful for various applications in both food industry and bio-electrochemistry.
Zusammenfassung


Wir benutzten rational protein design, semi-rational protein design und verschiedene Methoden der directed evolution, um Varianten der \( TmP2Ox \), die für verschiedene Anwendungen in der Lebensmittelindustrie und in der Bio-Elektrochemie geeignet sind, herzustellen.
Introduction

The complexity of all living things, which is apparent in the amazing diversity of whole organisms down to individual protein molecules, results from mutation and natural selection. Organisms, as well as polypeptides, evolved over billions of years through the application of environmental pressure. Repeating cycles of mutation and selection, a process called Darwinian evolution, enabled organisms to adapt to changes in the environment over time and resulted in a vast diversity of life. Spontaneous mutations, which occur during DNA replication or by recombination events, cause this genetic diversity. In the past few decades scientists have tried to mimic this natural Darwinian evolution in the test tube. There are, however, some fundamental differences in these two kinds of evolution. Natural evolution allows adaptability to ever changing environments and does not work towards any particular direction. In contrast, an evolution experiment in the laboratory has a defined goal and progresses at a far more rapid pace. Natural evolution occurs under variable selection pressures, whereas evolution experiments are done under controlled selection pressures. Non-natural functions can be obtained through the design of appropriate selection conditions in the laboratory, whereas natural evolution develops functions which only guarantee and facilitate the survival of the organism.

Natural enzymes are optimized and specialized for specific biological functions within the context of a living organism, and are therefore often not well suited for industrial applications. For example, they can just use certain substrates, they are not stable at high temperatures and in organic solvents or they are inhibited by their substrates or products. Generally, they show optimal activity in aqueous solution at neutral pH and moderate temperatures. In contrast, industry, especially white biotechnology, needs enzymes which show increased catalytic activity, accept different substrates and are stable and active in non-aqueous solvents and at high temperatures. For example, the use of high processing temperatures for enzymatic
conversions in industry avoids microbial contamination and is beneficial for substrate and product solubility, viscosity and process speed. The use of biotransformation and biocatalysis in industry is increasing and it has been claimed that the number of industrially established biocatalytic processes will double every decade (Straathof et al. 2002; Panke et al. 2004). Thus, rapid, systematic production of optimized designer enzymes would therefore alter both industry and medicine. Enzymes that have already been modified for commercial use include α-amylases, lipases and thermostable esterases and dehydrogenases.

The number of methods proposed for *in vitro* evolution is great and still increasing, thus the method and the strategy for each experiment have to be chosen carefully. In general, there are three ways to design new tailor-made biocatalysts. The first category describes rational protein design, in which specific mutations at certain sites are chosen rationally based on the crystal structure and computational predictions. Therefore structural data and knowledge about structure-function relationships have to be available. A significant drawback of this method is that knowledge of the structural basis of enzymatic catalysis is still incomplete.

The second category of methods includes techniques in which the copying of a DNA sequence is intentionally disturbed and mutations are generated at random positions distributed over the entire gene. These techniques are summarized as directed evolution approaches and include the use of physical and chemical mutagens, mutator strains, error prone PCR and others. The basic scheme of rational protein design and directed evolution is illustrated in Figure 1.
Figure 1. Usual steps in enzyme evolution by either rational protein design or directed evolution approaches.

The third category is referred to as semi-rational protein design and targets randomization to specific positions within the DNA sequence space. Sites for mutagenesis are chosen based on the crystal structure of the enzyme and then mutated by saturation mutagenesis, a method that allows the introduction of all possible 20 proteinogenic amino acids in one single step.

However, all these strategies are limited to the 20 proteinogenic amino acids. Currently, chemical modification of natural amino acids is used to overcome this limitation and allows unlimited alteration of amino acid side chain structures (Davis 2003).

Rational protein design

The term “rational protein design” describes two different strategies. First, it entails the traditional way of *in vitro* enzyme evolution, the re-design of existing proteins by site-directed mutagenesis. This requires structural as well as functional data of the enzymes of interest and is
therefore very information-intensive. Based on structural data, homology models and putative structure-function relationships, certain residues are chosen and mutated by site-directed mutagenesis. The simplest possible designs are tried first, followed by iterative additions of more mutations causing more complex interactions until an enzyme variant with the desired features is achieved. Another useful aspect of this method is the possibility to test general theories in protein chemistry. Theoretical structure-function relationships can be analyzed by mutating certain residues of the protein and analyze the resulting effects.

Second, “rational protein design” also means the \textit{de novo} creation of proteins. The first experiments of \textit{de novo} protein design described random combinations of amino acids, but only resulted in inactive, loosely packed structures. In 2001, Wilson and colleagues (Wilson et al. 2001) generated random polypeptides which were 88 amino acids in length. The screening of more than 1000 clones resulted in just 1 variant with the desired function. This demonstrates that the \textit{de novo} synthesis of proteins by completely random combinations of amino acids can be successful, but powerful selection methods have to be available to handle the huge libraries.

The use of chemical and structural information in the \textit{de novo} design method increased the probability to generate desired proteins. Based on computational algorithms using various parameters which describe packing interactions like van der Waals and electrostatic interactions, hydrogen bonds and hydrophobic interactions, sequences can be predicted to synthesize polypeptides. However, the number of conformations a polypeptide can potentially adopt is vast, making it difficult even for computational algorithms to seek out the preferred solutions. Therefore all computational programs use the same general approach for protein design. First the structure of a protein backbone is chosen and serves as a fixed framework. Then different amino acids are added to this framework by chemoselective ligation, resulting in branched chain structures that are called template-assembled synthetic proteins (TASP). This so called “inverse folding” is done until the polypeptide with the desired structure is designed
and shows the lowest free energy of all possible states. The *de novo* synthesized polypeptides are then tested for their activity in the laboratory (Mutter and Tuchscherer 1997).

*De novo* protein design aims for an amino acid sequence that folds to a specific structure, yet enzymatic function is mostly caused by conformational changes of the enzyme and not solely by a certain structure. Therefore, challenges in the *de novo* design of enzymes are the creation of novel protein folds, binding interfaces and enzymatic activities, as well as designing certain specificities. The term specificity describes the selective binding of the enzyme to certain ligands and the enzymatic activity with particular substrates. To date, it is difficult to design an enzyme with certain affinities while avoiding undesired interactions. Another common difficulty in *de novo* design is the accurate evaluation of electrostatic interactions in the polypeptide. Further factors that complicate calculations are the accommodation of the substrate, release of product, protein flexibility and dynamics, solvent and solvent-mediated effects and the presence and interactions of catalytic residues in the active site (Lippow and Tidor 2007). The ultimate goal of *de novo* protein design is to develop a fully automated method which can accommodate structural and functional specificity and is based on knowledge-based structure-function information as well as on physics-based energy functions.

**Directed evolution**

Rational protein design requires enormous input of structural, mechanistic and dynamic information and even if one trait is successfully designed, it is impossible to predict the cost to another characteristic. This makes it very difficult for rational protein design to successfully develop tailor-made biocatalysts within reasonable time. All of these hurdles of rational protein design are bypassed by directed evolution, which mimics Darwinian evolution in the test tube by using random mutagenesis and recombination, followed by screening or selection to
identify enzyme variants that have the desired properties. Random mutagenesis over the entire gene allows discovery of unanticipated solutions and novel combinations of mutations that would be inaccessible by rational design. Directed evolution methods have now been used for more than two decades as an alternative to rational design for protein engineering. The basic processes of rational protein design and directed evolution approaches are shown in Figure 2.

Figure 2. Basic processes in rational protein design and directed evolution (Bornscheuer and Pohl 2001).
In directed evolution experiments, the genes of interest are randomly mutated and cloned into a suitable expression host like *Escherichia coli*, *Bacillus subtilis* or *Saccharomyces cerevisiae*. Clones expressing improved enzymes are identified in a high-throughput screening assay or by selection. Screening assays are mostly based on *in vitro* detection of colored or fluorescent reaction products, whereas selection is done by *in vivo* life and death selection resulting from the modified target molecule. If a clone is identified to possibly have the desired characteristic, it is isolated, sequenced and tested for its catalytic activities. A directed evolution experiment is considered complete when the desired catalytic properties are achieved or when the screening/selection process can not show any further improvement in the phenotype.

Basically, there are 4 requirements for successful directed evolution:

1. the desired function must be physically possible
2. the creation of genetic diversity
3. the functional expression in a suitable host organism
4. a rapid and highly selective screening or selection that reflects the desired function

An attractive aspect of directed evolution is that improved variants selected from the first rounds of evolution are used as starting points for further rounds of mutagenesis to introduce additional mutations which might lead to further improvements (Figure 3).
A vast number of enzymes has already been optimized by directed evolution (Powell et al. 2001, Reetz et al. 2006, Toscano et al. 2007). Modified properties include stability, substrate specificity, tolerance to non-natural conditions and enantioselectivity (examples are given in Table 1).

**Table 1.** Examples for successfully evolved enzymatic features.

<table>
<thead>
<tr>
<th>target enzyme</th>
<th>target function</th>
<th>approach</th>
<th>effect</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>kanamycin nucleotidyltransferase</td>
<td>thermostability</td>
<td>mutator strain &amp; selection</td>
<td>200-fold increase of $\tau_{1/2}$ at 60°C</td>
<td>Liao et al. (1986)</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>substrate specificity</td>
<td>DNA shuffling &amp; screening</td>
<td>1000-fold increase</td>
<td>Zhang et al. (1997)</td>
</tr>
<tr>
<td>pNB esterase</td>
<td>thermostability</td>
<td>epPCR &amp; screening</td>
<td>14°C increase in $T_m$ &amp; increased activity</td>
<td>Giver et al. (1998)</td>
</tr>
<tr>
<td>cytochrome P450</td>
<td>substrate specificity</td>
<td>saturation mutagenesis</td>
<td>activity towards new substrate</td>
<td>Li et al. (2000)</td>
</tr>
</tbody>
</table>
Whether a directed evolution experiment can solve a problem depends to some extent on the strength of selection pressure that trait has already faced. If a particular trait has already been under selective pressure in nature it is unlikely that further improvements can be obtained in the laboratory by small mutational steps. In contrast, it is easy to improve features that are never required for biological function, such as stability or activity in a non-natural environment or activity towards a new substrate.

“You get what you screen for”

An enzyme consists of $N$ amino acids with 20 possible amino acids at each position in the chain. Thus, the amount of all possible protein-variants is far beyond imagination ($20^N$). This astronomically large number of possible polypeptides is an obstacle to identifying and isolating interesting variants. Therefore a directed evolution experiment must be designed carefully providing intelligently designed libraries and improved screening techniques. Identifying the few molecules that show improved characteristics in a vast population of clones and separating them from the rest is the greatest challenge in directed evolution. There are two fundamentally different ways to address this problem. Either each member of a library is tested for its features individually (screening) or certain conditions are applied which allow only desirable clones to appear (selection). Whether screening or selection can be applied depends on the target enzyme and its features. To check modified proteins for catalytic turnover, for example, is difficult to achieve by selection and therefore screening assays have to be developed. Most screening assays are based on the detection of colored or fluorescent reaction products and accommodate $10^2$ to $10^4$ clones. An example of a screening assay based on a colored reaction product is given in Figure 4.
Figure 4. Screening assay for improved pyranose 2-oxidase variants from *Trametes multicolor* in microtiter plates (Spadiut et al. 2008a).

Higher throughputs can be achieved by screening techniques like FACS, which can analyze $10^4$ to $10^5$ clones per second. Also display techniques, like phage display, cell surface display and ribosome display can be used to identify the variant of interest within a library. Many enzyme activities which are targets for evolution do not provide an obvious high-throughput assay and developing a new screening assay may take up to several months. Recent developments in screening and selection however facilitate this challenge. A quite new and very useful technique for example is “*in vitro* compartmentalization (IVC)” (Miller et al. 2006), which allows the analysis of $10^8$-$10^{11}$ clones in a single experiment. Genes together with the protein biosynthesis apparatus are encapsulated in water-in-oil droplets where they can be effectively screened for improved or novel functions. However, the selection technique is still superior to screening because it allows the simultaneous sampling of $>10^8$ clones in a single experiment and does not require any special equipment.
Tools for directed evolution

A large number of various techniques for generating genetic diversity and carrying out evolution experiments both within cells (in vivo) and in the test tube in the laboratory (in vitro) are available to date. A general scheme of in vivo and in vitro evolution is given in Figure 5.

Creating molecular diversity is the initial step in every directed evolution experiment. Mutator strains, mutator plasmids, subjection of DNA to UV radiation, addition of chemical mutagens, error prone PCR and a number of other methods introduce random mutations all over the entire gene and do not require any knowledge of the structure or the function of the protein. In vitro recombination techniques such as DNA shuffling, staggered extension process (StEP), random
chimeragenesis on transient templates (RACHITT), iterative truncation for the creation of hybrid enzymes (ITCHY) and recombined extension on truncated templates (RETT) have been developed to mimic and accelerate nature’s recombination strategy and also result in genetic diversity. The most important parameter in mutagenesis experiments is the random distribution of mutations over the entire gene. This can be estimated on the basis of binomial distribution. For a sequence of length $n$ which is mutagenized with an error rate $\epsilon$, the probability of introducing $k$ mutations is given by:

$$P = \frac{n!}{(n-k)!k!} \epsilon^k (1-\epsilon)^{n-k} \quad \text{(Joyce 2004)}$$

Most studies have shown that a low mutation frequency of about 1-3 nucleotide mutations per gene, distributed over the entire sequence space, leads to better results than higher mutation rates.

In conclusion, it is essential to plan each directed evolution experiment carefully by choosing a suitable mutagenesis method, an appropriate mutation rate and a reliable and selective screening or selection method.

**Directed evolution methods – how to generate genetic diversity?**

Error prone PCR (epPCR)

EpPCR is the most commonly used method to introduce random mutations in a sequence. There are several protocols available that use either altered reaction conditions or a mutant DNA-polymerase to incorporate mutations during PCR. EpPCR employs a non-proofreading polymerase (e.g. *Taq* polymerase), an increased concentration of MgCl$_2$ in order to stabilize non-complementary pairs, unbalanced concentrations of dNTPs (e.g. dCTP/dTTP:dGTP/dATP
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= 3:1) and sometimes MnCl₂, which is added in low concentrations to increase the error rate of
the polymerase. A standard epPCR protocol results in an error rate of 0.66% per nucleotide
position in 30 temperature cycles (Cline and Hogrefe 2000). Special hypermutagenic PCR
procedures which employ more heavily unbalanced concentrations of dNTPs (ratio up to 20:1)
and 50 rather than 30 temperature cycles result in an error rate of up to 10% per nucleotide.
Another strategy utilizes standard PCR conditions in combination with a mutant DNA
polymerase (mutazyme™) which provides an error rate of up to 0.7% per nucleotide position.
However, two different genes will exhibit different mutation frequencies depending on the
length and the base composition of the template DNA, even if the same error-prone PCR
conditions are applied. That means that for each single application the suitable epPCR
conditions leading to the desired mutation rate still have to be found. A further disadvantage of
this method is that at least 4 steps are required until clones can be screened: (1) epPCR; (2)
digestion of the PCR products and of the expression vector with suitable restriction enzymes;
(3) ligation; and finally (4) transformation into the host. In addition, different studies have
shown that the created libraries are often biased in the types of nucleotide mutations (error
bias), in the types of amino acids changes (codon bias) and in the distribution of specific
sequences in the library (amplification bias; Cirino et al. 2003).

Mutator strain

A mutator strain describes a highly efficient, rapid and reproducible tool for introducing
random mutations in a cloned gene of interest. The cloned gene is propagated in a bacterial
strain (e.g. E.coli XL1-Red; Greener et al. 1997), which exhibits unusually high rates of
spontaneous DNA mutagenesis. Mutator strains are typically characterized by genetic
deficiencies in their DNA proofreading and editing machinery. The most commonly occurring
deficiencies are mutations in the mutS, mutD and mutT genes. Mutations in mutS disable DNA
mismatch repair, *mutD* mutations prevent the 3′-5′ exonuclease activity of DNA polymerase III, thereby preventing the repair of incorrectly incorporated bases, and mutations in *mutT* disable the hydrolysis of 8-oxodGTP in mismatches involving A:G. The random mutation rate in a mutator strain is ~5000-fold higher than in the wildtype strain, resulting in a mutation rate of ~0.5 mutations per kb after 30 generations of growth. Using a mutator strain does not require extensive genetic or biochemical manipulations and can be easily performed in the laboratory without any specialized equipment. However, one disadvantage of this method is a large variation in the number of introduced mutations in the gene-library. In addition, the mutations are not just introduced in the target-gene but all over the plasmid, e.g. also in the antibiotic-resistance gene. Therefore no antibiotics can be used in the media, which makes the system very sensitive to microbial infections. Another disadvantage of the mutator strain is the fact that it can not be propagated on plates for prolonged periods of time because the rapid mutation rate affects the chromosome, and after prolonged growth the subsequent colonies are not genetically identical to the original strain any more (Greener et al.1997, Nguyen and Daugherty 2003).

**Mutator plasmid**

This technique allows the temporary conversion of a normal bacterial strain into a mutator strain by supplying the mutator gene on a plasmid (Figure 6). As a consequence the mutation rate of the strain increases up to 4000-fold. The mutator plasmid (“pmut”) encodes a non-functional mutant of the *mutD* gene, which encodes a subunit of DNA polymerase III. As a result the functional, chromosomal allele of *mutD* is displaced by this protein variant, which causes the introduction of mutations. The mutator plasmid further carries a temperature-sensitive origin of replication. Thus, it is easy to remove the mutator plasmid from the evolved
strain by simply elevating the temperature. However, like for the mutator strain, this method has several disadvantages.

**Figure 6.** Use of a mutator plasmid to generate genetic diversity. A mutator plasmid ("pmut") converts a normal bacterial strain into a mutator strain. By increasing the temperature pmut can be removed (Selifonova et al. 2001).

DNA shuffling

DNA shuffling describes the combination of *in vitro* recombination of homologous sequences and a low rate of random point mutagenesis in one experiment. The goal is to create gene libraries containing all possible combinations of mutations present in the parental genes. The method was established by Stemmer (1994) and includes (1) the digestion of genes with *DNasel* to a pool of random DNA fragments, (2) a reassembly PCR in which the different DNA fragments prime each other based on homology and (3) a final PCR employing certain primers which allow subsequent cloning into an expression vector (Figure 7). Either single genes carrying different mutations ("single gene shuffling") or naturally occurring homologous genes from different species, which provide functional diversity ("family shuffling"), can be shuffled. Family shuffling results in libraries of chimeras that have greater sequence divergence than libraries obtained from single gene shuffling. Only with DNA shuffling it is possible to remove neutral mutations by genetic backcrossing with wildtype DNA but retain
those that confer selective advantage. However, this method requires $>70\%$ of homology of the template DNA and the creation of error-free sequences can not be ruled out. Furthermore, $DNase I$ hydrolyzes double-stranded DNA preferentially at sites next to pyrimidine nucleotides which results in sequence bias in the DNA fragment pool.

Recently, also methods for recombination of genes with less homology than 70% and powerful $in vivo$ techniques have been described (e.g. ITCHY and Heteroduplex Recombination). In general, DNA shuffling and the variations of this method allow the creation of novel hybrid proteins by the means of genetic recombination.

**Figure 7.** Scheme of the DNA shuffling method (Joern 2003). Parental genes are randomly fragmented by $DNase I$. Several cycles of reassembly PCR, in which the different DNA fragments prime each other based on homology, are performed. After 20-50 cycles of reassembly, a final PCR amplification with primers is used to amplify full-length genes.
Random Chimeragenesis on Transient Templates (RACHITT)

In RACHITT single stranded DNA is first fragmented by DNasel and then fractionated by size. The different fragments are hybridized to a complementary synthetic single-stranded scaffold in the absence of a polymerase. The gaps between the fragments are filled and ligated, yielding a pool of full-length, diversified, single-stranded DNA molecules hybridized to a scaffold (Figure 8). The scaffold, which was synthesized with uracils in place of thymidines, is then fragmented by uracil-DNA glycosidases and replaced with PCR by a new strand which is complementary to the diversified strand (Pelletier 2001, Lee et al. 2003). RACHITT creates chimeric genes by aligning parental gene fragments on a full-length DNA template. It is similar to the Staggered Extension Process (StEP) and DNA shuffling, but produces chimeras with a much larger number of crossovers and thus more diverse libraries. Since just single stranded DNA molecules are fragmented, they can not re-anneal to their own complementary strands which guarantees no un-shuffled parental clones.

Figure 8. The processes of DNA shuffling and RACHITT (Pelletier 2001).
Staggered extension process (StEP)

StEP is an *in vitro* PCR-based recombination method, in which homologous DNA sequences are shuffled to yield highly mosaic chimeric sequences (Zhao et al. 1998). In this process various defined oligonucleotide primers bind on the DNA templates in a PCR like reaction. Priming is followed by repeated cycles of denaturation and very short annealing-extension cycles. During each cycle the fragments increase in size and randomly anneal to different templates. This switching of templates generates a library of chimeric sequences. Fragments are added in short steps to the end of growing DNA strands (Figure 9). The full-length recombination products are then amplified in a final PCR and prepared for cloning. StEP relies on the annealing of a growing DNA strand to a template, which can only happen if the sequences are similar enough. StEP is simpler and less labor intensive than DNA shuffling. However, crossovers will occur preferentially where the sequences are most similar, resulting in bias, and a homology of the parental sequences of at least 80% is required.

![Diagram of StEP recombination](image)

**Figure 9.** StEP recombination of two gene templates. After annealing to the templates, the primers are partially extended. Repeated cycles of denaturation, annealing and brief extension result in random priming of the partially-extended primers, and finally in the production of chimeric full-length genes (Zhao et al. 1998).
Random-Priming recombination (RPR)

Different random-sequence oligonucleotide primers are used to generate a large number of short DNA fragments which are complementary to different sections of the template genes. These short DNA fragments can also contain a controllable level of point mutations due to mispriming and base misincorporations. Recombination and reassembly of these short DNA fragments happen in repeated rounds of thermocycling until full-length genes are created (Figure 10). These sequences are cloned into expression vectors and the resulting polypeptides are screened for modified activities (Shao et al. 1998). The synthetic primers are random and therefore lack sequence bias. They can bind to the template DNA at many positions, showing no preference. RPR is independent of the length of the DNA template, but because of library size limitations it is usually not possible to explore all possible sequences. For example, the analysis of all possible sequences of a region of just 5 amino acids (= 15 nucleotides) would result in a library of $4^{15}$ (= 1.07x10$^9$) sequences. It is not possible to screen libraries of this size, which leaves the majority of sequence space unexplored. However, RPR can also target a specific region of the genes by adapting the oligonucleotide primers, which results in smaller libraries at the cost of diversity.
Figure 10. *In vitro* Random-Priming Recombination (RPR). Short single-stranded DNA fragments from random-sequence primers are synthesized. X’s indicate newly introduced mutations. The template is removed and full-length genes are amplified. These steps are repeated until the desired functional improvement is achieved (Shao et al. 1998).

**Heteroduplex Recombination**

Yeast cells possess an active system for recombination of linear, partially overlapping double-stranded DNA fragments. Cells efficiently join partially overlapping inserts together in regions of homology to yield a functional, covalently closed plasmid. However, the number of crossovers introduced in one recombination experiment is rather low. Heteroduplex Recombination describes a convenient hybrid *in vitro-in vivo* DNA recombination method (*in vitro* heteroduplex formation and *in vivo* repair) which generates multiple crossovers. This method is based on the ability of the host cell (yeast as well as bacteria) to repair mismatched heteroduplexes. Heteroduplexes of parental sequences are prepared *in vitro* and transformed into an appropriate host. Because each mismatch or region of non-identity between the parents is repaired independently in the cells, libraries of chimeric genes, composed of elements of
each parent, are generated (Volkov et al. 1999). Depending on the ability of the cells to take up large pieces of DNA, this \textit{in vivo} recombination approach is free from the size limitation associated with the PCR-based \textit{in vitro} recombination methods.

Recombined Extension of Truncated Templates (RETT)

RETT describes a method based on PCR to generate genetic diversity. Single stranded DNA is fragmented and pooled. The DNA-fragments can not prime each other but act as templates for different, synthetic oligonucleotide primers, which are added to the pool. Ensuing repeating cycles of thermocycling result in the creation of randomized full-length genes. Growing primers perform template-switching under normal PCR conditions (Lee et al. 2003, Sen et al. 2007). Since no enzymatic cleavage is necessary to obtain the shuffling blocks, no sequence bias is generated.

The Incremental Truncation for the Creation of Hybrid Enzymes (ITCHY)

ITCHY is a method for creating a library of every one base truncation of dsDNA. Two different parental genes are truncated by the enzyme exonuclease III (\textit{ExoIII}) under controlled conditions in a time-dependent reaction. Over the course of the truncation, small aliquots are removed and quenched at a frequency such that, theoretically, every single base deletion of the two gene fragments will be collected. Blunt ends are then prepared by treatment with a single-strand nuclease and a DNA polymerase. Finally, these fragments are ligated to each other with DNA ligase. The resulting “fusion-molecules” are then transformed into expression hosts and analyzed for their functions (Ostermeier et al. 1999). ITCHY does not require any homology of the parental sequences and still can create fusion-libraries of genes. However, time dependent truncation is quite difficult and requires extensive, multiple time-point sampling to achieve a comprehensive library. A useful variation of ITCHY is THIO-ITCHY, which uses the
incorporation of nucleotide triphosphate analogs such as α-phosphothioate dNTPs. α-Phosphothioate nucleotides are similar to standard nucleotides, except that a sulfur atom replaces an oxygen atom at the α-phosphate. Key to the procedure is the random, low frequency incorporation (“spiking”) of the target DNA segment with these α-phosphothioate nucleotides. The nucleotide analogs protect the DNA from exonuclease digestion, thus leading to the desired variation in truncation length upon nuclease treatment. The generation of diversity is no longer a function of timed exonuclease digestion but is instead based on the random distribution of the α-phosphothioate nucleotides (Lutz et al. 2001a). Spiking of the targeted DNA with α-phosphothioates can be achieved in two ways. The first approach involves an initial exonuclease treatment limited to the segment of DNA to be truncated, generating a single-stranded overhang. This overhang subsequently serves as a template in a DNA polymerase catalyzed fill-in reaction in the presence of α-phosphothioates (Figure 11A). Alternatively, α-phosphothioate dNTPs are incorporated during PCR amplification of the entire plasmid (Figure 11B). THIO-ITCHY is similar to ITCHY, but more efficient, less time-consuming and easier to perform.
Figure 11. Schematic overview of THIO-ITCHY using α-phosphothioate nucleotide incorporation by A) primer extension and B) PCR amplification. (a) Linearization of the plasmid by restriction at a unique site between the two parental genes. A.b1) Treatment with ExoIII produces single-stranded overhangs. A.b2) The single-stranded target region serves as template for polymerase catalyzed resynthesis of the complementary strand. The addition of α-phosphothioate dNTPs to the reaction mixture results in random, low frequency incorporation of the analogs into the newly synthesized strand. B.b) PCR amplification of the entire linearized vector in the presence of a mixture of dNTPs and α-phosphothioate dNTPs. (c) Incubation of the plasmid with ExoIII results in hydrolysis of standard dNMPs while the dNMP-analogs will block enzymatic degradation. (d) Blunt ends are generated by nucleases and (e) the constructs are recircularized by intramolecular ligation (Lutz et al. 2001a).
SCRATCHY

SCRATCHY is a combination of ITCHY and DNA shuffling. The main drawback of ITCHY is that members of the libraries contain only one crossover per gene. DNA shuffling of ITCHY libraries introduces multiple crossovers between the genes of interest by preserving ITCHY crossovers in the starting material and by recombining regions of homology between genes (Figure 12). First, hybrid proteins of two or more parental sequences are generated by ITCHY and then these libraries are shuffled with each other resulting in libraries of multiple-crossover hybrids. The ITCHY library serves as an artificial library of hybrid sequences that provides a variety of crossover-carrying templates. During DNA shuffling, these templates can anneal with one another, leading to the introduction of two or more crossovers per shuffled sequence (Lutz et al. 2001b).

**Figure 12.** Schematic overview of SCRATCHY. a) Individual incremental truncation libraries (ITCHY libraries) of two complementary constructs are created. b) After functional selection to recover in-frame hybrids of parental size, the libraries are mixed and c) submitted to DNA shuffling. d) A final selection identifies functional constructs (Lutz et al. 2001b).
Sequence Homology-Independent Protein Recombination (SHIPREC)

Like ITCHY, this method is capable of generating chimeric gene libraries independent of sequence homology, unlike other recombination methods (Udit et al. 2003a). The starting material for SHIPREC is a fusion of two parental genes of interest, with the C-terminus of the first gene and the N-terminus of the second gene joined through a linker-sequence. This linker-sequence contains a unique restriction site (e.g. PstI). The treatment of this gene fusion by DNaseI results in a library of fusion molecules of varying size. This library is then separated on an agarose gel and DNA corresponding to the length of the parental genes is isolated, purified and subsequently circularized by blunt end ligation. This size selection ensures that the generated chimeras retain proper sequence alignment with the parental genes. The resulting chimeras are linearized by cleaving at the unique restriction site in the linker-sequences and then cloned into an expression vector for screening (Figure 13). SHIPREC must be performed twice, geneA-geneB and geneB-geneA, in order to generate all possible single-crossover chimeras.
**Introduction**

**Figure 13.** SHIPREC overview. A gene fusion of two parental genes connected by a linker sequence, which contains a unique restriction site, is created. 1) This fusion molecule is randomly fragmented by DNase I. 2) Molecules corresponding to the length of the parental genes are isolated. 3) Single-gene length fragments are circularized by blunt end ligation. 4) Circular DNA is linearized at the unique restriction site (e.g., PstI). This yields a library of chimeric genes that encode for proteins with an N-terminal and a C-terminal region originating from different parental genes. 5) The chimeras are amplified and cloned into an expression host (Udit et al. 2003b).

**Sequence Saturation Mutagenesis (SeSaM)**

This four-step method saturates every single nucleotide position of the target sequence with all four standard nucleotides (Wong et al. 2004). At first, a pool of DNA fragments varying in length is generated. Then, a terminal transferase creates tails at the 3’ ends with universal bases. These elongated DNA fragments are then extended by PCR to full-length genes. In the fourth and final step a concluding PCR replaces the universal bases with standard nucleotides (Figure 14). Random mutations are created at universal sites due to the promiscuous base-
pairing property of universal bases. Using SeSaM, each nucleotide species can be exchanged in a controlled manner, avoiding the mutational bias of DNA polymerases. Moreover, SeSaM can target a nucleotide species of the selected sequence and each nucleotide species can be exchanged since SeSaM regulates the mutational spectra through a universal base.

**Figure 14.** Scheme of the SeSaM method that comprises four steps. 1) Creation a pool of DNA fragments with a random size distribution. 2) Enzymatic elongation of the DNA fragments with a universal base. 3) Full-length gene synthesis. 4) Universal base replacement by standard nucleotides (Wong et al. 2004).
Conclusion

Each of the methods described above has disadvantages and difficulties. Many of them have to be carefully optimized in numerous lab experiments, are limited by the DNA composition or are hard to control. Further studies have to be done to find the most efficient strategy for enzyme evolution, but there may not be one particular strategy or technique that will be commonly suitable for all purposes. However, in directed evolution experiments, screening and selection of the created libraries remain the most critical step.

Semi-rational protein design

Rational protein design was the earliest approach to make enzymes fit for industry by modifying some of their features. Besides the fact that for rational protein design knowledge of the structure of the protein is required, the complexity of structure-function relationships in enzymes has proven to be the limiting factor of this method. Directed evolution strategies, on the other hand, do not need any structural data of the protein. However, they are limited by the necessity of developing a reliable high-throughput screening assay. Another drawback of directed evolution is the fact that numerous random mutations are introduced in sequence space where they have no effect on the desired property of the enzyme. Therefore it is advantageous to use structural information, if available, for concentrating mutations where they might be most effective (Chockalingam et al. 2005). Semi-rational protein design describes a combination of methods of directed evolution with elements of rational protein design, bypassing certain limitations of both strategies (Chica et al. 2005). Rational input limits library size and thus the screening effort to obtain success is reduced. Specific residues are chosen based on molecular modeling or structural and functional data and mutated by saturation mutagenesis to create “smart” libraries. A comparison of the three techniques for enzyme
evolution - rational protein design, directed evolution and semi-rational protein design - is shown in Table 2.

**Table 2.** Comparison of the three strategies for enzyme engineering (Chica et al. 2005)

<table>
<thead>
<tr>
<th>strategy for enzyme evolution</th>
<th>rational protein design</th>
<th>directed evolution</th>
<th>semi-rational protein design</th>
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</thead>
<tbody>
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<td>high-throughput screen or selection</td>
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<td>essential</td>
<td>not essential</td>
</tr>
<tr>
<td>structural information</td>
<td>essential</td>
<td>not essential</td>
<td>either is sufficient</td>
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<td>essential</td>
<td>not essential</td>
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<tr>
<td>probability of synergistic mutations</td>
<td>moderate</td>
<td>low</td>
<td>high</td>
</tr>
</tbody>
</table>

Saturation mutagenesis, a method where one amino acid is replaced by all the other 19 proteinogenic ones in just a single reaction, is used in semi-rational protein design approaches to create smaller mutant libraries which no longer have to be screened in high-throughput screens. Saturation mutagenesis can be accomplished by several methods: cassette insertion, mutagenic oligonucleotide PCR amplification, sequence overlap extension (SOE-ing) or by mutagenic plasmid amplification (Georgescu et al. 2003). A very simple and efficient method which is commonly used is based on sequence overlap extension (Ho et al. 1989). Separate PCR reactions are used to amplify two DNA fragments which contain overlapping regions. Each primer at the target site is designed with a degenerated codon (e.g. NNN, where N = A, C, G or T) at the amino acid position of interest. After the initial PCR amplifications, another
PCR reaction is used to reassemble a new version of the original full-length sequence, where the target codon is effectively randomized. The library size which has to be screened to cover 95% of all possible combinations is determined by the degenerated codon and the number of target sites, which are simultaneously randomized (Georgescu et al. 2003).

Various studies have shown the successful application of this semi-rational approach for the modification of enzyme activities, for example. Specific residues, which interact with the bound substrate in the active site of an enzyme, are targeted and used for saturation mutagenesis studies. Furthermore, the simultaneous randomization of various positions in the active site can result in synergistic effects. Thus, this method allows the experimenter to focus mutations in areas which more likely yield the desired results. Two examples for the successful application of this semi-rational protein design strategy for changing the substrate specificity of an enzyme are given below.

First, Santoro and Schultz (2002) modified the substrate specificity of a Cre DNA recombinase from bacteriophage P1 by simultaneous randomization of 5 and 6 residues and screening the libraries using fluorescence-activated cell sorting (FACS). They isolated a mutant that efficiently recombined a new loxP site not recognized by the wildtype enzyme. Moreover, this variant also retained the ability to recognize the native loxP site. Semi-rational protein design resulted in an enzyme with modified substrate specificity.

The second example for the successful application of saturation mutagenesis in the context of semi-rational protein design can be found in this thesis. Based on the crystal structure (Hallberg et al. 2004, Kujawa et al. 2006) we targeted all the active site residues of pyranose 2-oxidase from *Trametes multicolor* (*Tm*P2Ox) which interact with the bound sugar substrate and modified them by saturation mutagenesis. The created libraries were screened in a 96 well plate screening assay based upon the formation of a colored reaction product, which revealed the mutant V546C. V546C showed highly changed substrate specificities for the sugar
substrates as well as for alternative electron acceptors and was used as the basis for further rounds of mutagenesis (Salaheddin et al. 2008, Spadiut et al. 2008b, Spadiut et al. 2008c). However, in certain cases where more than one residue is randomized simultaneously the created library which has to be screened exceeds the possibility of manual screening. Thus, computational methods based on protein design algorithms are combined with semi-rational approaches to facilitate the screening process. Recently, Hayes and colleagues (2002) developed a method for the computational screening of large libraries called Protein Design Automation (PDA). PDA allows all residues to be changed and chooses the optimal sequence based on the lowest conformational energy, thus decreasing the sequence space of interest by many orders of magnitude. This approach allows a fast prescreening of virtual mutant-libraries on the basis of conformational energy.

Perspectives and prospects

Natural evolution resulted in a vast amount of various proteins catalyzing various functions. Characteristic features of many enzymes, such as high chemo-, stereo- and regioselectivity make them superior to chemical catalysts. In addition, progress in genetic manipulation and heterologous expression enable the large-scale production of many enzymes at low cost. Today over 90% of industrial enzymes are produced recombinantly in fungal or bacterial hosts, which can yield protein levels of up to 40 g·L⁻¹. However, natural enzymes are often not well suited for industrial applications and therefore have to be modified. The three strategies to accomplish that are rational protein design, directed evolution and semi-rational protein design. The challenge for applying evolution to biocatalysis is to identify the best strategy to evolve the enzymes. The combination of computational and directed evolution methods is emerging as the most powerful strategy for generating functional macromolecules with the desired features
within reasonable time. The challenge for the future will be to use (directed) evolution methods for more complex problems like the evolution of biosynthetic pathways and signal transduction systems, or the creation of catalysts for reactions which can not be found in nature.

**Goal of this work**

The goal of this project was the modification of the enzyme pyranose 2-oxidase from *Trametes multicolor* (*TmP2Ox*) to make this enzyme useful for applications in the food industry and in bio-electrochemistry. For the food industry, a stable variant of *TmP2Ox*, which shows increased catalytic activity with the poor substrate D-galactose, would be interesting for the conversion of hydrolyzed lactose to yield equal amounts of D-fructose and D-tagatose. In bio-electrochemistry, *TmP2Ox* can be used as anodic bio-component in biofuel cells and biosensors. For these applications, variants with increased stability and catalytic activity with alternative electron acceptors like 1,4-benzoquinone and ferricenium ion, which can serve as electron mediators, would be of great interest. By applying rational protein design, semi-rational protein design and different methods of directed evolution we intended to increase the stability of the enzyme, to change the substrate specificity and to improve its catalytic activity with different sugar substrates and alternative electron acceptors, and therefore generate specific variants of *TmP2Ox* for various applications.
References


Chapter 1

Mutations of Thr169 affect substrate specificity of pyranose 2-oxidase from *Trametes multicolor*

Oliver Spadiut¹, Christian Leitner¹, Tien-Chye Tan², Roland Ludwig¹, Christina Divne² & Dietmar Haltrich¹

¹Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU_University of Natural Resources and Applied Life Sciences, Vienna, Austria
²School of Biotechnology, KTH, Albanova University Centre, Stockholm, Sweden

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Mutations of Thr169 affect substrate specificity of pyranose 2-oxidase from *Trametes multicolor*

OLIVER SPADIUT\textsuperscript{1}, CHRISTIAN LEITNER\textsuperscript{1}, TIEN-CHYE TAN\textsuperscript{2}, ROLAND LUDWIG\textsuperscript{1}, CHRISTINA DIVNE\textsuperscript{2}, & DIETMAR HALTRICH\textsuperscript{1}

\textsuperscript{1}Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU–University of Natural Resources and Applied Life Sciences, Vienna, A-1190 Vienna, Austria, and
\textsuperscript{2}School of Biotechnology, KTH, Albanova University Centre, SE-106 91 Stockholm, Sweden

Abstract

We used site-directed mutagenesis to enhance the catalytic activity of pyranose 2-oxidase (P2Ox) from *Trametes multicolor* with different substrates. To this end, the amino acid threonine at position 169 was replaced by glycine, alanine and serine, respectively. Using oxygen as electron acceptor the mutant T169G was equally active with D-glucose and D-galactose, whereas wild-type recombinant P2Ox only showed 5.2\% relative activity with the latter substrate. When D-galactose was used as electron donor in saturating concentrations, T169G showed a 4.5-fold increase in its catalytic efficiency $k_{\text{cat}}/K_M$ for the alternative electron acceptor 1,4-benzoquinone and a 9-fold increased $k_{\text{cat}}/K_M$ value with the ferricenium ion compared with wt recP2Ox. Variant T169S showed an increase in its catalytic efficiency both with 1,4-benzoquinone (3.7 times) as well as with the ferricenium ion (1.4 times) when D-glucose was the substrate.

Keywords: Pyranose oxidase, enzyme engineering, substrate specificity, galactose oxidation
Introduction

The flavoenzyme pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) catalyzes the oxidation of several aldopyranoses at position C-2 to yield the corresponding 2-ketoaldooses, while reducing molecular oxygen to hydrogen peroxide. It is widespread among wood-degrading basidiomycetes, where it is localized in the hyphal periplasmic space. P2Ox is thought to be involved in the ligninolysis of wood-rotting fungi by supplying lignin peroxidases with H₂O₂ (Daniel et al. 1994). In some fungi, P2Ox is involved in a secondary metabolic pathway leading from D-glucose via 2-keto-D-glucose as an intermediate to cortalcerone, a β-pyrene antibiotic (Volc et al. 1991).

P2Ox was first isolated and characterized from Polyporus obtusus (Janssen and Ruelius 1968), Phanerochaete chrysosporium (Artolozaga et al. 1997; Volc and Eriksson 1988) and Pleurotus ostreatus (Shin et al. 1993). In this study pyranose 2-oxidase from Trametes multicolor (TmP2Ox) was used (Leitner et al. 2001). TmP2Ox is homotetrameric with a molecular mass of 270 kDa, with each of the four 68-kDa subunits carrying one flavin adenine dinucleotide (FAD) bound covalently to Nε2 (i.e., N3) of His167 via its 8α-methyl group (Halada et al. 2003; Hallberg et al. 2004). The reaction catalysed by P2Ox is of the Ping Pong Bi Bi type typically found in flavoprotein oxidoreductases (Artolozaga et al. 1997; Ghisla and Massey 1989). In the reductive half-reaction an aldopyranose substrate reduces the FAD cofactor to yield reduced flavin FADH₂ and 2-dehydroaldose (2-ketoaldose) as the result of oxidation at position C-2 of the sugar substrate (reaction 1) (Freimund et al. 1998; Volc and Eriksson 1988). The ensuing oxidative half-reaction involves the reoxidation of FADH₂ by the second substrate. This can be oxygen, which is reduced to hydrogen peroxide (reaction 2), or alternative electron acceptors, including either two-electron acceptors such as benzoquinones (reaction 3) or one-electron acceptors such as chelated metal ions, e.g., the ferricenium ion, or radicals (Leitner et al. 2001).

\[
\begin{align*}
\text{FAD} + \text{aldopyranose} & \rightarrow \ FADH₂ + 2-\text{keto-aldopyranose} \\
\text{FADH₂} + \text{O₂} & \rightarrow \ FAD + \text{H₂O₂} \\
\text{FADH₂} + \text{benzoquinone} & \rightarrow \ FAD + \text{hydroquinone}
\end{align*}
\]
Recently, the structure of P2Ox from *T. multicolor* in complex with one of its slow substrates, 2-fluoro-2-deoxy-d-glucose, was reported (Kujawa et al. 2006), which showed the interactions between polypeptide and sugar substrate in the active site as well as the tentative determinants for C-2 oxidation by P2Ox. This structure also gives an explanation why D-glucose is a far better substrate than D-galactose [data from the fungal wild-type enzyme: D-glucose, \(K_m = 0.74\) mM, \(k_{cat} = 54\) s\(^{-1}\); D-galactose, \(K_m = 9.2\) mM, \(k_{cat} = 3.1\) s\(^{-1}\); (Leitner et al. 2001)]. D-Galactose differs from D-glucose by having the C-4 hydroxyl group in axial position rather than equatorial. Based on docking experiments of D-galactose in position for oxidation at C-2 in the active site of P2Ox, the axial C-4 hydroxyl group appears sterically hindered by the side chain of Thr169, thus providing a tentative explanation for the lower turnover of this monosaccharide by P2Ox.

Oxidation of D-galactose at position C-2 is highly interesting from an applied point of view. The product obtained in the transformation, 2-keto-D-galactose (2-dehydro-D-galactose, galactosone) can be reduced easily at position C-1 to yield D-tagatose (Haltrich et al. 1998), which is a ketose sugar with a significant potential as a noncariogenic, low caloric sweetener used in food applications. To improve the activity of P2Ox with D-galactose we decided to mutate position Thr169 to create additional space in the active site so that D-galactose could be more easily accommodated. The mutations chosen were replacement of Thr169 with Gly and Ala as well as the conservative substitution with Ser, which retains the OH-group in the amino-acid side chain, and which should enable the formation of a hydrogen bond similar to that reported for the Thr169 side chain and the flavin N5 and O4 (Kujawa et al. 2006). Here we report the catalytic properties of these variants for the oxidation of D-glucose and D-galactose as well as for some other substrates.

**Material and methods**

*Plasmids, bacterial strains and media*

Construction of the plasmid pHL2, which expresses the His-tagged wild-type recombinant pyranose 2-oxidase gene from *T. multicolor* under the control of the T7 promoter, has been described recently (Kujawa et al. 2006). The *E. coli* strain BL21 Star DE3 (Invitrogen;
Carlsbad, CA, U.S.A.) was used for the production of active P2Ox. *E. coli* cells were grown in TB<sub>amp</sub>-media (yeast extract 24 g l<sup>-1</sup>, peptone from casein 12 g l<sup>-1</sup>, glycerol 4 ml l<sup>-1</sup>; phosphate buffer 1 M, pH 7.5), for selection ampicillin was added to 100 mg l<sup>-1</sup>. The chemicals used were of the highest grade available and were purchased from Sigma (Vienna, Austria) unless otherwise stated.

**Generation of mutants**

Site-directed mutagenesis at the position Thr169 was performed by using the GeneTailor Site Directed Mutagenesis System (Invitrogen). The mutagenic forward primers 5'-GTCGTCGGAGGCGCATGTCTACGCACGGGATGCACCACACC-3' (T169G), 5'-GTCGTCGGAGGCGCATGTCTACGCACGGGATGCACCACACC-3' (T169A), 5'-GTCGTCGGAGGCGCATGTCTACGCACGGGATGCACCACACC-3' (T169S) and the reverse primer 5'-CCAGTGCGCGCAGCGCTCCGTACAGATGCGTGACC-3' (T169rev) were from VBC Biotech (Vienna, Austria). The plasmid pHL2 was used as template for mutagenic PCR using the following conditions: 95°C for 4 min, then 30 cycles of 94°C for 40 sec; 60°C for 40 sec; 72°C for 16 min, with a final 72°C for 10 min incubation. Each reaction contained 1x buffer (Fermentas; St. Leon-Rot, Germany), 2.5 units *Pfu* DNA polymerase (Fermentas), 1 μg of plasmid DNA, 10 μM of each dNTP and 5 pmol of each primer. The PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, U.S.A.). To obtain intact mutated plasmids 2 μl of each PCR product were transformed into chemically competent *E. coli* BL21 Star DE3 cells (Invitrogen). To confirm the presence of the correct mutations and the absence of further mutations, plasmid DNA was extracted and used as templates for DNA sequencing of the complete P2Ox-encoding gene using the forward primer 5'-AATACGACTCAGATAGGG -3' (T7promfwd) and the reverse primer 5'-GCTAGTTATTGCTCAGCGG -3' (T7termrev). The DNA sequencing was performed as a commercial service (VBC Biotech).
Protein expression and purification

Cultures (2 litres) of *E. coli* BL21 Star DE3 transformants were grown in shaken flask cultures at 37°C in TB<sub>amp</sub> until the cultures reached an OD<sub>600</sub> of 0.5 – 0.6. Protein expression was induced by adding lactose (0.5 %) and cultures were grown for a further 20 h at 25°C. Cells were harvested by centrifugation (13,000 rpm, 20 min), resuspended in phosphate buffer (50 mM, pH 6.5) containing phenylmethylsulfonylfluoride (PMSF; 0.1%) and lysed by using a French press. The crude extract was separated from cell debris by centrifugation (30,000 rpm, 30 min, 4°C), and the supernatant was used for protein purification by immobilised metal affinity chromatography (IMAC). P2Ox variants were purified using a His Trap HP column (Amersham Biosciences, Vienna, Austria) via the C-terminal His-tag. Enzymes were eluted with an elution buffer (Bis-Tris 20 mM, pH 6.5; NaCl 0.5 M, chloramphenicol 10 mg) and a gradient of 10–1000 mM imidazole. The eluted enzymes were concentrated by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (10 kDa cut-off; Millipore, Billeria, MA, U.S.A.).

Electrophoresis

Both native PAGE and SDS PAGE were performed to evaluate the purity of the enzyme preparations. Electrophoresis was done as described by Laemmli (Laemmli 1970) using a 10% separating gel and a 5% stacking gel. Samples were diluted to 1–2 mg of protein per litre, and aliquots of 5 µl were loaded per lane. Protein bands were stained with Coomassie blue.

Enzyme activity assays

To determine P2Ox activity, the standard chromogenic [2,2’-azinobis(3-ethylbenzthiazolinesulfonic acid)] (ABTS) assay (Danneel et al. 1993) was used. A sample of diluted enzyme (10 µl) was added to 980 µl assay buffer containing horseradish peroxidase (142 U), ABTS (14.7 mg) in phosphate buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM), and the absorbance change at 420 nm (ε = 42.3 mM<sup>-1</sup> cm<sup>-1</sup>) was recorded at 30°C for 180 sec. One unit of P2Ox activity was defined as the amount of enzyme
catalyzing the oxidation of 2 μmol of ABTS per min (which equals the consumption of 1 μmol of O₂ per min) under the assay conditions.

Protein concentrations were determined by the Bradford assay (Bradford 1976) using the BioRad Protein Assay Kit (BioRad; Vienna, Austria). Bovine serum albumin (BSA) was used as the standard.

**Steady-state kinetic measurements**

Kinetic constants for the two electron donors D-glucose and D-galactose were measured using the standard ABTS assay and air saturation (D-glucose, 0.1–50 mM; D-galactose, 0.1–200 mM). The kinetic constants for the electron acceptors 1,4-benzoquinone and the ferricenium Fc⁺ ion (using ferricenium hexafluorophosphate FcFP₆; Aldrich, Steinheim, Germany) were measured as previously described (Kujawa et al. 2007). In short, 10 μl of appropriately diluted enzyme was added to 990 μl of phosphate buffer (50 mM, pH 6.5) containing the sugar substrate (D-glucose, D-galactose) in a constant concentration of 100 mM, and which had been flushed with nitrogen for the removal of oxygen. 1,4-Benzoquinonnen was varied from 0.01–2.0 mM, and the absorbance change at 290 nm (ε = 2.24 mM⁻¹ cm⁻¹) was followed at 30°C for 180 sec. FcPF₆ was varied from 0.005–1.0 mM, and the absorbance change at 300 nm (ε = 4.3 mM⁻¹ cm⁻¹) was followed at 30°C for 180 sec. Kinetic constants were calculated by nonlinear least-square regression, fitting the measured data to the Henri-Michaelis-Menten equation.

**Results**

*Generation of mutants and protein purification*

Variants of P2Ox were produced by site-directed mutagenesis as described in the Material and Methods section. DNA sequence analysis confirmed the presence of the correct mutations at the amino acid position 169 in the P2Ox gene with no undesired mutations. *E. coli* transformants expressing active P2Ox variants were cultivated in shaken flasks (total volume of 2 litres) and recombinant protein expression was induced by the addition of lactose (0.5%) to the culture medium. Routinely, roughly 70 mg of P2Ox protein was obtained per litre culture medium in these cultivations. P2Ox proteins were purified from the crude extracts by IMAC.
using a His Trap HP column followed by ultrafiltration. The resulting proteins were apparently homogenous (>98%) as judged by native PAGE and SDS-PAGE (Fig. 1).

![Native PAGE (A) and SDS-PAGE (B) of different Thr169 variants of pyranose 2-oxidase from *Trametes multicolor*. Protein bands were stained with Coomassie blue. Lane 1, molecular mass standard (A, High Molecular Weight Calibration Kit for native electrophoresis (Amersham); B, Precision Plus Protein Dual Color (Biorad)); lane 2, variant T169G; lane 3, variant T169A; lane 4, variant T169S.]

**Figure 1.** Native PAGE (A) and SDS-PAGE (B) of different Thr169 variants of pyranose 2-oxidase from *Trametes multicolor*. Protein bands were stained with Coomassie blue. Lane 1, molecular mass standard (A, High Molecular Weight Calibration Kit for native electrophoresis (Amersham); B, Precision Plus Protein Dual Color (Biorad)); lane 2, variant T169G; lane 3, variant T169A; lane 4, variant T169S.

**Kinetic characterisation of mutational variants**

Initial rates of activity were recorded over a substrate rate of 0.1 to 50 mM D-glucose and 0.1 to 500 mM D-galactose for His6-tagged wild-type P2Ox expressed in *E. coli* and the mutational variants T169G, T169A and T169S using the standard ABTS assay and air saturation. Table I provides a summary of these kinetic data. The catalytic activity of each mutational variant of P2Ox was reduced for both substrates D-glucose and D-galactose. This effect was less pronounced for the variant with the conservative replacement Thr → Ser. In contrast, the Michaelis constant $K_M$ was reduced for variant T169G for both sugars, and for T169S for D-glucose, a result indicating improved binding of the sugars by these variants. Interestingly, the $K_M$ values were increased substantially for both substrates and variant T169A. While the wild-type enzyme clearly preferred D-glucose over D-galactose as its electron donor with catalytic
efficiencies $k_{cat}/K_M$ of 51.2 mM$^{-1}$s$^{-1}$ and 0.29 mM$^{-1}$s$^{-1}$, respectively, and a 20-fold higher catalytic constant for D-glucose, this clear preference was not observed for some of the variants. Notably, variant T169G was characterised by $k_{cat}$ values that are approximately equal for both sugars, in fact $k_{cat}$ is slightly higher for D-galactose than for D-glucose. Furthermore, this variant showed a $k_{cat}/K_M$ value that is only 3.5 times lower for D-galactose than for D-glucose, while this value is 177 times lower for D-galactose in the wild-type enzyme.

**Table I.** Kinetic properties of wild-type recombinant pyranose 2-oxidase from *T. multicolor* and mutational variants for D-glucose and D-galactose as electron donors, with the concentration of O$_2$ as electron acceptor held constant. Kinetic data were obtained using the standard ABTS assay and air saturation.

<table>
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<th>Variant</th>
<th>D-glucose</th>
<th>D-galactose</th>
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<tbody>
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<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>wt P2Ox</td>
<td>0.94 ± 0.04</td>
<td>48.1 ± 0.52</td>
</tr>
<tr>
<td>T169G</td>
<td>0.69 ± 0.11</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>T169A</td>
<td>6.42 ± 0.57</td>
<td>0.065 ± 0.002</td>
</tr>
<tr>
<td>T169S</td>
<td>0.27 ± 0.02</td>
<td>7.24 ± 0.09</td>
</tr>
</tbody>
</table>

In addition, the kinetic constants were determined for two electron acceptors, 1,4-benzoquinone and ferricenium ion, with either D-glucose or D-galactose as electron donor in saturating concentration (Tables II and III). Notably, two of these variants, T169G and T169S, showed significantly higher $k_{cat}$ and $k_{cat}/K_M$ values for both of these electron acceptors than the wild-type enzyme when D-galactose was used as sugar substrate. When using D-glucose as the saturating substrate these values were lower for the variants than for the wild-type enzyme with the exception of 1,4-benzoquinone and T169S. With D-galactose as the electron donor with variant T169G, the catalytic efficiency increased 3-fold and 8.6-fold for 1,4-benzoquinone and for ferricenium, respectively; and for variant T169S with the same electron-acceptor substrates, efficiencies were increased by 1.4- and 4.4-fold, respectively. These considerable increases in
\( k_{\text{cat}}/K_M \) are mainly the result of more favourable \( k_{\text{cat}} \) values. Again, variant T169A showed substantially decreased kinetic constants for both electron acceptors (Tables II and III).

**Table II.** Kinetic properties of wild-type recombinant pyranose 2-oxidase from *T. multicolor* and mutational variants for 1,4-benzoquinone as electron acceptor, with the concentration of D-glucose or D-galactose held constant (100 mM).

<table>
<thead>
<tr>
<th>Variant</th>
<th>( K_M (\text{mM}) )</th>
<th>( k_{\text{cat}} (\text{s}^{-1}) )</th>
<th>( k_{\text{cat}}/K_M ) (mM(^{-1})s(^{-1}))</th>
<th>( K_M (\text{mM}) )</th>
<th>( k_{\text{cat}} (\text{s}^{-1}) )</th>
<th>( k_{\text{cat}}/K_M ) (mM(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt P2Ox</td>
<td>0.24 ± 0.025</td>
<td>152 ± 6.0</td>
<td>633</td>
<td>0.065 ± 0.003</td>
<td>4.79 ± 0.06</td>
<td>74.2</td>
</tr>
<tr>
<td>T169G</td>
<td>0.026 ± 0.011</td>
<td>6.5 ± 0.62</td>
<td>252</td>
<td>0.10 ± 0.046</td>
<td>21.7 ± 3.62</td>
<td>219</td>
</tr>
<tr>
<td>T169A</td>
<td>0.081 ± 0.014</td>
<td>0.69 ± 0.03</td>
<td>8.48</td>
<td>0.013 ± 0.001</td>
<td>0.14 ± 0.002</td>
<td>11.3</td>
</tr>
<tr>
<td>T169S</td>
<td>0.044 ± 0.011</td>
<td>105 ± 7.7</td>
<td>2370</td>
<td>0.049 ± 0.005</td>
<td>5.05 ± 1.33</td>
<td>101</td>
</tr>
</tbody>
</table>

**Table III.** Kinetic properties of wild-type recombinant pyranose 2-oxidase from *T. multicolor* and mutational variants for the ferricenium ion as electron acceptor, with the concentration of D-glucose or D-galactose held constant (100 mM).

<table>
<thead>
<tr>
<th>Variant</th>
<th>( K_M (\text{mM}) )</th>
<th>( k_{\text{cat}} (\text{s}^{-1}) )</th>
<th>( k_{\text{cat}}/K_M ) (mM(^{-1})s(^{-1}))</th>
<th>( K_M (\text{mM}) )</th>
<th>( k_{\text{cat}} (\text{s}^{-1}) )</th>
<th>( k_{\text{cat}}/K_M ) (mM(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>recP2Ox</td>
<td>0.254 ± 0.099</td>
<td>151 ± 35.4</td>
<td>592</td>
<td>0.070 ± 0.008</td>
<td>5.37 ± 0.22</td>
<td>77.0</td>
</tr>
<tr>
<td>T169G</td>
<td>0.086 ± 0.07</td>
<td>25.9 ± 1.20</td>
<td>302</td>
<td>0.075 ± 0.032</td>
<td>49.4 ± 8.73</td>
<td>660</td>
</tr>
<tr>
<td>T169A</td>
<td>0.049 ± 0.006</td>
<td>1.13 ± 0.068</td>
<td>3.86</td>
<td>0.028 ± 0.012</td>
<td>0.40 ± 0.066</td>
<td>14.4</td>
</tr>
<tr>
<td>T169S</td>
<td>0.263 ± 0.058</td>
<td>213 ± 21.6</td>
<td>810</td>
<td>0.026 ± 0.011</td>
<td>8.66 ± 1.09</td>
<td>336</td>
</tr>
</tbody>
</table>

**Discussion**

The conversion of sugars by pyranose oxidase is gaining increased attention since a number of these oxidised sugars are attractive intermediates for several ensuing chemoenzymatic biotransformation reactions. Preparation of these ketosugars by an enzymatic approach is
clearly favourable as the corresponding chemical routes are laborious and characterised by low yields (Freimund et al. 1998). We reported on two of these possible P2Ox-catalysed biotransformations, the redox isomerisation of D-glucose to fructose, and D-galactose to tagatose via the corresponding 2-ketoaldoses (Haltrich et al. 1998; Leitner et al. 1998). Europe is one of the world-leading producers of the milk sugar lactose, originating from the dairy industry, and an interesting possibility is the concomitant oxidation of D-glucose/D-galactose mixtures as these can be easily obtained through hydrolysis of lactose. However, all P2Ox enzymes studied to date clearly prefer D-glucose over D-galactose as the electron-donor substrate, and hence D-glucose will be transformed more rapidly. We used an approach based on rational design and site-directed mutagenesis to improve the relative efficiencies of turnover of D-galactose and D-glucose by P2Ox. Rational protein design is a very powerful tool for the alteration and improvement of biocatalysts (Bornscheuer and Pohl 2001), but it requires detailed knowledge about the structure and function of a biocatalyst. Recently, we reported the structure of TmP2Ox in complex with acetate, a competitive inhibitor (Hallberg et al. 2004) and of a variant of the enzyme, H167A, liganded with a slow substrate, 2-fluoro-2-deoxy-D-glucose (Kujawa et al. 2006). Based on this latter structure we also proposed the binding mode for D-glucose positioned for regioselective oxidation at C-2. This proposed binding mode of D-glucose explains to some extent why D-galactose is a much poorer substrate, as the hydroxyl group at position C-4 of the sugar and the side chain of Thr169 show a steric clash (Fig. 2). In an attempt to relieve steric strain in the P2Ox active site during D-galactose binding, we constructed three P2Ox variants, T169G, T169A and T169S. These mutations were chosen in order to create additional space in the active site so that D-galactose might be accommodated more easily. These mutational variants were characterised pertaining to their catalytic properties with different substrates.
Figure 2. Model of D-galactose in position for C-2 oxidation in the P2Ox active site. The P2Ox model used is the H167A variant that was determined in complex with 2-fluoro-2-deoxy-D-glucose (Kujawa et al., 2006). The protein conformation shown is that observed during binding of electron-donor substrate, i.e. sugar (reductive half-reaction), during which the side-chain hydroxyl group of Thr169 is not hydrogen bonded to the flavin N5 atom. The axial C-4 hydroxyl group is sterically hindered by the side chain of Thr169 (D-Gal O4–Thr169 Oγ ~ 2.2 Å). Atom colouring scheme: oxygen, red; nitrogen, blue; carbon: beige (protein), yellow (FAD), green (sugar). The protein backbone is shown as beige coil omitting backbone atoms (i.e., N, Cα, C, O). Only relevant amino-acid side chains are shown. The picture was prepared using PyMOL v. 0.93 (http://pymol.sourceforge.net).

Replacing Thr169 with the amino acids Gly and Ser indeed showed preferred binding of both sugar substrates, D-glucose and D-galactose, as is indicated by the $K_M$ values which are in general lower for the variants than for the wild-type enzyme. The replacement of Thr $\rightarrow$ Ala, however, gave considerably higher Michaelis constants for both sugars. It is not clear why the presence of the methyl side chain in Ala leads to this impaired binding of both sugar substrates. Removing the Thr side chain at position 169 also has a considerable effect on the catalytic
constants for the sugar substrates of the variants, which were lower for all three mutational variants and both sugars measured. This negative effect was less pronounced for the conservative replacement Thr → Ser, which retains the hydroxyl functionality in the side chain. Again, the most drastic decrease in $k_{\text{cat}}$ was observed for the T169A variant with $k_{\text{cat}}$ being reduced almost 100-fold for both sugar substrates. The catalytic constant for D-glucose was affected considerably more than that for D-galactose, and as a result the ratio of $k_{\text{cat,Glc}}$ to $k_{\text{cat,Gal}}$ is 0.96 for T169G and 4.7 for T169S while it is 19 for the wild-type enzyme. Similarly, the ratio of $(k_{\text{cat}}/K_{\text{M}})_{\text{Glc}}$ to $(k_{\text{cat}}/K_{\text{M}})_{\text{Gal}}$ was altered for T169G to 3.5 from a values of 177 for the wild-type enzyme. This indicates that the relative activity with D-galactose was substantially improved in this variant, resulting in both sugars being oxidised with similar relative efficiencies. This could be of interest when the concomitant oxidation of mixtures of D-glucose and D-galactose is necessary.

In addition to studying the kinetic properties of the mutational variants with the electron donor (sugar) substrates, we evaluated the effect of the mutations on the steady-state kinetic constants of two electron acceptors, 1,4-benzoquinone (a two-electron acceptor) and the ferricenium ion Fe$^+$ (a one-electron acceptor), using either D-glucose or D-galactose as saturating sugar substrate. Again the variant T169A was found to be the least efficient variant, with $k_{\text{cat}}/K_{\text{M}}$ values decreased for all substrate combinations measured. This was mainly the effect of substantially lower catalytic constants. Variant T169G showed decreased $k_{\text{cat}}/K_{\text{M}}$ values for both electron acceptors when D-glucose was the sugar substrate, while the catalytic efficiency increased considerably for both electron acceptors when D-galactose was the saturating sugar substrate; these $k_{\text{cat}}/K_{\text{M}}$ values increased 3.0- and 8.6-fold for 1,4-benzoquinone and Fe$^+$, respectively. This is the result of increased $k_{\text{cat}}$ values since the Michaelis constants were approximately constant. Variant T169S showed increased $k_{\text{cat}}/K_{\text{M}}$ values for both 1,4-benzoquinone and Fe$^+$, regardless of the sugar substrate used. This significant increase in catalytic efficiencies for the mutational variants T169G and T169S with the electron acceptors 1,4-benzoquinone and Fe$^+$ when using D-galactose was an unexpected outcome, but most probably this reflects the higher reactivity with D-galactose. To some extent this increased efficiency could, however, result from a better accommodation of the substrates, both sugar
and electron acceptor, in the active sites (Alvarez-Icaza et al. 1995) as T169S showed higher \( k_{\text{cat}}/K_M \) values for both electron acceptors and sugar substrates used in the determination of the kinetic constants.

Sugar oxidising enzymes are of substantial importance for applications in biofuel cells and enzyme electrodes (Christenson et al. 2004; Wong and Schwaneberg 2003). For improved mediated electron transfer between these enzymes and the anode of such biofuel cells, high activity with the mediator is essential. Ferrocene derivatives are used frequently as mediators in biofuel cells, and therefore the improved catalytic efficiency with \( \text{Fc}^+ \) observed for variants T169G and T169S could be of significance for such applications. Recently, we showed that \( TmP2Ox \) can efficiently transfer electrons to a graphite electrode when using appropriate mediators (Tasca et al. 2007), and hence it can be a better alternative to the commonly used glucose oxidase for the anodic compartment of biofuel cells.

Thr169 occupies an interesting, and important, position in the \( TmP2Ox \) structure. During the oxidative half-reaction when electron-acceptor substrates such as dioxygen are bound, the dynamic substrate loop is fully closed (Hallberg et al. 2004) and Thr169 forms a hydrogen bond with the flavin N5 atom. During the reductive half-reaction, the substrate loop swings out of the active site to accommodate binding of electron-donor substrate (sugar). In this conformation, the Thr169 side chain adopts a different rotamer and discards of its hydrogen bond to the flavin cofactor (Kujawa et al. 2006). In a recent comparison of a number of different flavoproteins it was noted that a recurrent feature in most of these proteins is a hydrogen bond between N5 of their flavin prosthetic group and a hydrogen-bond donor, typically a backbone or side-chain nitrogen (Fraaije and Mattevi 2000). This proximity of the hydrogen-bond donor to the isoalloxazine ring is expected to increase the oxidative power of the cofactor. In human electron transfer flavoprotein ETF this hydrogen bond between flavin N5 and protein is formed through Thr residue 266 of its \( \alpha \) chain. The position of the threonine residue is highly conserved in various ETF from different sources, with a conservative replacement of this Thr residue by Ser in a high-potential ETF from \( \text{Methylophilus methylotrophus} \) (Roberts et al. 1996; Roberts et al. 1999). The loss of the hydrogen bond is observed in the mutant \( \alpha T266M \), which is a frequent mutation found in the inherited metabolic
disease glutaric acidemia type II (Salazar et al. 1997). The mutation has relatively little effect on the reductive half-reaction of human ETF while the $k_{\text{cat}}/K_M$ value of the oxidative half-reaction is reduced 33-fold.

Obviously, the possibility to form a protein-mediated hydrogen bond to the flavin N5 atom is abolished in the P2Ox variants T169G and T169A, while in the conservative mutation of T169S, the hydrogen bond to N5 can be retained by the serine hydroxyl group. This probably explains why $k_{\text{cat}}$ values for the sugar substrates are less drastically affected in the T169S variant than for the other two variants, T169G and T169A. Regardless of electron acceptor used, T169A is the most impaired variant. In context of a tentative, specific role of T169 in the oxidative half-reaction, this is expected since the alanine side chain has no possibility to form a hydrogen bond, whereas T169G can accommodate a water molecule close to N5. In human ETF the removal of the hydrogen bond between flavin N5 and protein affects the oxidative half-reaction specifically, i.e., its reoxidation by the electron acceptor. In our study we have only measured steady-state kinetic data, and these reflect both the reductive and the oxidative half-reaction. Hence it is not possible to identify unequivocally which of the two half-reactions is affected by the removal of the hydrogen bond between flavin N5 and Thr169 in $TmP2Ox$ from these data, and more detailed kinetic studies will be necessary to clearly identify which reaction step is influenced.

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References


Chapter 2

Improving thermostability and catalytic activity of pyranose 2-oxidase from
Trametes multicolor by rational and semi-rational design

Oliver Spadiut¹, Christian Leitner¹, Clara Salaheddin¹, Balázs Varga², Beata Vertessy², Tien-Chye Tan³, Christina Divne³ & Dietmar Haltrich¹

¹Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU_University of Natural Resources and Applied Life Sciences, Vienna, Austria
²Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapest, Hungary
³School of Biotechnology, KTH, Albanova University Centre, Stockholm, Sweden

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*Trametes multicolor* by rational and semi-rational design

Oliver Spadiut¹, Christian Leitner¹, Clara Salaheddin¹, Balázs Varga², Beata Vertessy², Tien-Chye Tan³, Christina Divne³, Dietmar Haltrich¹

¹ Department of Food Sciences and Technology, BOKU–University of Natural Resources and Applied Life Sciences, Vienna, A-1190 Vienna, Austria

² Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1518 Budapest, Hungary

³ School of Biotechnology, Royal Institute of Technology KTH, Albanova University Center, SE-106 91 Stockholm, Sweden

*Corresponding author:* Dietmar Haltrich, Department of Food Sciences and Technology, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Wien, Austria

Tel. +43-1-36006 6275; fax. +43-1-36006 6251; E-mail: dietmar.haltrich@boku.ac.at

Website: http://www.dlwt.boku.ac.at/lbt.html?&L=1

*Running title:* stabilisation of pyranose oxidase

*Database:* structural data are available in the Protein Data Base under the accession numbers 3BG6, 3BG7, and 3BLY
Abstract

The fungal homotetrameric flavoprotein pyranose oxidase (P2Ox; EC1.1.3.10) catalyses the oxidation of various sugars at position C2, while concomitantly electrons are transferred to oxygen as well as to alternative electron acceptors, e.g. oxidised ferrocenes. These properties make P2Ox an interesting enzyme for various biotechnological applications. Random mutagenesis has previously been used to identify variant E542K, which shows increased thermostability. In the present study we selected position Leu537 for saturation mutagenesis, and identified variants L537G and L537W, which are characterised by higher stability and improved catalytic properties. We report detailed studies on both thermodynamic and kinetic stability as well as on the kinetic properties of the mutational variants E542K, E542R, L537G and L537W, as well as on the respective double mutants (L537G/E542K, L537G/E542R, L537W/E542K, and L537W/E542R). The selected substitutions at positions Leu537 and Glu542 increase the melting temperature by approximately 10°C and 14°C, respectively, relative to the wild-type enzyme. While both wild-type and single mutants showed first-order inactivation kinetics, thermal unfolding and inactivation was more complex for the double mutants showing two distinct phases as was shown by microcalorimetry and circular dichroism spectroscopy. Structural information on the variants does not provide a definitive answer to the stabilising effects or the alteration of the unfolding process. Distinct differences, however, are observed for the P2Ox Leu537 variants at the interfaces between the subunits, which results in tighter association.

Keywords
Pyranose oxidase; stabilisation; enzyme engineering; stability; subunit interaction

Abbreviations
ABTS, azino-bis-(3-ethylbenzthiazolin-6-sulfonic acid); CD, circular dichroism; DSC, differential scanning calorimetry; Fe⁺, ferricenium ion; GMC, glucose–methanol–choline;
IMAC, immobilised metal affinity chromatography; Mes, 2-(N-morpholino) ethane sulfonic acid, or 4-morpholine ethane sulfonic acid; MET, mediated electron transfer; Mme PEG 2,000, monomethylether polyethylene glycol 2000; P2Ox, pyranose 2-oxidase; PsP2Ox, pyranose oxidase from _Peniophora_ sp.; _T_ _m_ , melting temperature; _TmP2Ox_, pyranose oxidase from _T. multicolor_; _TvP2Ox_, pyranose oxidase from _Trametes (Coriolus) versicolor_; _T_ _50_, temperature at which activity is reduced by 50%; _wt_, wild type; _τ_ _1/2_, half life of activity.

**Introduction**

The flavoenzyme pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10), a member of the glucose-methanol-choline (GMC) family of FAD-dependent oxidoreductases [1], catalyses the oxidation of several aldopyranoses at position C-2 to yield the corresponding 2-ketoaldoses and _H_ _2_ _O_ _2_ as products. The enzyme is found in wood-degrading basidiomycetes, where it is localized in the hyphal periplasmic space. Presumably, P2Ox supplies lignin and manganese peroxidases with _H_ _2_ _O_ _2_, an essential co-substrate for ligninolysis by wood-rotting fungi [2]. To date, P2Ox from _Trametes multicolor_ and _Peniophora gigantea_ are the best studied enzymes both from a biochemical and structural point of view [3-6]. Native P2Ox from _T. multicolor_ (_TmP2Ox_) is composed of four identical 68-kDa subunits resulting in a 270-kDa homotetramer [7]. It contains the prosthetic group flavin adenine dinucleotide (FAD) bound covalently _via_ its 8α-methyl group to each _His167 N^ε_ (i.e. N3) per subunit [8], which was also confirmed from the crystal structure of _TmP2Ox_ determined at 1.8 Å resolution [3]. Structurally, the homotetramer is described more accurately as a dimer of dimers, _i.e._, dimers formed by the subunits A and B, as well as C and D (Fig. 1).
Interaction between the interfaces is most extensive between these two dimers A–B and C–D with a large number of hydrogen bonds and hydrophobic contacts. These interactions are mainly through two distinct regions of the subunit termed the oligomerisation loop and oligomerisation arm. The latter is also involved in the interactions between subunits A and D (respectively B and C), while the weakest interaction surfaces are observed at the interface of the A–C (and B–D) pair. These latter interactions are mainly through hydrophobic contacts between residues 508–528 and 532–540 (segments H8 and B6, respectively) [3].

In accordance with other flavoprotein oxidoreductases the reaction mechanism of P2Ox is of the typical Ping Pong Bi Bi type [9, 10]. In the reductive half-reaction an aldopyranose is oxidised at position C-2 to yield a 2-ketoaldose (aldos-2-ulose), while FAD is reduced to FADH₂.
(reaction 1) [11, 12]. During the ensuing oxidative half-reaction FADH$_2$ is re-oxidised by the second substrate oxygen, yielding the oxidised prosthetic group and H$_2$O$_2$ (reaction 2). In addition, alternative electron acceptors, including either two-electron acceptors such as benzoquinones (reaction 3) or one-electron acceptors such as chelated metal ions, e.g. the ferricenium ion or radicals, are used efficiently by P2Ox instead of oxygen [7].

\[
\begin{align*}
FAD + \text{aldopyranose} & \rightarrow FADH_2 + 2\text{-keto-aldopyranose} \\
FADH_2 + O_2 & \rightarrow FAD + H_2O_2 \\
FADH_2 + \text{benzoquinone} & \rightarrow FAD + \text{hydroquinone}
\end{align*}
\]

P2Ox has become an interesting biocatalyst in biotransformations of carbohydrates as it can be used to synthesise various carbohydrate derivatives and rare sugars [12]. Amongst others, the oxidation of D-glucose and D-galactose to 2-keto-D-glucose and 2-keto-D-galactose is of applied interest as these oxidised intermediates can be subsequently reduced at position C-1 to obtain the ketoses D-fructose and D-tagatose [13, 14], which are of interest for food industry. P2Ox is not only useful for biotransformations of carbohydrates, but also for applications in sensors or biofuel cells [15, 16]. Recently, we could show the electrical wiring of P2Ox with an osmium redox polymer serving as a redox mediator on graphite electrodes [15]. Here, the redox polymer collects the electrons from the prosthetic groups of the enzyme and transfers them to the electrode. Other mediators that have been investigated for providing contact between P2Ox and the electrode include ruthenium or modified ferrocenes [16]. For this bioelectrochemical application, the reactivity of P2Ox with alternative electron acceptors, and notably with (complexed) metal ions such as the ferrocenes, is of significant importance.

As for many other enzymes applied in industry [17, 18], there is a need for more stable and active P2Ox. To date, few attempts to improve P2Ox by enzyme engineering have been reported. Studies on P2Ox from *Coriolus (Trametes) versicolor* (TvP2Ox) using random
mutagenesis revealed the importance of position Glu542, both for improved thermostability and catalysis, with variant E542K showing an increase in optimum temperature by 5°C and a decrease in the Michaelis constant $K_m$ for the two substrates D-glucose and 1,5-anhydro-D-glucitol [19]. Subsequent studies on P2Ox from *Peniophora gigantea* (*PgP2Ox*) and *Peniophora* sp. (*PsP2Ox*) confirmed the beneficial effects of the Glu→Lys mutation at position 542 (540 for *PgP2Ox*), and identified additional amino acid residues, e.g., Thr158 in *PsP2Ox*, which affect positively the $K_m$ values for a range of carbohydrate substrates [20, 21]. Here we report novel mutations of P2Ox at position Leu537, which affect beneficially both the turnover number and thermal stability, and give for the first time a detailed analysis of the effects of several mutations including the E542K variant on the kinetic and thermodynamic stability of *TmP2Ox*.

**Results**

**Generation of mutants**

Based on published results, we selected position Glu542 for mutational studies towards improved thermostability, as replacement of this residue by Lys was shown to be beneficial, increasing the temperature optimum of activity and lowering the Michaelis constant [19, 21]. In addition to variant E542K, which was shown previously to be advantageous, we also produced the variant E542R, again replacing Glu by a basic amino acid. DNA sequence analysis confirmed the presence of the correct mutations at the amino acid position 542 in the *TmP2Ox* sequence with no undesired mutations. Furthermore, we selected position Leu537 for mutational studies using saturation mutagenesis. As is evident from the structure of *TmP2Ox* [3], Leu537 is located on the surface of the P2Ox subunit as part of $\beta$-strand B6. Presumably, it takes part in the (weak) interaction between subunits A and C as well as B and D with Leu537 of monomer A positioned opposite Leu537’ of monomer C (Fig. 2 a, b).
Fig. 2. Ribbon drawings showing the position 537 at the A/C interface. The A and C subunits are coloured yellow and red, respectively. For clarity, subunits B and D have been omitted. a) Model 2IGO with Leu537 at the dyad axis between monomers A and C in the A/C interface. b) Zoomed in view of a). Zoom-in views of c) the L537G variant lacking a side chain at position 537, and d) the E542K/L537W mutant with tryptophan at position 537.
Replacement of this amino acid by a more suitable residue might therefore increase the interaction between the subunits and stabilise the quaternary structure of P2Ox. Saturation mutagenesis was done as described in the Experimental section. After screening of 190 colonies using a microtiter plate-based assay we selected the most thermostable mutants for sequencing; these were identified as variants L537G and L537W. Different codons for these two amino acids were found in the selected variants at position 537, which confirmed the successful procedure of saturation mutagenesis. After characterization of these four single mutants, the double-mutants L537G/E542K, L537G/E542R, L537W/E542K and L537W/E542R were constructed by site-directed mutagenesis to possibly combine the positive effects of the different single mutations on thermostability and catalytic activity. Again, DNA sequence analysis confirmed the presence of the correct replacements in the P2Ox gene with no undesired mutations.

**Protein expression and purification**

To express active P2Ox variants, the different transformants were cultivated in 2-L shaken flasks and recombinant protein expression was induced by the addition of lactose (0.5%) to the culture medium. Routinely, approx. 30 mg of P2Ox protein were obtained per litre culture medium in these cultivations. P2Ox variants were purified from the crude extracts by IMAC followed by ultrafiltration. This two-step purification procedure resulted into proteins that were apparently homogenous (>98%) as judged by native PAGE and SDS-PAGE (Fig. 3).
Fig. 3. Native PAGE (A) and SDS-PAGE (B) of different variants of P2Ox from *T. multicolor*. Lane 1, molecular mass standards (High Molecular Weight Calibration Kit for native electrophoresis, Amersham, and Precision Plus Protein Dual Color, Biorad, respectively); lane 2, wt *TmP2Ox*; lane 3, variant L537G; lane 4, L537W; lane 5, E542K; lane 6, E542R; lane 7, L537G/E542K; lane 8, L537G/E542R; lane 9, L537W/E542K; lane 10, L537W/E542R.

**Kinetic characterisation of mutational variants**

Steady-state kinetic constants for the different mutational variants of *TmP2Ox* were determined for the two sugar substrates D-glucose and D-galactose, which were varied over a range of 0.1–50 mM and 0.1–200 mM, respectively, using the standard ABTS assay and oxygen (air saturation). Prior to determination of the kinetic constants it was confirmed that introduction of the amino acid substitutions in the different variants did not affect the pH profile of P2Ox activity (data not shown). Table 1 provides a summary of the kinetic data for both D-glucose and D-galactose. For the presumed natural substrate of P2Ox, D-glucose, the two Leu537 variants
studied showed slightly decreased $K_m$ and increased $k_{cat}$ values. Mutations at Glu542 lowered significantly the Michaelis constant, while $k_{cat}$ was also decreased to some extent, especially for the E542R variant, when compared to the wt enzyme. These effects could be combined in the double mutants, which all showed noteworthy reduced $K_m$ values and turnover numbers that are comparable to wt P2Ox. Variant L537W/E542K showed the highest increase in catalytic efficiency $k_{cat}/K_m$, which more than doubled relative to the wt (Table 1). D-Galactose is a relatively poor substrate of P2Ox; apparently, the axial hydroxyl group at position C-4 is sterically hindered by the side chain of Thr169 in the active site [22]. In accordance with the results obtained for D-glucose, the Glu542 variants showed lower $K_m$ values while $k_{cat}$ is hardly affected by the mutations considered in this study. Variants E542K and L537W/E542K resulted in the highest increase in catalytic efficiency (2.3- and 1.7-fold, respectively), compared to the wt enzyme; this is mainly due to the decrease in $K_m$ (Table 1).

<table>
<thead>
<tr>
<th>Variant</th>
<th>D-glucose</th>
<th>D-galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>wt P2Ox</td>
<td>0.939 ± 0.037</td>
<td>48.1 ± 0.53</td>
</tr>
<tr>
<td>L537G</td>
<td>0.851 ± 0.035</td>
<td>52.1 ± 0.59</td>
</tr>
<tr>
<td>L537W</td>
<td>0.749 ± 0.022</td>
<td>59.0 ± 0.48</td>
</tr>
<tr>
<td>E542K</td>
<td>0.521 ± 0.019</td>
<td>35.9 ± 0.33</td>
</tr>
<tr>
<td>E542R</td>
<td>0.489 ± 0.032</td>
<td>28.5 ± 0.46</td>
</tr>
<tr>
<td>L537G/E542K</td>
<td>0.487 ± 0.027</td>
<td>43.9 ± 0.61</td>
</tr>
<tr>
<td>L537G/E542R</td>
<td>0.441 ± 0.021</td>
<td>33.1 ± 0.38</td>
</tr>
<tr>
<td>L537W/E542K</td>
<td>0.432 ± 0.012</td>
<td>46.5 ± 0.32</td>
</tr>
<tr>
<td>L537W/E542R</td>
<td>0.419 ± 0.015</td>
<td>31.7 ± 0.32</td>
</tr>
</tbody>
</table>

**Table 1.** Apparent kinetic constants of wild-type recombinant pyranose 2-oxidase from *T. multicolor* and mutational variants for either D-glucose or D-galactose as substrate, with the concentration of O$_2$ as electron acceptor held constant. Kinetic data were determined at 30°C using the standard ABTS assay and air saturation.
Steady-state kinetic constants were furthermore determined for alternative electron acceptors of P2Ox, the one-electron acceptor substrate ferricenium ion \( \text{Fe}^+ \), and the two-electron acceptor substrate 1,4-benzoquinone, using both D-glucose and D-galactose as the saturating substrate. Data are summarised in Tables 2–3. Here, replacing Leu537 with either Trp or Gly resulted in a significant increase in \( k_{\text{cat}} \) for both substrates, which is more pronounced for variant L537W than for L537G. Interestingly, all other variants had lower \( k_{\text{cat}} \) values for \( \text{Fe}^+ \) as substrate than the wt enzyme. Furthermore, all of the variants studied showed lower \( K_m \) values for 1,4-benzoquinone. As a result, the catalytic efficiencies increased considerably for some of these variants, which is most noteworthy for L537W, where \( k_{\text{cat}}/K_m \) increased 2.2- and 2.5-fold for \( \text{Fe}^+ \) and 1,4-benzoquinone with D-glucose as electron donor substrate (Tables 2 and 3).

### Table 2. Apparent kinetic constants of wild-type recombinant pyranose 2-oxidase from *T. multicolor* and mutational variants for the ferricenium ion \( \text{Fe}^+ \) as varied substrate, with the concentration of D-glucose or D-galactose as electron donor held constant at 100 mM. Kinetic data were determined at 30°C.

<table>
<thead>
<tr>
<th>Variant</th>
<th>D-glucose</th>
<th></th>
<th>D-galactose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>( k_{\text{cat}} ) (s(^{-1}))</td>
<td>( k_{\text{cat}}/K_m ) (M(^{-1})·s(^{-1}))</td>
<td>rel. ( k_{\text{cat}}/K_m ) (%)</td>
</tr>
<tr>
<td>wt P2Ox</td>
<td>0.254 ± 0.099</td>
<td>151 ± 35</td>
<td>592,000</td>
<td>100</td>
</tr>
<tr>
<td>L537G</td>
<td>0.289 ± 0.097</td>
<td>282 ± 49</td>
<td>975,000</td>
<td>164</td>
</tr>
<tr>
<td>L537W</td>
<td>0.253 ± 0.093</td>
<td>334 ± 61</td>
<td>1,320,000</td>
<td>223</td>
</tr>
<tr>
<td>E542K</td>
<td>0.290 ± 0.096</td>
<td>54.4 ± 9.2</td>
<td>187,000</td>
<td>31.6</td>
</tr>
<tr>
<td>E542R</td>
<td>0.319 ± 0.105</td>
<td>46.7 ± 8.1</td>
<td>147,000</td>
<td>24.8</td>
</tr>
<tr>
<td>L537G/E542K</td>
<td>0.296 ± 0.097</td>
<td>86.7 ± 14</td>
<td>294,000</td>
<td>49.6</td>
</tr>
<tr>
<td>L537G/E542R</td>
<td>0.320 ± 0.141</td>
<td>102 ± 23</td>
<td>309,000</td>
<td>52.2</td>
</tr>
<tr>
<td>L537W/E542K</td>
<td>0.408 ± 0.168</td>
<td>127 ± 29</td>
<td>310,000</td>
<td>52.4</td>
</tr>
<tr>
<td>L537W/E542R</td>
<td>0.281 ± 0.103</td>
<td>86.3 ± 16</td>
<td>307,000</td>
<td>51.9</td>
</tr>
</tbody>
</table>
Table 3. Apparent kinetic constants of wild-type recombinant pyranose 2-oxidase from *T. multicolor* and mutational variants for 1,4-benzoquinone as varied substrate, with the concentration of D-glucose or D-galactose as electron donor held constant at 100 mM. Kinetic data were determined at 30°C.

<table>
<thead>
<tr>
<th>Variant</th>
<th>D-glucose</th>
<th>D-galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>wt P2Ox</td>
<td>0.241 ± 0.025</td>
<td>152 ± 5.9</td>
</tr>
<tr>
<td>L537G</td>
<td>0.176 ± 0.025</td>
<td>184 ± 7.6</td>
</tr>
<tr>
<td>L537W</td>
<td>0.130 ± 0.013</td>
<td>205 ± 6.1</td>
</tr>
<tr>
<td>E542K</td>
<td>0.182 ± 0.025</td>
<td>189 ± 9.2</td>
</tr>
<tr>
<td>E542R</td>
<td>0.136 ± 0.015</td>
<td>127 ± 4.0</td>
</tr>
<tr>
<td>L537G/E542K</td>
<td>0.150 ± 0.015</td>
<td>173 ± 5.1</td>
</tr>
<tr>
<td>L537G/E542R</td>
<td>0.155 ± 0.032</td>
<td>173 ± 10.3</td>
</tr>
<tr>
<td>L537W/E542K</td>
<td>0.140 ± 0.018</td>
<td>181 ± 7.8</td>
</tr>
<tr>
<td>L537W/E542R</td>
<td>0.137 ± 0.024</td>
<td>175 ± 10.4</td>
</tr>
</tbody>
</table>

**Thermodynamic stability**

Wild-type *Tm*P2Ox and its variants were investigated by differential scanning calorimetry (DSC) in order to acquire thermodynamic data on heat-induced unfolding of these proteins and hence on their thermodynamic stability [23]. For each protein sample, cooperative unfolding peaks were observed for the first heating cycle (Fig. 4). Samples after the first heating cycle showed considerable precipitation, arguing for irreversible aggregation, and, therefore, no cooperative melting peaks could be observed in the second heating cycle. Because of the irreversible nature of the unfolding under the present circumstances, the thermodynamic values associated with the heat absorption curves, as calculated by equations based on reversible thermodynamic criteria, are only indicative. However, the melting temperature $T_m$ can be taken as an informative value, since irreversible aggregation is expected to occur only once the unfolding is complete, after the melting point has been reached. WtP2Ox from *T. multicolor* shows a $T_m$ of 60.7°C, and all
variants are characterised by significantly increased $T_m$ values and thermal stability (Fig. 4). The clear differences between the melting points of the single Leu537 and the Glu542 mutants (approx. 70 and 75°C, respectively) indicate that the replacement of Glu542 with a basic residue might introduce an ionic interaction exerting a greater stabilising effect on the tetramer than the mere alteration of an apolar residue by residues of comparable hydrophobicity (Fig. 4A).

Interestingly, the double mutants L537G/E542K, L537G/E542R, L537W/E542K and L537W/E542R all showed more complex melting curves with a first, shouldered peak around 65°C and a second peak around 75–77°C (Fig. 4B). Immediately after the second peak had been reached, a sudden drop was observed in the heat absorption signal, possibly indicating major aggregation, which was also confirmed by visual inspection of the samples. This behaviour prevented full analysis of the second heat absorption step, however, the two steps are clearly different from the single mutant and the wild-type proteins. The first transition appears to be cooperative, though irreversible, as determined by repeated heat cycles. However, the two peaks can also be measured in two subsequent heating cycles if the heating process is stopped once the end of the first transition has been reached, suggesting that the conformation associated with this first transition remains stable and does not undergo any irreversible changes at lower temperatures.
Fig. 4. (A) Denaturation thermograms of wt P2Ox from *T. multicolor* (solid line) and the single mutants L537W (dotted line), L537G (dashed line), E542R (dash-dotted line) and E542K (thick solid line). (B) Heat-induced unfolding of *TmP2Ox* double mutant variants L537G/E542K (solid line), L537G/E542R (dashed line), L537W/E542K (thick solid line) and L537W/E542R (dash-dotted line). Melting temperatures are indicated directly in the figure. As for the double mutants, the peaks of the second transitions occur at the following temperatures: L537G/E542K, 77.4ºC; L537G/E542R, 75.0ºC; L537W/E542K, 77.5ºC; L537W/E542R, 76.4ºC.

**Kinetic stability**

Kinetic stability, i.e., the length of time an enzyme remains active before undergoing irreversible inactivation [23], was measured for wtP2Ox and *TmP2Ox* variants at different temperatures and at a constant pH of 6.5, and the inactivation constants $k_{in}$ and half-life of denaturation $\tau_{1/2}$ were
determined (Table 4). The single mutants showed first-order inactivation kinetics when analysed in the ln(residual activity) versus time plot (Fig. 5).

Table 4 Kinetic stability of pyranose oxidase from T. multicolor at various temperatures

<table>
<thead>
<tr>
<th>Variant</th>
<th>Inactivation constant $k_{in,1}$ (min$^{-1}$)</th>
<th>Inactivation constant $k_{in,2}$ (min$^{-1}$)</th>
<th>Half-life $\tau_{1/2}$ (min)</th>
<th>Inactivation constant $k_{in}$ (min$^{-1}$)</th>
<th>Half-life $\tau_{1/2}$ (min)</th>
<th>Inactivation constant $k_{in}$ (min$^{-1}$)</th>
<th>Half-life $\tau_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt P2Ox</td>
<td>$-1040 \times 10^{-4}$</td>
<td>—</td>
<td>6.66</td>
<td>n.d.</td>
<td>&lt; 1 min</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>L537G</td>
<td>$-5.87 \times 10^{-4}$</td>
<td>—</td>
<td>1180</td>
<td>$-24.4 \times 10^{-2}$</td>
<td>2.84</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>L537W</td>
<td>$-5.20 \times 10^{-4}$</td>
<td>—</td>
<td>1330</td>
<td>$-46.4 \times 10^{-2}$</td>
<td>1.49</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>E542K</td>
<td>$-4.22 \times 10^{-4}$</td>
<td>—</td>
<td>1640</td>
<td>$-1.26 \times 10^{-2}$</td>
<td>55.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>E542R</td>
<td>$-4.12 \times 10^{-4}$</td>
<td>—</td>
<td>1680</td>
<td>$-2.25 \times 10^{-2}$</td>
<td>30.8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>L537G/E542K</td>
<td>$-81.2 \times 10^{-4}$</td>
<td>$-11.3 \times 10^{-4}$</td>
<td>241*</td>
<td>$-0.242 \times 10^{-2}$</td>
<td>5.5*</td>
<td>$-2.90 \times 10^{-1}$</td>
<td>2.39</td>
</tr>
<tr>
<td>L537G/E542R</td>
<td>$-154 \times 10^{-4}$</td>
<td>$-4.34 \times 10^{-4}$</td>
<td>132*</td>
<td>$-0.349 \times 10^{-2}$</td>
<td>7.2*</td>
<td>$-3.98 \times 10^{-1}$</td>
<td>1.74</td>
</tr>
<tr>
<td>L537W/E542K</td>
<td>$-61.1 \times 10^{-4}$</td>
<td>$-3.37 \times 10^{-4}$</td>
<td>934*</td>
<td>$-0.207 \times 10^{-2}$</td>
<td>105*</td>
<td>$-2.35 \times 10^{-1}$</td>
<td>2.95</td>
</tr>
<tr>
<td>L537W/E542R</td>
<td>$-75.9 \times 10^{-4}$</td>
<td>$-3.10 \times 10^{-4}$</td>
<td>727*</td>
<td>$-0.435 \times 10^{-2}$</td>
<td>71.1*</td>
<td>$-3.43 \times 10^{-1}$</td>
<td>2.02</td>
</tr>
</tbody>
</table>

The selected substitutions at both position 537 and 542 resulted in considerably stabilised P2Ox variants, with the replacement of Glu542 by either Lys or Arg showing a stronger effect (decreased $k_{in}$ and increased $\tau_{1/2}$ values) than the Leu$\rightarrow$Gly and Leu$\rightarrow$Trp replacements at position 537. At 60°C the half-life values were increased for the Leu537 and Glu542 variants approximately 200-fold and 250-fold, respectively, compared to the wt enzyme. Inactivation of the double mutants L537G/E542K, L537G/E542R, L537W/E542K, and L537W/E542R was a more complex process, showing two distinct phases, a first phase of relatively rapid inactivation that apparently followed first-order kinetics, and after an intermediate phase a second phase of first-order decay, with inactivation constants that were much lower than for the first phase. This

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*a* n.d., not determined

* inactivation did not follow apparent first-order kinetics but showed two distinct phases; $\tau_{1/2}$ values were calculated using the inactivation constant calculated by the regression analysis for the second phase, but are not true half-life values.
complex behaviour is in excellent agreement with the results obtained by microcalorimetry. At 60°C this first phase of inactivation lasted for about 45 min, while it was instantaneous (less than 2.5 min) at 70°C (Fig. 5). Interestingly, the second phase was characterised by inactivation constants that were even lower than those found for the single mutants. This is especially pronounced at 70°C with $k_{in}$ values for the double mutants being lower by one or two orders of magnitude than those of the single mutants. Because of this complex behaviour no true half-lives can be given, yet the values calculated by using the obtained inactivation constants show significant stabilisation, especially at higher temperatures.

**Fig. 5.** Inactivation kinetics of pyranose oxidase from *T. multicolor* at 60°C (A) and 70°C (B) and pH 6.5.

Symbols: (A): ■, wild-type pyranose oxidase; ●, variant L537G; ▲, variant L537W; □, variant E542K; △, variant E542R; (B): ■, variant L537W/E542K; ●, variant L537G/E542R; ▲, variant L537W/E542K; △, variant L537W/E542R.
Circular dichroism spectroscopy

In order to learn more about heat-induced conformational changes of the proteins studied and the nature of the residual fraction obtained for the double mutants after the first melting step, circular dichroism (CD) spectroscopy was applied using wtP2Ox as well as the L537W/E542K and L537W/E542R double mutants as protein samples. The far-UV CD spectrum of wtP2Ox at 25°C was typical for a protein composed of both α-helical and β-strand secondary structure elements, as also expected from the crystal structure of TmP2Ox [3]. This spectrum was essentially unchanged when the temperature was increased up to 55°C (Fig. 6), while a sharp loss in intensity was obtained near the melting point of wtP2Ox (60.7°C). It is worth noting that the spectrum recorded at 61°C showed weak but clearly observable double maxima at 208 and 222 nm, which is typical for an α-helical protein and suggests that the most stable region of wtP2Ox is an α-helical core. The highest CD signal in the CD spectrum was observed at 209 nm, and thermal unfolding was followed at this wavelength in a separate experiment. Intensity at 209 nm did not change significantly until approximately 60°C was reached, upon which it quickly diminished and became zero (Fig. 6, inset). This is in good agreement with the spectral CD measurements as well as with the DSC results.
Fig. 6. Temperature dependence of wt *Tm*P2Ox circular dichroism spectra. The inset shows the CD signal at 209 nm as a function of temperature. For the main panel, the sample was heated up to the different temperature values (25, 35, 45, 55, and 61°C), and full spectra were recorded at these temperatures, requiring 3–4 min for each single measurement. For the inset, the sample was heated using the constant rate of 0.9 °C/min. Therefore, the sample in the inset spent much shorter time at the elevated temperatures and this melting curve is more appropriate to evaluate the thermodynamic parameters of unfolding.

In the DSC experiment, two well-separated peaks could be observed for the double mutants, the first of which was also deconvoluted into two transitions. In the CD spectra of the double mutants, we observe two well-separated steps of intensity loss as well, and these occur at temperatures that agree well with those in the DSC experiment (Figures 4 and 7). Based on the behaviour of the L537W/E542K and L537W/E542R double mutants observed in the DSC experiments, the CD spectra of the protein samples heated to this plateau temperature (68–70°C) and then cooled to 25°C are expected to reflect the conformation of the partially melted protein (Fig. 7B). These partially melted samples showed a profile identical to that of native P2Ox, however, with lower intensity, suggesting that no drastic change in the composition of the
secondary structural elements occurred in the partially melted sample as compared to the native one. Since no stable dimeric or monomeric form of P2Ox is, however, available for comparative CD studies, we cannot unambiguously decide the oligomeric state of the species possessing the residual CD spectrum and activity associated with the first DSC transition.

Fig. 7. (A) Complete (two-step) thermal unfolding of the L537W/E542K and the L537W/E542R mutants in one single heating cycle. The spectra of the native proteins L537W/E542K (solid line) and L537W/E542R (dashed line), as well as the spectra of the completely unfolded proteins were recorded at 25°C. Inset: The CD signal at 209 nm was followed as a function of temperature (L537W/E542K – black, L537W/E542R – grey). (B) Circular dichroism spectra of the two-step thermal unfolding of the L537W/E542K and the L537W/E542R mutants recorded at 25°C. Initial spectra (L537W/E542K - solid line, L537W/E542R - dashed line) are those of the native proteins, the second set of spectra were recorded after partial thermal unfolding, while the final spectra show the loss of CD signal after complete unfolding. Inset: The CD signal at 209 nm was followed as a function of temperature (L537W/E542K – black, L537W/E542R – grey).
Structure of the P2Ox variants

Data collection and model statistics are given in Table 5. The final L537G and E542K models include two complete tetramers per asymmetric unit with each monomer consisting of residues 43 to 619, and one FAD molecule per monomer. The L537W/E542K mutant contains one monomer per asymmetric unit comprising residues 46 to 618 with one FAD and one Mes molecule per monomer. As shown in Table 5, all models have good $R$ values with residues that fall within the allowed regions of the Ramachandran plot [24].

Table 5 Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>E542K</th>
<th>L537G</th>
<th>E542K/L537W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength, $\lambda$ (Å)</td>
<td>0.918</td>
<td>1.042</td>
<td>0.931</td>
</tr>
<tr>
<td>Beamline / Temp (K)</td>
<td>BESSY 14.1 / 100</td>
<td>MAX-lab P911-2 / 100</td>
<td>ESRF ID14-3 / 100</td>
</tr>
<tr>
<td>Cell constants a, b, c (Å); $\beta$ (°) / Space group</td>
<td>168.9, 103.7, 169.3, 106.31 / $P2_1$</td>
<td>168.5, 103.2, 169.3, 106.45 / $P2_1$</td>
<td>103.4, 103.4, 118.6 / $P4_2_12$</td>
</tr>
<tr>
<td>Resolution range, nominal (Å)</td>
<td>40-1.70 (1.75-1.70)</td>
<td>40-2.10 (2.20-2.10)</td>
<td>51-1.90 (2.00-1.90)</td>
</tr>
<tr>
<td>Unique reflections</td>
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<td>321,136 (39,548)</td>
<td>51,240 (7,193)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.8 (3.2)</td>
<td>4.4 (3.3)</td>
<td>12.6 (12.7)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.2 (97.4)</td>
<td>99.0 (94.0)</td>
<td>99.9 (100)</td>
</tr>
<tr>
<td>$&lt;</td>
<td>I</td>
<td>/\sigma(I)&gt;$</td>
<td>9.7 (2.2)</td>
</tr>
<tr>
<td>$R_{sym}$ (%)</td>
<td>13.7 (58.8)</td>
<td>6.6 (26.2)</td>
<td>12.1 (62.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement</th>
</tr>
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<tbody>
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<tr>
<td>Completeness, all % (highest bin)</td>
</tr>
<tr>
<td>$R_{free}$/work reflns, all</td>
</tr>
<tr>
<td>$R_{free}$/free reflns, all</td>
</tr>
<tr>
<td>Non-hydrogen atoms all/protein</td>
</tr>
<tr>
<td>Mean $B$ (Å$^2$) protein all/mc/sc</td>
</tr>
<tr>
<td>Mean $B$ (Å$^2$) solvent / $N^o.$ molecules</td>
</tr>
<tr>
<td>Mean $B$ (Å$^2$) cofactor / $N^o.$ atoms</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Rmsd bond lengths (Å), angles (°)</td>
</tr>
<tr>
<td>Ramachandran: favored / allowed (%)</td>
</tr>
<tr>
<td>Protein Data Bank code</td>
</tr>
</tbody>
</table>

1 The outer shell statistics of the reflections are given in soft brackets. Shells were selected as defined in XDS [32] by the user.

2 \( R_{\text{sym}} = \left[ \frac{\sum_{hkl} \sum_i |I_i - \langle I \rangle|}{\sum_{hkl} \sum_i |I_i|} \right] \times 100 \% . \)

3 \( R_{\text{factor}} = \frac{\sum_{hkl} |F_{o} - F_{c}|}{\sum_{hkl} |F_{o}|} \)

4 As determined by MolProbity [24].

5 Protein Data Bank accession codes for atomic coordinates and structure factors deposited with the RCSB Protein Data Bank.

The overall tetramer structure (Fig. 1) of all mutants is identical to that previously reported for wild-type and recombinant P2Ox from *T. multicolor* [3, 4]. Typical r.m.s.d. values using all C\(\alpha\) atoms from all monomers of the tetramer fall in the range of 0.2–0.3 Å. The structures are also almost identical to the models of *Peniophora* P2Ox (PDB codes 1TZL, 2F5V and 2F6C; [5, 6]) with r.m.s.d. values of approximately 0.9 Å for the monomer structure. The only major difference observed between all *Trametes* and *Peniophora* P2Ox models is the precise conformation of the substrate loop. As discussed in detail elsewhere, we have shown that this loop is in an open conformation when no substrate is bound (e.g., unliganded recombinant P2Ox; PDB codes 2IGK, 2IGM, 2IGN; [4]) or when an electron-donor substrate is bound (e.g., monosaccharide as in P2Ox H167A in complex with 2-fluoro-2-deoxy-D-glucose, 2FG; PDB code 2IGO; [4]), and in a closed conformation when small electron-acceptor substrates (i.e., dioxygen) or small inhibitor molecules (e.g., acetate as in wt P2Ox in complex with acetate; PDB code 1TT0; [3]) are bound. In the existing *PsP2Ox* models (recombinant wild-type P2Ox and P2Ox E542K mutant; PDB codes 1TZL, 2F5V and 2F6C, respectively; [5, 6]), the substrate loop assumes a disordered conformation intermediate to the ordered open and closed conformers seen for *TmP2Ox*.

As expected for the active site in the absence of electron-donor monosaccharide substrate or electron-acceptor substrate, the substrate loop in the E542K and L537G variants is open and
slightly disordered, as indicated by partly weak electron density and elevated temperature factors. In the L537W/E542K variant, however, the substrate loop is open and fully ordered. In the E542K structure, the introduced Lys side chain has unambiguous electron density and points into the internal cavity at the centre of the homotetramer. In the L537G mutant structure, the elimination of the relatively large and hydrophobic Leu side chain results in remarkably small changes. In wt P2Ox, Leu537 is located in strand B6 close to the dyad axis between monomers A and C (or B and D) where the Cβ atoms of Leu537 of each monomer interact through a hydrophobic packing interaction (Fig. 2 a,b). Upon replacement of the Leu side chain by Gly (Fig. 2 c), the Ca-Cα distance at position 537 between monomers A and C (or B and D) increases from 6.2 Å to 6.4 Å. The mutation produces a relative Cα displacement at position 537 within the monomer of 0.6–0.7 Å. The largest displacement is, however, seen two residues away, where the backbone Cα atom of Gly535 is shifted 0.9–1.0 Å as a result of the Leu537→Gly substitution in the L537G mutant. At the interface between subunits A and C, solvent molecules substitute for the missing Leu side chain. In addition, the small, but distinct, displacement around position 537 is accompanied by backbone displacements in the substrate loop (0.8–1.0 Å at Cα position 453).

We chose to use P2Ox H167A in complex with 2FG (PDB code 2IGO; [4]) as reference for comparisons since this model has the substrate loop in an open and ordered conformation, the open conformer being also observed in the three P2Ox variants described here. The mutants show minor but distinct differences compared with 2IGO. With Trp residues introduced at position 537, as in L537W/E542K (Fig. 2 d), the 537 backbone of monomers A and C move 0.2 Å closer together (with a concomitant movement of helices H8 in A and C closer by 0.4 Å), whereas with Gly replacements at this position (as in L537G), the monomers move 0.4 Å further apart. However, two residues away at position 535, the backbones of the A and C monomers show tighter association in L537G by 1.4 Å, and only by 0.9 Å in L537W/E542K, compared with model 2IGO. As a result of these movements, the L537W/E542K variant also shows a
concomitant displacement of the substrate loop by 0.4–0.6 Å, as well as tighter association between the oligomerisation arm in monomers A and D by 0.6 Å at position 121. In the E542K and L537G mutants, the corresponding position is shifted 0.1 and 0.3 Å further apart, respectively, thus possibly weakening the A-D interaction compared with 2IGO. At the more detailed structural level, we observe that, compared with 2IGO, the A/C interface of the L537W/E542K variant shows improved hydrophobic stacking interactions between Trp537 of monomer A and Gln539 of monomer C, with a possibility of additional amino-aromatic interaction between Gln539 Ne2 and the Trp537 ring. In addition, this arrangement allows a shorter and more aligned hydrogen bond between Gln539 Ne2 and Trp537 O, which ought to be more stable.

When comparing the three mutants and 2IGO, the largest difference observed is the position of the “head” domain (Fig. 8). In the thermostable L537W/E542K double mutant, differences in the backbone position of the exposed head domain of up to 4.3 Å are observed, and of exposed parts of the Rossmann domain of up to 2.7 Å. For the rest of the homotetrameric assembly, only smaller backbone displacements of up to 1 Å occur. Although these differences might arise from different packing in the tetragonal space group of the double mutant, the amino-acid replacements may also be of importance.
Fig. 8. Ribbon drawing showing the superpositioning of the tetramers of 2IGO (red), L537G (yellow), and E542K/L537W (blue). As discussed in the text, the only significant difference in the overall tetramer structure is the relative displacement of the head domain in E542K/L537W.

Discussion

Pyranose oxidase is an enzyme of applied interest, and hence previous studies were aiming at improving this biocatalyst [19-22]. One residue that was identified in some of these studies as being important for both stability and reactivity is Glu542, which is located on the surface of the internal cavity, close to the entrance to the active site [6]. Replacement of that residue by Lys resulted in increased thermostability, i.e., for Tvp2Ox an increase in $T_{50,30\text{ min}}$ from $\sim55^\circ\text{C}$ for the wt to $\sim65^\circ\text{C}$ [19], or for Psp2Ox an increase in the optimum temperature from 50°C for the wt to 58°C [20], as well as in improved catalytic properties. However, the effect of this Glu→Lys replacement on stability has not been studied in full detail. Here, we present for the first time detailed investigations for the E542K and E542R variants pertaining to their stability. In addition, we selected position Leu537, which is located at the interface of two subunits, for
mutational studies. Leu537 of one subunit (A or B) interacts with Leu537’ of another subunit (C or D) through hydrophobic packing. Strengthening this interaction through the introduction of a better-suited residue might therefore improve subunit-subunit interactions and thereby stability. Saturation mutagenesis at this position and subsequent screening for thermostable variants identified the replacements Leu537→Gly and Leu537→Trp as beneficial.

Differential scanning microcalorimetry measurements of both the wt enzyme and these four single mutants (L537G, L537W, E542K, E542R) showed a significant increase in the melting temperatures $T_m$ for the variants. The replacements at position Glu542 proved to be more efficient for stabilisation as $T_m$ was increased by approximately 14°C for both E542K and E542R, while this increase was 10.4 and 8.3°C for L537G and L537W, respectively. These improvements in thermostability were further confirmed by inactivation studies at 60–75°C, where again the Glu542 variants showed smaller inactivation constants and hence higher half-life times $\tau_{1/2}$ than the two Leu537 variants. Interestingly, not only the Glu542→Lys replacement but also the corresponding exchange with the basic residue Arg resulted in considerably improved stabilisation to about an equal extent (e.g., $T_m$ of 74.7 and 74.3°C, $\tau_{1/2,60°C}$ of 1640 and 1680 min for E542K and E542R, respectively). Former studies had concluded that only the Lys but no other amino acid substitution at this position has a similar positive effect [21]. The four single mutants of $TmP2Ox$ showed considerably increased half-life times and hence much slower inactivation at higher temperatures than the wild-type enzyme. To possibly combine these stabilising effects we constructed the double mutants L537G/E542K, L537G/E542R, L537W/E542K, and L537W/E542R. Again, these P2Ox variants were more thermostable than the wt, but interestingly, the concomitant introduction of the substitutions at both Glu542 and Leu537 altered the unfolding process significantly. The single mutants showed one unfolding peak in DSC, and the inactivation kinetics followed a first-order equation, indicative of a simple one-step inactivation process, where the native, active form is transformed directly into the denatured, inactive form [25]. All of the double mutants studied showed two separate unfolding
peaks in the DSC measurements. Furthermore, the inactivation curves did not follow first-order kinetics but showed two distinct phases that can be described as two subsequent first-order reactions, a first phase of rapid activity loss, and after a short transition a prolonged second phase of moderate activity loss. This behaviour could indicate an inactivation procedure consisting of two consecutive processes, with the native, active form of the P2Ox double mutants being first transformed rapidly into an active intermediate species, which then inactivates slowly in a second, independent reaction. The second melting temperature $T_{m,2}$, resulting in the final denaturation step of the P2Ox double mutants, was increased by 14.3–16.8°C in comparison to the wild-type enzyme, which is even higher than for the P2Ox mutational variants with only one amino acid substitution. Based on CD studies, the first inactivation process leading to the active intermediate is not reversible. The nature of this intermediate species is yet unknown. Since the mutations are, however, mainly affecting the interactions between the subunits, it is conceivable that either active dimers or monomers of P2Ox are formed in the first denaturation process. This is further corroborated by the CD measurements of protein samples of the L537W/E542K and L537W/E542R variants heated only to the first transition temperature, thus obtaining the intermediate species, and then cooled to 25°C. These samples showed spectra identical to those of the native enzyme yet with lower intensity. This shows that no drastic changes in the secondary structure elements occurred in this first unfolding process.

In addition, we studied the effects of the replacements at Leu537 and Glu542 on the reactivity of P2Ox. In accordance with the previous studies [19, 21], the introduction of a basic amino acid at position Glu542 results in a decrease of the Michaelis constant for the two sugar substrates D-glucose and D-galactose but also in a decrease in the catalytic constant $k_{cat}$ to some extent. The variants L537G and L537W show almost unaltered $K_m$-values for both sugar substrates, while $k_{cat}$ was improved specifically for the substrate D-glucose. These improvements of the catalytic properties could be combined advantageously in the double mutants, especially those containing the E542K replacement, which were characterised by considerably reduced $K_m$
values combined with practically unchanged $k_{\text{cat}}$, and therefore improved catalytic efficiencies $k_{\text{cat}}/K_m$, notably for D-glucose. One aspect that has not been studied to date is the effect of these mutations on the second half reaction of P2Ox, the oxidative half reaction, in which electrons are transferred to an acceptor. P2Ox not only can transfer these electrons to oxygen but also to a range of other electron acceptors including substituted quinones, complex metal ions or certain organic radical species [7], some of which are considerably better substrates than oxygen. When benzoquinone was used as the substrate these mutations affected mainly $K_m$, which decreased by a factor of 2 for some of these variants. Most of the substitutions had a negative effect when the ferricenium ion was the varied substrate. It is conceivable that the introduction of a positive charge in the internal cavity of P2Ox, close to the entrance of the tunnel leading to the active site [3], as in the E542K and E542R variants, results in repulsion of the positively charged ferricenium ion $F_{\text{c}^+}$. In contrast, the replacements at position Leu537 showed a significant positive effect on $k_{\text{cat}, F_{\text{c}^+}}$, especially for L537W where $k_{\text{cat}, F_{\text{c}^+}}$ was increased more than two-fold when D-glucose was the saturating substrate. This could be of interest for the application of P2Ox in the anodic reaction in biofuel cells based on mediated electron transfer (MET). For MET, certain mediators such as ferrocenes, Os-redox polymers or other complexed metal ions collect electrons, resulting from the oxidation of the sugar, at the active site of the enzyme and transfer these to the electrode. In a biofuel cell based on an enzyme that is electrically wired to the electrode in this way, the current measured as an analytical response signal represents the actual turnover rate of the enzyme [15], and therefore a P2Ox variant with an increased $k_{\text{cat}}$ for the mediator (electron acceptor) will certainly be of interest. Recently it was shown that TmP2Ox can communicate efficiently with electrodes by using either ferrocenes or other complexed metal ions [15, 16]. It was further shown that the E542K variant, which is characterised by a lower catalytic constant for $F_{\text{c}^+}$ than the wild-type enzyme, also performs significantly worse in bioelectrochemical studies than the wt, confirming the results of the kinetic characterisation using $F_{\text{c}^+}$ in this study.
The crystal structures of $TmP2Ox$, both in the unliganded recombinant form and in complex with an electron-donor substrate, have been studied in detail previously [3, 4]. One characteristic feature is the substrate loop, which is in an open conformation when no substrate or an electron-donor substrate such as 2-fluoro-2-deoxy-D-glucose is bound, and in a closed conformation when small electron acceptor substrates are bound. The transition from the open to the closed active site involves a major reorganisation of the substrate loop, residues 451–461.

Two aromatic residues, Phe454 and Tyr456, which have no interaction with the active site in the open conformation, undergo major structural rearrangements during this transition. Notably, Tyr456 moves 9 Å (together with Ser455) to completely close off the active site from the internal cavity of the homotetramer. Concomitantly, Phe454 rotates and moves some 7 Å to fill the active site and pack against the FAD cofactor. In the loop between $\beta$-strand B6 in the substrate-binding domain and strand E2 in the hinge domain, two residues appear to act, at least partly, as structural determinants for the closed conformation of the substrate loop. The side chains of Met541 and Leu545 create a flat surface onto which Tyr456 stacks in its “swung-in” conformation observed in the closed state of the P2Ox active site [3, 4]. These two residues are intervened by Glu542, the O$_\varepsilon 2$ atom of which forms a hydrogen bond to Ser153 O$_\gamma$ located in the nearby loop preceding $\beta$-strand D2 at the start of the oligomerisation arm. The O$_\gamma$ atom of Ser153 in monomer A is also involved in a hydrogen-bond interaction with Asp124 in monomer D. The A-Ser153–D-Asp124 interaction helps to stabilise the association between the oligomerisation arms in monomers A and D, as well as B and C. Moreover, it appears as if the interaction between Glu542 and Ser153 serves to secure the position of the hydrophobic Met/Leu platform for Tyr456 when the active site adopts its closed state. However, the Met/Leu platform does not seem to have any apparent function when the active site is in the open conformation since Met541 and Leu545 are then completely exposed to the solvent of the internal void, and distant from the aromatic side chain of Tyr456 in the substrate loop.
When replacing Glu542 by Lys, the Glu542-Ser153 hydrogen bond is lost, and no additional hydrogen bond is offered to Lys542. The loss of the hydrogen bond may or may not affect the precise positioning of the aromatic ring of Tyr456 in the closed state. In the absence of a closed complex of E542K P2Ox, the effect of the loss of this hydrogen bond on the packing of the substrate loop is difficult to assess. However, any mutation that affects the structure and function of the substrate loop and/or the local environment of the flavin cofactor is likely to affect the kinetics of catalysis of either the reductive or oxidative half-reaction, or both. Such an effect could be either due to altered redox power of the cofactor and/or discrete conformational changes of amino acids critical for substrate binding and catalysis.

As mentioned above, the Lys side chain points into the internal cavity of the tetramer and forms no hydrogen bonds to either protein or ordered solvent. Furthermore, the Lys replacement does not introduce any significant structural changes in either the monomer or tetramer structure of TmP2Ox in the folded state, and the corresponding mutation in PsP2Ox shows a Lys with the same side-chain conformation as that observed here. For PsP2Ox, it was proposed that the increased thermostability of the E540K mutant may be due to an ionic effect assigned to the ability of the Lys side chain to relieve possible electrostatic repulsion between Glu542 and Asp124 in the wild-type enzyme at higher pH values [6], however since neither the pKa of Asp124 is known, nor the dependence of thermostability on pH, this hypothesis, although probable, remains unproven.

As for the increased thermostability of P2Ox variants where Leu537 is replaced by either Gly or Trp, we observe distinct differences at the A/C interface. By introducing either Trp or Gly at position 537, tighter association between the A and C monomers can be achieved, although at different locations in the interface. Compared with L537G, additional favourable interactions are seen in E542K/L537W where also the A/D interface is strengthened significantly by the tighter association of the oligomerisation arms of monomers A and D. This is likely to explain why, although both Gly and Trp replacements increase stability to thermal unfolding, the Trp
replacement is overall more thermostable. The largest difference between the structures is observed in the relative position of the head domain (Fig. 8), however, this is most likely due to differences in packing environment of the monoclinic and tetragonal crystal lattices rather than due to the amino-acid replacements per se. Thus, the subtle differences observed at the subunit interfaces discussed above are more likely determinants of increased thermostability.

**Experimental procedures**

**Bacterial strains, plasmids and media**

*E. coli* strain BL21 Star DE3 (Invitrogen; Carlsbad, CA, U.S.A.), well suited for the bacteriophage T7 promoter-based expression system pET, was used as host for the expression plasmids and consequently for the production of active P2Ox protein. The vector pET21d(+) was used throughout this work to express wtP2Ox and P2Ox variants containing a C-terminal His$_6$-tag. Construction of the plasmid pHL2, which expresses the His-tagged wtTmP2Ox under the control of the T7 promoter, has been recently described [4]. *E. coli* cells were grown in TB$_{amp^{-}}$media (yeast extract 24 g·L$^{-1}$, peptone from casein 12 g·L$^{-1}$, glycerol 4 mL·L$^{-1}$; phosphate buffer 1 M, pH 7.5) under appropriate selective conditions (ampicillin was added to 100 mg·L$^{-1}$). The chemicals used were of the purest grade available and were purchased from Sigma (Vienna, Austria). Nucleotides, buffers and enzymes for molecular biology were from Fermentas (St. Leon-Rot, Germany).

**Generation of mutants**

The *Tm*P2Ox gene was mutated by a two-step site-directed mutagenesis procedure using PCR and digestion with DpnI [26]. For the replacement of Glu542 with Lys the primers used were P2OE542K-for (5’-gcaattcatgaagcctggt-3’) and P2OE542K-rev (5’-accaggcttcatgaattgc-3’). The primers for constructing variant E542R were P2OE542R-for (5’-gcaattcatgcggcctggt-3’) and P2OE542R-rev (5’-accaggccgcatgaattgc-3’). For saturation mutagenesis of position Leu537 we
used the primers P2O-Wobble-537-for (5’-tcctacccggtccnnscgcaaa-3’) and P2O-Wobble-537-rev (5’-ttcgccsnncgccggccctcga-3’), where N = A, G, C or T, and S = G or C. To create double mutants at positions 537 and 542 we used the plasmids of variants L537G and L537W as templates for the PCR reactions, the forward primers P2OE542K-for and P2OE542R-for, and the reverse primers Double-G-rev (5’-catgaattgcggccggagccg-3’) and Double-W-rev (5’-catgaattgcggccaggagccg-3’). All primers were purchased from VBC Biotech (Vienna, Austria). The mutagenic PCR was performed under the following conditions: 95°C for 4 min, then 30 cycles of 94°C for 30 sec; 58°C for 30 sec; 72°C for 16 min, with a final 72°C for 10 min incubation. Each reaction contained 1× buffer (Fermentas), 0.1 μg of plasmid DNA, 2.5 U Pfu DNA polymerase (Fermentas), 10 μM of each dNTP and 5 pmol of each primer in a total volume of 50 μL. After PCR the methylated template DNA was degraded by digestion with 10 U of DpnI at 37°C for 3 hours. The remaining PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, U.S.A.). Five μL of each purified PCR product were transformed into chemically competent E. coli BL21 Star DE3 cells. The successful introduction of the desired mutations and the absence of further mutations were confirmed by DNA sequencing, which was done as a commercial service (VBC-Biotech). Plasmidic DNA was extracted and used as template for DNA sequencing of the complete P2Ox-encoding sequence using the forward primer T7promfwd (5’-aatagctactatagggg-3’) and the reverse primer T7termrev (5’-getagttattgctcagcgg-3’).

**Screening for improved P2Ox variants**

Position Leu537 of TmP2Ox was mutated by saturation mutagenesis, which allows the creation of a mutant library containing all possible codons at the target position. The size of the library, which subsequently has to be screened to cover all possible mutants, is determined by the mutagenic codon and the number of target sites. For mutation of position Leu537 by saturation
mutagenesis the primers used were of the NNS type, which defines the minimum library size to be screened to statistically cover 95% of all possible substitutions as 95 colonies [27]. To this end a screening assay based on 96-well plates was used. Transformed *E. coli* BL21 Star DE3 cells were transferred from LB-ampicillin plates into microtiter wells containing 200 μL of liquid LBamp medium (“masterplates”). Cells were grown on a shaking incubator (150 rpm) at 25°C for 20 h. To induce protein expression, 10 μL of the cell suspension of each well were transferred into another 96-well plate containing 200 μl of 2× LBamp / lactose (yeast extract 10 g·L⁻¹, peptone from casein 20 g·L⁻¹, sodium chloride 5 g·L⁻¹, lactose 5 g·L⁻¹; ampicillin 100 mg·L⁻¹) per well, and incubated at 25°C for another 20 h (“working plates”). The growth of the cells was measured in a plate reader (Sunrise Remote; Tecan, Gröding, Austria) at 600 nm. Induced cells were sedimented in the wells by centrifugation (3700 rpm, 15 min) and the supernatant was discarded. The cells were lysed by adding 200 μL of 1× lysis buffer (CellLytic B Cell Lysis Reagent; Sigma) and incubated at 4°C for 30 min. After this, the plates were frozen at −70°C for 1 h and then thawed at room temperature to increase the efficiency of the lysis. Cell debris was removed by centrifugation, and 10 μL of the supernatant were added to 80 μL of chromogenic assay mixture (0.035 mg·mL⁻¹ of horseradish peroxidase and 0.7 mg·mL⁻¹ of ABTS [2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] in 50 mM phosphate buffer, pH 6.5). The reaction was started by adding either 10 μL D-glucose or D-galactose (each 1 M), and recorded automatically at 420 nm and 30°C by the plate reader. To test for increased thermostability the microtiter plates containing the cell extracts were incubated at 65°C for 10 min before performing the activity assay.

**Protein expression and purification**

Cultures (2 litres) of *E. coli* BL21 Star DE3 transformants were grown in TBamp in shaken flasks at 37°C and 160 rpm. When the cultures reached OD₆₀₀ of 0.5–0.6, recombinant protein expression was induced by adding lactose to a final concentration of 0.5%. After cultivation at
25°C for additional 20 h, cells were harvested by centrifugation (5,000 rpm, 20 min),
resuspended in phosphate buffer (50 mM, pH 6.5) containing phenylmethylsulfonylfluoride
(PMSF; 0.1%), and lysed by using a continuous homogeniser (APV Systems, Denmark). The
crude cell extract was obtained by centrifugation (30,000 rpm, 30 min, 4°C) and then used for
protein purification by immobilised metal affinity chromatography (IMAC) using the ÄKTA
purifier system (Pharmacia; Uppsala, Sweden) and the BioRad Profinity IMAC Ni-Charged
Resin (10 mL; BioRad; Vienna, Austria). The column was pre-equilibrated with ten column
volumes (CV) of buffer (50 mM KH₂PO₄, 0.5 M NaCl, 20 mM imidazole; pH 6.5). After
applying the sample to the column it was washed with five CV of the same buffer at a flow rate
of 2 mL·min⁻¹. Proteins were then eluted with a linear gradient (flow rate 1.5 mL·min⁻¹ for 60
min) of the same buffer containing 1 M imidazole and monitored at 280 nm (protein content) and
456 nm (FAD content). The fractions containing eluted enzyme were pooled, and imidazole was
removed by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (Millipore; Billerica,
MA, U.S.A.) with a 10-kDa cut-off membrane. The eluted, concentrated enzymes were washed
three times with 10 mL of phosphate buffer (50 mM, pH 6.5), and finally diluted in this buffer to
a protein concentration of 10–20 mg·L⁻¹.

**Electrophoresis**

Electrophoresis was done principally as described by Laemmli [28]. Both native PAGE and
SDS-PAGE were performed using a 5% stacking gel and a 10% separating gel on the
PerfectBlue vertical electrophoresis system (Peqlab; Erlangen, Germany) and using the
molecular mass standards High Molecular Weight Calibration Kit for native electrophoresis
(Amersham) and the Precision Plus Protein Dual Color Kit (BioRad) for SDS-PAGE. Gels were
stained with Coomassie blue.
Enzyme activity assays

P2Ox activity was measured with the standard chromogenic ABTS [2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)] assay [29]. A sample of diluted enzyme (10 μL) was added to 980 μL of assay mixture containing horseradish peroxidase (142 U), ABTS (14.7 mg) and phosphate buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM). The absorbance change at 420 nm was recorded at 30°C for 180 sec (ε_{420} = 42.3 mM^{-1} cm^{-1}). One unit of P2Ox activity was defined as the amount of enzyme necessary for oxidation of 2 μmol of ABTS per min (which equals the consumption of 1 μmol of O₂ per min) under assay conditions. Protein concentrations were determined by the Bradford assay [30] using the BioRad Protein Assay Kit (BioRad) with bovine serum albumin (BSA) as standard.

Steady-state kinetic measurements

Catalytic constants were measured for the two electron donors D-glucose (0.1–50 mM) and D-galactose (0.1–200 mM) using the standard ABTS assay and oxygen (air saturation). In addition, steady-state kinetic constants were determined for the alternative electron acceptors 1,4-benzoquinone and the ferricenium ion Fc⁺ (using ferricenium hexafluorophosphate Fc⁺PF₆⁻ as substrate; Aldrich, Steinheim, Germany). Appropriately diluted enzyme sample (10 μL) was added to 990 μL of assay buffer containing either D-glucose or D-galactose in a constant concentration of 100 mM, phosphate buffer (50 mM, pH 6.5) and 1,4-benzoquinone, which was varied from 0.01–1.5 mM. The absorbance change at 290 nm was recorded at 30°C for 180 sec, and the extinction coefficient ε_{290} used was 2.24 mM⁻¹ cm⁻¹. FcPF₆ was varied from 0.005–0.5 mM and the absorbance change at 300 nm was recorded at 30°C for 180 sec (ε_{300} = 4.3 mM⁻¹ cm⁻¹). Kinetic constants were calculated by non-linear least-square regression, fitting the data to the Henri-Michaelis-Menten equation.
Thermal stability

Kinetic stability of the *Tm*P2Ox variants was determined by incubating the enzymes in appropriate dilutions in 50 mM phosphate buffer (pH 6.5) at 60°C, 70°C, and 75°C, respectively, and by subsequent measurements of the enzyme activity (*A*) at various time points (*t*) using the standard ABTS assay and glucose as the substrate. A thermal cycler (thermocycler T3, Biometra; Göttingen, Germany) and thin-wall PCR tubes were used for all thermostability measurements. Residual activities (*A_t/A_0*, where *A_t* is the activity measured at time *t* and *A_0* is the initial P2Ox activity) were plotted versus the incubation time. For those experiments where the inactivation followed apparent first order kinetics the inactivation constant *k_{in}" was obtained by linear regression of ln (activity) versus time. The half-life values of thermal inactivation *τ_{1/2}" were calculated using *τ_{1/2} = ln 2/k_{in} [23].

Thermodynamic stability, i.e., melting temperature *T_m" [23], was measured by differential scanning microcalorimetry (DCS) using a MicroCal VP-DSC instrument (MicroCal; Northampton, MA, U.S.A.) between 15 and 80°C at a scan rate of 1°C per min on 0.2 g·L⁻¹ protein samples in 50 mM phosphate buffer, pH 6.4. Solutions were degassed by stirring under vacuum for 15 min at room temperature immediately before measurements. The solutions in the measuring cells were kept under pressure to prevent degassing during heating. The baseline was determined in an identical experiment with buffer in both cells and was subtracted. Data processing and evaluation were performed using the Origin 7.5 software (OriginLab Corporation; Northampton, MA, U.S.A.).

Circular dichroism measurements

Far-UV circular dichroism (CD) spectra (190–240 nm) were recorded at 25°C on a Jasco J-720 spectropolarimeter (Jasco International Co.; Tokyo, Japan) using protein samples at 8 µM concentration in 50 mM phosphate buffer (pH 6.4), 1-mm pathlength, thermostatted cuvettes and a Neslab RTE-100 computer-controlled thermostat. Spectra were averaged over three scans.
Processing of spectral data was done by using the built-in Jasco software of the spectropolarimeter. Thermal unfolding measurements were measured at 209 nm over a temperature range of 25–80°C. After reaching 80°C, samples were cooled back to 25°C. Data processing and evaluation were performed using the Origin 7.5 software.

**Crystallization, Data Collection and Refinement of the E542K and L537G mutants**

Crystals of the E542K, L537G and E542K/L537W mutants were produced using the hanging drop vapour diffusion method [31], essentially as described previously for recombinant P2Ox [4]. Drops were set up by mixing equal volumes of protein (4 mg·mL⁻¹ in 20 mM Mes pH 5.2) and reservoir [12–16% (w/v) mme PEG 2,000, 0.1 M Mes (pH 5.2), 50 mM MgCl₂, 25% glycerol]. Microseeding was used routinely. Prior to data collection, the crystals were frozen and vitrified in liquid nitrogen. Data for the E542K mutant were collected using synchrotron radiation (\(\lambda=0.918\) Å) at beamline 14.1 at BESSY, Berlin, Germany; and data for the L537G mutant (\(\lambda=1.042\) Å) at MAX-lab (Lund, Sweden), beamline J911-2 (100 K). Data for the E542K/L537W mutant (\(\lambda=0.931\) Å) were collected at beam line ID14-3 at ESRF, Grenoble, France (100 K). All data were processed using XDS [32]. The E542K and L537G mutants crystallize in space group \(P2_1\) with eight monomers forming two tetramers in the asymmetric unit, whereas the E542K/L537W mutant crystallizes in space group \(P4_2_2_1_2\) with one monomer in the asymmetric unit. Phases were obtained by means of Fourier synthesis using the H167A P2Ox variant (PDB code 2IGO; [4]) as starting model. Crystallographic refinement was performed with REFMAC5 [33], and included anisotropic scaling, calculated hydrogen scattering from riding hydrogens, and atomic displacement parameter refinement using the translation, libration, screw-rotation (TLS) model. In the case of E542K and L537G, for each of the eight monomers (two tetramers) in the asymmetric unit, individual TLS groups were defined as follows: the Rossmann domain (residues 44–79, 254–353, 552–618); the substrate-binding domain (residues 159–253, 354–551); the oligomerisation arm (residues 111–158); and the lid (residues 80–110).
For the E542K/L537W mutant the TLS model was determined using the TLS Motion Determination server (TLSMD; [34]). Corrections of the models were done manually with the guidance of $\sigma_A$-weighted $2F_o-F_c$ and $F_o-F_c$ electron-density maps. The same set of $R_{\text{free}}$ reflections was used throughout all refinements, and NCS restraints/constraints were not used. Model building, coordinate manipulation and least-squares comparisons were made with the program O [35] and Coot [36]. Figures 1 and 8 were prepared using PyMOL v. 0.93 [37].

Acknowledgements

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References


Chapter 3

Probing active-site residues of pyranose 2-oxidase from *Trametes multicolor* by semi-rational protein design

Clara Salaheddin\(^1\), Oliver Spadiut\(^1\), Tien-Chye Tan\(^2\), Christina Divne\(^2\), Dietmar Haltrich\(^1\), Clemens Peterbauer\(^1,^4\) *

\(^1\) Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU-University of Natural Resources and Applied Life Sciences, Vienna, Austria

\(^2\) School of Biotechnology, Royal Institute of Technology KTH, Albanova University Centre, Stockholm, Sweden

\(^3\) Research Centre Applied Biocatalysis, Graz, Austria

\(^4\) Vienna Institute of BioTechnology VIBT, Vienna, Austria

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Clara Salaheddin$^{1,3}$, Oliver Spadiut$^1$, Tien-Chye Tan$^2$, Christina Divne$^2$, Dietmar Haltrich$^{1,4}$, Clemens Peterbauer$^{1,4}$ *

$^1$ Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU University of Natural Resources and Applied Life Sciences, Vienna, Austria

$^2$ School of Biotechnology, Royal Institute of Technology KTH, Albanova University Centre, Stockholm, Sweden

$^3$ Research Centre Applied Biocatalysis, Graz, Austria

$^4$ Vienna Institute of BioTechnology VIBT, Vienna, Austria

*to whom correspondence should be addressed at:
Abteilung für Lebensmittelbiotechnologie, Universität für Bodenkultur, Muthgasse 18, A-1190 Wien, Austria
clemens.peterbauer@boku.ac.at, tel.: +43-1-36006-6274, fax +43-1-36006-6251
Abstract

D-Tagatose is a sweetener with low caloric and non-glycemic characteristics. It can be produced by an enzymatic oxidation of D-galactose specifically at C2 followed by chemical hydrogenation. Pyranose 2-Oxidase (P2Ox) from *Trametes multicolor* catalyzes the oxidation of many aldopyranoses to their corresponding 2-keto derivatives. Due to the fact that D-galactose is not the preferred substrate of P2Ox, semi-rational design was employed to improve the catalytic efficiency with this poor substrate. To probe the direct vicinity of the substrate-binding site, saturation mutagenesis was applied on all positions in the active site of the enzyme, resulting in a library of mutants. These variants were expressed in a 96-well screening assay and activity was determined by an ABTS [2,2’-azinobis(3-ethyl-benzthiazoline-6-sulfonic acid)] peroxidase coupled assay. Mutants with higher activity than wild-type P2Ox were chosen for further kinetic investigations. Variant V546C was found to show a 2.5-fold increase of $k_{cat}$ with both sugars, D-glucose and D-galactose, when oxygen was used as electron acceptor. Furthermore, variant T169S shows a higher turn-over rate with D-galactose and a better $K_M$ value with D-glucose as substrate.
1 Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase, EC 1.1.3.10) is occurring frequently among lignocellulose-degrading basidiomycete fungi [1,2]. It plays a role in the decomposition process of this complex material by providing the co-substrate hydrogen peroxide for lignin-degrading peroxidases [3,4]. The enzyme was first isolated from *Polyporus obtusus* [5] followed by various fungal sources, of which *Trametes multicolor* is the best studied [6].

P2Ox from *Trametes multicolor* (*Trametes ochracea*) [6] is a homotetrameric 270-kDa flavoenzyme. It is a member of the glucose-methanol-choline (GMC) structural family of flavin-adenine-dinucleotide (FAD) dependent oxidoreductases [7,8]. Each subunit contains an FAD molecule covalently bound to H167 in the active centre [9,10]. The subunits are symmetrically arranged around a pore, into which the substrates have to enter to reach the active sites in the direct vicinity of the FAD. The active centre is protected by an active-site loop, which blocks the entrance to the substrate-binding pocket and can undergo dynamic changes enabling the substrate to enter the active site from the internal void [10].

P2Ox catalyzes the C2-oxidation of several aldopyranoses commonly found in lignocellulose, preferentially D-glucose, to their corresponding 2-keto derivatives [11]. In the reductive half reaction electrons from the oxidized substrate are transferred to the co-factor. Re-oxidation of the FADH$_2$ follows in the oxidative half reaction by reducing molecular oxygen to hydrogen peroxide. The reaction mechanism is of the type Ping Pong Bi Bi typically found in flavoprotein oxidoreductases [12,13].

P2Ox is the key biocatalyst in the Cetus process, in which pure crystalline D-fructose is produced from D-glucose via the intermediate 2-keto-D-glucose. By using the same process D-galactose can be oxidized to 2-keto-D-galactose and subsequently hydrogenated to D-tagatose [14]. This sugar has interesting properties for the food industry such as low caloric and
nonglycemic characteristics. Additionally, D-tagatose can be used as prebiotic food additive. 2-Keto-D-glucose is also the key intermediate of a secondary metabolic pathway leading to the antibiotic cortalcerone [15].

Recently, the crystal structure of *T. multicolor* P2Ox (*Tm*P2Ox) in complex with one of its slow carbohydrate substrates, 2-fluoro-2-deoxy-D-glucose, was reported [16]. This structure gave detailed information about residues interacting with the sugar substrate in the active site. As a consequence, site-directed mutagenesis at these residues allows analysis of structure-function relationships, and also possible improvements by (semi-)rational protein design. Mutations of active-site residues are known to often dramatically change the properties of an enzyme. Frequently, these changes are simply unfavorable or even inactivating, however, the altered enzymes can also show improved properties such as broadened substrate specificities or increased activity. As was shown by the structural studies on *Tm*P2Ox, the active-site loop undergoes dynamical changes in the presence of a carbohydrate substrate, and the sugar can dock into the active site. Two amino acids, H548 and N593, are thought to control the catalytic event by supporting the electron transfer from the substrate C2 (or C3) to the N5 of the flavin.

According to the hypothetical binding mode for the C2-oxidation [16] (Fig. 1), side chains of D452, an amino acid of the active-site loop, and R472 form hydrogen bonds with the O6 of the substrate, with additional hydrogen bonds being formed by Q448 and V546. Position T169 has been proposed to play an important role in the re-oxidation of the reduced flavin. The hydroxyl group of Thr forms a hydrogen bond to the N5-O4 of the FAD and supports the transfer of electrons to the electron acceptor [16]. This position also seems to partly explain substrate specificity and the poor reactivity with D-galactose. D-Galactose differs from D-glucose by the axial rather than equatorial orientation of the C4 hydroxyl group, which results in a steric clash with the side chain of T169, making binding of D-galactose rather unfavorable [17].
In this study, we chose the individual active-site residues of the TmP2Ox interacting with the bound sugar substrate as targets for saturation mutagenesis to probe the effect of mutations on the activity of this enzyme with both the substrates D-glucose and D-galactose. The aim was to identify important active-site residues for better understanding of structure/function relationships of this enzyme as well as to improve the catalytic efficiency with D-galactose as a substrate.

2 Material and Methods

**Organisms and plasmids:** P2Ox from *T. multicolor* was heterologously expressed in *E.coli* BL21* (DE3) (Fermentas, St. Leon-Roth, Germany) using the pET21-d(+) expression vector (Novagen/Merck, Darmstadt, Germany) [16]. The recombinant P2Ox has a C-terminal His$_6$-tag for one-step purification via immobilized metal affinity chromatography (IMAC).

**Saturation mutagenesis:** Saturation mutagenesis was done by the overlap extension method (OEM). OEM is based on four primers using two flanking (T7 primer) and two internal primers (Table 1). Both internal primers must contain the mutation of interest and have overlapping nucleotide sequences [19]. Internal primers with one random codon each (NNN or NNS; N=A, U, G, C; S=G, C) were designed, and two fragments were amplified by PCR using one flanking and one internal primer. These two overlapping fragments were then used as template for a third PCR reaction using the flanking primers to amplify the full-length gene. The mutagenized full-length gene was subsequently inserted into the expression vector pET 21-d(+) as described previously [16].
Table 1. Oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7term</td>
<td>5’- gctagttatgctcagcgg -3’</td>
</tr>
<tr>
<td>T7prom</td>
<td>5’- aatacgactcatatagggg -3’</td>
</tr>
<tr>
<td>T169wobble_fwd</td>
<td>5’- ctacgcactggnntgcgccacac -3’</td>
</tr>
<tr>
<td>T169wobble_rev</td>
<td>5’- gtgtggecgannccagtgcgtag -3’</td>
</tr>
<tr>
<td>Q448wobble_fwd</td>
<td>5’- ccgtggacactnnatcaacgc -3’</td>
</tr>
<tr>
<td>Q448wobble_rev</td>
<td>5’- cgggtgccannntagtgtgccacgg -3’</td>
</tr>
<tr>
<td>D452wobble_fwd</td>
<td>5’- cagatcaccgennngetttcagttacg -3’</td>
</tr>
<tr>
<td>D452wobble_rev</td>
<td>5’- cegtaactgaaagnnnccgggtagctg -3’</td>
</tr>
<tr>
<td>R472wobble_fwd</td>
<td>5’- atcgtggaactggnntcttctcg -3’</td>
</tr>
<tr>
<td>R472wobble_rev</td>
<td>5’- gcggaagaasnnccagtccacga -3’</td>
</tr>
<tr>
<td>V546wobble_fwd</td>
<td>5’- agctggtgtennntcactttgtagt -3’</td>
</tr>
<tr>
<td>V546wobble_rev</td>
<td>5’- ccaccaagttgaagnnaagacccagtct -3’</td>
</tr>
<tr>
<td>H548wobble_fwd</td>
<td>5’- ggtcttgcttenntcactttgtaga -3’</td>
</tr>
<tr>
<td>H548wobble_rev</td>
<td>5’- tgcgtaccccaacaaggnaagacacc -3’</td>
</tr>
<tr>
<td>N593wobble_fwd</td>
<td>5’- cgtaagggcggunncgcagct -3’</td>
</tr>
<tr>
<td>N593wobble_rev</td>
<td>5’- gacgctgguannccggccgtac -3’</td>
</tr>
</tbody>
</table>

**Microtiter-plate cultivation and induction:** Depending on the type of the mutagenic codon (NNS or NNN), the size of the library to be screened was determined to be at least 190 (NNN) or 95 (NNS) colonies to achieve a 95% probability of “success” (appearance of Trp and Met) [20]. Routinely a double number of colonies were screened, in order to achieve a higher probability. Microtiter (96-well) master plates (MP) were prepared with 200 µL LB-Amp (5 g/L yeast extract, 10 g/L peptone from casein, 10 g/L sodium chloride, 100 µg/ml ampicillin) medium and inoculated with single colonies using sterile toothpicks. Six wells, randomly spread over each plate, were inoculated with clones expressing wild-type P2Ox. The MP were shaken at 140 rpm overnight at 25 °C in a closed tray with water-soaked paper towels to prevent desiccation of the wells. Screening plates (SP) were prepared with 200 µL of inducing LB-Amp + IPTG (isopropyl-β-D-thiogalactopyranoside) media (0.1 mM IPTG). Cells were
transferred into the SPs from the MPs using sterile dry replicators, MPs were stored at 4 °C and SP were shaken at 140 rpm overnight at 25 °C as described above.

**Cell lysis:** The screening plates were centrifuged in a 96-well plate centrifuge (Eppendorf AG, Hamburg, Germany) at 3700 rpm for 15 min. The supernatant was discarded by inverting the plates on dry paper towels. Cell pellets were lysed by adding 100 µL of 0.5-fold BugBuster Protein Extraction Reagent (Novagen) in KH$_2$PO$_4$ buffer (pH 7, 25 mM) per well and shaking at room temperature for 1 h. After lysis the plates were centrifuged again as above, and the supernatant was used for further determination.

**Enzyme assay:** The activity of P2Ox was determined spectrophotometrically at 420 nm and 30 °C by a coupled chromogenic assay. Enzyme variants were screened for D-galactose and D-glucose activity to compare differences in substrate specificity. Eighty µL of ABTS reagent (0.7 mg/mL ABTS, 0.035 mg/mL horseradish peroxidase type II [181 U/mg], KH$_2$PO$_4$ buffer: 50 mM pH6.5) were pipetted into every well of the 96-well microtiter plate followed by 10 µL of the enzyme crude extract. The reaction was started with 10 µL of the sugar substrate (1 M). D-Glucose oxidation was immediately measured spectrophotometrically in a Sunrise Remote plate reader (Tecan Austria, Grödig, Austria) for 180 sec at room temperature. For D-galactose two one-point measurements were done after 15 min and 24 hours. Mutants with a higher activity compared to wild-type P2Ox were transferred from the MP by streaking on a LB-Amp agar plate and cultivated over night in 3 mL of liquid LB-Amp media at 37 °C and 150 rpm. Plasmids were isolated using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO) and sequenced (AGOWA GmbH, Berlin, Germany) to identify the mutations in the gene.
**Cultivation:** Selected mutant strains were pre-cultivated, each in four small shaking flasks using 30 mL of LB-Amp medium. After four h the cell suspensions were transferred into baffled shaking flasks containing 250 mL of LB-Amp + 5% Lactose. The flasks were incubated overnight at 25 °C and 110 rpm. Cells were harvested by centrifugation for 15 min at 8000 g and the pellets resuspended in a threefold volume of Buffer A (50 mM KH$_2$PO$_4$, 20 mM imidazole, 0.5 M sodium chloride). Homogenization was done in a continuous homogenizer (APV Systems, Albertslund, Denmark) adding PMSF as a serine-protease inhibitor. The homogenates were ultra-centrifuged at 35,000 x g for 30 min at 4 °C, and the recombinant protein was purified from the supernatants.

**Purification:** Purification was done in one step employing an Äkta Purifier System (Amersham Pharmacia Biotech, Uppsala, Sweden). Crude extract was passed through a 100 mL Ni$^{2+}$ immobilized column, using Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) as a chromatographic support. After washing the column with Buffer A, bound protein was eluted with an imidazole gradient using Buffer A and Buffer B (50 mM KH$_2$PO$_4$, 0.5 M sodium chloride, 1 M imidazole). Fractions containing P2Ox activity were pooled and concentrated by ultrafiltration using an Amicon Ultra Centrifugal Filter Device with a 10-kDa cut-off membrane (Millipore, Billerica, MA). The purity of the enzyme preparations was checked by SDS-PAGE using the Precision Plus Protein Dual Color Kit (BioRad Laboratories, Hercules, CA) as molecular mass standard (Fig. 2).

**Characterisation:** P2Ox variants were characterized in terms of kinetic constants for different sugar substrates using the routine ABTS-peroxidase assay and air saturation as described previously [6]. D-Glucose and D-galactose were varied over a range of 0.1–50 mM and 0.1–
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110 mM, respectively, and protein concentrations were determined using a Bradford assay [21] (Table 3).

3 Results and Discussion

Based on structural data of *TmP2Ox* with its bound sugar substrate [10, 16] we decided to perform saturation mutagenesis of amino acid residues directly positioned in the active site and interacting with the sugar substrate, aiming at studying the modulation of substrate specificity and promiscuity of P2Ox. The following positions were chosen for this semi-rational approach: T169, Q448, D452, R472, V546, H548, and N593 (Fig. 1). All of these residues are located in highly conserved regions of the *p2ox* gene, and are presumably involved in the reduction half-reaction, i.e., sugar oxidation, either by being involved in the electron transfer to the flavin (H548, N593) or by forming hydrogen bonds to the sugar substrate (Q448, D452, R472, V546).

![Figure 1](image)

**Figure 1.** Theoretical models showing the presumed binding of β-D-glucose in the active site of *T. multicolor* pyranose 2-oxidase. The protein chosen for modeling was the crystal structure of pyranose 2-oxidase H167A in complex with 2-fluoro-2-deoxy-D-glucose (PDB code 2IGO; [16]). The FAD cofactor is colored yellow, and the monosaccharide is shown in green. For clarity, protein backbone atoms have been omitted. The monosaccharide is
oriented for oxidation at C2. Modeling was done using the program O [23], and the picture was made using MacPyMOL [22].

The p2ox gene was mutagenized in three steps by an overlap extension method and ligated into the expression vector. Colonies (total number of 360) were picked for every single position to be screened for substrate specificity by using a newly developed microtiter plate-based screening assay based on the routine spectrophotometric ABTS assay. Depending on the amino acid position mutated and studied, varying numbers of active mutants were found in this screening and used for further studies. Variants showing activity with both sugar substrates D-glucose and D-galactose in the initial screening were selected for DNA sequence analysis to identify the mutation at the desired position and to exclude undesired mutations in other regions. Several variants, exhibiting higher or comparable activity relative to recombinant wild-type TmP2Ox, were expressed in E. coli, purified and characterized in terms of kinetic constants by using the spectroscopic ABTS assay under standard conditions (Table 2). The one-step purification procedure based on IMAC yielded homogenous protein preparations as proven by SDS-PAGE (Fig. 2).

Table 2. Fraction of variants showing activity with the poor P2Ox substrate D-galactose (within 24 hours) identified in the screening assay after saturation mutagenesis of the respective positions.

<table>
<thead>
<tr>
<th>Position</th>
<th>Number of variants showing activity with D-galactose [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>T169</td>
<td>22.5</td>
</tr>
<tr>
<td>Q448</td>
<td>17.8</td>
</tr>
<tr>
<td>D452</td>
<td>6.5</td>
</tr>
<tr>
<td>R472</td>
<td>18.4</td>
</tr>
<tr>
<td>V546</td>
<td>73.9</td>
</tr>
<tr>
<td>H548</td>
<td>6.6</td>
</tr>
<tr>
<td>N593</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Figure 2. SDS-PAGE analysis of mutational variants of pyranose 2-oxidase from *T. multicolor*. Lane 1, molecular mass marker (Precision Plus Protein Dual Color, Biorad); lane 2, 5 and 8, crude extract of *E.coli* BL21 Star DE3 expressing variants H548I, H548R and N593R; lane 3, 6 and 9, H548I, H548R and N593R purified by IMAC; lane 4, 7, 10, flow through from IMAC of H548I, H548R and N593R.

Determination of catalytic constants of the selected active mutants revealed one variant with noticeable properties, V546C. V546 is positioned directly in the active site, interacting with the C1 hydroxyl group of the sugar substrate by forming a hydrogen bond through its carbonyl oxygen [16]. When replacing V546 by a cysteine, $k_{cat}$ values for both substrates investigated, D-glucose and D-galactose, were approximately 2.5-fold increased compared to wild-type P2Ox (Table 3).
Table 3. Kinetic constants of wild-type P2Ox from *Trametes multicolor* and mutational variants for D-glucose and D-galactose as substrates and O₂ (air saturation) as electron acceptor.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Substrate</th>
<th>$K_M$ [mM]</th>
<th>$k_{cat}$ [s⁻¹]</th>
<th>$k_{cat}/K_M$ [mM⁻¹ s⁻¹]</th>
<th>$k_{cat}/K_M$ relative to wtP2Ox [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>D-glucose</td>
<td>0.698</td>
<td>35.4</td>
<td>50.8</td>
<td>100</td>
</tr>
<tr>
<td>wild-type</td>
<td>D-galactose</td>
<td>8.09</td>
<td>2.73</td>
<td>0.337</td>
<td>100</td>
</tr>
<tr>
<td>T169G</td>
<td>D-glucose</td>
<td>0.691</td>
<td>0.262</td>
<td>0.379</td>
<td>74</td>
</tr>
<tr>
<td>T169G</td>
<td>D-galactose</td>
<td>2.48</td>
<td>0.273</td>
<td>0.110</td>
<td>32.6</td>
</tr>
<tr>
<td>T169N</td>
<td>D-glucose</td>
<td>0.327</td>
<td>2.41</td>
<td>7.30</td>
<td>14.4</td>
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<tr>
<td>T169N</td>
<td>D-galactose</td>
<td>3.56</td>
<td>1.96</td>
<td>0.551</td>
<td>164</td>
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<tr>
<td>T169S</td>
<td>D-glucose</td>
<td>0.394</td>
<td>21.8</td>
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<tr>
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<td>12.9</td>
<td>5.53</td>
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<tr>
<td>Q448N</td>
<td>D-glucose</td>
<td>28.0</td>
<td>4.73</td>
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</tr>
<tr>
<td>Q448N</td>
<td>D-galactose</td>
<td>940</td>
<td>0.433</td>
<td>0.000461</td>
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<tr>
<td>Q448S</td>
<td>D-glucose</td>
<td>30.0</td>
<td>5.42</td>
<td>0.181</td>
<td>0.36</td>
</tr>
<tr>
<td>Q448S</td>
<td>D-galactose</td>
<td>750</td>
<td>1.53</td>
<td>0.00204</td>
<td>0.61</td>
</tr>
<tr>
<td>Q448C</td>
<td>D-glucose</td>
<td>100</td>
<td>1.51</td>
<td>0.0151</td>
<td>0.03</td>
</tr>
<tr>
<td>Q448C</td>
<td>D-galactose</td>
<td>1260</td>
<td>0.622</td>
<td>0.000494</td>
<td>0.15</td>
</tr>
<tr>
<td>R472L</td>
<td>D-glucose</td>
<td>0.886</td>
<td>30.9</td>
<td>34.9</td>
<td>68.7</td>
</tr>
<tr>
<td>R472L</td>
<td>D-galactose</td>
<td>7.21</td>
<td>1.69</td>
<td>0.236</td>
<td>70.0</td>
</tr>
<tr>
<td>R472G</td>
<td>D-glucose</td>
<td>0.773</td>
<td>32.2</td>
<td>41.7</td>
<td>82.0</td>
</tr>
<tr>
<td>R472G</td>
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<td>6.38</td>
<td>2.07</td>
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<td>96.1</td>
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<tr>
<td>V546G</td>
<td>D-glucose</td>
<td>5.70</td>
<td>23.6</td>
<td>4.13</td>
<td>8.13</td>
</tr>
<tr>
<td>V546G</td>
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<td>4.51</td>
<td>0.0226</td>
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<td>V546P</td>
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<td>57.0</td>
</tr>
<tr>
<td>V546C</td>
<td>D-galactose</td>
<td>46.2</td>
<td>6.57</td>
<td>0.142</td>
<td>41.2</td>
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<tr>
<td>H548I</td>
<td>D-glucose</td>
<td>0.811</td>
<td>31.6</td>
<td>39.5</td>
<td>77.8</td>
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<tr>
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<td>79.8</td>
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<td>20.6</td>
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<td>7.39</td>
<td>1.57</td>
<td>0.213</td>
<td>63.2</td>
</tr>
</tbody>
</table>
However, this increase in the turnover number comes at the cost of substrate binding, since the introduction of Cys also lowered the ability of TmP2Ox to bind or interact with these substrates, as indicated by elevated $K_M$ values. Hence, the catalytic efficiency $k_{cat}/K_M$ of V546C for the sugar substrates was lower than that of the wild-type P2Ox. The high number of active variants at this position (73.9%) that were found in the screening assay indicates that position 546 does not have to be strictly conserved, which is also obvious from the sequence alignment shown in Figure 3.

**Figure 3.** Sequence alignment of pyranose oxidases from different organisms.

Abbreviations used and accession numbers: T. Mats., *Tricholoma matsutake* (BAC24805); L.s., *Lyophyllum shimeji* (Q75ZP8); P.c., *Phanerochaete chrysosporium* (AAS93628); T.m., *Trametes multicolor* (AAX09279); P. sp., *Peniophora sp.* (AAO13382); T.v., *Trametes versicolor* (P79076); T.p., *Trametes pubescens* (AAW57304); P.g., *Phlebiopsis gigantea* (AAQ72486); E.n., *Emericella nidulans* (Q5B2E9).

Several of the naturally occurring $p2ox$ genes, e.g., from *Phanerochaete chrysosporium* or *Lyophyllum shimeji*, carry an alanine residue at this position rather than the valine found in the $p2ox$ genes of various *Trametes* or *Peniophora* species. Two other variants at this position, showing significant activity in the screening as well, were also selected and further
characterized (V546G and V546P). These did not show the increase in turnover number as observed for V546C but again showed elevated $K_M$ values for both sugar substrates. Therefore, position 546 has a strong impact on substrate binding and on the formation of the enzyme-substrate complex. Interestingly, the V546A variant, naturally occurring in the above-mentioned enzymes, was not identified among the isolated active variants in this saturation mutagenesis study of $TmP2Ox$. It should, however, be noticed that not all variants showing activity in the initial screening could be studied in detail.

Q448, forming hydrogen bonds to the hydroxyl groups at C3 and C4 of the sugar substrate, is another active-site residue that has a considerable effect on the Michaelis constant and hence on the formation of the enzyme-substrate complex. In this case, however, substrate binding as expressed by the $K_M$ values as well as turnover numbers $k_{cat}$ changed for the worse with both substrates for all three amino acid substitutions studied at this position (Q448N, Q448S, Q448C). This negative effect is especially pronounced for the $K_M$ values, which were increased 40- to 150-fold when compared to the wild-type enzyme.

For N593, only seven active mutants were found in the saturation mutagenesis experiment at this position, and one of them was identified as N593R, which was subsequently characterized. N593 takes part in hydrogen bonding of the hydroxyl groups at C2 and C3 of the sugar substrate [16]. Presumably, Arg can provide these H bonds as no changes in the Michaelis constants for both sugars were observed, however, the turnover number was reduced by a factor of approximately 2, indicating that the introduction of a negative charge at this position in the direct vicinity of the isoalloxazine ring might have a negative effect on the catalytic mechanism. The low number of active variants found in the screening indicates that this position is relatively important for P2Ox activity.

The arginine residue at position 472 on the active site loop has been shown to be involved in substrate binding through a H-bond with the hydroxyl group at position C5 and thereby in the
correct binding of the sugar substrate for C-2 oxidation [16]. Two of the active variants identified in the screening were R472G and R472L, which obviously cannot form the hydrogen bond to C5-OH. Interestingly, the Michaelis constants for both sugars stay almost unchanged for these two mutants, and $k_{\text{cat}}$ was reduced only to a slight extent (Table 3). The relatively high number of active variants found in the screening also points to a lesser importance of this residue with respect to activity.

As mentioned above, the side chain of Thr at position 169 clashes with the axially orientated C4-OH group of D-galactose. Furthermore, Thr169 occupies an interesting position in the $Tm$P2Ox structure. During the oxidative half-reaction the dynamic substrate loop is fully closed and Thr169 forms a hydrogen bond with the flavin N5 atom [10]. During the reductive half-reaction, the substrate loop swings out of the active site to accommodate the electron-donor substrate, i.e., the sugar. In this conformation, the Thr169 side chain adopts a different rotamer, which disable hydrogen bond formation to the flavin cofactor [16]. This amino acid position had been studied previously by our group using rational protein design, introducing glycine and alanine at this position to create additional space, as well as serine as a conservative substitution that retains the hydroxyl functionality in the side chain [17]. To study whether other amino acid substitutions at this position that might not be an obvious choice in rationally designed experiments can have a positive effect on P2Ox reactivity, saturation mutagenesis was again applied, with a subsequent screening for variants with improved properties for D-galactose oxidation. One of the variants with high activity found in the screening assay, T169S, had already been proposed and investigated in our previous study [17]. Serine is a smaller amino acid than threonine, but also provides the hydroxyl group and thus can form a hydrogen bond to the FAD. Presumably due to reduced steric hindrances in T169S, this mutant showed improved binding of D-glucose ($K_M$ is reduced almost twofold compared to the wild-type) and higher turnover rates with D-galactose (increased twofold compared to
Two other T169 variants identified in the screening, T169G and T169N, showed particularly interesting results. T169G exhibited the lowest $K_M$ value for D-galactose of all the mutants studied, reduced by more than 3-fold compared to wild-type P2Ox, albeit $k_{cat}$ is also reduced significantly. The Thr $\rightarrow$ Asn substitution at position 169 is also of interest for the oxidation of D-galactose as was revealed by the kinetic characterization, since this substitution results in a significantly reduced $K_M$ for this sugar, while $k_{cat}$ is hardly affected, resulting in the P2Ox variant with the highest $k_{cat}/K_M$ for D-galactose identified in this approach (1.6-fold higher than the wild-type). Apparently, Asn can provide the hydrogen bond to the N5 of the isoalloxazine ring of the flavin, which is a recurrent and presumably an essential feature in flavoproteins [24]. N5 of the isoalloxazine directly takes part in substrate dehydrogenation, and any interaction involving this atom can affect catalysis. Fraaije and Mattevi even proposed a more subtle effect of this hydrogen bond. Upon reduction, N5 becomes protonated and so the hydrogen-bond interaction might become energetically less favorable in the reduced than in the oxidized state. Therefore, the proximity of a hydrogen-bond donor is generally expected to increase the oxidative power of the cofactor [24, 25]. The relatively high number of active variants found at position 169 (Table 2) indicates that abolishing this H bond does not result in complete loss of enzyme activity, and that several of the variants retain at least a certain level of activity.

4 Conclusion

An analysis of enzyme engineering results indicates that the majority of mutations that beneficially affect enzyme properties such as substrate selectivity or catalytic promiscuity are located in or close to the active site, and in particular near residues that are involved in catalysis or substrate binding. For other properties, such as stability or activity, both close and distant mutations seem similarly effective in engineering enzymes [26, 27]. Using this
approach of probing active-site residues of P2Ox by saturation mutagenesis we could identify several amino acid substitutions that show improved activity of this biocatalytically interesting enzyme with its poor substrate D-galactose. Especially variants V546C, T169G and T169N are of interest because of certain improved properties. Further studies and combinations of these mutations showed the appropriateness of a semi-rational approach for the improvement of P2Ox with regard to the conversion of D-galactose [28].

Acknowledgements

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Declaration

All authors declare the absence of financial or commercial conflicts of interest.
5 References


Chapter 4

A thermostable triple mutant of pyranose 2-oxidase from *Trametes multicolor* with improved properties for biotechnological applications

Oliver Spadiut¹, Katrin Radakovits¹, Ines Pisanelli¹, Clara Salaheddin¹, Montarop Yamabhai², Tien-Chye Tan³, Christina Divne³ & Dietmar Haltrich¹,⁴*

¹Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

²School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

³School of Biotechnology, KTH, Albanova University Centre, SE-106 91 Stockholm, Sweden

⁴Vienna Institute of BioTechnology VIBT, A-1190 Vienna, Austria

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Oliver Spadiut\(^1\), Katrin Radakovits\(^1\), Ines Pisanelli\(^1\), Clara Salaheddin\(^1\), Montarop Yamabhai\(^2\), Tien-Chye Tan\(^3\), Christina Divne\(^3\) & Dietmar Haltrich\(^{1,4*}\)

\(^1\)Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

\(^2\)School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

\(^3\)School of Biotechnology, KTH, Albanova University Centre, SE-106 91 Stockholm, Sweden

\(^4\)Vienna Institute of BioTechnology VIBT, A-1190 Vienna, Austria

*to whom correspondence should be addressed at:

Abteilung für Lebensmittelbiotechnologie, Universität für Bodenkultur, Muthgasse 18, A-1190 Wien, Austria
dietmar.haltrich@boku.ac.at, tel.: +43-1-36006-6275, fax +43-1-36006-6251
Abstract

In order to increase the thermal stability and the catalytic properties of pyranose oxidase (P2Ox) from *Trametes multicolor* towards its poor substrate D-galactose and the alternative electron acceptor 1,4-benzoquinone (1,4-BQ), we designed the triple-mutant T169G/E542K/V546C. Whereas the wild-type enzyme clearly favors D-glucose as its substrate over D-galactose [substrate selectivity \( \frac{V_{cat}/K_M}{V_{cat}/K_M} = 172 \)], the variant oxidizes both sugars equally well [\( \frac{V_{cat}/K_M}{V_{cat}/K_M} = 0.69 \)], which is of interest for food biotechnology. Furthermore, the variant showed lower \( K_M \) values and approx. tenfold higher \( k_{cat} \) values for 1,4-BQ when D-galactose was used as the saturating sugar substrate, which makes this enzyme particularly attractive for use in biofuel cells and enzyme-based biosensors.

In addition to the altered substrate specificity and reactivity, this mutant also shows significantly improved thermal stability. The half-life time at 60°C was closed to 10 hrs, compared to 7.6 minutes for the wild-type enzyme. We performed successfully small-scale bioreactor pilot conversion experiments of D-glucose/D-galactose mixtures at both 30°C and 50°C, showing the usefulness of this P2Ox variant in biocatalysis as well as the enhanced thermal stability of the enzyme. Moreover, we determined the crystal structure of the mutant in its unliganded form at 1.55 Å resolution. Modeling D-galactose in position for oxidation at C2 into the mutant active site shows that substituting Thr for Gly at position 169 favorably accommodates the axial C4 hydroxyl group that would otherwise clash with Thr169 in the wild-type.

**Keywords:** pyranose oxidase; rational protein design; enzyme engineering; thermal stability; biofuel cell; enzymatic batch conversion
1 Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10), a flavoprotein found widespread in wood-degrading basidiomycetes, catalyzes the oxidation of different aldopyranoses at C2 to the corresponding 2-ketoaldoses, producing H$_2$O$_2$ as a by-product [1–3]. It is a homotetrameric enzyme, typically of a molecular mass of 270 kDa with each of the four 68-kDa subunits carrying one flavin adenine dinucleotide (FAD) covalently bound [4]. The crystal structure of P2Ox from *Trametes multicolor* was determined at 1.8 Å resolution [5]. An intriguing structural feature of P2Ox is its large internal cavity, from which the active sites are accessible. Four solvent channels lead from the surface of the polypeptide into this cavity through which substrates must enter before accessing the narrow active-site channels of P2Ox. The monosaccharide D-glucose is its preferred substrate and it shows high activities with e.g. D-xylose and L-sorbose, whereas D-galactose is a rather poor substrate with only 5.7% relative activity [6]. Oxidation of D-galactose at position C2 is interesting from an applied point of view, since 2-keto-D-galactose can be easily reduced at position C1 to yield D-tagatose [7], which is used as a non-cariogenic, low caloric sweetener in food industry. Lactose, which is available in large amounts as a by-product of the cheese and dairy industry, can be hydrolyzed enzymatically, and thus provides an amply supply of D-glucose and D-galactose. For effective biotechnological applications, the catalytic activity of P2Ox with D-galactose, however, is too low, leading to either very long conversion times or disproportionate amounts of required enzyme. Additionally, a conversion at elevated temperatures is desirable, as catalytic activities increase with higher temperatures and also undesired microbiological growth is avoided during conversions at increased temperatures. Besides applications in food industry, P2Ox is increasingly getting attractive for enzymatic biosensors and biofuel cells. Biofuel cells convert sugars into electrical energy by employing oxidoreductases as an anodic biocomponent, and coupling this with a suitable enzyme on the
cathode [8]. In mediated electron transfer, certain mediators such as quinones [9] or osmium redox polymers [10] collect the electrons from the prosthetic group of the enzymes and transfer them to a graphite electrode. To improve the performance of biofuel cells it is crucial to increase both the catalytic activity and the stability of the enzymes applied. Rational protein design to change substrate specificity and reactivity has already been successfully performed for oxidoreductases, hydrolases and transferases [11], and thus provides an excellent tool for adapting P2Ox to the above-mentioned biotechnological applications. In order to improve P2Ox from *Trametes multicolor* with respect to its catalytic activity with the poor substrate D-galactose and the alternative electron acceptor 1,4-benzoquinone as well as thermal stability, we combined different mutations, which had previously shown positive effects with respect to the properties of the respective single mutants [12–14]. Here we report on the detailed biochemical and structural characterization of the resulting P2Ox variant T169G/E542K/V546C, and its possible application for the conversion of D-glucose/D-galactose mixtures at elevated temperatures. To examine the details of the structural effects of the amino-acid substitutions, we also determined the crystal structure of T169G/E542K/V546C in the absence of a ligand at 1.55 Å resolution.

### 2 Material and methods

#### 2.1 Plasmids, microorganism and media

The construction of the pET21d+/P2Ox vector (pHL2), which expresses the His-tagged pyranose 2-oxidase gene from *Trametes multicolor* under control of the T7 promoter, has been described previously [15]. Active, recombinant wild-type P2Ox and the mutant T169G/E542K/V546C were expressed in the *E. coli* strain BL21 Star DE3 (Invitrogen; Carlsbad, CA, USA). TB$_{amp}$-medium (yeast extract 24 g/L, peptone from casein 12 g/L,
glycerol 4 mL/L; KH₂PO₄-buffer 1 M, pH 7.5) was used for cultivation and protein expression under appropriate selective conditions (ampicillin was added to 0.1 g/L). All chemicals used were from Sigma (Vienna, Austria) and were of the highest grade available.

### 2.2 Generation of mutants

The P2Ox gene was mutated by site-directed mutagenesis using PCR and digestion with *DpnI* [16]. The plasmid pHL2 as template and primers T169G_fwd (5’-gtctgctggggcatgtctacgcacctggggatgcgccacacc-3’), T169Grev (5’-ccagtgcgcgcagcagcctccgtacagatgcgtgacc-3’), E542K_V546C_fwd (5’-gaagcctggtctttgccttcaccttggtgg-3’), E542K_V546C_rev (5’-aagaccaggtctcatgaattgcgggagg-3’) were used for mutagenic PCRs. The PCR reaction mix contained 2.5 U *Pfu* DNA polymerase (Fermentas; St. Leon-Rot, Germany), 100 ng of plasmid DNA, 5 pmol of each primer, 10 µM of each dNTP and 1× PCR buffer (Fermentas) in a total volume of 50 µL. The following conditions were used for mutagenic PCRs: 95°C for 4 min, then 30 cycles of 94°C for 30 s; 56°C for 30 s; 72°C for 16 min, with a final incubation at 72°C for 10 min. After PCR, the methylated template-DNA was degraded by digestion with 10 U of *DpnI* at 37°C for 3 h. The remaining PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, USA). PCR products (5 µL) were transformed into electro-competent *E. coli* BL21 Star DE3 cells. To prove, that only the desired mutations had occurred, the P2Ox-encoding gene was sequenced using primers T7promfwd (5’-aatagcactcataggg-3’) and T7termrev (5’-gctagttattgctcagcgg-3’).

### 2.3 Protein expression and purification

Cultures (2 liters) of *E. coli* BL21 Star DE3 transformants were grown in TB*amp in shaken flasks at 37°C and 160 rpm. Protein expression was induced at an OD₆₀₀ of ~0.5 by adding
lactose to a final concentration of 0.5% (w/v). After incubation at 25°C for further 20 h, approximately 20 g of wet biomass per liter were harvested by centrifugation at 10,000×g for 20 min and 4°C, and resuspended in KH$_2$PO$_4$-buffer (50 mM, pH 6.5) containing the protease inhibitor PMSF (0.1% w/v). After disruption in a continuous homogenizer (APV Systems; Silkeborg, Denmark) the crude cell extract was separated from cell debris by centrifugation (70,400×g, 4°C) and used for protein purification by immobilized metal affinity chromatography (IMAC) with a 10-mL BioRad Profinity IMAC Ni-Charged Resin (Biorad; Vienna, Austria). The column was equilibrated with 10 column volumes (CV) of buffer (0.05 M KH$_2$PO$_4$, pH 6.5, 0.5 M NaCl, 20 mM imidazole). After the protein sample had been applied to the column it was washed with 3 CV of the same buffer, before proteins were eluted with a linear gradient of 10 CV starting buffer containing 1 M imidazole. Active fractions were combined and imidazole was removed by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (Millipore; Billerica, MA, USA) with a 10-kDa cut-off membrane. The concentrated enzymes were washed 3 times with 10 mL of KH$_2$PO$_4$-buffer (50 mM, pH 6.5) and finally diluted in the same buffer to a protein concentration of 5–10 mg/mL.

2.4 Enzyme activity assay

P2Ox activity was measured with the standard chromogenic ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] assay [6]. A sample of diluted enzyme (10 μL) was added to 980 μL of assay buffer containing horseradish peroxidase (142 U), ABTS (14.7 mg) and KH$_2$PO$_4$-buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM). The absorbance change at 420 nm was recorded at 30°C for 180 s. The chromophore $\varepsilon_{420}$ used was 42.3 mM$^{-1}$·cm$^{-1}$. One unit of P2Ox activity was defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS per min, corresponding to the consumption of 1 μmol of O$_2$ per min, under assay conditions. Protein concentrations were determined by the Bradford assay [17] using the BioRad Protein Assay Kit with BSA as standard.
2.5 Steady-state kinetic measurements

Kinetic constants were calculated by non-linear least-square regression, fitting the data to the Henri-Michaelis-Menten equation. The catalytic constants were measured for the electron donor substrates D-glucose (0.1–50 mM) and D-galactose (0.1–200 mM) using the standard ABTS assay and air saturation. Additionally, catalytic constants were measured for the alternative electron acceptor of this oxidase, 1,4-benzoquinone (1,4-BQ), with 100 mM of either D-glucose or D-galactose as saturating substrate by adding 10 µL of diluted enzyme to 990 µL of assay buffer containing either of the two sugar substrates, KH2PO4-buffer (50 mM, pH 6.5) and 1,4-BQ (0.01–2 mM). The absorbance change at 290 nm was recorded at 30°C for 180 s. The chromophore ε290 used was 2.24 mM⁻¹·cm⁻¹. Steady-state kinetics measurements were carried out both at 30°C and 50°C.

2.6 Electrophoresis

To check the purity of the purified P2Ox variants, electrophoresis was done as described by Laemmli et al. [18]. SDS-PAGE was performed using a 5% stacking gel and a 10% separating gel. The system used was the PerfectBlue vertical electrophoresis apparatus (Peqlab; Erlangen, Germany). The mass standard used was the Precision Plus Protein Dual Color (Biorad). Gels were stained with Coomassie blue.

2.7 Kinetic stability

Kinetic stability, as expressed by the half-life time of thermal inactivation τ₁/₂, of the purified wild-type enzyme and the triple mutant was determined by incubating the proteins in appropriate dilutions in 50 mM KH2PO4-buffer (pH 6.5) at both 60°C and 70°C for various time intervals and by subsequent measurements of the residual enzyme activity using the standard ABTS assay and D-glucose as substrate. A thermal cycler (thermocycler T3, Biometra; Göttingen, Germany) and thin-walled PCR tubes were used for all thermostability
measurements. Residual activities were plotted versus the incubation time and the half-life values of thermal inactivation ($\tau_{1/2}$) were calculated using $\tau_{1/2} = \ln 2/k_{in}$, where $k_{in}$ is the observed rate of inactivation.

2.8 Batch conversion experiments

Wild-type P2Ox and the variant T169G/E542K/V546C were compared in terms of their ability to oxidize D-glucose and D-galactose to the corresponding 2-ketoaldoses with oxygen as electron acceptor at both 30°C and 50°C. Four batch conversion experiments (each with a volume of 300 mL) using equimolar amounts of D-glucose and D-galactose were designed in a way to guarantee a complete conversion of D-galactose within 20 hours for the mutated enzyme and a complete conversion of D-glucose for the wild-type enzyme within reasonable times. The experiments were performed in parallel in a multifermenter (Infors; Bottmingen, Switzerland); these were initial bioreactor studies proving the applicability of the enzyme variant developed and not aiming at process optimization. The conversion experiments were conducted in 100 mM KH$_2$PO$_4$-buffer (pH 6.5) at 400 rpm, a DO$_2$ concentration of 15%, both at 30°C and 50°C. Catalase was used in excess (100,000 U) to decompose H$_2$O$_2$. Depending on the catalytic activity of the enzymes with D-galactose different amounts of an equimolar mixture of the sugar substrates and biocatalyst concentrations were used for the conversion experiments (Table 5). The specific activity of wild-type P2Ox with D-galactose was determined with 330 mU·mg$^{-1}$, of variant T169G/E542K/V546C with 12.2 mU·mg$^{-1}$, both at 30°C. For batch conversions (total volume of 300 mL) at 30°C 1600 mU wild-type P2Ox and 400 mU mutated enzyme (measured under standard assay conditions with D-galactose as substrate) were used. The kinetic characterization of the enzymes at 50°C revealed a specific activity of wild-type P2Ox with D-galactose of 500 mU·mg$^{-1}$, of variant T169G/E542K/V546C with 20.4 mU·mg$^{-1}$. Conversions at 50°C were conducted with 2400 mU of wild-type and 750
mU T169G/E542K/V546C, respectively. Samples (2 mL) from the bioconversion experiments were taken periodically, held at 95°C for 5 min to inactivate the enzymes and centrifuged. The supernatants were analyzed for their sugar content using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which was carried out using a Dionex DX-500 system (Dionex; Sunnyvale, CA, USA) and a CarboPac PA-1 column (4 × 250 mm) at 27°C [19].

2.9 X-ray crystallographic analysis

Crystals of the P2Ox variant T169G/E542K/V546C were produced using the hanging drop vapor diffusion method [20]. Drops were prepared by equal volumes of 5 mg/mL protein and reservoir [10% (w/v) monomethylether polyethylene glycol 2000 (mme PEG), 0.1 M Mes (pH 5.2), 50 mM MgCl$_2$, 25% glycerol]. Prior to data collection, the crystals were stabilized using their respective reservoir solution where the PEG concentration had been increased to 50% (stabilizing solution) followed by vitrification in liquid nitrogen. The protein crystallizes in space group $P4_22_12$ with one molecule in the asymmetric unit. Data were collected using synchrotron radiation at MAX-lab, beamline I911-3 ($\lambda =1.0$ Å; 100 K). Data were processed using XDS [21]. Phases for the T169G/E542K/V546C structure were obtained by means of Fourier synthesis using the refined model of P2Ox variant H167A as starting model (PDB code 2IGO; [15]. Crystallographic refinement was performed with REFMAC5 [22], including anisotropic scaling, calculated hydrogen scattering from riding hydrogens, and atomic displacement parameter refinement using the translation, libration, screw-rotation (TLS) model. The TLS models were determined using the TLS Motion Determination server (TLSMD; [23]). Corrections of the models were done manually based on $\sigma_A$-weighted $2F_o-F_c$ and $F_o-F_c$ electron density maps. The $R_{\text{free}}$ reflection sets were kept throughout refinement. All model building was performed with the program O [24] and Coot [25]. Data collection and model refinement statistics are given in Table 6.
Table 6. Data collection and refinement statistics

<table>
<thead>
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<th>Data collection(^1)</th>
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<td>Unique reflections</td>
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<td>Multiplicity</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
<td>&lt;I / σI&gt;</td>
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<td>R(_{sym})(^2) (%)</td>
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</table>

<table>
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<td>R(_{factor})(^3)/work reflns, all</td>
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<tr>
<td>R(_{free})/free reflns, all</td>
</tr>
<tr>
<td>Non-hydrogen atoms</td>
</tr>
<tr>
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<td>Mean B (Å(^2)) solvent /N°. molecules</td>
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<tr>
<td>Rmsd bond lengths (Å), angles (°)</td>
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<td>Ramachandran: favored / allowed (%)(^4)</td>
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</table>

\(^1\) The outer shell statistics of the reflections are given in soft brackets. Shells were selected as defined in XDS [21] by the user.

\(^2\) \(R_{sym} = \left[ \sum_{hklt} \sum_i |I_i| - \langle|I|\rangle \right] / \sum_{hklt} \sum_i |I_i| \times 100\%.

\(^3\) \(R_{factor} = \sum_{hklt} |F_o| - |F_c| / \sum_{hklt} |F_o|.

\(^4\) As determined by MolProbity [29]
3 Results

3.1 Generation of mutants

After site-directed mutagenesis was performed as described in the Material and Methods section, the presence of the correct and the absence of undesired mutations in the P2Ox gene were confirmed by DNA sequence analysis. Wild-type P2Ox and the variant T169G/E542K/V546C were expressed in *E. coli*, purified to apparent homogeneity and concentrated by ultrafiltration. The purity of the resulting enzyme preparations was confirmed by SDS-PAGE (Fig. 1). Routinely, approx. 20 mg of P2Ox protein were obtained per liter culture medium by this procedure.

![SDS-PAGE analysis](image)

**Figure 1.** SDS-PAGE analysis of purified wild-type P2Ox from *Trametes multicolor* and the mutational variant T169G/E542K/V546C. Lane 1, molecular mass marker proteins; lane 2, wild-type P2Ox; lane 3, T169G/E542K/V546C after purification by IMAC.

3.2 Kinetic characterization

For determination of the steady-state kinetic constants, initial rates of substrate turnover were recorded over a substrate range of 0.1–50 mM D-glucose and 0.1–200 mM D-galactose for wild-type P2Ox and the mutational variant T169G/E542K/V546C using the standard ABTS
assay and oxygen (air saturation), both at 30°C and 50°C. Kinetic data are summarized in Table 1.

**Table 1.** Steady-state kinetic constants of wild-type P2Ox and variant T169G/E542K/V546C with either D-glucose or D-galactose as substrate and O₂ (air) as electron acceptor at pH 6.5 and at the temperatures indicated.

<table>
<thead>
<tr>
<th></th>
<th>30°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s⁻¹)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.76 ± 0.05</td>
<td>34.0 ± 0.43</td>
</tr>
<tr>
<td>T169G/E542K/V546C</td>
<td>0.64 ± 0.10</td>
<td>0.072 ± 0.003</td>
</tr>
<tr>
<td>D-Galactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s⁻¹)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>7.94 ± 0.39</td>
<td>2.10 ± 0.03</td>
</tr>
<tr>
<td>T169G/E542K/V546C</td>
<td>1.66 ± 0.70</td>
<td>0.27 ± 0.02</td>
</tr>
</tbody>
</table>

Prior to determination of the kinetic constants it was confirmed that the introduction of the amino acid substitutions in the triple-mutant did not affect the pH profile of P2Ox activity (data not shown). T169G/E542K/V546C showed an approx. 5-fold decreased Michaelis constant $K_M$ for D-galactose compared to the wild-type enzyme when air oxygen was the electron acceptor, whereas $K_M$ for D-glucose was hardly altered. Yet, turnover numbers $k_{cat}$ for both sugar substrates were decreased considerably as well, regardless of the temperature used for activity measurements. As a result, the catalytic efficiency $k_{cat}/K_M$ of the mutant was similar to that of the wild-type enzyme with D-galactose but was decreased ≈400-fold with D-glucose, resulting in an enzyme that showed an equal or even higher $k_{cat}/K_M$ value for D-galactose than for D-glucose. In contrast, the wild-type enzyme clearly prefers D-glucose over D-galactose as its sugar substrate as is also expressed by the substrate selectivity values, i.e., the ratio of the catalytic efficiencies $k_{cat}/K_M$ for the two substrates. This value is 172 for the wild-type, while it is 0.69 for T169G/E542K/V546C at 30°C (Table 2).
Table 2. Substrate selectivity of wild-type P2Ox from *Trametes multicolor* and the variant T169G/E542K/V546C at 30°C and at 50°C.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$(k_{\text{cat}}/K_M)_{\text{Glc}}$</th>
<th>$(k_{\text{cat}}/K_M)_{\text{Gal}}$</th>
<th>$(k_{\text{cat}}/K_M)<em>{\text{Glc}} / (k</em>{\text{cat}}/K_M)_{\text{Gal}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtP2Ox</td>
<td>172</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>T169G/E542K/V546C</td>
<td>0.69</td>
<td>1.11</td>
<td></td>
</tr>
</tbody>
</table>

In addition, the kinetic constants were determined for the alternative electron acceptor 1,4-benzoquinone (1,4-BQ) with either D-glucose or D-galactose in saturating concentrations at 30°C and 50°C (Table 3), and it was found that the three amino acid substitutions dramatically affect the catalytic properties. The $K_M$ value of the mutant for 1,4-BQ was reduced two to three times compared to the wild-type enzyme, regardless of the sugar substrate used. While the turnover number with D-glucose as saturating substrate was reduced significantly, it increased considerably with D-galactose (≈10-fold). Compared to the wild-type enzyme, mutant T169G/E542K/V546C showed a 24- and 15-fold increase in its catalytic efficiency at 30°C and 50°C, respectively, for 1,4-BQ and D-galactose as saturating substrate.

Table 3. Steady-state kinetic constants of wild-type P2Ox and variant T169G/E542K/V546C for 1,4-benzoquinone as electron acceptor with either D-glucose or D-galactose as saturating substrate. Data were determined at pH 6.5 and at the temperatures indicated.

<table>
<thead>
<tr>
<th></th>
<th>30°C</th>
<th>50°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D-Glucose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>0.40 ± 0.05</td>
<td>0.78 ± 0.07</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (s$^{-1}$)</td>
<td>349 ± 17.8</td>
<td>615 ± 32.6</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_M$ (mM$^{-1}$s$^{-1}$)</td>
<td>863</td>
<td>785</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T169G/E542K/V546C</td>
<td>0.22 ± 0.10</td>
<td>0.31 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>21.16 ± 3.7</td>
<td>79.3 ± 16.6</td>
</tr>
<tr>
<td></td>
<td>94.5</td>
<td>258</td>
</tr>
<tr>
<td><strong>D-Galactose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_M$ (mM)</td>
<td>0.25 ± 0.03</td>
<td>0.23 ± 0.037</td>
</tr>
<tr>
<td>$k_{\text{cat}}$ (s$^{-1}$)</td>
<td>6.61 ± 0.34</td>
<td>14.6 ± 0.69</td>
</tr>
<tr>
<td>$k_{\text{cat}}/K_M$ (mM$^{-1}$s$^{-1}$)</td>
<td>26.3</td>
<td>62.1</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T169G/E542K/V546C</td>
<td>0.093 ± 0.04</td>
<td>0.19 ± 0.084</td>
</tr>
<tr>
<td></td>
<td>59.6 ± 7.59</td>
<td>171 ± 28.2</td>
</tr>
<tr>
<td></td>
<td>622</td>
<td>911</td>
</tr>
</tbody>
</table>
3.3 Thermal stability

Kinetic stability, the length of the time in which an enzyme remains active before undergoing irreversible inactivation, of wtP2Ox and of variant T169G/E542K/V546C was determined at 60°C and at 70°C and a constant pH of 6.5. The inactivation constants $k_{in}$ and the half-lives of denaturation $\tau_{1/2}$ were determined (Table 4), and both enzymes showed first-order inactivation kinetics when analyzed in the ln(residual activity) versus time plot (Fig. 2). The mutation E542K in combination with T169G and V546C stabilized P2Ox significantly. At 60°C the half-life was increased 76-fold compared to the wild-type enzyme. The effect of the mutations on stability is even more pronounced at 70°C, where $\tau_{1/2}$ was increased 350-fold.

Table 4. Kinetic stability of pyranose oxidase from *T. multicolor* at various temperatures. The inactivation constants $k_{in}$ and half-life times of inactivation $\tau_{1/2}$ are given for 60°C and 70°C.

<table>
<thead>
<tr>
<th>Variant</th>
<th>60°C</th>
<th>70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{in}$ (min$^{-1}$)</td>
<td>$\tau_{1/2}$ (min)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>$-9.15 \cdot 10^{-2}$</td>
<td>7.6</td>
</tr>
<tr>
<td>T169G/E542K/V546C</td>
<td>$-1.20 \cdot 10^{-3}$</td>
<td>578</td>
</tr>
</tbody>
</table>
Figure 2. Inactivation kinetics of P2Ox variants from *Trametes multicolor* at pH 6.5 and various temperatures. A: wtP2Ox and the variant T169G/E542K/V546C at 60°C; B/C: ●, wild-type P2Ox/variant T169G/E542K/V546C at 70°C. Symbols: ●, wtP2Ox; ■, T169G/E542K/V546C

3.4 Enzymatic batch conversion experiments

In order to assess the effects of the selected amino acid substitutions on the biocatalytic performance of P2Ox, batch conversion experiments using equimolar mixtures of D-glucose and D-galactose were performed with oxygen as electron acceptor (Fig. 3). Reaction conditions were chosen to guarantee reasonable process times in each reactor and were not aimed at
process optimization; hence different amounts of enzyme and sugar substrates were used (Table 5).

**Table 5.** Batch conversion experiments of wild-type pyranose oxidase from *T. multicolor* and the variant T169G/E542K/V546C using equimolar mixtures of D-glucose and D-galactose at 30°C and at 50°C.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>batch A</th>
<th>batch B</th>
<th>batch C</th>
<th>batch D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild-type</td>
<td>variant</td>
<td>wild-type</td>
<td>variant</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>30°C</td>
<td>30°C</td>
<td>50°C</td>
<td>50°C</td>
</tr>
<tr>
<td>Enzyme activity applied (mU)</td>
<td>1600</td>
<td>400</td>
<td>2400</td>
<td>750</td>
</tr>
<tr>
<td>Initial sugar concentration (g·L⁻¹)</td>
<td>0.8</td>
<td>0.3</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>Conversion rate D-glc (g·L⁻¹·h⁻¹)</td>
<td>2.0</td>
<td>0.051</td>
<td>8.7 / 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.117</td>
</tr>
<tr>
<td>Conversion rate D-gal (g·L⁻¹·h⁻¹)</td>
<td>0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.065</td>
<td>0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.124</td>
</tr>
</tbody>
</table>

<sup>a</sup> D-gal was not converted until D-glc was completely oxidized

<sup>b</sup> during the first 45 min the average conversion rate was high with 8.7 g·L⁻¹·h⁻¹, inactivation resulted in a lower average conversion rate of 2.7 g·L⁻¹·h⁻¹ over the subsequent 45 min period, wild-type enzyme was completely inactived after 90 minutes

At 30°C the wild-type enzyme clearly preferred D-glucose compared to D-galactose with a conversion rate of 2.0 g·L⁻¹·h⁻¹. Only when D-glucose was oxidized completely, D-galactose was converted at a very low rate of 0.02 g·L⁻¹·h⁻¹. In contrast to that, mutant T169G/E542K/V546C showed similar conversion rates of 0.054 and 0.065 g·L⁻¹·h⁻¹ for D-glucose and D-galactose. The engineered variant did not prefer any of the sugars as its substrate but converted both of them simultaneously. As is also expressed by the slightly higher *k*ₘₐₜ for D-galactose, this monosaccharide was converted at a somewhat faster rate than D-glucose. When the conversion experiments were performed at 50°C, the wild-type enzyme oxidized D-glucose initially at a high rate of 8.7 g·L⁻¹·h⁻¹ for the first phase of the conversion (up to 45 min). Yet, thermal inactivation of the enzyme resulted in a rapid drop of the conversion rate over time and P2Ox activity was completely lost after 90 min, as was evident from residual D-
glucose left in the reaction mixture and the complete lack of 2-keto-D-galactose. In contrast, variant T169G/E542K/V546C converted both sugar substrates at an almost equal rate of 0.12 g·L⁻¹·h⁻¹ resulting in complete conversion of both sugar substrates within 20 h.

**Figure 3.** Batch conversion experiments of equimolar mixtures of D-glucose and D-galactose and oxygen as electron acceptor at both 30°C and 50°C using wild-type TmP2Ox or the variant T169G/E542K/V546C as biocatalyst. A, wtP2Ox at 30°C; B, T169G/E542K/V546C at 30°C; C, wtP2Ox at 50°C; D, T169G/E542K/V546C at 50°C. Symbols: ●, D-glucose; ▲, D-galactose

**4 Discussion**

Pyranose oxidase is an enzyme of interest for use in biofuel cells and enzyme-based biosensors as well as for applications in food industry. In several previous studies the improvement of P2Ox both in terms of stability and reactivity were reported. The mutation E542K, which is located on the surface of the internal cavity, was found to kinetically stabilize the enzyme as
well as to improve its catalytic properties to some extent [12, 26]. Other studies showed the positive effects of the mutations V546C [13] and T169G [14] with respect to kinetic properties, especially for the oxidation of the substrate D-galactose. The replacement of Val by Cys at position 546 in the direct vicinity of the active site of P2Ox resulted in significantly increased turnover rates for both the sugar substrate and the alternative electron acceptor, albeit at the costs of an increased $K_M$. We determined the crystal structure of the T169G/E542K/V546C mutant at 1.55 Å resolution and performed theoretical modeling of $\beta$-D-glucose and $\beta$-D-galactose in the active site (Fig. 4).

**Figure 4.** Theoretical models showing the presumed binding of a) $\beta$-D-glucose, and b) $\beta$-D-galactose in the active site of *T. multicolor* P2Ox variant T169G/E542K/V546C based on the crystal structure of P2Ox variant H167A in complex with 2-fluoro-2-deoxy-D-glucose (PDB code 2IGO; [15]). The triple mutant is shown in yellow, and the protein model of
2IGO in light blue (ligand removed). The modeled monosaccharides (glucose and galactose) are shown in light green. For clarity, protein backbone atoms and water molecules have been omitted. The covalent linkage between the FAD $\alpha\text{methyl group}$ and His167 $N^2$ is indicated. The monosaccharides are oriented for oxidation at C2, and their C4 atoms are marked by an asterisk (*). Modeling was performed using the program O [24], and the picture was made using MacPyMOL v. 0.98 [28].

The axial C4 hydroxyl in $\beta$-D-galactose cannot be accommodated easily in the active site and clashes with the side chain of Thr169, whereas the $\beta$-D-glucose C4 hydroxyl fits well. In the mutant, Gly169 relieves steric hindrance and provides space for the galactose C4 hydroxyl group to give a relative decrease in $K_M$ value. This, at least partly, explains why $\beta$-D-galactose is a poor substrate for wild-type P2Ox, and performs relatively better as substrate for P2Ox T169G/E542K/V546C. By introducing this mutation we intended to counteract the negative effects on $K_M$ observed for the V546C mutation. By combining these three different mutations we aimed at creating a thermostable variant of P2Ox, which converts D-galactose and D-glucose concomitantly and at equal rates. This simultaneous conversion of D-glucose and D-galactose is important when e.g. lactose hydrolysates are used as a starting material for the envisaged bioconversion. P2Ox is known to overoxidize its primary reaction product, 2-keto-D-glucose, thus forming 2,3-diketo-D-glucose [27]. Simultaneous conversion of the two sugar substrates will obviously avoid this overoxidation and thus the formation of the undesired byproduct. We were further interested in increasing the turnover number for 1,4-benzoquinone, which can be used as electron mediator in biofuel cells and biosensors, in combination with D-galactose as the saturating substrate. In biofuel cells based on mediated electron transfer, suitable mediators gather electrons from the prosthetic group of an enzyme and transfer them to the electrode. In these applications, the measured current represents the actual turnover rate of
the immobilized enzyme, and, consequently, an enzyme with increased turnover rates for the mediator will boost the power output of biofuel cells [8, 10].

Kinetic characterization and comparison of variant T169G/E542K/V546C showed that the substrate selectivity was indeed changed significantly for the mutant. Whereas wtP2Ox clearly prefers D-glucose as its substrate, as indicated by a considerably higher $k_{cat}/K_M$ value, T169G/E542K/V546C does not show any clear preference for either sugar substrate as is evident from comparable catalytic efficiencies. This change in substrate selectivity, however, comes at a cost in $k_{cat}$, which is reduced for the triple-mutant for both sugar substrates. The altered sugar selectivity is also obvious when performing small-scale conversion experiments, using equimolar mixtures of D-glucose and D-galactose, as found in lactose hydrolysates, as the starting material.

Here, the variant oxidized both sugars simultaneously, while the wild-type enzyme converted D-galactose only when D-glucose was exhausted from the reaction mixture. Introducing the E542K mutation in the variant also enabled conversions at higher temperatures, which is preferable because of higher reaction rates and a decreased possibility of microbial contamination. The triple-mutant showed considerably increased thermostability as is evident from the remarkable increase in half-life times, at both 60°C and 70°C, which were improved 76-fold and 350-fold, respectively, when compared to the wild-type. Thus, bioconversions based on the thermostable variant will be feasible at temperatures of up to 60°C.

The triple-mutant T169G/E542K/V546C also showed significantly improved catalytic properties for its substrate 1,4-BQ when D-galactose was the saturating sugar. Compared to the wild-type enzyme, the turnover numbers for 1,4-BQ with D-galactose as saturated substrate at 30°C and at 50°C were increased 9-fold and 12-fold, respectively, for the variant. In combination with a lowered $K_M$ value for the electron acceptor the resulting catalytic efficiency was 24 times and 15 times higher, respectively, compared to the wild-type enzyme.
This property, together with its considerably increased stability, makes this variant particularly promising for applications in biofuel cells. The bioelectrochemical properties of T169G/E542K/V546C are currently studied in our laboratory.

**Acknowledgements**

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5 References


Chapter 5

Engineering of pyranose 2-oxidase: improvement for biofuel cell and food applications through semi-rational protein design

Oliver Spadiut\textsuperscript{1}, Ines Pisanelli\textsuperscript{1}, Thomas Maischberger\textsuperscript{1}, Clemens Peterbauer\textsuperscript{1}, Lo Gorton\textsuperscript{2}, Pimchai Chaiyen\textsuperscript{3} & Dietmar Haltrich\textsuperscript{1}

\textsuperscript{1}Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

\textsuperscript{2}Department of Analytical Chemistry, Lund University, P.O.Box 124, SE-22100 Lund, Sweden

\textsuperscript{3}Department of Biochemistry and Center for Excellence in Protein Structure and Function, Mahidol University, Rajthevi, Bangkok 10400, Thailand

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Engineering of pyranose 2-oxidase: improvement for biofuel cell and food applications through semi-rational protein design

Oliver Spadiut\textsuperscript{1}, Ines Pisanelli\textsuperscript{1}, Thomas Maischberger\textsuperscript{1}, Clemens Peterbauer\textsuperscript{1}, Lo Gorton\textsuperscript{2}, Pimchai Chaiyen\textsuperscript{3} & Dietmar Haltrich\textsuperscript{1}

\textsuperscript{1}Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria
\textsuperscript{2}Department of Analytical Chemistry, Lund University, P.O.Box 124, SE-22100 Lund, Sweden
\textsuperscript{3}Department of Biochemistry and Center for Excellence in Protein Structure and Function, Mahidol University, Rajthevi, Bangkok 10400, Thailand

Corresponding author: Dietmar Haltrich; Department of Food Sciences and Technology, BOKU – University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria
email: dietmar.haltrich@boku.ac.at;
tel.: +43-1-36006-6275, fax: +43-1-36006-6251

Running title: Engineering of pyranose 2-oxidase
Abstract

Pyranose 2-oxidase (P2Ox) has several proposed biotechnological applications, amongst others as a bio-component in biofuel cells or for carbohydrate transformations. To improve some of the catalytic properties of P2Ox from *Trametes multicolor*, we selected a semi-rational approach of enzyme engineering, saturation mutagenesis of active-site residues and subsequent screening of mutant-libraries for improved activity. One of the active-site mutants with improved catalytic characteristics identified was V546C, which showed catalytic constants increased by up to 5.7-fold for both the sugar substrates (D-glucose, D-galactose) and alternative electron acceptors [1,4-benzoquinone (BQ), ferricenium ion (Fc⁺)], albeit at the expense of increased Michaelis constants. By combining V546C with other amino acid replacements, we obtained P2Ox variants that are of interest for biofuel cell applications due to their increased $k_{\text{cat}}$ for both BQ and Fc⁺, e.g., V546C/E542K showed 4.4- and 17-fold increased $k_{\text{cat}}$ for BQ compared to the wild-type enzyme when D-glucose and D-galactose, respectively, were the saturating substrates, while V546C/T169G showed approx. 40- and 50-fold higher $k_{\text{cat}}$ for BQ and Fc⁺, respectively, with D-galactose in excess. This latter variant also shows significantly modulated sugar substrate selectivity. While the wild-type enzyme strongly prefers D-glucose over D-galactose as its substrate, V546C/T169G converts both sugars equally well as was shown by the kinetic constants determined as well as by biotransformation experiments.

Keywords: pyranose oxidase; saturation mutagenesis; enzyme engineering; biofuel cells; anodic bio-component
1 Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10) is a flavin adenine dinucleotide (FAD) dependent enzyme widespread in wood-degrading basidiomycetes (Vole et al. 1985; Danneel et al. 1992; Leitner et al. 1998). The reaction catalyzed by P2Ox is of the Ping Pong Bi Bi type typically found in flavoprotein oxidoreductases (Ghisla and Massey 1989; Artolozaga et al. 1997) and consists of the reductive half-reaction, in which an aldopyranose substrate reduces the FAD cofactor to yield FADH₂ and 2-dehydroaldose (2-ketoaldose) as the result of oxidation at position C-2 (Freimund et al. 1998), and the ensuing oxidative half-reaction, which involves the reoxidation of FADH₂ by the electron acceptor. Possible electron acceptors include molecular oxygen as well as various quinones, complexed metal ions and radicals (Leitner et al. 2001). As judged from the catalytic efficiency some of these alternative electron acceptors are in fact better substrates for the enzyme than oxygen, suggesting that P2Ox can play an important role in the reduction of quinones during the process of ligninolysis (Ander and Marzullo 1997; ten Have and Teunissen 2001). This excellent reactivity of P2Ox with alternative electron acceptors and a range of sugar substrates can be employed in various attractive applications. One possible field of application is as a bio-element in sensors and biofuel cells, where it could replace glucose oxidase, which is typically used but shows certain disadvantages. In these applications, the enzyme communicates with an electrode through small redox-active compounds, so-called mediators, in a process referred to as mediated electron transfer (MET). Recently, we could show that P2Ox can be electrically wired to graphite electrodes through the use of osmium redox polymers (Tasca et al. 2007), which gather the electrons from the enzyme and transfer them to the electrode. Additional mediators that have been successfully tested for providing contact between P2Ox or other oxidases and an electrode include ruthenium ion complexes and
modified ferrocenes as well as various quinones (Zhu et al. 2006; Nazaruk and Bilewicz 2007; Tamaki et al. 2007).

In this study we used P2Ox from *Trametes multicolor* (*Tm*P2Ox), which was first isolated and characterized by Leitner et al. (2001). The crystal structure of *Tm*P2Ox was determined at 1.8 Å resolution (Hallberg et al. 2004). It is a homotetrameric enzyme with a molecular mass of 270 kDa with each of the four 68-kDa subunits carrying one flavin adenine dinucleotide (FAD) covalently bound (Halada et al. 2003; Hallberg et al. 2004). Recently, the crystal structure of *Tm*P2Ox in complex with one of its slow carbohydrate substrates, 2-fluoro-2-deoxy-D-glucose, was reported (Kujawa et al. 2006). This structure gave detailed information about residues interacting with the sugar substrate in the active site. As a consequence, site-directed mutagenesis at these residues allows analysis of structure-function relationships, and also possible improvements by (semi-)rational protein design. Mutations of active-site residues are known to often dramatically change the properties of an enzyme. Frequently, these changes are simply unfavorable or even inactivating, however, the altered enzymes can also show improved properties such as broadened substrate specificities or increased activity (Toscano et al. 2007).

The monosaccharide D-glucose is the preferred substrate of *Tm*P2Ox, whereas D-galactose performs poorly with only 5.7% relative activity compared to D-glucose (Leitner et al. 2001). Oxidation of D-galactose at position C-2 is interesting from an applied point of view because the product obtained in this transformation, 2-keto-D-galactose (2-dehydro-D-galactose, galactosone), can be reduced easily at position C-1 to yield D-tagatose (Haltrich et al. 1998), which is a ketose sugar with significant potential as a non-cariogenic, low caloric sweetener in food applications. Lactose, which is available in large quantities as a by-product of dairy industry, can be hydrolyzed by β-galactosidases and thus provides an ample supply of D-galactose (Nakayama and Amachi 1999). For effective biotechnological applications, the catalytic activity of P2Ox with D-galactose, however, is too low, leading to either very long
conversion times or disproportionate amounts of required enzyme. Also, an increased catalytic activity with the alternative electron acceptors such as the ferricenium ion or quinones is desirable for biofuel cell applications. Based on the crystal structure we decided to use saturation mutagenesis to exchange all of the active-site residues one by one, and screen for improvements in the catalytic activity with D-galactose and alternative electron acceptors. In this work we report on one of these active-site variants, V546C, together with its combinations with other mutations, which show significantly improved catalytic properties and which are attractive for various biotechnological applications.

2 Material and Methods

2.1 Plasmids, microorganisms and media

The construction of the pET21d+/P2Ox vector (pHL2), which expresses the His-tagged P2Ox gene from Trametes multicorl under the control of the T7 promoter, has already been described (Kujawa et al. 2006). The plasmids carrying the T169G as well as the E542K mutation have also been described (Spadiut et al. 2008a; Spadiut et al. 2008b).

Active, recombinant TmP2Ox and P2Ox mutants were expressed in E. coli strain BL21 Star DE3 (Invitrogen, Carlsbad, CA, U.S.A.). TBamp-medium (yeast extract 24 g/L, peptone from casein 12 g/L, glycerol 4 mL/L; KH2PO4-buffer 1 M, pH 7.5) was used to cultivate E. coli cells for protein expression under appropriate selective conditions (ampicillin was added to 0.1 g/L). LBamp-medium (yeast extract 5 g/L, peptone from casein 10 g/L, NaCl 5 g/L, ampicillin 0.1 g/L) was used to cultivate E. coli cells in 96-well screening plates. For induction of protein expression cells were grown in 2x LBamp/lactose (yeast extract 10 g/L, peptone from casein 20 g/L, NaCl 5 g/L, lactose 5 g/L; ampicillin 0.1 g/L). All chemicals used were purchased from Sigma (Vienna, Austria) and were of the highest grade available.
2.2 Generation of mutants

The P2Ox gene was mutated by a two-step saturation mutagenesis using PCR and digestion with \textit{DpnI} (Li and Wilkinson 1997). Plasmid pHIL2 was used as template for saturation mutagenesis at position V546 with primers P2OxV546\textunderscore fwd (5´- GCCTGGTCTTNNSCTTCACCTTGGTG-3´) and P2OxV546\textunderscore rev (5´- AAGACCAGGCTCCATGAATTGC-3´). The forward primer carried the variable position NNS, where N = A, G, C or T and S = G or C. To create double mutants, combining the Val $\rightarrow$ Cys substitution at position 546 with known mutations affecting the kinetic properties of \textit{TmP2Ox}, the plasmids carrying the mutations E542K and T169G were used as templates for the PCR reactions. Mutation V546C was introduced with primers V546C\_E542K\_fwd (5´- GAAGCCTGGTCTTTGCTTCACCTTGGTG–3´) and V546C\_E542K\_rev (5´- AAGACCAGGCTCCATGAATTGCGGAGG-3´). Variant V546C/T169G was constructed by using the primers V546C\_fwd (5´-GCCTGGTCTTTGCTTCACCTTGGTG–3´) and V546C\_rev (5´-AAGACCAGGCTCCATGAATTGCAGG-3´). The mutant V546C/T169G was further mutated by using the primers L537W\_fwd (5´-ACCCGGCTCTTGCTGGCCGCAATTCC–3´) and L537W\_rev (5´-GGAGCCCGGTAGGAAGCCACC–3´) to additionally introduce tryptophane at position 537. The PCR reaction mix contained 2.5 Units \textit{Pfu} DNA polymerase (Fermentas; St. Leon-Rot, Germany), 100 ng of plasmid DNA, 5 pmol of each primer, 10 $\mu$L of each dNTP and 1x PCR buffer in a total volume of 50 $\mu$L. The mutagenic PCRs were done using the following conditions: 95°C for 4 min, then 30 cycles of 94°C for 30 sec; 55°C for 30 sec; 72°C for 16 min, and a final incubation at 72°C for 10 min. After PCR, the methylated template DNA was degraded by digestion with 10 Units of \textit{DpnI} at 37°C for 2 h. The remaining PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, U.S.A.). Five $\mu$L of the PCR products were transformed into electro-competent \textit{E. coli} BL21 Star DE3 cells. To
confirm the presence of the correct mutations and the absence of undesired mutations, the P2Ox-encoding insert was sequenced using primers T7promfwd (5´-AATACGACTCCTATAGGG–3´) and T7termrev (5´-GCTAGTTATTGCTCAGGG-3´).

2.3 Screening assay for improved P2Ox variants in 96-well plates

Position V546 was mutated by saturation mutagenesis, which allowed the creation of a mutant library containing all possible codons at this target position. The size of the library, which has to be screened to cover all possible mutants, is determined by the mutagenic codon. To mutate the amino acid V546 by saturation mutagenesis we used a primer of the NNS type, which defines the minimum library size with 95 colonies to be screened to statistically cover 95% of all possible substitutions (Georgescu et al. 2003). To cover all possible amino acid substitutions with very high probability, we screened 360 mutants for their catalytic activity with either D-glucose or D-galactose. A screening assay based on 96-well microtiter plates as previously described (Spadiut et al. 2008a) was used to this end. Six wells per plate were inoculated with E. coli expressing wild-type enzyme for comparison. The average value of specific activity with either of the two sugar substrates D-glucose or D-galactose of the wild-type P2Ox wells was determined and compared with the values of the mutants.

2.4 Protein expression and purification

Cultures (1 liter) of E. coli BL21 Star DE3 transformants were grown in TBamp in shaken flasks at 37°C and 160 rpm. When OD600 of ~ 0.5 was reached, recombinant protein expression was induced by adding lactose to a final concentration of 5 g/L. After incubation at 25°C for further 20 h, about 20 g wet biomass per liter were harvested by centrifugation at 10,000×g for 20 min, resuspended in KH2PO4-buffer (50 mM, pH 6.5) containing the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF; 1 g/L) and disrupted in a continuous. The crude extract was
separated from cell debris by centrifugation (70,400×g, 4°C) and used for protein purification by immobilized metal affinity chromatography (IMAC) with a 10-mL BioRad Profinity IMAC Ni-Charged Resin (Biorad; Vienna, Austria). The column was equilibrated with 10 column volumes (CV) of buffer (0.05 M KH₂PO₄, 0.5 M NaCl, 20 mM imidazole; pH 6.5), and washed with 5 CV of the same buffer after loading. Enzymes were eluted with a linear gradient of 10 CV with a buffer containing 1 M imidazole. The active fractions were combined and imidazole was removed by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (Millipore; Billerica, MA, U.S.A.) with a 10-kDa cut-off membrane. The eluted, concentrated enzymes were washed 3 times with 10 mL of KH₂PO₄-buffer (50 mM, pH 6.5) and finally diluted in the same buffer to a protein concentration of 10–20 mg/mL.

2.5 Enzyme activity assays

P2Ox activity was measured with the standard chromogenic ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] assay (Danneel et al. 1992). A sample of diluted enzyme (10 μL) was added to 980 μL of assay buffer containing horseradish peroxidase (142 U), ABTS (14.7 mg) and KH₂PO₄-buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM). The absorbance change at 420 nm was recorded at 30°C for 180 s. The chromophore $\varepsilon_{420}$ used was 42.3 mM⁻¹·cm⁻¹. One Unit of P2Ox activity was defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS per min, which equals the consumption of 1 μmol of O₂ per min, under assay conditions. Protein concentrations were determined by the Bradford assay (Bradford 1976) using the BioRad Protein Assay Kit with bovine serum albumin as standard.
2.6 Steady-state kinetic measurements

Kinetic constants were calculated by non-linear least-square regression, fitting the data to the Henri-Michaelis-Menten equation. These constants were measured for the two electron donors D-glucose (0.1–50 mM) and D-galactose (0.1–200 mM) using the standard ABTS assay and oxygen (air saturation). In addition, the catalytic constants were measured for the two-electron proton acceptor 1,4-benzoquinone and the one-electron non-proton acceptor ferricenium ion Fc⁺ (ferricenium hexafluorophosphate FcPF₆) at a saturating concentration of 100 mM of either D-glucose or D-galactose. The kinetic parameters for 1,4-benzoquinone were determined by adding 10 µL of diluted enzyme to 990 µL of assay buffer containing the sugar substrate (100 mM), KH₂PO₄-buffer (50 mM, pH 6.5) and 1,4-benzoquinone (0.01–2 mM). The absorbance change at 290 nm was recorded at 30°C for 180 s. The chromophore ε₂₉₀ used was 2.24 mM⁻¹·cm⁻¹. FcPF₆ was varied from 0.005–1.5 mM and the absorbance change at 300 nm was recorded at 30°C for 180 s. The chromophore ε₃₀₀ used was 4.3 mM⁻¹·cm⁻¹.

2.7 Electrophoresis

To check the purity and the correct molecular mass of the purified enzymes electrophoresis was done as described by Laemmli (1970). Both native PAGE and SDS-PAGE were performed using a 5% stacking gel and a 10% separating gel in a PerfectBlue vertical electrophoresis system (Peqlab; Erlangen, Germany). Samples were diluted to 1–2 mg of protein per mL and aliquots of 5 µL were loaded per lane. The High Molecular Weight Calibration Kit (Amersham; NJ, U.S.A.) and the Precision Plus Protein Dual Color Kit (Biorad) were used as mass standards for native electrophoresis and SDS-PAGE, respectively. Gels were stained with Coomassie blue.
2.8 Bioreactor cultivations

To produce sufficient amounts of the wild-type and the mutated enzyme V546C/T169G two batch cultivations were carried out in a 5-L Biostat MD stirred tank reactor with a working volume of 4 liters. Both cultivations were done in TBamp-medium. A preculture grown in an Erlenmeyer flask containing 200 mL of sterile TBamp-medium (37°C, 140 rpm) was transferred to the bioreactor at an OD600 of ~ 0.5. The temperature was set to 25°C, the culture pH was maintained at pH 7.0 by automatic addition of sterile NaOH (4 M), and the dissolved oxygen concentration (DO2) was set to 30%. DO2 levels were maintained by supplying filtered air automatically (0–4 L/min) and adjusting the stirrer velocity. The parameters were controlled by the IMCS-2000 digital control unit (PCS AG; Wetzikon, Switzerland). The medium was supplemented with 5 g/L lactose for induction of P2Ox expression from the beginning of the cultivation. Samples were taken every 2 h to measure the optical density, total intracellular protein concentration and P2Ox activity to monitor the enzyme production. When the volumetric activity reached a maximum the cells were harvested by centrifugation for 10 min at 16,000×g. Cells were homogenized and enzymes were purified as described above.

2.9 Batch conversion experiments

Wild-type P2Ox and the variant V546C/T169G were compared in terms of their ability to concomitantly oxidize D-glucose and D-galactose to the corresponding 2-ketoalldoses with either oxygen or 1,4-benzoquinone as electron acceptor. Four batch conversion experiments (300 mL) were performed in parallel in a multifermenter (Infors; Bottmingen, Switzerland). Specific activity of wild-type P2Ox with D-glucose was determined with 7.6 U/mg, of variant V546C/T169G with 0.08 U/mg. For 300-mL batch conversions 45 U of wild-type P2Ox and 5 U of mutated enzyme were used. Catalase was used in excess (100,000 Units) to decompose H2O2. When 1,4-benzoquinone was applied as electron acceptor in excess (5 mM), laccase was
used in a three times higher activity than P2Ox (150 Units and 15 Units, respectively) to reoxidize the formed hydroquinone (Ludwig et al. 2004). Laccase from *Trametes pubescens* MB 89 was prepared according to (Galhaup et al. 2002) and purified to a specific activity of 64.5 U·mg⁻¹. The conversion experiments were conducted in 100 mM sodium citrate buffer (pH 5.0) at 30°C, 300 rpm and a DO₂ concentration of 20%. As calculated from the volumetric activity of the wild-type enzyme with its substrate D-galactose, both conversion reactions were performed with 0.75 g·L⁻¹ of both D-glucose and D-galactose to ensure complete conversion within reasonable time for the selected activities. For the batch experiments with the mutated variant 1.4 g·L⁻¹ of each sugar were used, again based on the volumetric activity of the mutant enzyme.

Samples (2 mL) were taken periodically, held at 95°C for 3 min to inactivate the enzymes and centrifuged. The supernatants were analyzed for their sugar content using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), which was carried out using a Dionex DX-500 system (Dionex; Sunnyvale, CA, USA) and a CarboPac PA-1 column (4 × 250 mm) at 27°C (Splechtna et al. 2006).

### 3 Results

#### 3.1 Generation of mutants

The presence of different mutations at position V546 in the *TmP2Ox* gene after saturation mutagenesis was confirmed by DNA sequence analysis of 10 randomly picked clones, proving the successful application of this technique. Six variants, which showed the highest activity with D-galactose in the 96-well plate screening assay, were isolated and were all identified as V546C encoded by either of the two possible codons for the amino acid cysteine (TGT, TGC). Variant V546C was expressed with a C-terminal His-tag added in *E.coli*, purified by IMAC and concentrated by ultrafiltration as previously described (Hallberg et al. 2004; Spadiut et al.
The purity of the protein preparations was confirmed by native PAGE and SDS-PAGE (Figure 1). Subsequently, this variant was combined with already described mutations of *Tm*P2Ox to possibly combine their positive effects. T169G shows dramatically decreased $K_M$ values for both sugar substrates D-glucose and D-galactose (Spadiut et al. 2008b), and the variants E542K and L537W are characterized by elevated $k_{cat}$ and reduced $K_M$ values for some electron acceptors and significantly increased stability (Spadiut et al. 2008a). Thus, the P2Ox variants V546C/T169G, V546C/E542K and V546C/T169G/L537W were produced, purified to apparent homogeneity and characterized.

**Figure 1.** Native PAGE (lanes 1, 2) and SDS-PAGE (lanes 3, 4) analysis of *Trametes multicolor* P2Ox mutational variant V546C. Lanes 1 and 4, molecular mass marker proteins; lanes 2 and 3, V546C after purification by IMAC.

### 3.2 Kinetic characterization of mutational variants

Initial rates of activity were recorded over a substrate range of 0.1 to 50 mM D-glucose and 0.1 to 200 mM D-galactose for wild-type P2Ox and the mutational variants V546C, V546C/T169G, V546C/E542K and V546C/T169G/L537W, using the standard ABTS assay and air oxygen. Kinetic data are summarized in Table 1. The turnover number $k_{cat}$ of mutational variant V546C increased significantly for both substrates D-glucose and D-galactose compared to the wild-type enzyme (1.8- and 2.6-fold, respectively). Albeit, $K_M$ values
increased as well (3- and 5-fold, respectively) resulting in an overall reduced catalytic efficiency $k_{cat}/K_M$. In order to lower $K_M$ we introduced several additional amino acid substitutions that are known to affect the kinetic properties of $TmP2Ox$ into the V546C variant. The Glu $\rightarrow$ Lys substitution at position 542 is known to decrease the $K_M$ value for sugar substrates considerably while lowering $k_{cat}$ to some extent (Masuda-Nishimura et al. 1999; Spadiut et al. 2008a). This was confirmed when comparing the V546C/E542K double mutant with V546C. The $K_M$ values for both d-glucose and d-galactose decreased significantly (by approx. 50% compared to V546C), while $k_{cat}$ was hardly affected. Overall, V546C/E542K shows catalytic efficiencies that are comparable to the wild-type enzyme, yet with a two-fold increased turnover number for both sugar substrates.

**Table 1.** Kinetic properties of wild-type P2Ox from *Trametes multicolor* and mutational variants with either D-glucose or D-galactose as substrate and O$_2$ (air) as electron acceptor.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>D-glucose</th>
<th>D-galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.94 ± 0.04</td>
<td>48.1 ± 0.5</td>
</tr>
<tr>
<td>V546C</td>
<td>3.06 ± 0.14</td>
<td>88.6 ± 1.3</td>
</tr>
<tr>
<td>E542K$^a$</td>
<td>0.52 ± 0.02</td>
<td>35.9 ± 0.3</td>
</tr>
<tr>
<td>T169G$^b$</td>
<td>0.69 ± 0.11</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>L537W$^a$</td>
<td>0.75 ± 0.02</td>
<td>59.0 ± 0.5</td>
</tr>
<tr>
<td>V546C/E542K</td>
<td>1.52 ± 0.13</td>
<td>82.2 ± 1.9</td>
</tr>
<tr>
<td>V546C/T169G</td>
<td>0.44 ± 0.02</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>V546C/T169G/L537W</td>
<td>0.49 ± 0.11</td>
<td>0.27 ± 0.01</td>
</tr>
</tbody>
</table>

$^a$ (Spadiut et al. 2008a)  
$^b$ (Spadiut et al. 2008b)
The Thr → Gly substitution at position 169 is known to lower the \( K_M \) values dramatically, especially for D-galactose, while \( k_{cat} \) is also lowered significantly (Spadiut et al. 2008b). Again, this was confirmed when comparing V546C/T169G and V546C/T169G/L537W with V546C. Despite this reduction in \( k_{cat} \), variant V546C/T169G shows interesting properties. Wild-type P2Ox as well as V546C strongly prefer D-glucose over D-galactose as their substrate, as is expressed by their substrate specificity, i.e., the ratio of the specificity constants \( k_{cat}/K_M \) for the two substrates (Morley and Kazlauskas 2005). This value is 180 for the wild-type (Table 2), while it is 1.05 for V546C/T169G. In addition, \( K_M \) and \( k_{cat} \) for both sugar substrates D-glucose and D-galactose are almost identical for this mutational variant, indicating that both sugars are equally good substrates.

**Table 2.** Substrate selectivity of wild-type P2Ox from *Trametes multicolor* and some of its mutational variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>( (k_{cat}/K_M)<em>{glc} / (k</em>{cat}/K_M)_{gal} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtP2Ox</td>
<td>180</td>
</tr>
<tr>
<td>V546C</td>
<td>210</td>
</tr>
<tr>
<td>E542K</td>
<td>100</td>
</tr>
<tr>
<td>T169G</td>
<td>3.5</td>
</tr>
<tr>
<td>L537W</td>
<td>250</td>
</tr>
<tr>
<td>V546C/E542K</td>
<td>220</td>
</tr>
<tr>
<td>V546C/T169G</td>
<td>1.05</td>
</tr>
<tr>
<td>V546C/T169G/L537W</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Furthermore, the kinetic constants were determined for both 1,4-benzoquinone (BQ; a two electron, H\(^+\) acceptor) and ferricenium ion (Fc\(^+\); a one electron, no H\(^+\) acceptor) with either D-glucose or D-galactose in saturating concentrations (Table 3 and 4). Here the Val → Cys replacement at position 546 of *TmP2Ox* showed comparable effects as with the electron donors, it significantly increased the turnover number by a factor of two to six depending on
Chapter 5

the substrates, yet also the Michaelis constant was increased. Overall, V546C showed improved catalytic efficiencies for all substrate combinations (electron acceptor and donor) investigated. Compared to the wild-type enzyme, variant V546C/T169G had a two-fold higher catalytic efficiency with 1,4-benzoquinone and D-glucose and a nearly nine-fold increased \( k_{\text{cat}}/K_M \) with D-galactose, the latter is due to the 39-fold increased \( k_{\text{cat}} \).

Table 3. Kinetic properties of wild-type P2Ox from Trametes multicolor and mutational variants with 1,4-benzoquinone as electron acceptor and either D-glucose or D-galactose as saturating substrate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>D-glucose</th>
<th></th>
<th></th>
<th>D-galactose</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_M )</td>
<td>( k_{\text{cat}} )</td>
<td>( k_{\text{cat}}/K_M )</td>
<td>( K_M )</td>
<td>( k_{\text{cat}} )</td>
<td>( k_{\text{cat}}/K_M )</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.24 ± 0.03</td>
<td>152 ± 6.0</td>
<td>632</td>
<td>0.060 ± 0.003</td>
<td>4.79 ± 0.06</td>
<td>74.2</td>
</tr>
<tr>
<td>V546C</td>
<td>0.37 ± 0.08</td>
<td>520 ± 49</td>
<td>1410</td>
<td>0.19 ± 0.05</td>
<td>15.1 ± 1.1</td>
<td>78.9</td>
</tr>
<tr>
<td>E542K(^a)</td>
<td>0.182 ± 0.025</td>
<td>189 ± 9.2</td>
<td>1040</td>
<td>0.049 ± 0.009</td>
<td>5.52 ± 0.22</td>
<td>113</td>
</tr>
<tr>
<td>T169G(^b)</td>
<td>0.026 ± 0.011</td>
<td>6.50 ± 0.62</td>
<td>252</td>
<td>0.101 ± 0.046</td>
<td>21.7 ± 3.6</td>
<td>219</td>
</tr>
<tr>
<td>L537W(^a)</td>
<td>0.134 ± 0.013</td>
<td>205 ± 6.0</td>
<td>1580</td>
<td>0.036 ± 0.004</td>
<td>5.37 ± 0.13</td>
<td>150</td>
</tr>
<tr>
<td>V546C/E542K</td>
<td>0.37 ± 0.07</td>
<td>664 ± 54</td>
<td>1800</td>
<td>1.52 ± 0.13</td>
<td>82.2 ± 1.9</td>
<td>53.9</td>
</tr>
<tr>
<td>V546C/T169G</td>
<td>0.043 ± 0.020</td>
<td>42.1 ± 4.9</td>
<td>1040</td>
<td>0.28 ± 0.11</td>
<td>189 ± 54</td>
<td>663</td>
</tr>
<tr>
<td>V546C/T169G/ L537W</td>
<td>0.072 ± 0.048</td>
<td>24.1 ± 6.9</td>
<td>345</td>
<td>1.18 ± 0.91</td>
<td>103 ± 50.3</td>
<td>87.1</td>
</tr>
</tbody>
</table>

\(^a\) (Spadiut et al. 2008a)
\(^b\) (Spadiut et al. 2008b)

The positive effect of these mutations on the catalytic activity is even more obvious with the electron acceptor Fc\(^+\) and the sugar D-galactose. Here, the turnover number was increased 49-fold resulting in a catalytic efficiency that was increased more than 11-fold compared to the wild-type enzyme (Table 4). Variant V546C/E542K showed higher \( k_{\text{cat}} \) values for both electron acceptors in combination with both sugars, but overall its catalytic performance was not
competitive with the wild-type enzyme because of worsened substrate binding. Mutant V546C/T169G/L537W showed higher $k_{cat}$ values with D-galactose and either of the two electron acceptors, whereas for the reaction with D-glucose $k_{cat}$ was reduced. Regarding the catalytic efficiency, increased values for the reactions with D-galactose are noteworthy, but are not comparable to the improvements of variant V546C/T169G.

**Table 4.** Kinetic properties of wild-type P2Ox from *Trametes multicolor* and mutational variants with the ferricenium ion Fc$^+$ as electron acceptor and either D-glucose or D-galactose as saturating substrate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>D-glucose</th>
<th>D-galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.25 ± 0.10</td>
<td>151 ± 35</td>
</tr>
<tr>
<td>V546C</td>
<td>0.93 ± 0.14</td>
<td>866 ± 94.3</td>
</tr>
<tr>
<td>E542K$^a$</td>
<td>0.290 ± 0.096</td>
<td>54.4 ± 9.2</td>
</tr>
<tr>
<td>T169G$^b$</td>
<td>0.086 ± 0.07</td>
<td>25.9 ± 1.2</td>
</tr>
<tr>
<td>L537W$^a$</td>
<td>0.253 ± 0.093</td>
<td>334 ± 61</td>
</tr>
<tr>
<td>V546C/E542K</td>
<td>1.06 ± 0.18</td>
<td>246 ± 32</td>
</tr>
<tr>
<td>V546C/T169G</td>
<td>0.50 ± 0.31</td>
<td>221 ± 98</td>
</tr>
<tr>
<td>V546C/T169G/L537W</td>
<td>0.092 ± 0.057</td>
<td>56.4 ± 14.9</td>
</tr>
</tbody>
</table>

$^a$ (Spadiut et al. 2008a)
$^b$ (Spadiut et al. 2008b)

### 3.3 Enzyme production and substrate conversion experiments

Batch cultivations of *E. coli* BL21DE3 carrying either the wild-type or the mutated enzyme V546C/T169G, which shows comparable kinetic properties for both D-glucose and D-galactose, were carried out to obtain sufficient amounts of protein for the following conversion experiments. Biomass was harvested at an OD$_{600}$ of 8.0, when the volumetric activity of wild-
type P2Ox with D-glucose reached a maximum level of 240 U·L⁻¹, corresponding to approximately 30 mg protein per liter. Then the purified enzymes were used for batch conversion experiments using an equimolar mixture of D-glucose and D-galactose as substrate, and either oxygen or 1,4-benzoquinone as electron acceptor (Figure 2). Six mg of purified wild-type enzyme, corresponding to 45 D-glucose Units under standard assay conditions, and 60 mg of purified mutant protein, corresponding to 5 D-glucose Units, were used for each batch conversion. When oxygen was the electron acceptor the wild-type enzyme clearly preferred D-glucose over D-galactose as substrate. Only when D-glucose was completely consumed, D-galactose was converted at a very low rate of 0.02 g·L⁻¹·h⁻¹ during the batch conversion. This conversion rate was 100-fold lower than the value of 2.0 g·L⁻¹·h⁻¹ calculated for D-glucose, again stressing the considerable discrimination of D-glucose oxidation versus that of D-galactose. In contrast, variant V546C/T169G showed identical conversion rates of 0.2 g·L⁻¹·h⁻¹ for both D-glucose and D-galactose under the reaction conditions. The mutant did not prefer any of the sugars as its substrate but oxidized both of them concomitantly in equal rates as was also predicted by the kinetic constants determined.

When 1,4-benzoquinone was the electron acceptor the catalytic activity of both enzymes with either D-glucose or D-galactose was increased 2- to 3-fold compared to oxygen (air saturation), resulting in significantly faster turnover of the sugars. Again, the wild-type enzyme reached a more than 100-fold higher conversion rate for D-glucose (6.65 g·L⁻¹·h⁻¹) than for D-galactose (0.047 g·L⁻¹·h⁻¹), whereas the conversion rates for the sugars were identical (0.54 g·L⁻¹·h⁻¹) when the mutated variant was used.
Figure 2. Batch conversion experiments of an equimolar mixture of the sugars D-glucose and D-galactose and either oxygen or 1,4-benzoquinone as electron acceptor, using wild-type *TmP2Ox* or the variant V546C/T169G as biocatalyst. A, wtP2Ox and oxygen; B, V546C/T169G and oxygen; C, wtP2Ox and 1,4-benzoquinone; D, V546C/T169G and 1,4-benzoquinone. Symbols: ●, D-glucose; ■, D-galactose

4 Discussion

During the last years, both rational design and directed evolution have emerged as powerful methods for engineering, redesigning and improving both functions and properties of enzymes (Penning and Jez 2001; Williams et al. 2004; Toscano et al. 2007; Jäckel et al. 2008). Analysis of enzyme engineering results indicates that the majority of mutations that beneficially affect enzyme properties such as substrate selectivity or catalytic promiscuity are located in or close to the active site, and in particular near residues that are involved in catalysis or substrate binding. For other properties, such as stability or activity, both close and distant mutations
seem similarly effective in engineering enzymes (Chica et al. 2005; Morley and Kazlauskas 2005). In order to improve the enzyme pyranose oxidase from *Trametes multicolor* with respect to its substrate promiscuity and selectivity (i.e., increased activity with a broader range of electron acceptors and increased activity with its poor substrate D-galactose) we used a semi-rational approach, saturation mutagenesis of active-site residues involved in binding of the carbohydrate substrate of *Tm*P2Ox and subsequent screening in 96-well plates. While the structures of P2Ox with its bound slow substrate 2-fluoro-2-deoxy-D-glucose (Kujawa et al. 2006) and its product 2-keto-D-glucose (Bannwarth et al. 2006) are known, the structure of P2Ox liganded with an electron acceptor such as benzoquinone or a ferrocene is not known to date. In accordance with structural models of glucose oxidase, however, one can assume that at least the less bulky of these molecules also enter the active site of P2Ox and bind in direct vicinity of the isoalloxazine ring and hence interact with at least some of the active-site residues (Alvarez-Icaza et al. 1995; Wohlfahrt et al. 2004; Leskovac et al. 2005). When screening the libraries of *Tm*P2Ox variants, which were obtained after saturation mutagenesis at position Val546, we found several mutants that reacted faster with D-galactose in the microtiter-plate based screening assay than the wild-type. Val546 is positioned directly in the active site, interacting with the C1 hydroxyl group of the bound sugar substrate by forming a hydrogen bond through its carbonyl oxygen. Six of these improved variants were selected for sequencing, and all contained a cysteine at this position. Cys was coded by either of the two possible codons (TGT, TGC), which proved the successful application of saturation mutagenesis. In proteins Cys are frequently found either in disulfide linkages or in the solvent-inaccessible interior, but typically not in the active site (Bulaj et al. 1998). In this study the replacement of the hydrophobic amino acid Val by the small, polar amino acid Cys positively affected the turnover rates for both the sugar substrates and the alternative electron acceptors investigated. However, the introduction of Cys also lowered the ability of *Tm*P2Ox to bind
these substrates, as indicated by elevated $K_M$ values. Hence, the catalytic efficiency $k_{cat}/K_M$ of V546C for the sugar substrates was lower than that of the wild-type P2Ox.

Recombinations of beneficial mutations are often used to improve enzymes subsequent to saturation mutagenesis studies (Chica et al. 2005). Therefore, the additional mutations T169G, E542K and L537W, which had shown positive effects on the catalytic properties of TmP2Ox before (Spadiut et al. 2008a; Spadiut et al. 2008b), were introduced into V546C for further improvements of the catalytic efficiency. These different mutational variants were characterized pertaining to their kinetic properties and compared to the wild-type. Some of the properties of these new TmP2Ox variants make them highly interesting for various biotechnological applications. As mentioned above, enzymes can transfer electrons from their prosthetic group to an electrode in MET by using so-called mediators, rather small molecules such as organic complexes of iron, osmium or ruthenium as well as benzoquinones, in biofuel cell and biosensor applications. The successful wiring of P2Ox to graphite electrodes was recently shown using an osmium redox polymer as well as a ferrocene (Nazaruk and Bilewicz 2007; Tasca et al. 2007), and hence P2Ox could be of interest as the bio-component in biofuel cell anodes. In biofuel cells, the current output depends directly on the actual turnover rate of the enzyme, and consequently an enzyme with an increased catalytic constant will also boost the power output of the biofuel cell, which is especially attractive for implantable miniature biofuel cells (Heller 2004). In this respect, some of the studied variants exhibit promising catalytic properties. V546C shows increased $k_{cat}$ values for all the substrates and substrate couples tested (D-glucose, D-galactose, BQ, Fc$^+$). For some of these $k_{cat}$ increased 3.4-fold (D-glucose with air oxygen) or even up to 5.7-fold (Fc$^+$ with D-glucose as the saturating substrate) relative to the wild-type. Variant V546C/E542K showed high $k_{cat}$ values for 1,4-benzoquinone regardless of the sugar used, with a 4.4-fold and a 17-fold increase compared to the wild-type when D-glucose and D-galactose, respectively, were present in excess. In turn, highest $k_{cat}$
values for both mediators and d-galactose as saturating substrate were obtained for V546C/T169G, with approximately 40-fold and 50-fold increases for BQ and Fe\(^{+}\), respectively. Depending on the envisaged set-up of a biofuel cell and the available sugar substrates, different TmP2Ox active-site variants show clearly improved and advantageous catalytic properties.

A second area of applications of P2Ox has been proposed in carbohydrate conversions (Giffhorn 2000), and for this purpose variant V546C/T169G is of interest. Wild-type TmP2Ox clearly prefers d-glucose (\(k_{\text{cat}}/K_{M} = 51,200 \text{ M}^{-1}\cdot\text{s}^{-1}\)) over d-galactose (\(k_{\text{cat}}/K_{M} = 2,900 \text{ M}^{-1}\cdot\text{s}^{-1}\)), and therefore the substrate selectivity of wild-type TmP2Ox for these two substrates can be calculated as 180. This ratio changes to 1.05 for V546C/T169G, a significant reduction of the selectivity, however, comes at a cost in \(k_{\text{cat}}\), which is reduced for both sugar substrates. Again, mutations of active-site residues proved to be very efficient in altering substrate selectivity as was previously shown for other examples (Morley and Kazlauskas 2005). In contrast to the wild-type enzyme, V546C/T169G catalyzes the concomitant oxidation of both sugars equally well, as was confirmed in small-scale bioconversion experiments.

D-Galactose differs from D-glucose by having the C-4 hydroxyl group in axial position rather than equatorial. As described elsewhere, we presume that the axial C-4 hydroxyl group of D-galactose appears sterically hindered by the side chain of Thr169, thus providing a tentative explanation for the lower turnover and the relatively high \(K_{M}\) of this monosaccharide by P2Ox (Spadiut et al. 2008b). The replacement of Thr by Gly creates additional space in the active site so that the sugar substrates can be accommodated more easily, resulting in lowered \(K_{M}\) values, as is for example seen by the reduced \(K_{M}\)-value for D-galactose of the T169G variant compared to that of the wild-type (2.48 and 8.79 mM, respectively). Combining T169G with the V546C mutation results in an additional drastic decrease of \(K_{M}\) to 0.40 mM, which is more than 100-fold lower than for the single V546C variant. Apparently, the creation of extra space in the
active site, as in T169G, alleviates some of the unfavorable interactions that cause the increase in $K_M$ for carbohydrate substrates observed for the V546C variant.

In conclusion, the semi-rational approach selected for the engineering of P2Ox with respect to its substrate specificity and promiscuity proved very successful. By combining the V546C variant with other mutations, some of which are also located in the active site, we obtained a set of biocatalysts that show clearly improved catalytic properties compared to the wild-type. These variants can be used for example in carbohydrate biotransformations or as the anodic bio-component in biofuel cells. This latter application is currently studied in our laboratories.

Acknowledgements

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5 References


Chapter 6

Mutations in the active site loop of pyranose 2-oxidase from Trametes multicolor dramatically affect substrate specificity and catalytic activity with sugar substrates and electron acceptors

Oliver Spadiut\textsuperscript{1}, Dagmar Brugger\textsuperscript{1}, Tien-Chye Tan\textsuperscript{2}, Christina Divne\textsuperscript{2} & Dietmar Haltrich\textsuperscript{1}

\textsuperscript{1}Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

\textsuperscript{2}School of Biotechnology, KTH, Albanova University Centre, SE-106 91 Stockholm, Sweden

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Mutations in the active site loop of pyranose 2-oxidase from *Trametes multicolor* dramatically affect catalytic substrate specificity and activity with sugar substrates and electron acceptors

Oliver Spadiut\(^1\), Dagmar Brugger\(^1\), Tien-Chye Tan\(^2\), Christina Divne\(^2\) & Dietmar Haltrich\(^1\)

\(^1\)Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

\(^2\)School of Biotechnology, KTH, Albanova University Centre, SE-106 91 Stockholm, Sweden

*Corresponding author:* Dietmar Haltrich; Department of Food Sciences and Technology, BOKU – University of Natural Resources and Applied Life Sciences, A-1190 Vienna, Austria

e-mail: dietmar.haltrich@boku.ac.at; tel.: +43-1-36006-6275, fax: +43-1-36006-6251
Abstract

Pyranose 2-oxidase from *Trametes multicolor* (*TmP2Ox*) is a homotetrameric enzyme which oxidizes various aldopyranoses regioselectively at C2 to the corresponding 2-keto sugars. The crystal structure of *TmP2Ox* was refined at 1.8 Å resolution and revealed that each of the four active sites is blocked by a loop. This active site loop is dynamic and only allows the various substrates to enter the active site in an open conformation. The loop is important for substrate binding and catalysis and is also the discriminating factor of C2 versus C3 oxidation of the sugar substrates. The analysis of this loop by alanine-scanning substitutions, site-saturation mutagenesis and by removing parts of the loop showed that the different amino acids in the loop have different significant effects on the catalytic activity as well as on the substrate specificity of the enzyme. Especially mutant H450G draws attention in terms of a 3 times decreased catalytic efficiency (*k*<sub>cat</sub>/*K*<sub>M</sub>) with the substrate D-glucose, but a more than 5 times increased *k*<sub>cat</sub>/*K*<sub>M</sub> with D-galactose. Besides, the activity of this variant with both alternative electron acceptors, 1,4-benzoquinone and ferricenium ion, with either of the two sugar substrates in saturating concentrations, was increased significantly making this variant interesting for applications in biofuel cells. We further showed that mutations in the active site loop can even increase the kinetic stability of the enzyme; variant Y456W showed a 34-fold increased stability at 60°C compared to the wild-type enzyme.

**Keywords:** pyranose 2-oxidase; active site loop; catalytic activity; site-saturation mutagenesis; loop deletion; crystal structure
Abbreviations: CV, column volume; BQ, 1,4-benzoquinone; FAD, flavin adenine dinucleotide; Fe⁺, ferricenium ion; IMAC, immobilized metal affinity chromatography; P2Ox, pyranose 2-oxidase; TmP2Ox, pyranose 2-oxidase from *Trametes multicolor*

1 Introduction

Pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; glucose 2-oxidase; EC 1.1.3.10) from *Trametes multicolor* (*Tm*P2Ox; synonym = *Trametes ochracea*) is a flavin adenine dinucleotide (FAD) dependent oxidoreductase (Daniel et al. 1994, Volc et al. 1985, Leitner et al. 1998). It catalyzes the regioselective oxidation of several aldopyranoses at position C2 by molecular oxygen to the respective 2-ketoaldoses and hydrogen peroxide (H₂O₂). This reaction mechanism is of the typical Ping Pong Bi Bi type, which is common in flavoprotein oxidoreductases (Artolozaga et al. 1997; Ghisla & Massey 1989). After a reductive half-reaction, in which an aldopyranose is oxidized at position C-2 to the corresponding 2-ketoalldose and the cofactor FAD is reduced to FADH₂ (Freimund et al. 1998), the cofactor is reoxidized by an electron acceptor in an ensuing oxidative half-reaction. Judged by the catalytic efficiency (*k*_cat_/K*_M*_), D-glucose is the best substrate for *Tm*P2Ox whereas D-galactose, which is interesting for technical applications, performs poorly with only 5.7% relative activity (Leitner et al. 2001). Oxidation of D-galactose at position C-2 leads to the interesting intermediate 2-keto-D-galactose (2-dehydro-D-galactose, galactosone), which can easily be reduced at C-1 to yield D-tagatose (Haltrich et al. 1998), which is a ketose sugar with significant potential as a non-cariogenic, low caloric sweetener in food industry. Other applications of P2Ox for the synthesis of rare sugars, fine
chemicals and drugs have recently been reviewed (Giffhorn 2000). To avoid the formation of inhibiting H₂O₂, alternative electron acceptors like 1,4-benzoquinone (BQ) and ferrocenium hexafluorophosphate (FcPF₆) can be used. These electron acceptors also serve as electron mediators in biofuel cells and biosensors (Zhu et al. 2007, Tamaki et al. 2007, Nazaruk and Bilewicz 2007). Biofuel cells convert sugars into electrical energy by employing oxidoreductases on electrodes. Recently, we could show that TmP2Ox can be electrically wired to graphite electrodes through the use of osmium redox polymers (Tasca et al. 2007), which gather the electrons from the enzyme and transfer them to the electrode. We further demonstrated that the measured current represents the actual turnover rate of the immobilized enzymes. Consequently, higher catalytic activities are required for boosting the power output of these biofuel cells.

The crystal structure of TmP2Ox, which was determined at 1.8 Å resolution in 2004 (Hallberg et al. 2004), revealed that the enzyme is a homotetrameric protein with a molecular mass of 270 kDa with each of the four 68-kDa subunits carrying one FAD covalently bound (Halada et al. 2003, Hallberg et al. 2004). Four solvent channels lead from the surface of the enzyme into a large internal cavity, from which the four active sites are accessible through narrow active site channels. Each of the four active sites is blocked by a flexible active site loop (residues 450-HRDAFSYG-457). The loop is dynamic and only allows the substrates to enter the active site in an open conformation. In silico docking of D-glucose in the active site has shown that monosaccharides can not be accommodated when the loop is closed and that this restriction is mainly due to the position of the Phe454 and Tyr456 side chains. Thus, a major conformational change of the active site loop is required to allow the entry and the binding of the carbohydrate substrates. Recently, we reported the crystal structure
of *Tm*P2Ox in complex with one of its slow substrates, 2-fluor-2-deoxy-D-glucose (Kujawa et al. 2006). This structure gave information about the amino acids which interact with the sugar substrate in the active site and elucidated the function of the active site loop and its huge impact on substrate binding and catalysis. We showed that the tentative determinant for discriminating between the two binding modes of the monosaccharide substrates (C2 versus C3) is the position of the C6 hydroxyl group of the sugar substrate, which, only in the C2-oxidation mode, makes favourable interactions with the side chain of Asp452 in the active site loop. This interaction is just possible because of a total loop rearrangement. As the active site loop rearranges to allow binding of the electron-donor (i.e. aldopyranose), Asp452 is brought into the active site as a potential discriminator in the mechanism of regioselectivity and its side chain becomes accessible for interactions. The importance of this aminoacid in the active site loop for substrate binding and catalysis was further shown by kinetic studies on the variant Asp452Ala.

This study was initiated to investigate the crucial role of the active site loop of P2Ox from *Trametes multicolor* in the catalytic function of the enzyme by alanine-scanning mutagenesis, site-saturation mutagenesis and by removing parts of the loop. The fact that active site loops are very important for substrate binding and catalysis was shown before for different enzymes (Sampson et al. 1998, Lauhon et al. 2004, Venkitakrishnan et al. 2004, Shinoda et al. 2005). In the present work we show that the active site loop of *Tm*POx is crucial for substrate binding, catalysis and substrate specificity for different sugar substrates and electron acceptors, and that mutations at different sites in the active site loop also influence the thermal stability of the enzyme.
2 Material and Methods

2.1 Plasmids, microorganisms and media

The construction of the pET21d’/P2Ox vector (pHL2), which expresses the His tagged pyranose 2-oxidase gene from *Trametes multicolor* under the control of a T7 promoter, has already been described (Kujawa et al. 2006). Active, recombinant *Tm*P2Ox and P2Ox mutants were expressed in the *E. coli* strain BL21 Star DE3 (Invitrogen, Carlsbad, CA, U.S.A.). TB<sub>amp</sub>-medium (yeast extract 24 g·L<sup>-1</sup>, peptone from casein 12 g·L<sup>-1</sup>, glycerol 4 mL·L<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub>-buffer 1 M, pH 7.5) was used to cultivate *E. coli* cells for protein expression under appropriate selective conditions (ampicillin was added to 0.1 g·L<sup>-1</sup>). LB<sub>amp</sub>-medium (yeast extract 5 g·L<sup>-1</sup>, peptone from casein 10 g·L<sup>-1</sup>, NaCl 5 g·L<sup>-1</sup>, ampicillin 0.1 g·L<sup>-1</sup>) was used to cultivate *E. coli* cells in 96-well screening plates. For induction of protein expression cells were grown in 2x LB<sub>amp</sub>/lactose-medium (yeast extract 10 g·L<sup>-1</sup>, peptone from casein 20 g·L<sup>-1</sup>, NaCl 5 g·L<sup>-1</sup>, lactose 5 g·L<sup>-1</sup>; ampicillin 0.1 g·L<sup>-1</sup>). All chemicals used were purchased from SIGMA (Vienna, Austria) and were of the purest grade available.

2.2 Generation of mutants

The plasmid pHL2 was always used as template for mutagenic PCRs. Alanine mutants were prepared by the sequence overlap extension method (Ho et al. 1989) using the primers F454A_Y456A_fwd, F454A_S455A_Y456A_fwd, F454A_S455A_Y456A_rev and the flanking primers T7fwd and T7rev. The other mutants were prepared by a two-step mutagenesis method using PCR and digestion with the restrictase *DpnI* (Li and Wilkinson 1997). The aminoacids F454, S455 and Y456 were removed to create the loop deletion variant. For site-saturation mutagenesis the forward primers carried the variable position NNN, where N = A, G,
C or T, at the position of interest. The nucleotide sequences of the various synthetic oligonucleotides are listed in Table 1.

**Table 1.** Nucleotide sequences of synthetic oligonucleotides used for the creation of different TmP2Ox mutants.

<table>
<thead>
<tr>
<th>Oligonucleotide Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F454A_Y456A_fwd</td>
<td>5’-gcatgctgccagcggcgcag-3’</td>
</tr>
<tr>
<td>F454A_S455A_Y456A_fwd</td>
<td>5’-gcagctgctgccggcgcag-3’</td>
</tr>
<tr>
<td>F454A_S455A_Y456A_rev</td>
<td>5’-ctgtagcgcagcgcgcagctg-3’</td>
</tr>
<tr>
<td>T7_fwd</td>
<td>5’-agatctcgtccgagaattatagctcacttag-3’</td>
</tr>
<tr>
<td>T7_rev</td>
<td>5’-cccaagggtttatgcattttgcgcgtg-3’</td>
</tr>
<tr>
<td>H450wobble_fwd</td>
<td>5’-acactcagatcnnncgcgatgctttcagttacgg-3’</td>
</tr>
<tr>
<td>H450wobble_rev</td>
<td>5’-ccgtaactgaaagcatcgcgnnngatcgtagtgt-3’</td>
</tr>
<tr>
<td>D452wobble_fwd</td>
<td>5’-cagatcaccgcnnngccttcagttacgg-3’</td>
</tr>
<tr>
<td>D452wobble_rev</td>
<td>5’-ccgtaactgaaagcnnngcggtggatctg-3’</td>
</tr>
<tr>
<td>F454wobble_fwd</td>
<td>5’-accgcgatgctnnnagttacggcg-3’</td>
</tr>
<tr>
<td>F454wobble_rev</td>
<td>5’-cgcggtaactinnngcctgcgggt-3’</td>
</tr>
<tr>
<td>Y456wobble_fwd</td>
<td>5’-atgctttcagtnnnngcgcaggtgc-3’</td>
</tr>
<tr>
<td>Y456wobble_rev</td>
<td>5’-gcacgtgcctgcaactgcaagcat-3’</td>
</tr>
<tr>
<td>loop_del_fwd</td>
<td>5’-tgccacactcagatcaccgcgcgcgcgcagttgcgcgtgc-3’</td>
</tr>
<tr>
<td>loop_del_rev</td>
<td>5’-tcgccgcgggtgatcgtgcgtgctgcacgggcgtgc-3’</td>
</tr>
</tbody>
</table>

The PCR reaction mix contained 2.5 Units Phusion DNA polymerase (New England Biolabs; Ipswich, MA, U.S.A.), ~100 ng of plasmidic DNA, 5 pmol of each primer, 10 µM of each dNTP and 1x PCR buffer in a total volume of 50 µl. The mutagenic PCRs were done using the following conditions: 98°C for 20 sec, then 30 cycles of 98°C for 10 sec; 55°C for 20 sec; 72°C for 4 min, followed by a final incubation at 72°C for 7 min. After PCR, the methylated template-DNA was degraded by digestion with 10 Units of the restriction enzyme DpnI at 37°C for 3 hours. The remaining PCR products were separated by an agarose gel electrophoresis and purified using the
Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, U.S.A.). Five µL of the PCR products were transformed into electro-competent *E.coli* BL21 Star DE3 cells. The P2Ox-encoding insert was sequenced using primers T7fwd and T7rev to confirm the presence of the correct mutations and the absence of undesired mutations.

### 2.3 Screening assay for P2Ox variants in 96-well plates

The positions H450, D452, F454 and Y456 were mutated by site-saturation mutagenesis, which allowed the creation of a mutant-library containing all possible codons at the target positions. The size of the library, which had to be screened to statistically cover 95% of all possible mutants, was determined by the mutagenic codon NNN (Georgescu et al. 2003). Instead of the required 190 colonies, we screened 360 to cover more than 95% of all possible combinations with very high probability. A screening assay based on 96-well plates, required for that purpose, has been described elsewhere (Spadiut et al. 2008). Six wells per plate were inoculated with *E. coli* containing the wild-type enzyme for comparison. The average value of the specific activity of the wild-type P2Ox with different substrates was determined and compared with the values of the mutants. The screening assay was performed with both sugar substrates D-glucose and D-galactose and air saturation. The mutated enzymes were allowed to react with the sugar substrates at room temperature in the 96-well plates for 24 hours. If there was still no activity observable then, these variants were assumed to be inactive. Ten active mutants (showing high, moderate or low activity with either of the two sugar substrates) were picked randomly and sent for sequencing to prove the success of site-saturation mutagenesis. Interesting variants were further analyzed.
2.4 Protein expression and purification

Cultures (1 litre) of *E. coli* BL21 Star DE3 transformants were grown in TB<sub>amp</sub>-medium in shaken flasks at 37°C and 160 rpm. Recombinant protein expression was induced by adding lactose to a final concentration of 5 g·L<sup>−1</sup> at an OD<sub>600</sub> of ~0.5. After incubation at 25°C for further 20 hours, about 20 g wet biomass per litre were harvested by centrifugation at 10,000xg in a Sorvall Evolution centrifuge (Kendro; Vienna, Austria) for 20 min, resuspended in KH<sub>2</sub>PO<sub>4</sub>-buffer (50 mM, pH 6.5) containing the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF; 1 g·L<sup>−1</sup>) and disrupted in a continuous homogenizer (APV Systems; Silkeborg, Denmark). After separation of the crude extract from the cell debris by centrifugation (70,400xg, 4°C) in a Beckman L70 Ultracentrifuge (Beckmann Instruments; Vienna, Austria), the crude extract was used for protein purification by immobilized metal affinity chromatography (IMAC) with a 10-mL BioRad Profinity IMAC Ni-Charged Resin (Biorad; Vienna, Austria). The column was equilibrated with 10 column volumes (CV) of buffer (0.05 M KH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, 20 mM imidazole; pH 6.5), and washed with 5 CV of the same buffer after loading. A linear gradient of 10 CV with a buffer containing 1 M imidazole was applied to elute the enzymes. Active fractions were combined and imidazole was removed by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (Millipore; Billerica, MA, U.S.A.) with a 10-kDa cut-off membrane. After 3 washing steps with 10 mL of KH<sub>2</sub>PO<sub>4</sub>-buffer (50 mM, pH 6.5) the enzymes were finally diluted in the same buffer to a protein concentration of 5 – 10 mg·mL<sup>−1</sup>.
2.5 Enzyme activity assays

P2Ox activity was measured with the standard chromogenic ABTS [2,2’-azinobis(3-ethylbenzthiazolesulfonic acid)] assay (Danneel et al. 1992). The exact procedure has already been described elsewhere (Spadiut et al. 2008). One Unit of P2Ox activity was defined as the amount of enzyme necessary for the oxidation of 2 μmol of ABTS per min under the assay conditions. Kinetic constants were calculated by non-linear least-square regression, fitting the data to the Henri-Michaelis-Menten equation. Protein concentrations were determined by the Bradford assay (Bradford M. 1976) using the BioRad Protein Assay Kit with bovine serum albumin as standard.

2.6 Steady-state kinetic measurements

The catalytic constants were determined for the two electron donors D-glucose (0.1-50 mM) and D-galactose (0.1-200 mM) using the standard ABTS assay and oxygen (air saturation). The constants were calculated by non-linear least-square regression, fitting the data to the Henri-Michaelis-Menten equation. Additionally, the catalytic constants were measured for the two-electron proton acceptor 1,4-benzoquinone (BQ) and the one-electron non-proton acceptor ferricenium ion Fc⁺ (ferricenium hexafluorophosphate FePF₆) at a saturating concentration of 100 mM of either D-glucose or D-galactose. The exact procedure has already been described by Spadiut and colleagues (2008).

2.7 Thermal stability

Kinetic stability of the TmP2Ox variants was determined by incubating the enzymes in appropriate dilutions in KH₂PO₄-buffer (50 mM, pH 6.5) at 60°C and 70°C, respectively, and by subsequent measurements of the enzyme activity at various time
points using the standard ABTS assay and D-glucose as sugar substrate at a constant concentration of 20 mM. A thermal cycler (thermocycler T3, Biometra; Göttingen, Germany) and thin-wall PCR tubes were used for all thermostability measurements. The inactivation constant $k_{in}$ was obtained by linear regression of ln(residual activity) versus time (Polizzi et al. 2007). The half-life values of thermal inactivation ($\tau_{1/2}$) were calculated using $\tau_{1/2} = \ln 2/kin$.

2.8 pH optima

The pH optima of the different enzyme variants were determined by measuring the activity with the standard ABTS assay and D-glucose as sugar substrate at a constant concentration of 20 mM at different pH values. To cover a broad range of pH values 3 different buffers were used for the activity measurements, namely citrate-buffer (50 mM; pH 2.5 – 6.0), phosphate-buffer (50 mM; pH 6.0 – 8.0) and borate-buffer (50 mM; pH 8.0 – 10.0).

2.9 FAD spectra

The covalently bound FAD cofactor of $TmP2Ox$ displays typical absorption maxima at 275, 345 and 456 nm. FAD absorption spectra of all variants and the wild-type enzyme were recorded using a DU 7400 spectrophotometer (Beckmann Instruments; Vienna, Austria) with diode array detection at room temperature. The enzymes were reduced by using D-glucose (10 mM) and sodium dithionite (5 mM) in the absence of oxygen and the spectra of the reduced states were recorded again. To check if the introduced mutations negatively affected the covalently binding of the cofactor FAD, trichloroacetic acid was added to the enzyme dilutions to a final concentration of 5% (v/v) followed by a heat treatment at 100°C for 10 min to completely denature the
enzymes. After separation of the denatured polypeptides from the supernatants by centrifugation at 13,200 rpm for 30 min, the FAD absorption spectra of the supernatants were recorded.

2.10 Electrophoresis

To check the molecular mass of the purified enzymes and the formation of inclusion bodies electrophoresis was done as described by Laemmli (1970). SDS-PAGE was performed using a 5% stacking gel and a 10% separating gel in a PerfectBlue vertical electrophoresis system (Peqlab; Erlangen, Germany). Enzyme samples were diluted to 0.5-1.0 mg of protein per mL and aliquots of 5 µL were loaded per lane. To check the formation of inclusion bodies, 50 mg of cell debris of each enzyme variant were washed 3 times with KH$_2$PO$_4$-buffer (50 mM, pH 6.5), resuspended in 100 µL of Laemmli buffer and treated at 95°C for 5 minutes. Aliquots of 5 µL were loaded per lane. The Precision Plus Protein Dual Color (Biorad) was used as mass standard. Gels were stained with Coomassie blue.

3 Results

3.1 Generation of mutants

Sequence comparison revealed that the aminoacids of the putative active site loops (H450-G457) of pyranose oxidases isolated from different organisms are highly conserved (Figure 1), suggesting that all these enzymes employ these active site loops for common roles in their catalytic reactions and that this structural element plays a crucial role in the function of these enzymes. Just P2Ox from *Tricholoma matsutake* differs in its aminoacid composition of this region. The other six organisms show exactly the same, highly conserved sequence of their putative active site loops.
Therefore we decided to analyze the active site loop of \( \text{TmP2Ox} \) by mutational studies.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Accession Number</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. multi.</td>
<td>AAP40332</td>
<td>PEPQVTTLFQPSHPWHTQIHRDAFSYGAQVQSIDSRILDWRFFG</td>
</tr>
<tr>
<td>Pha.chrys.</td>
<td>AAS93628</td>
<td>PEPQVTIKFTEEHPWHVQIHRDAFSYGAENMDTRVIVDYRFFG</td>
</tr>
<tr>
<td>Peni. gig.</td>
<td>AAQ72486</td>
<td>PEPQVTTLFQPSHPWHTQIHRDAFSYGAQVQSIDSRILDWRFFG</td>
</tr>
<tr>
<td>T. vers.</td>
<td>BAA11119</td>
<td>PEPQVTTLFQPSHPWHTQIHRDAFSYGAQVQSIDSRILDWRFFG</td>
</tr>
<tr>
<td>T. pub.</td>
<td>AAW57304</td>
<td>PEPQVTTLFQPSHPWHTQIHRDAFSYGAQVQSIDSRILDWRFFG</td>
</tr>
<tr>
<td>Lyo. shim.</td>
<td>BAD12079</td>
<td>PEPQVTTPFTEEHWPHTQIHRDAFSYAEGPEVDSRKVIVDWRFG</td>
</tr>
<tr>
<td>Trich. mat.</td>
<td>BAC24805</td>
<td>PEPQVMIFYTSDFPWHVQHRYAFGD---VGPKADPRVVDLRFFF</td>
</tr>
</tbody>
</table>

**Figure 1.** Sequence alignment of the putative active site loop region of selected pyranose oxidases.

Abbreviations and accession numbers used are: *T. multi.*, *Trametes multicolor* (AAP40332); *Pha. chrys.*, *Phanerochaete chrysosporium* (AAS93628); *Peni. gig.*, *Peniphora gigantea* (AAQ72486); *T. vers.*, *Trametes versicolor* (BAA11119); *T. pub.*, *Trametes pubescens* (AAW57304); *Lyo. shim.*, *Lyophyllum shimeij* (BAD12079); *Trich. mat.*, *Tricholoma matsutake* (BAC24805).

The presences of alanines at different positions in the active site loop in the context of alanine-scanning, the correct deletion of parts of the loop (\(^{\text{del}}454-456\)) and the introduction of different mutations at positions H450, D452, F454 and Y456 in the TmP2Ox gene by saturation mutagenesis was confirmed by DNA sequencing. Ten clones of each saturation mutagenesis library were picked randomly and analyzed; the chosen positions were altered in an unbiased fashion with each nucleotide position being fully randomized proving the success of saturation mutagenesis. Variants, which showed the highest activity with D-galactose in the 96-well plate screening assay, were isolated and were all identified as H450G encoded by one of the four possible codons for the amino acid glycine (GGT, GGC, GGA, GGG). Besides this variant, the loop deletion variant and the alanine-mutants, we decided to further analyze variants H450Q, F454P, F454N and Y456W. The percentage of inactive mutants in the 96 well plate screening assays demonstrated the importance and the
grade of conservation of the various positions for enzymatic activity and/or proper enzyme folding (Table 2).

**Table 2.** Number of inactive P2Ox variants found in 96 well plate screening assays; different amino acid positions of the active site loop of P2Ox from *Trametes multicolor* were mutated by site-saturation mutagenesis and libraries of 360 variants were screened in a 96 well plate screening assay. Variants which did not show any activity with either D-glucose or D-galactose and air saturation at 30°C after 24 hours of reaction time were considered inactive.

<table>
<thead>
<tr>
<th>aminoacid</th>
<th>position</th>
<th>number of screened mutants</th>
<th>inactive mutants</th>
<th>percentage of inactive mutants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H450</td>
<td>360</td>
<td>216</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>D452</td>
<td>360</td>
<td>158</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>F454</td>
<td>360</td>
<td>202</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Y456</td>
<td>360</td>
<td>173</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

Interestingly, position H450, which is not directly located near the active site, turned out to be the most sensitive one towards mutagenesis; 60% of all created mutants were inactive. Mutagenesis at the other positions resulted in libraries where about half of the variants were also inactive, showing the importance of the conservation of these aminoacids in the active site loop.

Interesting variants were expressed with a C-terminal His-tag added, purified to apparent homogeneity and concentrated by ultrafiltration as described before. Routinely, approx. 15 - 30 mg of P2Ox protein were obtained per litre culture medium in the cultivations, only the loop deletion variant and the alanine-mutants were expressed at significant lower levels (0.2 – 0.8 mg protein per litre culture medium). The purity and correct molecular mass of the enzymes were checked by SDS PAGE (Figure 2).
Figure 2. SDS-PAGE analysis of purified *Trametes multicolor* P2Ox variants. Lane 1, molecular mass marker proteins; lane 2, H450G; lane 3, F454P; lane 4, Y456W; lane 5, loop deletion mutant; lane 6, F454A/S455A/Y456A; lane 7, F454A/Y456A after purification by IMAC.

The increased formation of inclusion bodies, caused by the introduced mutations F454A/Y456A, F454A/S455A/Y456A and delF454-Y456 (loop deletion), was also shown by SDS PAGE (Figure 3).

Figure 3. SDS-PAGE analysis of cell debris to check for formed inclusion bodies. Lane 1, F454A/Y456A; lane 2, F454A/S455A/Y456A; lane 3, loop deletion variant; lane 4, molecular mass marker proteins; lane 5, wild-type enzyme; lane 6, H450G.
3.2 Kinetic characterization of mutational variants

Initial rates of activity were recorded over a substrate rate of 0.1 to 50 mM D-glucose, 0.1 to 200 mM D-galactose and 5.0 to 500 mM melibiose for wild-type P2Ox and all the mutational variants using the standard ABTS assay and air oxygen. Besides, initial rates of activity were recorded over a substrate rate of 1.0 to 300 mM gentiobiose for the wild-type enzyme and variants Y456W and F454A/S455A/Y456A. Kinetic data are summarized in Tables 3, 5 and 6.

$k_{cat}$ values of all variants for the substrate D-glucose and air oxygen decreased dramatically compared to the wild-type enzyme and $K_M$ values were increased 1.5 to 3 times leading to enzyme variants with a lowered catalytic efficiency ($k_{cat}/K_M$) for D-glucose. Just variant Y456W showed a similar turnover number ($k_{cat}$) for D-glucose, but a more than doubled $K_M$ value resulted in a 3-fold decreased $k_{cat}/K_M$.

Turnover numbers for D-galactose and air oxygen were increased for the variants H450G, H450Q and Y456W. The other variants, especially the alanine-substituted mutants and the loop deletion variant, showed a 3- to 12-fold lowered catalytic activity. The $K_M$ value of H450G for D-galactose was decreased 2.5 times, whereas the other mutants showed elevated $K_M$ values up to 5.7-fold. The reduced $K_M$ value in combination with the elevated $k_{cat}$ value of variant H450G for D-galactose leads to a 5.3-fold increased catalytic efficiency of this variant compared to the wild-type enzyme. These results indicate that this aminoacid in the active site loop influences the substrate specificity of \textit{Tm}P2Ox.
Table 3. Kinetic properties of P2Ox mutants and wild-type P2Ox from *Trametes multicolor* with either D-glucose or D-galactose as substrate and O₂ (air) as electron acceptor.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>D-glucose</th>
<th>D-galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>K</em>₉</td>
<td><em>k</em>₉</td>
</tr>
<tr>
<td></td>
<td>(mM)</td>
<td>(s⁻¹)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.76 ± 0.048</td>
<td>32.8 ± 0.43</td>
</tr>
<tr>
<td>H450G</td>
<td>0.99 ± 0.053</td>
<td>12.5 ± 0.15</td>
</tr>
<tr>
<td>H450Q</td>
<td>2.48 ± 0.34</td>
<td>17.4 ± 0.69</td>
</tr>
<tr>
<td>F454P</td>
<td>1.84 ± 0.30</td>
<td>0.99 ± 0.043</td>
</tr>
<tr>
<td>F454N</td>
<td>1.47 ± 0.077</td>
<td>12.0 ± 0.16</td>
</tr>
<tr>
<td>Y456W</td>
<td>1.72 ± 0.27</td>
<td>26.3 ± 1.09</td>
</tr>
<tr>
<td>F454A/Y456A</td>
<td>1.53 ± 0.15</td>
<td>7.13 ± 0.18</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>2.12 ± 0.31</td>
<td>0.20 ± 0.008</td>
</tr>
<tr>
<td>loop deletion</td>
<td>1.43 ± 0.046</td>
<td>3.09 ± 0.025</td>
</tr>
</tbody>
</table>

The wild-type enzyme clearly prefers D-glucose over D-galactose as its electron donor, as is expressed by the ratio of the specificity constants *k*₉/*K*₉ for the two substrates (Morley and Kazlauskas 2005). This value is 160 for the wild-type enzyme, while it is just 8.9 for the variant H450G (Table 4), indicating that the variant does not prefer D-glucose over D-galactose to the same extent as the wild-type any more, and that H450G can use the sugar substrate D-galactose more efficient than the wild-type enzyme, which is interesting from an applied point of view.
Table 4. Substrate selectivity of wild-type P2Ox from Trametes multicolor and some of its mutational variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>( \frac{(k_{\text{cat}}/K_M)<em>{\text{Glc}}}{(k</em>{\text{cat}}/K_M)_{\text{Gal}}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtP2Ox</td>
<td>160</td>
</tr>
<tr>
<td>H450G</td>
<td>8.9</td>
</tr>
<tr>
<td>H450Q</td>
<td>117</td>
</tr>
<tr>
<td>F454P</td>
<td>27</td>
</tr>
<tr>
<td>F454N</td>
<td>164</td>
</tr>
<tr>
<td>Y456W</td>
<td>204</td>
</tr>
<tr>
<td>F454A/Y456A</td>
<td>78</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>4.5</td>
</tr>
<tr>
<td>loop deletion</td>
<td>54</td>
</tr>
</tbody>
</table>

Except for H450G, all the other variants showed improved binding of the disaccharide melibiose compared to the wild-type enzyme (Table 5). \( K_M \) values were lowered up to 66 times and 30 times for variants F454A/S455A/Y456A and F454P, respectively. The conversion of the disaccharide melibiose, though, was worsened by introducing the mutations into the active site loop of P2Ox. In terms of catalytic efficiency variant Y456W showed a 3.3-fold increase compared to the wild-type enzyme in terms of a better substrate binding and a just slightly reduced \( k_{\text{cat}} \) value.
Table 5. Kinetic properties of P2Ox mutants and wild-type P2Ox from *Trametes multicolor* with melibiose as substrate and O\(_2\) (air) as electron acceptor.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>(k_M) (mM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/k_M) (mM(^{-1})·s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtP2Ox</td>
<td>1528 ± 329</td>
<td>7.58 ± 1.31</td>
<td>4.96</td>
</tr>
<tr>
<td>H450G</td>
<td>2033 ± 1050</td>
<td>1.81 ± 0.79</td>
<td>0.89</td>
</tr>
<tr>
<td>H450Q</td>
<td>386 ± 46.8</td>
<td>3.26 ± 0.21</td>
<td>8.43</td>
</tr>
<tr>
<td>F454P</td>
<td>50.3 ± 3.76</td>
<td>0.29 ± 0.005</td>
<td>5.68</td>
</tr>
<tr>
<td>F454N</td>
<td>240 ± 40.6</td>
<td>2.72 ± 0.20</td>
<td>11.3</td>
</tr>
<tr>
<td>Y456W</td>
<td>264 ± 14.5</td>
<td>4.38 ± 0.11</td>
<td>16.6</td>
</tr>
<tr>
<td>F454A/Y456A</td>
<td>352 ± 113</td>
<td>1.31 ± 0.22</td>
<td>3.71</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>23.2 ± 3.92</td>
<td>0.19 ± 0.006</td>
<td>7.99</td>
</tr>
<tr>
<td>loop deletion</td>
<td>211 ± 62.7</td>
<td>0.28 ± 0.035</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Similar results were obtained for the disaccharide gentiobiose and the variant Y456W; \(K_M\) was reduced significantly, while the \(k_{cat}\) value was just slightly decreased resulting in a doubled \(k_{cat}/k_M\) (Table 6). Interestingly, the alanine-mutant F454A/S455A/Y456A provided the best binding conditions for the disaccharides; \(K_M\) values were reduced 66 times for melibiose and 42 times for gentiobiose.
Table 6. Kinetic properties of some interesting P2Ox mutants and wild-type P2Ox from *Trametes multicolor* with gentiobiose as substrate and O₂ (air) as electron acceptor.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>KM  (mM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s⁻¹)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/KM (mM⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtP2Ox</td>
<td>165 ± 74.4</td>
<td>9.82 ± 2.13</td>
<td>74.5</td>
</tr>
<tr>
<td>Y456W</td>
<td>56.9 ± 22.9</td>
<td>8.32 ± 1.13</td>
<td>146</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>3.96 ± 2.62</td>
<td>0.18 ± 0.023</td>
<td>44.3</td>
</tr>
</tbody>
</table>

Furthermore the kinetic constants were determined for both electron acceptors 1,4-benzoquinone (BQ; a 2-electron, proton acceptor) and ferricenium ion (Fe⁺; a one-electron, no-proton acceptor) with either D-glucose or D-galactose in saturating concentrations (Tables 7 and 8). Variant H450G showed a nearly doubled $k_{cat}/K_M$ in terms of an increased turnover number for the combination BQ/D-glucose. Variant Y456W was characterized by an improved binding of the substrate BQ and an elevated $k_{cat}$ leading to a nearly 3-fold increased $k_{cat}/K_M$. The alanine-substituted mutants and the loop deletion variant showed decreased $K_M$ values for BQ but also dramatically decreased $k_{cat}$ values. Similar results were obtained for the conversions of D-galactose with BQ. Variant H450G turned out to be the most promising one for this reaction due to a reduced $K_M$, an elevated $k_{cat}$ and a catalytic efficiency which was 7.6-fold increased compared to the wild-type enzyme (Table 7).
Table 7. Kinetic properties of P2Ox mutants and wild-type P2Ox from *Trametes multicolor* with 1,4-benzoquinone as electron acceptor and D-glucose or D-galactose as saturating substrate.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>D-glucose</th>
<th>D-galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.14 ± 0.015</td>
<td>163 ± 7.09</td>
</tr>
<tr>
<td>H450G</td>
<td>0.14 ± 0.042</td>
<td>291 ± 40.8</td>
</tr>
<tr>
<td>H450Q</td>
<td>0.24 ± 0.076</td>
<td>221 ± 34.7</td>
</tr>
<tr>
<td>F454P</td>
<td>0.072 ± 0.029</td>
<td>30.4 ± 4.05</td>
</tr>
<tr>
<td>F454N</td>
<td>0.052 ± 0.013</td>
<td>134 ± 9.91</td>
</tr>
<tr>
<td>Y456W</td>
<td>0.072 ± 0.015</td>
<td>218 ± 14.8</td>
</tr>
<tr>
<td>F454A/Y456A</td>
<td>0.029 ± 0.004</td>
<td>61.5 ± 2.14</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>0.029 ± 0.011</td>
<td>14.9 ± 1.24</td>
</tr>
<tr>
<td>loop deletion</td>
<td>0.041 ± 0.004</td>
<td>24.6 ± 0.60</td>
</tr>
</tbody>
</table>

Comparable results were obtained for the combination of either D-glucose or D-galactose in saturating concentrations and the alternative electron acceptor ferricenium ion $Fc^+$. The alanine-substituted mutants and the loop deletion mutant showed lowered $K_M$ values, but again significantly reduced turnover numbers. Variant Y456W showed a 3-fold increased catalytic efficiency for $Fc^+$ in combination with either of the two sugar substrates due to reduced $K_M$ and slightly increased $k_{cat}$ values. Again variant H450G turned out to be to most useful variant in terms of a better substrate binding of $Fc^+$ and elevated turnover numbers ($k_{cat}/K_M$ was increased 4 and 9.4 times, respectively; see Table 8). Thus, H450G shows promising characteristics for applications in biofuel cells.
Table 8. Kinetic properties of P2Ox mutants and wild-type P2Ox from *Trametes multicolor* with ferricenium ion Fc as electron acceptor and D-glucose or D-galactose as saturating substrate.

<table>
<thead>
<tr>
<th>enzyme</th>
<th>D-glucose</th>
<th></th>
<th></th>
<th>D-galactose</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_M)</td>
<td>(k_{cat})</td>
<td>(k_{cat}/K_M)</td>
<td>(K_M)</td>
<td>(k_{cat})</td>
<td>(k_{cat}/K_M)</td>
</tr>
<tr>
<td>wtP2Ox</td>
<td>0.40 ± 0.11</td>
<td>207 ± 26.7</td>
<td>513</td>
<td>0.10 ± 0.067</td>
<td>6.59 ± 1.77</td>
<td>63.3</td>
</tr>
<tr>
<td>H450G</td>
<td>0.27 ± 0.057</td>
<td>544 ± 55.0</td>
<td>1995</td>
<td>0.043 ± 0.012</td>
<td>25.2 ± 2.02</td>
<td>593</td>
</tr>
<tr>
<td>H450Q</td>
<td>0.38 ± 0.11</td>
<td>470 ± 72.0</td>
<td>1252</td>
<td>0.049 ± 0.015</td>
<td>16.8 ± 1.50</td>
<td>341</td>
</tr>
<tr>
<td>F454P</td>
<td>0.14 ± 0.06</td>
<td>66.9 ± 11.8</td>
<td>480</td>
<td>0.023 ± 0.014</td>
<td>4.47 ± 0.66</td>
<td>197</td>
</tr>
<tr>
<td>F454N</td>
<td>0.35 ± 0.21</td>
<td>422 ± 111</td>
<td>1205</td>
<td>0.025 ± 0.010</td>
<td>7.29 ± 1.09</td>
<td>290</td>
</tr>
<tr>
<td>Y456W</td>
<td>0.24 ± 0.04</td>
<td>395 ± 28.5</td>
<td>1636</td>
<td>0.041 ± 0.009</td>
<td>7.28 ± 0.39</td>
<td>177</td>
</tr>
<tr>
<td>F454A/Y456A</td>
<td>0.147 ± 0.029</td>
<td>73.9 ± 4.88</td>
<td>505</td>
<td>0.016 ± 0.007</td>
<td>2.88 ± 0.28</td>
<td>184</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>0.28 ± 0.069</td>
<td>106 ± 10.7</td>
<td>373</td>
<td>0.015 ± 0.009</td>
<td>5.37 ± 0.67</td>
<td>360</td>
</tr>
<tr>
<td>loop deletion</td>
<td>0.30 ± 0.062</td>
<td>15.1 ± 1.32</td>
<td>50.4</td>
<td>0.011 ± 0.001</td>
<td>1.33 ± 0.050</td>
<td>121</td>
</tr>
</tbody>
</table>

3.3 Thermal stability

In former studies it was shown that loops, as structural elements of enzymes, stabilize proteins and subunit interactions (Fetrow et al. 1997). Consequently, mutations in loops can either positively or negatively affect the thermal stability of enzymes. Mainly, loops located at the surface of enzymes or at the interface between subunits influence the stability. Here we wanted to check if mutations in a loop, which is located in the interior of the enzyme, can also affect the stability. The length of the time in which an enzyme remains active before undergoing irreversible inactivation (kinetic stability) was measured for wtP2Ox and P2Ox variants at a constant pH of 6.5 at 60°C and 70°C. The inactivation constants \(k_{in}\) and the half-lives of denaturation \(\tau_{1/2}\) were determined (Table 9). All the mutants showed first-order inactivation kinetics when analyzed in the ln(residual activity) versus time plot (Figure 3 and 4).
Figure 3. Inactivation kinetics of P2Ox variants from *Trametes multicolor* at 60°C and pH 6.5. Symbols: A: ●, wild-type P2Ox; ▲, variant H450G; ○ variant H450Q; ▽, variant F454P; ▼, variant F454N; ■, variant Y456W; B: ■, variant F454A/Y456A; Δ, variant F454A/S455A/Y456A; ▼, loop deletion variant.
Figure 4. Inactivation kinetics of P2Ox variants from *Trametes multicolor* at 70°C and pH 6.5. Symbols as in Figure 3.

Just one variant, H450Q, showed a significantly decreased thermal stability, whereas the other variants showed similar or improved stability. The substitutions of both positions Phe454 by Asn and Tyr456 by Trp resulted in considerably stabilized P2Ox variants; half-lives at 60°C were increased 29-fold and 34-fold, respectively, compared to the wild-type enzyme. Interestingly, also the alanine-substituted variants were characterized by significantly increased half-lives at 60°C; $\tau_{1/2}$ was increased 12-fold and 23-fold, respectively. The increased kinetic stabilities of various enzyme variants were confirmed at 70°C (Table 9).
Table 9. Kinetic stability of pyranose 2-oxidase from *T. multicolor* at various temperatures.

<table>
<thead>
<tr>
<th>variant</th>
<th>Inactivation constant $k_{in}$ (min$^{-1}$)</th>
<th>Half-life $\tau_{1/2}$ (60°C) (min)</th>
<th>Inactivation constant $k_{in}$ (min$^{-1}$)</th>
<th>Half-life $\tau_{1/2}$ (70°C) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>$-57.5 \cdot 10^{-3}$</td>
<td>12.1</td>
<td>$-9.89$</td>
<td>0.07</td>
</tr>
<tr>
<td>H450G</td>
<td>$-28.9 \cdot 10^{-3}$</td>
<td>24.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>H450Q</td>
<td>$-85.8 \cdot 10^{-3}$</td>
<td>8.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>F454P</td>
<td>$-66.9 \cdot 10^{-3}$</td>
<td>10.4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>F454N</td>
<td>$-2.0 \cdot 10^{-3}$</td>
<td>347</td>
<td>$-1.77$</td>
<td>0.39</td>
</tr>
<tr>
<td>Y456W</td>
<td>$-1.7 \cdot 10^{-3}$</td>
<td>408</td>
<td>$-1.10$</td>
<td>0.63</td>
</tr>
<tr>
<td>F454A/Y456A</td>
<td>$-4.8 \cdot 10^{-3}$</td>
<td>144</td>
<td>$-1.09$</td>
<td>0.64</td>
</tr>
<tr>
<td>F454A/S455A/Y456A</td>
<td>$-2.5 \cdot 10^{-3}$</td>
<td>277</td>
<td>$-1.15$</td>
<td>0.60</td>
</tr>
<tr>
<td>loop deletion</td>
<td>$-12.6 \cdot 10^{-3}$</td>
<td>55.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*a n.d., not determined

### 3.4 pH optima

pH optima of all the variants were determined by measuring the activity with the standard ABTS assay and D-glucose as sugar substrate at a saturating concentration of 20 mM at different pH values. All the variants showed a pH optimum at pH 6.5 (data not shown), demonstrating that the introduction of the mutations in the active site loop did not change the pH optimum of any of the variants.

### 3.5 FAD-spectra

P2Ox preparations were bright yellow and produced a typical flavo-protein spectrum with absorption maxima $\lambda_{max}$ at 345 nm and 456 nm (Figure 5). The UV/VIS spectra of the mutated enzymes had the same values for the FAD ring as the wild-type enzyme. Reduction of the enzymes by addition of D-glucose (10 mM) and sodium dithionite (5 mM) in the absence of oxygen resulted in disappearance of the peak at
456 nm. Treatment of the enzyme with 5% trichloroacetic acid at 100°C for 10 min did not release the flavin moiety from the enzyme, suggesting that this moiety was covalently linked to the protein. The introduction of the mutations into the active site loop did not negatively influence the covalent binding of FAD in the active site.

**Figure 5.** Absorption spectra of the oxidized (solid line) and reduced (dotted line) states of P2Ox from *Trametes multicolor*. Reduction was performed with D-glucose (10 mM) and sodium dithionite (5 mM) in the absence of oxygen.

**4 Discussion**

In this work we undertook an assessment of the functional importance of the active site loop (residues H450 – G457) of pyranose 2-oxidase from *Trametes multicolor* (*TmP2Ox*) in catalysis and binding of different substrates. Former studies of different enzymes have shown that active site loops play an important role in binding and catalysis and have an effect on substrate specificity (Shinoda et al. 2005, Doyle et al. 2000, Sampson et al. 1998). To analyze the importance of various aminoacids for catalytic activity, alanine-scanning substitutions in the active site loop have been
performed in former studies (Lauhon et al. 2004). Also the technique of deleting some aminoacids at the tip of an active site loop to analyze their importance for catalysis has been performed for various enzymes (Pompliano et al. 1990, Sampson et al. 1998). Sampson and colleagues deleted 5 aminoacids at the tip of the active site loop of the enzyme cholesterol oxidase, but despite this deletion the enzyme was still properly folded and catalytically active. Sampson found out that the truncation of the loop negatively affected the binding of the substrate but that the movement of the substrate and the product into and out of the active site was facilitated. Sequence alignments of putative active site loop regions of pyranose oxidases, isolated from different organisms, revealed a highly conserved region (Fig.6); just *Tricholoma matsutake* differed in its sequence at positions 452, 455, 456 and 457. This high conservation of the active site loop sequences indicates a functional and structural importance of this region for the enzymes. Considering former studies on active site loops and the sequence alignment we decided to use site-saturation mutagenesis and alanine-scanning substitutions at various important positions of the active site loop. Besides, we deleted the tip of the loop, which directly covers the active site \( \text{del}454-456 \), and analyzed the resulting loop-deletion variant.

We used site-saturation mutagenesis at positions H450, D452, F454 and Y456. To prove the success of the method, we picked 10 variants of each library, showing high, moderate or low activity with either D-glucose or D-galactose and air saturation, and sequenced the encoding genes. Interesting mutations like F454P, F454N and Y456W were further analyzed, but in general no improved variants were found at positions D452, F454 and Y456, suggesting that these aminoacids are the only acceptable aminoacids at these positions for standard activity of *TmP2Ox*. This indicates that these positions play a specific role in substrate binding and catalysis. The screening of
360 enzyme variants which resulted from site-saturation mutagenesis at position H450, however, revealed variants with higher activity with the sugar substrate D-galactose and air saturation. All these variants were identified as H450G, where glycine was coded by either of the four codons, which again proved the success of site-saturation mutagenesis. Interestingly, H450G showed a higher $K_M$ and a lower $k_{cat}$ value for D-glucose but provided better conditions for binding of D-galactose and a more than 2-fold increased turnover number for this sugar. Apparently, this amino acid position affects the substrate specificity of TmP2Ox, either directly by interacting with the substrate or indirectly by interactions with Q448, which interacts with the C4 of the bound aldopyranose in the active site (Kujawa et al. 2006). Glycine, being the smallest amino acid with a very flexible nature, obviously provides more flexibility at this important pivotal point of the active site loop.

The alanine-substituted variants and the loop deletion mutant were characterized by increased $K_M$ and lowered $k_{cat}$ values for both D-glucose and D-galactose. This shows that the tip of the loop covering the active site and directly interacting with the bound substrates, is essential for binding and catalysis, which is different to the results of Sampson and colleagues.

Also the enzyme variants H450Q, F454P, F454N and Y456W showed a decreased catalytic activity and a worsened substrate binding for both monosaccharides. Obviously, the natural active site loop of TmP2Ox is especially designed to limit the substrate specificity of this enzyme to monosaccharides, leaving polysaccharides to hydrolases, transferases and amylases in the fungus, and even small changes in its highly conserved amino acid composition dramatically reduce the activity of this biocatalyst with the natural substrates. However, using the disaccharides melibiose and gentiobiose as substrates, these variants were characterized by decreased $k_{cat}$, but
also significantly lowered $K_M$ values compared to the wild-type enzyme. This result was also seen for the alanine-substituted variants F454A/Y456A and F454A/S455A/Y456A and for the loop-deletion variant, indicating that the positions F454, S455 and Y456, which directly cover the active site, have a huge impact on substrate binding and catalysis of disaccharides. By substituting the large, aromatic aminoacid Phe454 by the small aminoacids Pro, Asn or Ala more space in the active site was created, allowing larger substrates a facilitated binding. However, the aromatic side chain of F454, which interacts with the bound substrate when the loop is closed, is important for a fast turnover and deleting this side chain results in variants with dramatically decreased $k_{cat}$ values. The interesting variant Y456W was characterized by increased $K_M$ but just slightly decreased $k_{cat}$ values for D-glucose and D-galactose. For melibiose and gentiobiose $K_M$ values were reduced 5.8- and 2.9-fold, whereas $k_{cat}$ was again just slightly affected. The aromatic side chain of Y456 is quite far away from the bound sugar substrate when the loop is open (Kujawa et al. 2006) and does not really interact with the monosaccharide. By contrast, if a disaccharide binds to the active site, the 2nd sugar moiety might interact with the side chain of Y456. The lower $K_M$ values of Y456W for the disaccharides melibiose and gentiobiose probably result from enhanced hydrophobic interactions caused by a closer proximity between the galactose-moiety of the disaccharide and the large aromatic side-chain of tryptophane. The turnover number for both monosaccharides and disaccharides was just slightly affected by the replacement of Tyr456 by Trp, because the $k_{cat}$-influencing reaction happens at the sugar moiety bound directly to the active site.

For the reactions with the alternative electron acceptors BQ and Fc$^+$ again variant H450G turned out to be the most promising one due to reduced $K_M$ values for both
electron acceptors and increased turnover numbers for all combinations possible. The
mutants F454P, F454N, Y456W, the alanine substituted variants as well as the loop
deletion variant were characterized by lowered $K_M$ and $k_{cat}$ values for BQ and either
of the two monosaccharide substrates tested. Besides F454N and Y456W, which
showed an increased turnover number for Fe$^+$ and both sugars, these results were also
obtained for Fe$. Reducing the size of the active site loop and of the side chains of its
aminoacids results in a facilitated binding of the large alternative electron acceptors
but at cost of high turnover.

Besides the kinetic constants with various substrates we also determined the kinetic
stability (thermal stability) of the mutants. The thermal stability gives an idea of
increased or decreased structural stability of the enzyme. The variants with
significantly improved thermal stability were F454N, Y456W, both alanine-
substituted variants as well as the loop-deletion mutant. Y456W showed a 34-fold
increased kinetic stability at 60°C compared to the wild-type enzyme. The crystal
structure shows that there are possible interactions between the side chain of Tyr456
and Phe474. The replacement of Tyr456 by Trp introduces a larger aromatic side
chain missing the OH group, and therefore enforces the hydrophobic stacking of the
two aromatic side chains of Phe474 and Trp456.

These results are mainly important for basic research to elucidate the impact of the
active site loop on substrate binding and specificity as well as catalysis. Besides,
aminoacids in the active site loop turned out to be promising targets for mutations to
enhance the stability of the enzyme. Variant H450G also shows promising
characteristics for application in food industry for the synthesis of tagatose and
fructose from hydrolyzed lactose. The strong preference for D-glucose over D-
galactose is not given any more resulting in a possible, more efficient conversion of
lactose-hydrolysate into its products. Variant H450G also shows useful features for an application in a galactose-biosensor. The catalytic efficiency for either BQ or Fe\(^{3+}\) in combination with D-galactose was improved 7.6- and 9.4-fold, respectively.

**Acknowledgements**

Financial support from the Austrian Science Fund (FWF, Translational Project L213-B11) and The Swedish Research Council are gratefully acknowledged.
5 References


Appendix
Mutations of Thr169 affect substrate specificity of pyranose 2-oxidase from *Trametes multicolor*

Oliver Spadiut a, Christian Leitner a, Tien-Chye Tan b, Roland Ludwig a, Christina Divne b, Dietmar Haltrich a

a Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU-University of Natural Resources and Applied Life Sciences, Vienna, Austria
b School of Biotechnology, KTH, Albanova University Centre, Stockholm, Sweden

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PLEASE SCROLL DOWN FOR ARTICLE
Mutations of Thr169 affect substrate specificity of pyranose 2-oxidase from Trametes multicolor

OLIVER SPADIUT1, CHRISTIAN LEITNER1, TIEN-CHYE TAN2, ROLAND LUDWIG1, CHRISTINA DIVNE2, & DIETMAR HALTRICH1

1Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU-University of Natural Resources and Applied Life Sciences, Vienna, Austria and 2School of Biotechnology, KTH, Albanova University Centre, Stockholm, Sweden

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Abstract
Site-directed mutagenesis was used to enhance the catalytic activity of pyranose 2-oxidase (P2Ox) from Trametes multicolor with different substrates. To this end, threonine at position 169 was replaced by glycine, alanine and serine, respectively. Using oxygen as electron acceptor the mutant T169G was equally active with D-glucose and D-galactose, whereas wild-type recombinant P2Ox only showed 5.2% relative activity with the latter substrate. When D-galactose was used as electron donor in saturating concentrations, T169G showed a 4.5-fold increase in its catalytic efficiency $k_{cat}/K_M$ for the alternative electron acceptor 1,4-benzoquinone and a nine-fold increased $k_{cat}/K_M$ value with the ferricenium ion compared with wt recP2Ox. Variant T169S showed an increase in its catalytic efficiency both with 1,4-benzoquinone (3.7 times) as well as with the ferricenium ion (1.4 times) when D-glucose was the substrate.

Keywords: Pyranose oxidase, enzyme engineering, substrate specificity, galactose oxidation

Introduction
The flavoenzyme pyranose 2-oxidase (P2Ox; pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) catalyses the oxidation of several aldopyranoses at position C-2 to yield the corresponding 2-ketoaldoses, while reducing molecular oxygen to hydrogen peroxide. It is widespread among wood-degrading basidiomycetes, where it is localized in the hyphal periplasmic space. P2Ox is thought to be involved in the ligninolysis of wood rot fungi by supplying lignin peroxidases with H$_2$O$_2$ (Daniel et al. 1994). In some fungi, P2Ox is involved in a secondary metabolic pathway leading from D-glucose via 2-keto-D-glucose as an intermediate to cortalcerone, a $\beta$-pyrone antibiotic (Volc et al. 1991).

P2Ox was first isolated and characterized from Polyporus obtusus (Janssen & Ruelius 1968), Phanerochaete chrysosporium (Artolozaga et al. 1997; Volc & Eriksson 1988) and Pleurotus ostreatus (Shin et al. 1993). In this study, pyranose 2-oxidase from Trametes multicolor (TmP2Ox) was used (Leitner et al. 2001). TmP2Ox is homotetrameric with a molecular mass of 270 kDa with each of the four 68-kDa subunits carrying one flavin adenine dinucleotide (FAD) bound covalently to N$_{\text{o2}}$ (i.e. N3) of His167 via its 8$\alpha$-methyl group (Halada et al. 2003; Hallberg et al. 2004). The reaction catalysed by P2Ox is of the Ping Pong Bi Bi type typically found in flavoprotein oxidoreductases (Artolozaga et al. 1997; Ghisla & Massey 1989). In the reductive half-reaction an aldopyranose substrate reduces the FAD cofactor to yield reduced flavin FADH$_2$ and 2-dehydroaldose (2-ketoaldose) as the result of oxidation at position C-2 of the sugar substrate (reaction 1; Freimund et al. 1998; Volc & Eriksson 1988). The ensuing oxidative half-reaction involves the reoxidation of FADH$_2$ by the second substrate. This can be oxygen, which is reduced to hydrogen peroxide (reaction 2), or alternative electron acceptors, including either two-electron acceptors such as benzoquinones (reaction 3) or one-electron acceptors such as chelated metal ions,
Recently, the structure of P2Ox from *T. multicolor* in complex with one of its slow substrates, 2-fluoro-2-deoxy-D-galactose, has been reported (Kujawa et al. 2006), which showed the interactions between polypeptide and sugar substrate in the active site, as well as the tentative determinants for C-2 oxidation by P2Ox. This structure also explains why D-glucose is a far better substrate than D-galactose 

\[ \text{FAD} + \text{aldopyranose} \rightarrow \text{FADH}_2 + 2\text{-keto-aldopyranose} \]  

(1)

\[ \text{FADH}_2 + \text{O}_2 \rightarrow \text{FAD} + \text{H}_2\text{O}_2 \]  

(2)

\[ \text{FADH}_2 + \text{benzoquinone} \rightarrow \text{FAD} + \text{hydroquinone} \]  

(3)

\[ \text{2-oxidase gene from } T. \text{ multicolor} \] under the control of the T7 promoter, has been described recently (Kujawa et al. 2006). The *E. coli* strain BL21 Star DE3 (Invitrogen; Carlsbad, CA, USA) was used for the production of active P2Ox. *E. coli* cells were grown in TB$_{amp}$-media (yeast extract 24 g l$^{-1}$, peptone from casein 12 g l$^{-1}$, glycerol 4 ml l$^{-1}$; phosphate buffer 1 M, pH 7.5), for selection ampicillin was added to 100 mg l$^{-1}$. The chemicals used were of the highest grade available and were purchased from Sigma (Vienna, Austria) unless otherwise stated.

**Generation of mutants**

Site-directed mutagenesis at the position Thr169 was performed by using the GeneTailor Site Directed Mutagenesis System (Invitrogen). The mutagenic forward primers 5'-GTGGTCGGAGGCCATG TCTACGACTGGGATGCGCCACACC-3' (T169G), 5'-GTGGTCGGAGGCCATGCTCTACGCC TGGCGCATGCCACACC-3' (T169A), 5'-GT CGTCGGAGGCCATGCTCTACGCATGGCTTGGCCACACC-3' (T169S) and the reverse primer 5'-CCAGTGCGGCAGGCACCTCGCTAC AGATCGTGACC-3' (T169rev) were from VBC Biotech (Vienna, Austria). The plasmid pHL2 was used as template for mutagenic PCR using the following conditions:

- 95°C for 4 min, then 30 cycles of 94°C for 40 s;
- 60°C for 40 s;
- 72°C for 16 min, with a final 72°C for 10 min incubation.

Each reaction contained 1× buffer (Fermentas; St. Leon-Rot, Germany), 2.5 units *Pfu* DNA polymerase (Fermentas), 1 μg of plasmid DNA, 10 μM of each dNTP and 5 pmol of each primer. The PCR products were separated by agarose gel electrophoresis and purified using the Wizard SV Gel and PCR-Clean-Up System (Promega; Madison, WI, USA). To obtain intact mutated plasmids 2 μl of each PCR product were transformed into chemically competent *E. coli* BL21 Star DE3 cells (Invitrogen). To confirm the presence of the correct mutations and the absence of further mutations, plasmid DNA was extracted and used as templates for DNA sequencing of the complete P2Ox-encoding gene using the forward primer 5'- AATACGACTCATAAGGG G-3' (T7promfwd) and the reverse primer 5'- GCT AGTTATTGCTCAGCGG -3' (T7termrev). The DNA sequencing was performed as a commercial service (VBC Biotech).
Protein expression and purification

 Cultures (2 litres) of E. coli BL21 Star DE3 transformants were grown in shaken flask cultures at 37°C in TBamp until the cultures reached an OD600 of 0.5–0.6. Protein expression was induced by adding lactose (0.5%) and cultures were grown for a further 20 h at 25°C. Cells were harvested by centrifugation (12,000 rpm, 20 min, 4°C; Sorvall RC26, rotor SLA-3000; Dupont, New York, NY, USA), resuspended in phosphate buffer (50 mM, pH 6.5) containing phenylmethylsulfonylfluoride (PMSF; 0.1%) and lysed using a French press. The crude extract was separated from cell debris by centrifugation (30,000 rpm, 30 min, 4°C; Beckman ultracentrifuge L-70, rotor 45 Ti; Beckman Palo Alto, CA, USA), and the supernatant was used for protein purification by immobilized metal affinity chromatography (IMAC). P2Ox variants were purified using a His Trap HP column (Amersham Biosciences, Vienna, Austria) via the C-terminal His-tag. Enzymes were eluted with an elution buffer (Bis-Tris 20 mM, pH 6.5; NaCl 0.5 M, chloramphenicol 10 mg) and a gradient of 10–1000 mM imidazole. The eluted enzymes were concentrated by ultrafiltration using an Amicon Ultra Centrifugal Filter Device (10 kDa cut-off; Millipore, Billeria, MA, USA).

Electrophoresis

Both native PAGE and SDS PAGE were performed to evaluate the purity of the enzyme preparations. Electrophoresis was done as described by Laemmli (Laemmli 1970) using a 10% separating gel and a 5% stacking gel. Samples were diluted to 1–2 mg of protein ml⁻¹, and aliquots of 5 μl were loaded per lane. Protein bands were stained with Coomassie blue.

Enzyme activity assays

To determine P2Ox activity, the standard chromogenic [2,2’-azinobis(3-ethylbenzthiazolinesulfonic acid)] (ABTS) assay (Danneel et al. 1993) was used. A sample of diluted enzyme (10 μl) was added to 980 μl assay buffer containing horseradish peroxidase (142 U), ABTS (14.7 mg) in phosphate buffer (50 mM, pH 6.5). The reaction was started by adding D-glucose (20 mM) and the absorbance change at 420 nm (ε = 42.3 mM⁻¹ cm⁻¹) was recorded at 30°C for 180 s. One unit of P2Ox activity was defined as the amount of enzyme catalysing the oxidation of 2 μmol of ABTS per min (which equals the consumption of 1 μmol of O₂ per min) under the assay conditions.

Protein concentrations were determined by the Bradford assay (Bradford 1976) using the BioRad Protein Assay Kit (BioRad; Vienna, Austria). Bovine serum albumin (BSA) was used as the standard.

Steady-state kinetic measurements

Kinetic constants for the two electron donors D-glucose and D-galactose were measured using the standard ABTS assay and air saturation (D-glucose, 0.1–50 mM; D-galactose, 0.1–200 mM). The kinetic constants for the electron acceptors 1,4-benzoquinone and the ferricenium Fc⁺ ion (using ferricenium hexafluorophosphate FcPF₆; Aldrich, Steinheim, Germany) were measured as previously described (Kujawa et al. 2007). In short, 10 μl of appropriately diluted enzyme was added to 990 μl of phosphate buffer (50 mM, pH 6.5) containing the sugar substrate (D-glucose, D-galactose) in a constant concentration of 100 mM. 1,4-benzoquinone was varied from 0.01–2.0 mM and the absorbance change at 290 nm (ε = 2.24 mM⁻¹ cm⁻¹) was followed at 30°C for 180 s. FcPF₆ was varied from 0.005–1.0 mM and the absorbance change at 300 nm (ε = 4.3 mM⁻¹ cm⁻¹) was followed at 30°C for 180 s. Kinetic constants were calculated by non-linear least-square regression, fitting the measured data to the Henri-Michaelis-Menten equation.

Results

Generation of mutants and protein purification

Variants of P2Ox were produced by site-directed mutagenesis as described. DNA sequence analysis confirmed the presence of the correct mutations at the amino acid position 169 in the P2Ox gene with no undesired mutations. E. coli transformants expressing active P2Ox variants were cultivated in shake flasks (2 l) and recombinant protein expression was induced by the addition of lactose (0.5%) to the culture medium. Routinely, roughly 70 mg of P2Ox protein was obtained per litre culture medium in these cultivations. P2Ox proteins were purified from the crude extracts by IMAC using a His Trap HP column followed by ultrafiltration. The resulting proteins were apparently homogenous as judged by native PAGE and SDS-PAGE (Figure 1).

Kinetic characterization of mutational variants

Initial rates of activity were recorded over a substrate range of 0.1–50 mM D-glucose and 0.1–500 mM D-galactose for His₆-tagged wild-type P2Ox expressed in E. coli and the mutational variants T169G, T169A and T169S using the standard ABTS assay and air saturation. Table I provides a summary of
Figure 1. Native PAGE (A) and SDS-PAGE (B) of different Thr169 variants of pyranose 2-oxidase from Trametes multicolor. Protein bands were stained with Coomassie blue. Lane 1, molecular mass standard; A, High Molecular Weight Calibration Kit for native electrophoresis (Amersham); B, Precision Plus Protein Dual Color (Biorad). Lane 2, variant T169G; lane 3, variant T169A; lane 3, variant T169S.

these kinetic data. The catalytic activity of each mutational variant of P2Ox was reduced for both substrates D-glucose and D-galactose. This effect was less pronounced for the variant with the conservative replacement Thr → Ser. In contrast, the Michaelis constant KM was reduced for variant T169G for both sugars, and for T169S for D-galactose, a result indicating improved binding of the sugars by these variants. Interestingly, the KM values were increased substantially for both substrates and variant T169A. While the wild-type enzyme clearly preferred D-glucose over D-galactose as its electron donor with catalytic efficiencies kcat/KM of 51.2 mM⁻¹ s⁻¹ and 0.29 mM⁻¹ s⁻¹, respectively, and a 20-fold higher catalytic constant for D-glucose, this clear preference was not observed for some of the variants. Notably, variant T169G was characterized by kcat values that are approximately equal for both sugars, in fact kcat was slightly higher for D-galactose than for D-glucose. Furthermore, this variant showed a kcat/KM value that was only 3.5 times lower for D-galactose than for D-glucose, while this value was 177 times lower for D-galactose in the wild-type enzyme.

In addition, the kinetic constants were determined for two electron acceptors, 1,4-benzoquinone and ferricenium ion, with either D-glucose or D-galactose as electron donor in saturating concentration (Tables II and III). Interestingly, two of these variants, T169G and T169S, showed significantly higher kcat and kcat/KM values for both of these electron acceptors than the wild-type enzyme when D-galactose was used as sugar substrate. When using D-glucose as the saturating substrate these values were lower for the variants than for the wild-type enzyme with the exception of 1,4-benzoquinone and T169S. With D-galactose as the electron donor with variant T169G, the catalytic efficiency increased three-fold and 8.6-fold for 1,4-benzoquinone and for ferricenium, respectively; and for variant T169S with the same electron-acceptor substrates, efficiencies were increased by 1.4- and 4.4-fold, respectively. These considerable increases in kcat/KM are mainly the result of more favourable kcat values. Again, variant T169A showed substantially decreased kinetic constants for both electron acceptors (Tables II and III).

### Discussion

The conversion of sugars by pyranose oxidase is gaining increased attention as a number of these oxidized sugars are attractive intermediates for chemo-enzymatic biotransformation reactions. Preparation of these ketosugars by an enzymatic approach is favourable as the corresponding chemical routes are laborious and characterized by low yields (Freimund et al. 1998). We reported on two of these possible P2Ox-catalysed biotransformations, the redox isomerization of D-glucose to fructose and D-galactose to tagatose via the corresponding 2-ketoaldoses (Haltrich et al. 1998; Leitner et al. 1998). Europe is one of the leading producers of the milk sugar lactose, originating from the dairy industry, and an interesting possibility is the conco-enzymatic biotransformation reactions. Preparation of these ketosugars by an enzymatic approach is favourable as the corresponding chemical routes are laborious and characterized by low yields (Freimund et al. 1998). We reported on two of these possible P2Ox-catalysed biotransformations, the redox isomerization of D-glucose to fructose and D-galactose to tagatose via the corresponding 2-ketoaldoses (Haltrich et al. 1998; Leitner et al. 1998). Europe is one of the leading producers of the milk sugar lactose, originating from the dairy industry, and an interesting possibility is the conco-enzymatic biotransformation reactions.

### Table I. Kinetic properties of wild-type recombinant pyranose 2-oxidase from T. multicolor and mutational variants for D-glucose and D-galactose as electron donors, with the concentration of O2 as electron acceptor held constant. Kinetic data were obtained using the standard ABTS assay and air saturation.

<table>
<thead>
<tr>
<th>Variant</th>
<th>D-glucose KM (mM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/KM (mM⁻¹ s⁻¹)</th>
<th>D-galactose KM (mM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/KM (mM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt P2Ox</td>
<td>0.94 ± 0.04</td>
<td>48.1 ± 0.52</td>
<td>51.2</td>
<td>8.79 ± 0.54</td>
<td>2.51 ± 0.05</td>
<td>0.29</td>
</tr>
<tr>
<td>T169G</td>
<td>0.69 ± 0.11</td>
<td>0.26 ± 0.01</td>
<td>0.38</td>
<td>2.48 ± 0.04</td>
<td>0.27 ± 0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>T169A</td>
<td>6.42 ± 0.57</td>
<td>0.063 ± 0.002</td>
<td>0.010</td>
<td>39.6 ± 2.61</td>
<td>0.017 ± 0.001</td>
<td>0.00045</td>
</tr>
<tr>
<td>T169S</td>
<td>0.27 ± 0.02</td>
<td>7.24 ± 0.09</td>
<td>26.6</td>
<td>10.1 ± 0.24</td>
<td>1.54 ± 0.01</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Mutations of Thr169 of pyranose oxidase

Table II. Kinetic properties of wild-type recombinant pyranose 2-oxidase from T. multicolor and mutational variants for 1,4-benzoquinone as electron acceptor, with the concentration of D-glucose or D-galactose held constant (100 mM).

<table>
<thead>
<tr>
<th>Variant</th>
<th>D-glucose $K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
<th>D-galactose $K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt P2Ox</td>
<td>0.24 ± 0.025</td>
<td>152 ± 5.96</td>
<td>633</td>
<td>0.065 ± 0.003</td>
<td>4.79 ± 0.06</td>
<td>74.2</td>
</tr>
<tr>
<td>T169G</td>
<td>0.026 ± 0.011</td>
<td>6.5 ± 0.62</td>
<td>252</td>
<td>0.10 ± 0.046</td>
<td>21.7 ± 3.62</td>
<td>219</td>
</tr>
<tr>
<td>T169A</td>
<td>0.081 ± 0.014</td>
<td>0.69 ± 0.03</td>
<td>8.48</td>
<td>0.013 ± 0.001</td>
<td>0.14 ± 0.002</td>
<td>11.3</td>
</tr>
<tr>
<td>T169S</td>
<td>0.044 ± 0.011</td>
<td>105 ± 7.73</td>
<td>2370</td>
<td>0.049 ± 0.046</td>
<td>5.05 ± 1.33</td>
<td>101</td>
</tr>
</tbody>
</table>

Rational protein design is a very powerful tool for the alteration and improvement of biocatalysts (Bornscheuer & Pohl 2001), but it requires detailed knowledge about the structure and function of a biocatalyst. Recently, we reported the structure of TmP2Ox in complex with acetate, a competitive inhibitor (Hallberg et al. 2004) and of a variant of the enzyme, H167A, complexed with a slow substrate, 2-fluoro-2-deoxy-D-glucose (Kujawa et al. 2006). Based on the latter structure we proposed the binding mode for D-glucose positioned for active site during D-galactose binding, we considered why D-galactose is a much poorer substrate, as the hydroxyl group at position C-4 of the sugar and the side chain of Thr169 show a steric clash (Figure 2).

In an attempt to relieve steric strain in the P2Ox active site during D-galactose binding, we constructed three P2Ox variants, T169G, T169A and T169S. These mutations were chosen in order to create additional space in the active site so that D-galactose might be accommodated more easily. The catalytic properties of these mutants were characterised.

Replacing Thr169 with the amino acids Gly and Ser showed improved binding of both sugar substrates, D-glucose and D-galactose, as indicated by the $K_M$ values, which are in general lower for the variants than for the wild-type enzyme. The replacement of Thr $\rightarrow$ Ala, however, gave considerably higher Michaelis constants for both sugars. It is not clear why the presence of the methyl side chain in Ala leads to this impaired binding of both sugar substrates. Removing the Thr side chain at position 169 also has a considerable effect on the catalytic constants for the sugar substrates of the variants, which were lower for all three mutational variants and both sugars measured. This negative effect was less pronounced for the conservative replacement Thr $\rightarrow$ Ser, which retains the hydroxyl functionality in the side chain. Again, the most drastic decrease in $k_{cat}$ was observed for the T169A variant with $k_{cat}$ being reduced almost 100-fold for both sugar substrates. The catalytic constant for D-glucose was affected considerably more than that for D-galactose, and as a result the ratio of $k_{cat,Glc}$ to $k_{cat,Gal}$ is 0.96 for T169G and 4.7 for T169S, while it is 19 for the wild-type enzyme. Similarly, the ratio of $(k_{cat,Glc}/K_M)_Glc$ to $(k_{cat,Gal}/K_M)_Gal$ was altered for T169G to 3.5 from a value of 177 for the wild-type enzyme. This indicates that the relative activity with D-galactose was substantially improved in this variant, resulting in both sugars being oxidized with similar relative efficiencies. This could be of interest when the concomitant oxidation of mixtures of D-glucose and D-galactose is necessary.

In addition to studying the kinetic properties of the mutational variants with the electron donor (sugar) substrates, we evaluated the effect of the mutations on the steady-state kinetic constants of two electron acceptors, 1,4-benzoquinone (a two-electron acceptor) and the ferricenium ion Fc$^+$ (a one-electron acceptor), using either D-glucose or D-galactose as saturating sugar substrate. Again, the variant T169A was found to be the least efficient variant, with $k_{cat}/K_M$ values decreased for all substrate combinations measured. This was mainly the effect of substantially lower catalytic constants. Variant T169G showed decreased $k_{cat}/K_M$ values for both electron acceptors when D-glucose was the sugar substrate, while the catalytic efficiency increased considerably for both electron acceptors when D-galactose was the sugar substrate; these $k_{cat}/K_M$ value increased 3.0- and 8.6-fold for

Table III. Kinetic properties of wild-type recombinant pyranose 2-oxidase from T. multicolor and mutational variants for the ferricenium ion as electron acceptor, with the concentration of D-glucose or D-galactose held constant (100 mM).

<table>
<thead>
<tr>
<th>Variant</th>
<th>D-glucose $K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
<th>D-galactose $K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>recP2Ox</td>
<td>0.254 ± 0.099</td>
<td>151 ± 35.44</td>
<td>592</td>
<td>0.070 ± 0.008</td>
<td>5.37 ± 0.22</td>
<td>77.0</td>
</tr>
<tr>
<td>T169G</td>
<td>0.086 ± 0.07</td>
<td>25.89 ± 11.97</td>
<td>302</td>
<td>0.075 ± 0.032</td>
<td>49.4 ± 8.73</td>
<td>660</td>
</tr>
<tr>
<td>T169A</td>
<td>0.049 ± 0.06</td>
<td>1.13 ± 0.068</td>
<td>3.86</td>
<td>0.028 ± 0.012</td>
<td>0.40 ± 0.066</td>
<td>14.4</td>
</tr>
<tr>
<td>T169S</td>
<td>0.263 ± 0.058</td>
<td>213 ± 21.65</td>
<td>810</td>
<td>0.026 ± 0.011</td>
<td>8.66 ± 1.09</td>
<td>336</td>
</tr>
</tbody>
</table>
1,4-benzoquinone and FeC², respectively. This is the result of increased \( k_{\text{cat}} \) values since the Michaelis constants were approximately constant. Variant T169S showed increased \( k_{\text{cat}}/K_M \) values for both 1,4-benzoquinone and FeC², regardless of the sugar substrate used. This significant increase in catalytic efficiencies for the mutational variants T169G and T169S with the electron acceptors 1,4-benzoquinone and FeC² when using D-galactose was an unexpected outcome, but most probably this reflects the higher reactivity with D-galactose. To some extent this increased efficiency could, however, result from a better accommodation of the substrates, both sugar and electron acceptor, in the active sites (Alvarez-Icaza et al. 1995) as T169S showed higher \( k_{\text{cat}}/K_M \) values for both electron acceptors and sugar substrates used in the determination of the kinetic constants.

Sugar oxidizing enzymes are important for applications in biofuel cells and enzyme electrodes (Christenson et al. 2004; Wong & Schwaneberg 2003). For improved mediated electron transfer between these enzymes and the anode of such biofuel cells, high activity with the mediator is essential. Ferrocene derivatives are used frequently as mediators in biofuel cells, and therefore the improved catalytic efficiency with FeC² observed for variants T169G and T169S could be of significance for such applications. Recently, we showed that TmP2Ox can efficiently transfer electrons to an electrode when using appropriate mediators (Tasca et al. 2007).

Thr169 occupies an interesting, and important, position in the TmP2Ox structure. During the oxidative half-reaction when electron-acceptor substrates such as dioxygen are bound, the dynamic substrate loop is fully closed (Hallberg et al. 2004) as T169S showed higher \( k_{\text{cat}}/K_M \) values for both electron acceptors and sugar substrates used in the determination of the kinetic constants.

Figure 2. Model of D-galactose in position for C-2 oxidation in the P2Ox active site. The P2Ox model used is the H167A variant that was determined in complex with 2-fluoro-2-deoxy-D-glucose (Kujawa et al., 2006). The protein conformation shown is that observed during binding of electron-donor substrate, i.e. sugar (reductive half-reaction), during which the side-chain hydroxyl group of Thr169 is not hydrogen bonded to the flavin N5 atom. The axial C-4 hydroxyl group is sterically hindered by the side chain of Thr169 (D-Gal O4–Thr169 O\(^{-}\) ~ 2.2 Å). Atom colouring scheme: oxygen atoms, red; nitrogen atoms, blue; protein carbon atoms, beige; FAD carbon atoms, yellow; carbohydrate carbon atoms, green. The protein backbone is shown as beige coil omitting backbone atoms (i.e. N, C, O). Only relevant amino-acid side chains are shown. The picture was prepared using PyMOL v. 0.93 (http://pymol.sourceforge.net).
recent comparison of a number of different flavoproteins it was noted that a recurrent feature in most of these proteins is a hydrogen bond between N5 of their flavin prosthetic group and a hydrogen-bond donor, typically a backbone or side-chain nitrogen (Fraaije & Mattevi 2000). This proximity of the hydrogen-bond donor to the isoalloxazine ring is expected to increase the oxidative power of the cofactor. In human electron transfer flavoprotein ETF this hydrogen bond between flavin N5 and protein is formed through Thr residue 266 of its N chain. The position of the threonine residue is highly conserved in various ETF from different sources, and there is a conservative replacement of this Thr residue by Ser in a high-potential ETF from Methylphilus methylotrophus (Roberts et al. 1996, 1999). The loss of the hydrogen bond is observed in the mutant αT266M, which is a frequent mutation found in the inherited metabolic disease glutaric acidemia type II (Salazar et al. 1997). The mutation has relatively little effect on the reductive half-reaction of human ETF, while the $k_{cat}/K_M$ value of the oxidative half-reaction is reduced 33-fold.

The possibility of forming a protein-mediated hydrogen bond to the flavin N5 atom is abolished in the P2Ox variants T169G and T169A, while in the conservative mutation of T169S, the hydrogen bond to N5 can be retained by the serine hydroxyl group. This probably explains why $k_{cat}$ values for the sugar substrates are less drastically affected in the T169S variant than for the other two variants, T169G and T169A. Regardless of electron acceptor used, T169A is the most impaired variant. Given the proposed specific role of T169 in the oxidative half-reaction, this is expected since the alanine side chain is unable to form a hydrogen bond, whereas T169G can accommodate a water molecule close to N5. In human ETF the removal of the hydrogen bond between flavin N5 and protein affects the oxidative half-reaction specifically, i.e., its reoxidation by the electron acceptor. In our study, we have only measured steady-state kinetic data, and these reflect both the reductive and the oxidative half-reaction. Hence, it is not possible to identify unequivocally which of the two half-reactions is affected by the removal of the hydrogen bond between flavin N5 and Thr169 in TmP2Ox from these data, and more detailed kinetic studies will be necessary to clearly identify, which reaction step is influenced.

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References


Curriculum vitae

Personal Details
Name: Ph.D. Oliver Spadiut
Address: Burggasse 94/12
A-1070 Vienna, Austria
E-mail: oliver.spadiut@gmx.at
Telephone: +43 650 42 742 96
Date of birth: 07.05.1980
Place of birth: Vienna
Nationality: Austria

Education
11/08 Ph.D. in “Food Science and Biotechnology” graduated with honours
Thesis: “Pyranose 2-oxidase: playground for enzyme evolution”

01/06–12/08 Ph.D. thesis at the Division of Food Biotechnology, Department of
Food Sciences and Technology, University of Natural Resources and
Applied Life Sciences BOKU, Vienna
Project coordinator: Prof. Dietmar Haltrich
Topic: “Tailoring of pyranose oxidase from Trametes multicolor for its
application in fuel cells”; Funded by the Austrian Science Fund (FWF
Translational-Research-Program L213-B11)

06/07–11/07 Visiting scholar at the Chemistry Department, University of British
Columbia UBC, British Columbia, Canada
Supervisor: Prof. Stephen G. Withers
Tasks: creation of mutant libraries of the enzyme LgtC from Neisseria
meningitidis using different directed evolution methods, screening for
increased catalytic activity using FACS, purification and
characterisation of mutated enzymes

06/06 Participation and successful completion of the “Practical Course on
Directed Enzyme Evolution” at the Institute of Biotechnology,
Vilnius, Lithuania
Coordinator: Prof. Uwe Bornscheuer

07/05 Master of Science in “Food Science and Biotechnology” graduated
with honours

05/05 Recipient of the scholarship for “Outstanding success in study” by the
University of Natural Resources and Applied Life Sciences BOKU,
Vienna
11/04–04/05  **Visiting scholar** at the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology SUT, Nakhon Ratchasima, Thailand  
Supervisor: Prof. Montarop Yamabhai  
Tasks: development of a screening assay based on 96 well plates for improved TmP2Ox variants, DNA shuffling experiments

08/04–04/05  **Master thesis** at the Division of Food Biotechnology, Department of Food Sciences and Technology, University of Natural Resources and Applied Life Sciences BOKU, Vienna  
Project coordinator: Prof. Dietmar Haltrich  
Topic: “Modification of pyranose 2-oxidase from *Trametes multicolor* by different directed evolution methods“

10/99–07/05  **Master study** in “Food Science and Biotechnology”, University of Natural Resources and Applied Life Sciences BOKU, Vienna

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**Professional Experience**

05/06–12/08  **Teaching assistant** for the “Laboratory Course in Enzyme Technology”, Department of Food Sciences and Technology, University of Natural Resources and Applied Life Sciences BOKU, Vienna

04/08  **Teaching assistant** for the laboratory course “Enzyme Engineering”, Department of Food Sciences and Technology, University of Natural Resources and Applied Life Sciences BOKU, Vienna

10/05–12/05  **Research assistant** for the „Research Centre Applied Biocatalysis Kplus”, Graz, Austria  
Tasks: creation of mutant libraries of the enzyme pyranose 2-oxidase from *Trametes multicolor* using different techniques of directed evolution, screening for modified substrate specificity and development of suitable high-throughput screening assays

02/04–06/04  **Research assistant** in the “Plant-Microbe Interaction Group” at the Institute of Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences BOKU, Vienna  
Group coordinator: Prof. Gerhard Adam  
Tasks: construction of DON-glycosyltransferase mutants and screening of NIV resistant mutants, construction of gene hybrids to increase resistance of different grains against mycotoxins, maintenance of stock cultures

03/04–04/05  **Teaching assistant** in the laboratory “Molecular Biology I”, Institute of Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences BOKU, Vienna
International Collaborations

Prof. Christina Divne, Royal Technical University Stockholm, Sweden
Prof. Beata Vertessy, Hungarian Academy of Sciences, Budapest, Hungary
Prof. Lo Gorton, Lund University, Sweden
Prof. Stephen G. Withers, UBC, Canada

Summary of Technical Experience

Biochemistry standard techniques
Microbiological and molecular biological standard techniques
Directed evolution methods: epPCR, gene shuffling, saturation mutagenesis, mutator strain, family shuffling, development of screening assays, FACS
Protein purification and characterization
Fermentation
Bioconversion experiments and HPLC analysis

Tutoring Experience
Supervising master students, including planning of master theses and corresponding experiments, supervising the students in the lab, correction of master theses.

Johannes Zöchling  10/05–05/06
Michael Weber  10/06–05/07
Jürgen Ebner  09/06–04/07
Petter Norberg  11/06–01/07
Sebastian Schmeißer  02/07–04/07
Gerald Posch  02/07–09/07
Katrin Radakovits  01/08–07/08
Dagmar Brugger  01/08–07/08
Dominika Prokurat  07/08–12/08
Christian Zingerle  07/08–12/08
Language and Computer Skills

Languages
- German mother tongue
- English fluent (oral and written)
- French intermediate level
- Italian intermediate level

Computer Skills
- Application of bioinformatics software: Vector NTI, SigmaPlot, FastPCR, Chromas
- Microsoft Office (MS Word, Excel, PowerPoint)

List of Publications

Scientific Papers


Spadiut O., Leitner C., Salaheddin C., Varga B., Vertessy B., Tan TC., Divne C. and Haltrich D. “Improving thermostability and catalytic activity of pyranose 2-oxidase from Trametes multicolor by rational and semi-rational design”, 2008, FEBS accepted


Spadiut O., Radakovits K., Tan TC., Divne C. and Haltrich D. “A thermostable variant of pyranose 2-oxidase from Trametes multicolor shows promising characteristics for applications in biofuel cells and for the conversion of hydrolysed lactose”, 2008, Biotechnol. J. accepted


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Scientific Posters


Spadiut O. and Haltrich D. (2008) “Rational protein design of pyranose 2-oxidase from Trametes multicolor (TmP2Ox) for applications in food industry and biofuel cells”, Gordon Research Conference GRC on Biocatalysis, Jul 06-11 2008, Bryant University, Smithfield, RI, USA

Presentations

Spadiut O. „Genetic engineering of the carbohydrate oxidase pyranose 2-oxidase from Trametes multicolor“, 12th Austrian Carbohydrate Workshop, Feb 21 2008, University of Natural Resources and Applied Life Sciences BOKU, Vienna