HMF oxidase
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Towards biotechnological applications of flavoprotein oxidases

Willem P. Dijkman, Gonzalo de Gonzalo, Andrea Mattevi and Marco W. Fraaije

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Selective oxidations are often key in synthetic routes towards valuable chemicals. Chemical oxidation methods typically require harsh conditions and polluting reagents and often suffer from poor chemo- and enantioselectivity. Nature, on the other hand, has come up with a versatile set of enzymes performing a wide variety of selective oxidation reactions. Known oxidative enzyme classes are the oxidases, oxygenases and dehydrogenases. Oxygenases typically require reducing equivalents, *e.g.* NAD(P)H, and molecular oxygen for activity, while dehydrogenases utilize organic coenzymes (for example pyrroloquinoline, quinones or NAD\(^+\)) as electron acceptors. The coenzyme dependence of these enzymes can compromise cost-effective biotechnological applications. Instead of (often expensive) coenzymes, oxidases merely require molecular oxygen as oxidant (electron acceptor) for catalysis. This feature makes them valuable enzymes for industrial applications that range from oxidase-based biosensors to their application as biocatalysts in the synthesis of valuable chemicals. For an organism, however, oxidases are much less favorable. By the direct reduction of molecular oxygen, electrons are lost and cannot be used in subsequent metabolic events, as in the case of dehydrogenases. Another physiological relevant issue concerning oxidases is their inherent reactivity to produce reduced dioxygen species. While some oxidases are capable to reduce dioxygen to harmless water, most oxidases generate hydrogen peroxide as by-product, and even in some cases, the even more toxic superoxide \(\text{O}_2^-\) is formed. The features above provide logic for why oxidases are relatively rare enzymes.

Most oxidases rely on a tightly bound cofactor for their activity, and only a few examples of cofactor independent oxidases have been described.\[^1\] This is due to the fact that amino acids are very poor in mediating redox reactions. To equip enzymes with oxidizing power, nature has evolved several different redox cofactors. For oxidases, there is a bias towards two types of cofactors resulting in two main oxidase families. One family utilizes copper in mono- and trinuclear centers or a copper atom combined with a quinone cofactor. The reader is refereed to many reviews on this topic.\[^2,3\] This chapter will focus on the other major oxidase family: the flavin-containing oxidases.

Flavin cofactors can be present as flavin adenine dinucleotide (FAD) or, less often, as flavin mononucleotide (FMN).\[^4\] In most flavoprotein oxidases, the flavin is the sole cofactor, but in some oxidases either FAD or FMN works in concert with another cofactor. The catalytic cycle of flavoprotein oxidases consists of two half-reactions. In the reductive half-reaction (step 1, Scheme 1), the organic substrate is oxidized by a two-electron transfer, which results in a fully reduced flavin (hydroquinone), and the oxidized product or product intermediate. For this half-reaction, and analogous to nicotinamide-catalyzed oxidations, a proper positioning of the substrate with respect to the reactive N5 atom of the flavin cofactor is required.\[^5\] Regeneration of the oxidized cofactor by dioxygen takes places in the subsequent oxidative half-reaction, which is a far-from-trivial reaction (steps 2 and 3, Scheme 1). In fact, the ability to use molecular oxygen as electron acceptor sets flavoprotein oxidases apart from all other flavoproteins. The reaction is spin forbidden because the electrons in dioxygen are in a triplet state. This restriction is overcome by a stepwise electron transfer.\[^6\] The redox potential of the oxidized flavin-hydroquinone flavin couple varies between -400 and +120 mV in a protein environment, which is much lower than the potential of the dioxygen-hydrogen peroxide couple. As a result, flavin-mediated oxidations that involve dioxygen as electron acceptor are thermodynamically favorable. Yet, the protein microenvironment around the flavin cofactor...
The success of flavin-containing oxidases in biotechnological applications is for a large part due to their interesting catalytic properties, as they can perform a wide variety of different oxidation reactions with exquisite chemo-, regio- and/or enantioselectivity while merely using molecular oxygen as oxidant. The best-known flavoprotein oxidase is probably glucose oxidase which has been produced and applied already for several decades. Other known examples of applied oxidases are γ-amino acid oxidase, employed in synthesis of antibiotics, and monoamine oxidases, used for the preparation of enantiopure fine chemicals. However, in the last decade a large number of flavoprotein oxidases with other substrate scopes and reactivities have been discovered. From detailed biochemical studies on these novel oxidases, new insights have been obtained on the catalytic properties of these enzymes. Besides oxidases that are able to catalyze relatively simple oxidations (for example the oxidation of alcohols to aldehydes or ketones), also more complex oxidative reactions can be catalyzed for example oxidative C-C bond formation by reticuline oxidase. The architecture of the active site of each oxidase clearly determines its substrate acceptance profile and oxidation reactivity. Moreover, the active site also contains the structural requirements to allow oxygen to reach the flavin and to facilitate dioxygen reduction by the reduced cofactor. In some cases, the active site entails even an additional catalytic property: it catalyzes the formation of a covalent flavin-protein bond. By this, the flavin cofactor is covalently tethered to the protein, preventing dissociation and increasing the redox potential.

Comparison of the currently available flavoprotein oxidase sequences and structures reveals that the oxidases belong to several structurally distinct flavoprotein families. In this chapter, an inventory of currently known flavoprotein oxidases is provided. Based on sequence homology and the available structural information, a comprehensive classification is proposed (Table 1). The distinct flavoprotein oxidase families will be described in more detail in the next paragraphs.

**Scheme 1.** Catalytic cycle of the flavin cofactor in flavoprotein oxidases.
Table 1. Classification of the major flavoprotein oxidase families.

<table>
<thead>
<tr>
<th>family</th>
<th>cofactor type</th>
<th>cofactor binding mode</th>
<th>typical substrates</th>
<th>example (PDB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMC</td>
<td>FAD</td>
<td>+</td>
<td>+</td>
<td>glucose oxidase (1CF3)</td>
</tr>
<tr>
<td>VAO</td>
<td>FAD</td>
<td>+</td>
<td>+</td>
<td>vanillyl alcohol oxidase (1VAO)</td>
</tr>
<tr>
<td>AO</td>
<td>FAD</td>
<td>+</td>
<td>+</td>
<td>D-amino acid oxidase (1KIF)</td>
</tr>
<tr>
<td>SO</td>
<td>FAD</td>
<td>+</td>
<td>-</td>
<td>Erv2p sulfhydryl oxidase (1RA)</td>
</tr>
<tr>
<td>ACO</td>
<td>FAD</td>
<td>+</td>
<td>-</td>
<td>acyl-CoA oxidase (1IS2)</td>
</tr>
<tr>
<td>HAO</td>
<td>FMN</td>
<td>+</td>
<td>-</td>
<td>glycolate oxidase (1GOX)</td>
</tr>
</tbody>
</table>

The GMC-type oxidase family

The glucose-methanol-choline oxidoreductase (GMC) flavoprotein family contains several well-known oxidases. The protein sequence of each member of this family entails a conserved N-terminal FAD binding domain, called GMC_oxred_N (Pfam00732) in the Pfam database. This domain includes the typical GxGxxG/A sequence motif, which is indicative for the Rossmann fold that is involved in binding the ADP moiety of the FAD. Less conserved is the C-terminal region, which forms the substrate binding domain. It contains only one generally conserved residue, an active-site histidine, which act as base in substrate oxidation and FAD reoxidation by molecular oxygen. Mutagenesis studies on glucose oxidase has revealed that protonation of this histidine (H516 in GO) is essential for catalyzing dioxygen reduction. Nevertheless, exceptions to this rule have already been discovered. For instance, in choline oxidase, the function of the histidine has been taken over by the positively charged amine moiety of the choline substrate molecule.

Known GMC-type oxidases act on primary and secondary alcohols, forming the corresponding aldehydes or ketones. Examples are glucose oxidase (EC 1.1.3.4) (Figure 1A), cholesterol oxidase (EC 1.1.3.6), aryl alcohol oxidase (EC 1.1.3.7), pyranose oxidase (EC 1.1.3.10), methanol oxidase (EC 1.1.3.13), choline oxidase (EC 1.1.3.17) and 5-(hydroxymethyl)furfural oxidase (EC 1.1.3.47). Aldehyde products which are hydrated to the gem-diol in solution can often undergo a second oxidation. An example is choline oxidase, which performs two sequential oxidations on choline, yielding the carboxylic acid glycine betaine (N,N,N-trimethylglycine), as shown in Scheme 2. In chapter 2 and 3 it is demonstrated that 5-(hydroxymethyl)furfural oxidase is also able to perform oxidations of aldehydes by acting on the respective gem-diols. In fact, work described in chapter 3 reveals that the bacterial oxidase is exceptional by catalyzing a cascade reaction that involves four oxidations which includes the oxidation of two aldehyde product intermediates.

Scheme 2. Choline oxidase catalyzed sequential oxidation of choline into trimethylglycine.
The majority of the GMC-type oxidases is active towards primary alcohols, but glucose oxidase, cholesterol oxidase and pyranose oxidase are able to oxidize one, or multiple, secondary hydroxyl groups on their carbohydrate or sterol substrates.\cite{17,21} Aryl alcohol oxidase,\cite{22} and 5-(hydroxymethyl)furfural oxidase (chapter 4) are active on primary alcohols while displaying no significant activity on secondary alcohols. Yet, it has been shown by us (chapter 4) and others\cite{22} that activity towards secondary alcohols can be introduced by enzyme engineering. To introduce secondary alcohol oxidation activity in these enzymes, a tryptophan residue adjacent to the active site histidine was mutated to the smaller residues phenylalanine or alanine. The created mutant enzymes of aryl alcohol oxidase and 5-(hydroxymethyl)furfural oxidase were found to be enantioselective towards secondary alcohols. This allows their use for kinetic resolution of chiral alcohols.\cite{23} The oxidation of secondary alcohols is however still considerably slower than the oxidation of primary alcohols.

All GMC-type oxidases oxidize alcohol groups and this was long thought to be the only activity of these enzymes. However, in chapter 6 of this thesis it is shown that GMC-type oxidases are not restricted to alcohols, but are also active towards the thiol analogues of their alcohol substrates. Methanol oxidase, glucose oxidase and 5-(hydroxymethyl)furfural oxidase were all found to convert thiol substrates.\cite{24} In all cases the conversion of the thiol substrates is less efficient than the oxidation of alcohol substrate, both due to a slightly lower $k_{cat}$ and a higher $K_M$ value. The oxidation of primary thiols leads to the formation of thioaldehydes, analogues to the oxidation of alcohols to aldehydes, and the oxidation mechanism is likely
to be the same in both cases. Thioaldehydes can serve as a starting point for the synthesis of thioamides,\textsuperscript{[25]} and this functional group is commonly found in drugs and other biologically active compounds.\textsuperscript{[26,27]} Overall, these findings disclose new possibilities for the use of oxidases and show that the activity of GMC-type oxidases is more versatile than previously thought.

Several oxidases were found to contain a covalently linked FAD cofactor (choline oxidase and pyranose oxidase), where the flavin is attached via an $\alpha$-$N_3$-histidyl bond. Nevertheless, the majority of GMC-type oxidases contain dissociable FAD cofactor that is tightly bound to the protein. The N5 of the FAD cofactor serves as the hydride acceptor, enabling a one-step oxidation of alcohols. A second recurrent element of the active site of a GMC-type oxidase is a strictly conserved active site histidine which serves as a base and is positioned close to the FAD ring. In most GMC-type oxidases this histidine has been mutated, rendering the enzyme inactive or catalytically impaired.\textsuperscript{[28–31]} A second conserved residue is an asparagine (N510 in choline oxidase, N593 in pyranose oxidase) or histidine (H546 in aryl alcohol oxidase) serving as hydrogen bond donor. An exception is pyridoxine 4-oxidase (EC 1.1.3.12) which contains a proline at this position.\textsuperscript{[32]} Other active site residues are less conserved and often substrate specific. For example, a glutamic acid to position the positive charge of choline is found in choline oxidase,\textsuperscript{[33]} while phenylalanine residues in aryl alcohol oxidase position the aromatic substrates oxidized by this enzyme.\textsuperscript{[34]}

Mechanistic details have been established for a number of GMC-type oxidases, including glucose oxidase, pyranose oxidase, aryl alcohol oxidase and choline oxidase. The mechanism by which GMC-type oxidases oxidize their substrates involves a direct hydride transfer from the substrate to the N5 atom of the FAD cofactor. This reaction is facilitated by proton abstraction, typically promoted by a histidine acting as a general base.\textsuperscript{[17]} During substrate oxidation, hydride transfer to the FAD cofactor can occur either after proton abstraction (stepwise mechanism), or simultaneously with proton abstraction (concerted mechanism). Detailed analysis of several GMC-type oxidases shows that both mechanisms occur in this family and even within a single enzyme.\textsuperscript{[21,30,35]} In the wild type choline oxidase for example, breaking of the C-H and O-H bonds occurs in a stepwise fashion. The substitution of asparagine 510 to alanine results in a mutant enzyme displaying a concerted mechanism.\textsuperscript{[35]} Upon oxidation of the substrate and concomitant reduction of the flavin cofactor, there are two options to complete the catalytic cycle. In a ping-pong mechanism, the product is released from the active site before the cofactor is reoxidized. In a ternary complex mechanism, the reduced FAD is reoxidized while the product is still bound. Both mechanism have been observed for GMC-type oxidases.\textsuperscript{[36–39]} It has been shown for aryl alcohol oxidase that not the enzyme, but the substrate dictates which mechanism applies.\textsuperscript{[34]}

In addition to oxidases, the GMC flavoprotein family also contains other types of flavoenzymes, for example dehydrogenases. Functionally related to GMC-type oxidases are pyranose dehydrogenase and choline dehydrogenase,\textsuperscript{[40,41]} which catalyze the same oxidation reaction as their oxidase siblings pyranose oxidase and choline oxidase. Dehydrogenases are, unlike oxidases, unable to use molecular oxygen as electron acceptor. The molecular details behind this differentiation were recently investigated for pyranose oxidase and pyranose dehydrogenase.\textsuperscript{[42,43]} The GMC-family even contains non-redox flavoproteins, for example hydroxynitrile lyase.\textsuperscript{[44]} At present, there is no sequence motif known that can discriminate between GMC-type oxidases and other GMC-type enzymes. The same holds true for most of the flavoprotein oxidase families below. More knowledge on the precise mechanism by which
flavoproteins can tune their oxygen reactivity may change this situation and would perhaps enable more reliable annotation of flavoprotein-encoding genes into oxidases, facilitating effective genome mining for novel flavoprotein oxidases.

The VAO-type oxidase family

The second flavoprotein oxidase family, is the vanillyl alcohol oxidase (VAO) flavoprotein family, which was named after the fungal oxidase VAO (EC 1.1.3.38). Similar to GMC-type flavoproteins, all VAO-type flavoproteins contain a distinct FAD binding domain (FAD-binding_4 domain, Pfam01565) in the N-terminal half of the protein (Figure 1B). This domain includes the so-called PP loop where the pyrophosphate moiety of the FAD interacts with the protein. The vanillyl alcohol oxidase crystal structure revealed for the first time how a FAD cofactor is covalently tethered to a protein via a histidine residue. Indeed, the VAO flavoprotein family is relatively rich in covalent flavoproteins (estimated 25 percent contain a histidyl-FAD). It has been established that, by covalent attachment of the FAD cofactor to a histidine, the redox potential of the cofactor is increased to a large extend. As a result, the number of feasible electron acceptors is limited, leaving molecular oxygen as one of the few candidates. This explains why many VAO-type flavoproteins act as oxidases. It has been discovered that several VAO-type oxidases even contain a bicovalently linked FAD cofactor. A recent engineering study on 6-hydroxy-d-nicotine oxidase (6HDNO) showed that it is possible to introduce a second covalent bond, changing the FAD cofactor from monocovalently to bicovalently bound. All bicovalently linked FAD cofactors are tethered at the 6 position to a cysteine, in addition to the histidyl bond. Except for an effect on the redox potential, it is hypothesized that the second covalent bond allows the protein to create a very open active site, while retaining the FAD cofactor in a proper position for catalysis through the bicovalent anchoring. This is in line with the observation that the described bicovalent flavoprotein oxidases all accept rather bulky substrates.

For VAO-type oxidases, some details on the oxygen reactivity have been elucidated. For the VAO-type cholesterol oxidase and alditol oxidase (EC 1.1.3.41) specific oxygen channels have been described, guiding dioxygen to the FAD. In alditol oxidase, all proposed channels are situated in the C-terminal part of the protein and lead to one oxygen entry site in the active site. Based on this finding, a mutant of l-galactonolactone dehydrogenase (EC 1.3.2.3) has been engineered in which such an oxygen entry in the active site was created. The mutation indeed resulted in the creation of an oxidase.

In the last decade, a large number of novel VAO-type oxidases have been discovered and studied. In addition to alcohol oxidations, leading in most cases to aldehydes or ketones, many other bonds can be oxidized. Vanillyl alcohol oxidase itself was shown to perform alcohol and amine oxidations, hydroxylations of alkyl phenols and ether bond cleavage. Alditol oxidase was shown to be active toward thiols, in addition to its natural alcohol substrates, which is presented in chapter 6 of this thesis. Another remarkable example of a VAO-type oxidase is reticuline oxidase, also known as berberine bridge enzyme (EC 1.21.3.3). This enzyme is able to form a C-C bond, creating the intramolecular bond between a methyl group and an aromatic carbon in the conversion from (S)-reticuline to (S)-scoulerine, a plant alkaloid. Elucidation of the crystal structure of this plant enzyme has revealed that the reaction is triggered by hydride transfer from the substrate to the flavin cofactor.
VAO-type oxidases that can catalyze an oxidative C-C bond formation are not restricted to plants. Prosolanapyronell oxidase (EC 1.1.3.42) from the fungus *Alternariasolani* performs an intramolecular Diels-Alder reaction, a [4+2] cycloaddition, in which a conjugated diene reacts with a carbon-carbon double bond. In addition, other microbial C-C and C-O forming VAO-type oxidases have been described in recent literature.

The amine oxidase (AO) family

The amine oxidase (AO)-type oxidases belong to another distinct structural FAD-containing flavoprotein family. As the name suggests, most enzymes are solely active on amines. Prototype oxidases for this family of flavoprotein oxidases are microbial amino acid oxidases (Figure 1C) and eukaryotic monoamine oxidases. Beside C-N oxidizing oxidases, a few alcohol and thioether oxidases have been found that belong to this family. This shows that AO-type oxidases cover a wide range of different reactions. The individual enzymes, however, can in most cases perform only one type of reaction.

All members of this family (Pfam01593 and Pfam01266) share a similar domain in the N-terminal half of the protein, responsible for FAD binding. Covalent binding through a histidyl or cysteinyl bond on the 8α position of FAD is frequently observed. Most AO-type oxidases act on a carbon-nitrogen bond of a primary or secondary amine. The catalytic cycle of these oxidases starts with proton abstraction from the amine or ammonium group by an active side base like histidine or a catalytic dyad of histidine and tyrosine. Hydride transfer to the enzyme-bound FAD results in the formation of an imine product intermediate, which can spontaneously hydrolyze to an aldehyde and amine.

Among the known AO-type oxidases, also thioether oxidases have been identified: prenylcysteine lyase (EC 1.8.3.5) and farnesylcysteine lyase (EC 1.8.3.6). The proposed catalytic mechanism for prenylcysteine oxidase is quite distinct from other FAD-catalyzed oxidations. The C-S bond is not activated by an active site base; the first step is hydride transfer to FAD. The resulting thiocarbenium is hydrolyzed to farnesal, an aldehyde, and cysteine.

![Scheme 3](image-url)

**Scheme 3.** Proposed mechanism for the formation of farnesal and cysteine as final products in the prenylcysteine-lyase-catalyzed oxidation of prenylcysteine.
Another unusual AO-type oxidase is nikD which is involved in the biosynthesis of the antibiotic nikkomycin. This oxidase produces the aromatic compound picolinate by a 4-electron oxidation of Δ-piperideine-2-carboxylate (Scheme 4). In the proposed mechanism, the reaction is initiated with oxidation of a carbon-nitrogen bond. After tautomerisation and isomerisation of the formed imine, the same carbon-nitrogen bond is oxidized again resulting in the final, aromatic product.\[^{60}\]

In contrast to all other members of this flavoprotein oxidase family, including monomeric sarcosine oxidase (EC 1.5.3.1), heterotetrameric sarcosine oxidase does not rely only on FAD as cofactor. The α-subunit of this tetrameric enzyme binds NAD\(^+\), but has no described catalytic function. The β-subunit resembles monomeric sarcosine oxidase, but contains both FAD and covalently bound FMN. In this oxidase subunit, sarcosine is oxidized to the corresponding imine by FAD. With the help of the γ and δ subunits, heterotetrameric sarcosine oxidase can interact with 5,10-CH\(_2\)-H\(_4\)-folate synthase, forming a channel, which prevents hydrolysis of the imine. The reaction of the imine product reacts with tetrahydrofolate resulting in the formation of 5,10-CH\(_2\)-tetrahydrofolate and glycine.\[^{61}\]

The sulfhydryl oxidase family

A number of FAD-containing oxidases acting on thiols have been discovered in the last decade. The most important role of these so-called sulfhydryl (SO) oxidases is the formation of disulfide bonds in proteins by oxidizing cysteine residues. Crystal structures of several sulfhydryl oxidases have been solved, including human, viral and yeast variants (Figure 1D).\[^{62,63}\] Based on this, two subfamilies can be identified: Erv-like (Pfam04777) and Ero-like (Pfam04137) sulfhydryl oxidases. The first subfamily is named after Erv1 (EC 1.8.3.2), a protein that was discovered in yeast as essential for respiration and viability. The Ero-like subfamily contains homologs of yeast Ero1, which provided insight in the cellular maintenance of a relatively high oxidation potential in the ER compared to the strongly reducing cytosol in which it is embedded.\[^{64}\] Although Ero and Erv oxidases differ in many aspects, including size and substrate spectrum, they belong to the same structural family. Both types of sulfhydryl oxidases have an all-alpha fold, in contrast to all other flavoprotein oxidases. In addition, the atypical FAD binding mode is similar in both subfamilies: the isoalloxazine ring is located between a “barrel” of four helices, with the adenine moiety exposed on the surface. FAD is neither extended (isoalloxazine and adenine far apart) nor bend (both moieties close together): the molecule is bound in an intermediate form.\[^{65}\] Covalent binding of the flavin cofactor has not been observed in any member of this flavoprotein oxidase family.

Erv1 from *Saccharomyces cerevisiae* has only 106 residues, which fold around the FAD cofactor, forming five alpha helices.\[^{65}\] This is different from most other FAD-containing...
oxidases, which usually have an N-terminal FAD binding domain and a C-terminal substrate binding domain. The ability of sulfhydryl oxidases to use dioxygen as electron acceptor has been explained by accessibility of the active site. In yeast Ery a hydrophobic channel is observed leading to the N5-C4a of the FAD, the oxygen-reactive locus of the flavin cofactor.\textsuperscript{[66]} Rat Erv1 does not have this channel and has poor reactivity towards dioxygen. The inactivity toward dioxygen of some Erv members points in the direction of another physiological terminal electron acceptor. For these enzymes transfer of electrons to cytochrome c oxidase has been proposed, leading to water instead of hydrogen peroxide, or alternatively, the electrons are shuttled in the respiratory chain instead of direct reduction of dioxygen.\textsuperscript{[66]}

Sulfhydryl oxidases do not form disulphide bonds directly on target proteins. For Ero, this is achieved via action of protein disulphide isomerase (PDI), the enzyme which introduces disulphide bonds in proteins in the ER.\textsuperscript{[67]} The catalytic disulphide bond in PDI is regenerated by action of Ero1p. This oxidation is not performed directly by the FAD cofactor but rather by a set of conserved cysteines, which form a relay path to transport the electrons to the core of the protein where the isoalloxazine is buried.\textsuperscript{[68]} Erv1 act on target proteins by forming a complex with Mia40.\textsuperscript{[62]} Erv1 contains a flexible loop, which functions as electron shuttle, bringing electrons to the active site. In general, as sulfhydryl oxidases do not catalyze direct cysteine oxidations of proteins, their industrial applicability seems to be limited.

### The acyl-CoA oxidase-type oxidase family

Acyl-CoA oxidase represents the prototype oxidase of another flavoprotein oxidase family. Acyl-CoA oxidase-type (ACO) oxidases contain an N-terminal domain of only \( \alpha \)-helices (Pfam02771), a middle domain formed by a \( \beta \)-barrel (Pfam02770), and a C-terminal domain of \( \alpha \)-helices (Pfam00441) (Figure 1E). These oxidases employ FAD as only cofactor, which is located between the middle and the C-terminal \( \alpha \)-domain. ACOs (EC 1.3.3.6) catalyze the \( \alpha \)-C\( \beta \) oxidation of fatty acids. The \( \alpha \)-proton is abstracted by an active site glutamate to trigger a hydride transfer from the \( \alpha \) to the N5 of the FAD cofactor.\textsuperscript{[69]} As for most other flavoprotein oxidase families, ACO sequence-related acyl-CoA dehydrogenases exist. Whereas the oxidases are predominantly found in peroxisomes, fatty acid degradation in the mitochondria is performed by acyl-CoA dehydrogenases.\textsuperscript{[70]}

Another recently discovered ACO-type oxidase is the fungal nitroalkane oxidase (EC 1.7.3.1). This FAD-containing oxidase oxidizes primary and secondary nitroalkanes to the corresponding aldehydes and ketones with the release of nitrite (Scheme 5). The first step, abstraction of the \( \alpha \)-proton from the nitroalkane, is similar to the acyl-CoA oxidase mechanism. The carbanion obtained forms a covalent adduct with the N5 of the flavin, which generates a cationic flavin imine. Attack by water leads to the reduced flavin and the final product, the aldehyde or ketone.\textsuperscript{[71]} Reoxidation of the reduced flavin cofactor by molecular oxygen completes the catalytic cycle.

\[
\text{Scheme 5. General scheme of the oxidation of primary nitroalkanes by nitroalkane oxidase in order to obtain aldehydes.}
\]
The 2-hydroxyacid oxidase family

A number of flavoprotein oxidases use FMN as prosthetic group. The majority of these oxidases belong to one distinct structural flavoprotein family. Many members of this oxidase family oxidize aromatic or aliphatic 2-hydroxy acids (grouped in EC 1.1.3.5), forming the respective 2-oxoacids. Known examples of this oxidase family are glycolate oxidase and L-lactate oxidase. The structure of several members of this family has been solved, providing insights into the fold and the catalytically important residues. The proteins have a β8/α8 TIM barrel structure (Figure 1F). A conserved arginine in the active site typically accommodates the carboxyl group of the substrate. The catalytic mechanism of the reductive half-reaction for 2-hydroxyacid oxidase (HAO)-type is still under debate. Studies seem to support both the formation of a carbanion and a hydride transfer mechanism. In both cases, however, a histidine (H265 in lactate oxidase) is involved, abstracting the Cα-proton in the case of the carbanion mechanism and the proton from the Cα-hydroxyl group in the hydride transfer mechanism.

The crystal structure of a FMN-containing nitroalkane oxidase has recently been elucidated. It revealed no structural resemblance with the above-mentioned ACO-type oxidases but rather to the HAO-type flavoprotein oxidase family. This shows that members of the HAO family can perform other reactions than the oxidation of 2-hydroxyacids. The active site of this FMN-containing TIM barrel protein also contains a histidine residue that was found to be crucial for catalysis.

Unique flavoprotein oxidases

Several flavoprotein oxidases have been identified for which no or only a few homologs with other activities have been described. These individual oxidases are discussed below.

Pyruvate oxidase is an exceptional flavin-containing oxidase as it contains two cofactors, the vitamin B1 and B2 derivatives, thiamine diphosphate (ThDP) and FAD, respectively. The combination of ThDP and FAD in one enzyme is rare, but not restricted to pyruvate oxidase. Acetolactate synthase, also acting on pyruvate, is another example in which both cofactors are bound. The crystal structure of pyruvate oxidase from Lactobacillus plantarum has revealed that each monomer of the homotetramer consist of three domains. The FAD cofactor is not buried within the enzyme but is located at the surface between the N-terminal α-domain and the middle β-domain. ThDP is coordinated by a magnesium ion and located at the C-terminal γ-domain. The α- and γ-domains have a similar topology, whereas the β-domain differs. Pyruvate oxidase catalyzes the decarboxylation of pyruvate to acetyl phosphate and carbon dioxide, consuming oxygen and yielding hydrogen peroxide. The proposed catalytic mechanism involves both cofactors, ThDP and FAD. The activated C2 carbanion of the thiazolium ring of ThDP attacks pyruvate, leading to decarboxylation. FAD subsequently oxidizes the ThDP-substrate adduct. The attack by phosphate releases acetyl phosphate from ThDP, and FADH₂ is oxidized by dioxygen to complete the catalytic cycle.

Pyridoxal 5′-phosphate oxidase (PNPO, EC 1.4.3.5) is an essential enzyme in the metabolism of vitamin B6. The enzyme oxidizes the amine group of pyridoxamine 5′-phosphate to an aldehyde, a reaction similar to those catalyzed by the AO-type oxidases. The enzyme is also active on pyridoxine 5′-phosphate in which the alcohol is oxidized to the aldehyde. In
addition, activity on imine and ester moieties has been described. PNPO contains a FMN as flavin cofactor and displays no structural resemblance with any of the above discussed flavoprotein oxidases. The crystal structures of human, yeast and two bacterial homologs have been solved. All PNPOs are dimers, with each monomer composed of two domains. The N-terminal domain (Pfam01243) forms a six stranded $\beta$-barrel with antiparallel strands. The smaller C-terminal domain (Pfam 10590) adopts a helix-loop-helix conformation. FMN binds in the $\beta$-barrel of the protein with the phosphate moiety near the N-terminus. Mechanistic studies have revealed that, as for many other flavoprotein oxidases, oxidation of the substrate is achieved by a direct hydride transfer from the substrate to the N5 of the FMN cofactor.

Another FAD-containing oxidase that catalyzes only one type of reaction is NADPH oxidase (NOX). This enzyme is implicated in a large number of physiological processes in humans. A crucial catalytic property is the ability to generate superoxide as reduced oxygen species. The enzyme is composed of multiple subunits and is membrane bound. Although the crystal structure of NOX has not yet been elucidated, a structure of the subunit gp91$^{\text{phox}}$ has been proposed based on sequence alignments and homology models. This shows that the protein contains an N-terminal membrane domain composed of six $\alpha$-helices. The C-terminal domain is exposed in the cytosol and contains both the NADPH and the FAD binding sites. NOXs have also been described in prokaryotes, being both FAD and FMN-dependent enzymes depending on the microbial source. These enzymes can be produced as recombinant enzymes, being valuable biocatalysts for regeneration of the NAD(P)$^{+}$ cofactor.

Xanthine oxidase (XO, EC 1.17.3.2) represents yet another flavin-containing oxidase type. The oxidase does not only rely on FAD for activity, but also contains two non-identical [2Fe-2S] clusters and a molybdenum cofactor. In fact, the oxidation of the organic substrate occurs at the molybdopterin. The [2Fe-2S] clusters assist in catalysis by facilitating electron transfer from the molybdenum site to the flavin cofactor. The FAD cofactor is merely used to transfer the electrons to a suitable electron acceptor. This also explains the atypical oxidations that are catalyzed by XO: it can catalyze hydroxylations of a large number of compounds, including pyrimidines, purines and pterins. In contrast with typical oxygenases, the inserted oxygen is derived from water. XO is an excellent example of the thin line between oxidases and dehydrogenases. The oxidase can efficiently use dioxygen as electron acceptor, but is unable to reduce NAD$. This is in sharp contrast with xanthine dehydrogenase (XDH) for which the opposite is true. Yet, both XO and XDH are essentially the same protein. XDH can reversibly be interconverted to XO when two disulfide bonds are formed. The disulfide bond formation causes a conformational change which disrupts the cavity needed for NAD$ to reach FAD. Irreversible formation of XO from XDH happens upon proteolytic cleavage of the linker between the FAD and the molybdenum binding domain, again changing the environment of the NAD$ binding site, leaving the protein only active towards dioxygen. Another special feature of XO is that, depending on the conditions, it produces hydrogen peroxide or superoxide as reduced dioxygen species.

**Flavoprotein oxidases in biotechnology**

Flavoprotein oxidases have been widely employed in biocatalytic processes. In most cases, the targeted reactions involved the selective oxidation of alcohols or amines. In the last decade, amine-oxidizing enzymes have become very popular as biocatalysts. Both monoamine oxidases
and amino acid oxidases have been extensively applied in the selective oxidation of amines and amino acids under mild reaction conditions. For instance, a D-amino acid oxidase from yeast has been employed in the industrial preparation of 7-aminocephalosporanic acid\cite{88} and an engineered bacterial glycine oxidase could be engineered to convert N-(phosphonomethyl) glycine (glyphosate).\cite{89} Another development is the use of monoamine oxidases and amino acid oxidases in chemo-enzymatic deracemization of racemic amines. This concept relies on the ability of the oxidases to oxidize only one enantiomer into the corresponding imine or imino acid, which is chemically (non-selectively) reduced back into the amine. By performing this cycle of enzymatic oxidation and chemical reduction in on pot (cyclic deracemization), a 100% theoretical yield of an optically pure amine is feasible. For examples of this elegant approach for deracemization of amines, the reader is referred to a recent review of Turner.\cite{10}

Amine-oxidizing oxidases have also been combined with other enzymes for the preparation of enantiopure amines.\cite{90} For example, in 2011, enantiopure (S)-amino-3-(3-(6-(2-methylphenyl))pyridyl)-propionic acid was synthesized in 72% yield from the racemic amino acid by combining the (R)-amino acid oxidase from *Trigonopsis variabilis* with (S)-aminotransferase from *Sporosarcina urea*.\cite{91} The racemic amino acid mixture was converted by the oxidase into the (S)-enantiomer and a keto acid, of which the latter was converted by the aminotransferase into the (S)-amino acid. An (S)-aminotransferase from *Burkholderia* sp. was also employed with a global yield of 85% (Scheme 6). The process was scaled up to 100 L. An (S)-amino acid oxidase has been used for production of 2-amino-3-(7methyl-1-H-indazol-5-yl)-propionic acid, a key intermediate for the preparation of anti-migraine drugs. Enantiopure (R)-amino acid was prepared with 79% global yield by the selective oxidation with the (S)-amino acid oxidase from *Proteus mirabilis* followed by the transamination of the keto acid intermediate by a commercially available (R)-transaminase.\cite{92}

One recent example of a flavoprotein oxidase that can be used for synthetic chemistry is the plant flavoprotein oxidase, reticuline oxidase. This VAO-type oxidase enzyme catalyzes the biotransformation of (S)-reticuline into (S)-scoulerine through the formation of an intramolecular C-C bond at the expense of molecular oxygen. Scoulerine and related compounds often exhibit interesting biological properties.\cite{93} Therefore, reticuline oxidase has been thoroughly studied for its use in the synthesis of such alkaloids. The flavoenzyme has been shown to be a potent biocatalyst for performing oxidative coupling reactions to form a range of benzylisoquinolines derivatives with high selectivity. Initial experiments showed that catalase was required in the reaction medium, as the formation of hydrogen peroxide during the oxidative process led to inactivation of the oxidase. Nevertheless, the enzyme is able to maintain its activity in a broad

![Scheme 6. Enzymatic synthesis of (S)-amino-3-(3-(6-(2-methylphenyl))pyridyl)-propionic acid by cyclic deracemization using two biocatalysts.](image-url)
range of pH values (8 to 11) and temperatures (30-60 °C). As most of the reticuline oxidase substrates are poorly soluble in water, the effect of different organic solvents and ionic liquids on the biocatalyst properties was studied. This revealed that the oxidase is highly tolerant towards solvents. It enabled working at solvent concentrations of 70% v/v and substrate concentrations of 20 g/L.[94] Thus, a set of racemic 1-substituted tetrahydroisoquinolines were synthesized starting from the corresponding N-phenylmethylamines and phenylacetic acid derivatives and subjected to reticuline oxidase-catalyzed resolutions.[94] These oxidase-mediated conversions yielded optically pure products. Oxidation of (S)-reticuline catalyzed by wild-type reticuline oxidase occurs with total regioselectivity, leading to the formation of the 9-hydroxy functionalized regioisomer (S)-scoulerine, while the 11-hydroxy regioisomer (S)-coreximine was not formed (Scheme 7). The reticuline oxidase E417Q mutant was able to produce a 30% of the 11-hydroxy regioisomer, at the expense of a huge decrease in the reaction rate for the major regioisomer, due to the substrate hydroxyl group deprotonation and phenyl moiety activation induced by the residue Glu417. As some of the 11-hydroxy functionalized products present biological activity, such as (S)-isocoreximine, (S)-corytenchine and phellodendrine, reticuline oxidase and its mutant were employed in the oxidative coupling of reticuline analogues, but only traces of the non-expected regioisomers were found. Only substrate modification, by introducing fluoro substituents, led to the synthesis of the 11-hydroxy compounds as sole products with good yields and excellent selectivities.[95]

Oxidases have also attractive features for use in biosensors. A well-known example of a flavoprotein oxidase that has been used for biosensor development is glucose oxidase,[96] which has been extensively applied to monitor the blood glucose levels in diabetics due to its catalytic ability to oxidize glucose. The generated electrons can be used in a direct or indirect manner for readout of a sensor. Another example of an oxidase that can be used for diagnostic purposes is fructosyl amino acid oxidase which oxidizes the C-N bond of an amino acid-carbohydrate adduct, resulting in formation of the respective glucosone and amino acid. The oxidase is of interest for detecting glycated proteins, a marker for hyperglycemia in diabetes patients.[97] Also D-amino acid oxidases are widely used for the quantification of D-amino acids in biological samples by virtue of their strict stereospecificity.[98,99]

Scheme 7. Reticuline oxidase-catalyzed oxidative C-C coupling leading to the 9-hydroxy (major) or 11-hydroxy (minor) stereoisomer.

Concluding remarks

Oxidases are widespread among a large number of structural flavoprotein families. This shows that nature has found many ways to exploit the ability of the reduced flavin cofactor to use
molecular oxygen as oxidant. Interestingly, depending on the structural fold, certain oxidation reactions are preferred. For example, for GMC-type oxidases, there is a strong bias towards alcohol oxidation while most members of the amine oxidase (AO) family exclusively act on amino groups. Yet, oxidases from different structural families can have similar activities. A notable example is cholesterol oxidase for which two structurally distinct representatives have been discovered, one belonging to the VAO oxidase family and the other is a GMC-type oxidase. Another interesting case of shared substrate specificity among two flavoprotein oxidase families has been found for 6-hydroxynicotine oxidases. For each enantiomer of this compound, a specific and structurally distinct oxidase has been found: (S)- and (R)-6-hydroxynicotine oxidase (resp. EC 1.5.3.5 and EC 1.5.3.6). These enzymes oxidize the pyrrolidine ring in the substrate to form the enamine 6-hydroxy-N-methylmyosmine 6-hydroxy-N-methylmyosmine. Hydration leads to the final product (R)-6-hydroxy-pseudooxynicotine. Interestingly, the other enantiomer (S)-6-hydroxynicotine is oxidized by a AO-type oxidase.

The flavoprotein oxidases that encompass the broadest range of oxidation activities are the VAO-type oxidases. Reactions of these oxidases include alcohol oxidations, amine oxidation, thiol oxidation, oxidative ether-bond cleavage, hydroxylations and oxidative C-C and C-O bond formation. Moreover, the recently discovered VAO-type oxidases that harbor a bicovalently bound FAD have shown to be able act on relatively large substrates. This makes the VAO-type oxidases an interesting oxidase family when looking for new oxidative biocatalysts. With the available sequence and structure data, it has become feasible to discover new oxidases by genome mining. In the last decade several novel flavoprotein oxidases have been discovered by this approach: alditol oxidase, eugenol oxidase, putrescine oxidase and chitoologosaccharide oxidase, and in this thesis, the identification and characterization of 5-(hydroxymethyl)furfural oxidase will be presented. Genome mining has also shown to be effective in identifying thermostable variants of known oxidases, as shown in L-aspartate oxidase or alditol oxidase. This shows that it is becoming relatively easy to identify and produce new oxidases for biotechnological applications. For fine tuning or improving the enzyme properties, ample methods are available to engineer oxidases. It has been demonstrated that glucose oxidase can be optimized for biosensor and biofuel cells, whereas glycine oxidase has been engineered towards converting glyphosate. We have shown that, by enzyme engineering, it is feasible to introduce peroxidase activity into an oxidase and to turn a dehydrogenase into an oxidase. These examples show that the number of available flavoprotein oxidases is rapidly expanding which will fuel the development of oxidase-based biotechnological applications.

**Aim and outline of the thesis**

The work described in this thesis aims at one specific application of a flavoprotein oxidase, the oxidation of 5-(hydroxymethyl)furfural (1). In this reaction, the sugar derivative 5-(hydroxymethyl)furfural (1) is converted into the diacid 2,5-furandicarboxylic acid (4) (Scheme 8). The latter compound is a very promising candidate for the synthesis of biobased and renewable polyesters, making the studied reaction of particular interest.
Scheme 8. The route from fructose to polyethylene furanoate (PEF) via 5-(hydroxymethyl)furfural (1) and its oxidation product 2,5-furandicarboxylic acid (4) as intermediates.

5-(Hydroxymethyl)furfural (1) is present in lignocellulosic hydrolysates, were it is formed from sugars, especially during autohydrolysis and acid-catalyzed pretreatment.\textsuperscript{[109]} The presence of 5-(hydroxymethyl)furfural (1) is not desired because it inhibits the growth of microorganisms used in the fermentation of the pretreated biomass.\textsuperscript{[110]} Because of this, several studies aimed at the discovery of micro-organisms which are resistant to 5-(hydroxymethyl)furfural (1) or can metabolize it.\textsuperscript{[111–113]} While it is an unwanted compound for fermentation approaches, 5-(hydroxymethyl)furfural (1) can serve as a precursor for a wide variety of polymers and biofuels.\textsuperscript{[114–117]} Because of this, numerous studies have been dedicated to develop an effective process for the synthesis of 5-(hydroxymethyl)furfural (1) from hexoses like glucose and fructose.\textsuperscript{[118–121]} Although 5-(hydroxymethyl)furfural (1) can serve as the precursor for different building blocks, its oxidation to 2,5-furandicarboxylic acid (4) is of particular interest. This dicarboxylic acid can be polymerized with ethylene glycol to form polyethylene furanoate (PEF). This polyester has similar, or even better properties, than its petroleum based counterpart polyethylene terephthalate (PET).\textsuperscript{[122]} Given the market volume of PET, the production of its renewable alternative PEF has great potential. Like PET, PEF will not be degraded in nature, although 2,5-furandicarboxylic acid (4) based polymers containing phototriggers can be completely decomposed using UV radiation.\textsuperscript{[123]}

The oxidation of 5-(hydroxymethyl)furfural (1) to 2,5-furandicarboxylic acid (4) includes three 2-electron oxidations. In the past few years, a wide variety of chemical methods have been described to perform the full oxidation of 5-(hydroxymethyl)furfural into 2,5-furandicarboxylic acid (4). In most methods 2,5-furandicarboxylic acid (4) is formed with high yield, but often some of the intermediate oxidation products remain present.\textsuperscript{[124–127]} In addition to chemical methods, the biochemical oxidation of 5-(hydroxymethyl)furfural (1) is described in a few studies.\textsuperscript{[128–131]} In most of these studies, multiple enzymes are needed to form 2,5-furandicarboxylic acid (4) from 5-(hydroxymethyl)furfural (1) or only intermediates can be obtained. The work by Koopman et al. shows the formation of 2,5-furandicarboxylic acid (4) in a whole cell system, which depends on the activity of multiple enzymes. The role of these enzymes in all three oxidation steps were largely unknown when starting the research described in this thesis.

This thesis describes the first, and up to now only, enzyme which can on its own convert 5-(hydroxymethyl)furfural (1) into 2,5-furandicarboxylic acid (4). The discovery of the respective enzyme, 5-(hydroxymethyl)furfural oxidase (HMFO), is described in chapter 2. HMFO is a flavin dependent enzyme and belongs to the GMC-family of oxidases, which typically act on alcohols. How this enzyme can perform both the alcohol and aldehyde oxidations required to form 2,5-furandicarboxylic acid (4) is described in chapter 3. The penultimate step of the cascade oxidation reaction, 5-formylfuran-2-carboxylic acid (3) to 2,5-furandicarboxylic acid (4), is not well catalyzed by HMFO. Solving the crystal structure of HMFO helped in guiding an engineering study which resulted in the design of a HMFO mutant with an increased activity towards 5-formylfuran-2-carboxylic acid (3) (chapter 4). A detailed analysis of the kinetic...
mechanism of HMFO is presented in chapter 5. Pre-steady state and steady state experiments are combined to give insights in both the reductive and oxidative half-reaction. The broad substrate scope of HMFO, already discussed in chapter 2, even includes thiols. Chapter 6 reveals that classical alcohol oxidases are also effective in catalyzing thiol oxidations. In short, this thesis describes the flavoprotein HMFO, all the way from its identification of a plain string of letters in a genome sequence database to an engineered enzyme which catalyzes the formation of the bioplastic monomer 2,5-furandicarboxylic acid (4).
References

characterization, application and engineering of 5-(hydroxymethyl)furfural oxidase


