Functions and applications of NMOs

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Abstract

The hydroxylation of amino groups is performed by different types of enzymes. Some of these types are metal dependent whereas others need a flavin cofactor. These flavoproteins (NMOs, for N-hydroxylating monooxygenases) can be assigned to subclass B of the external flavoproteins, an enzyme class consisting of enzymes with a broad range of monooxygenating activity. NMOs are mostly found in the biosynthesis of siderophores of different bacteria. Recently a NMO has been identified in the biosynthesis of a kutzneride. NMOs have a narrow substrate range, making them less applicable in industry. NMOs in siderophore biosynthesis can however be targeted to slow down bacterial growth.

Contents

Introduction

In nature, many enzymes with monooxygenase activity are known. Monooxygenation reactions can introduce a wide variety of functional groups: epoxides are formed from carbon-carbon double bonds, esters and lactones can be formed from ketones in a Baeyer-Villiger reaction, carbon and nitrogen atoms are hydroxylated and heteroatoms like sulfur, phosphorus and selenium can be oxidized. The reaction between molecular oxygen (O_2) and an organic compound is spin forbidden and therefore hard to achieve without enzymes [1]. The above mentioned conversions achieved by chemical means often require high temperatures and strong, and therefore hazardous reagents. In nature, enzymes which perform these monooxygenations often contain metal atoms. Different types of enzymes are known. P450 enzymes as well as non-heme monooxygenases contain iron [2] and copper-dependent enzymes also perform monooxygenations. Not all enzymes depend on a (metallic) cofactor for their activity: flavin-dependent monooxygenases make use of a flavin group instead of a metal ion and recently enzymes without any cofactor have shown monooxygenase activity [3,4].

In this article one type of monooxygenation will be described, focusing on one group of monooxygenating enzymes. The oxidation of an amine to a hydroxylamine will be described, performed by NMOs. NMOs are flavin dependent enzymes similar to some Baeyer-Villiger monooxygenases and therefore classified to the same subclass of flavin dependent monooxygenases. Because the wide variety of flavoproteins, an overview of the subclasses of external flavin dependent monooxygenases will be given first. Also the different states of the flavin cofactor during catalysis will be discussed. After this, the role of NMOs in nature will be shown and some NMOs will be discussed in detail. Substrate and cofactor preferences will be mentioned as well is the effects of mutations on these enzymes. The article will be concluded with perspectives of NMOs in industrial and pharmaceutical applications.

Classification of flavoproteins

Many attempt have been done to make a satisfactory classification for different types of flavoproteins, depending on the type of chemical reaction, the nature of the oxidizing and reducing substrates and more recently the structural motifs determined by X-ray crystallography [5]. Recently a new classification has been made, depending on sequence and structural data [1]. Using this classification, flavoproteins can be divided into 6 classes (table 1). The first two subclasses (A and B) consist of one polypeptide chain, subclass C-F depend on more components for their catalytic activity.

Enzymes in subclass A generally perform an electrophilic attack on activated hydroxyl and amino groups on aromatic compounds. Members from this class have a narrow substrate range. They are usually involved in the microbial degradation of aromatic compounds by hydroxylation. Other subclass A flavin-dependent monooxygenases are involved in the biosynthesis of ubiqinone and the modification of aromatic polyketides. Other members of this subclass perform epoxidations instead of hydroxylations.
One well studied enzyme from this subclass is 4-hydroxybenzoate 3-monooxygenase, a protein found in *Pseudomonas* species [6]. Several other enzymes have been studied as well.

Enzymes in subclass B can be divided according to their specificity. The first group is the flavin-containing monooxygenases (FMOs). These enzymes can catalyze the monooxygenation of carbon-bound reactive heteroatoms like nitrogen, phosphorus, sulfur, selenium or iodine. FMOs are present in all eukaryotes and are important in detoxification processes, in prokaryotes these enzymes are quite rare.
The second group in subclass B consist of Type I BVMOs, or Type I Baeyer-Villiger Monooxygenases, now called class B BVMOs. This name clearly indicates the reaction they perform: a Baeyer-Villiger oxidation. In this reaction a ketone (or aldehyde) is converted to an ester or a lactone. Several proteins of this group have been studied.
The last group belonging to the subclass B flavin-dependent monooxygenases is the N-hydroxylating monooxygenases (NMOs). NMOs catalyze the hydroxylating of primary amines mainly in the biosynthesis of siderophores. This article will focus on NMOs and therefore more information will be provided later in the article.

In the two subclasses just described, the reduction of flavin adenine dinucleotide (FAD) is done by the same enzyme as the oxidation of the substrate, enzymes in subclass A and B are therefore one component systems. In all other subclasses, one enzyme uses reduced flavin to incorporate an oxygen atom in the substrate and a separate enzyme uses NAD(P)H to reduce the flavin. All upcoming subclasses therefore are two component systems.

The C subclass contains enzyme complexes with one are two monooxygenase and one reducing component. This is the only subclass in the classification of Van Berkel et al.[1] using flavin mononucleotide (FMN) as flavin, all other classes use FAD.
The most extensively studied members of this subclass are bacterial luciferases. These enzymes emit light when oxidizing long-chain aliphatic aldehydes. Luciferases consist of a reductase component and a hetero dimeric oxygenase component. This hetero dimer consists of two quite similar peptide chains, but only one chain has an active site.
Next to luciferases, Type II BVMOs (class C BVMOs) belong to this subclass. Although class B and C BVMOs perform the same type of reaction, they are different in structure and sequence. It is shown that luciferases can also perform Baeyer-Villiger oxidations. In contrast, light emission during catalyzes of class C BVMOs has never been reported.
In addition, some enzymes performing a sulfur oxidation have also been assigned to this C subclass, based on similarities in structure and sequence.

Subclass D members catalyze reactions on similar substrates to the substrates in subclass A: aromatic compounds. But unlike subclass A enzymes, this enzymes only perform hydroxylation, epoxilations are not observed (yet). Several of these enzymes are involved in dechlorination. For example 4-chlorophenol is hydroxylated and thereby dechlorinated.

Table 1. Classification of external flavoprotein monooxygenases by Van Berkel et al.[1].

Subclass	Prototype	Reactions	Subunits	Cofactor	Coenzyme
A	4-OH-benzoate hydroxylase	Hydroxyaltion epoxilation	α	FAD	NAD(P)H
B	Cyclohexanone monooxygenase	Baeyer-Villiger; N-oxidation	α	FAD	NADPH
C	Luciferase	Light emision; Baeyer-Villiger; S-oxidation	α+β		FMN NAD(P)H
D	4-OH-pennylacetate hydroxylase	Hydroxylation	α+β		FAD NAD(P)H
E	Styrene monooxygense	Epoxylation	α+β		FAD NAD(P)H
F	Tryptophan 7-halogenase	Halogenation	α+β		FAD NAD(P)H

Table taken from Van Berkel et al. 2006 [1]. In the column 'Reactions' the most commonly oxidations found in vivo are listed

In subclass E only a very limited number of enzymes are found. All of them are styrene monooxygenases. The enzymes oxidize styrene and some derivates on a highly enantioselective way, these oxidized compounds are used as building blocks in the fine-chemical industry.

The last subclass (F) contains enzymes which perform halogenations. Therefore these enzymes are not oxygenases, but they perform a reaction which is mechanistically similar to the reaction of flavoprotein monooxygenases. These halogenase enzymes are found in biosynthetic routes for antitumor agents and antibiotics.

Flavin-dependent monooxygenases form a major group and cover a wide range of oxygenation reactions.
The use of these enzymes has been reported in different areas. Styrene oxidase can be made using subclass E monooxygenases and are important in pharmaceutical industry. Desulfurization of fuels could be obtained when using subclass C monooxygenases. BVMOs can perform a Baeyer-Villiger oxygenation, without the use of very reactive oxidators used in the classical chemical approach.
A very important feature of flavin-dependent monooxygenases is their highly regio- and/or enantioselectivity . Enantiomeric excess lager than 99% has been reported, which is very important in for example the (pharmaceutical) industry.

The catalytic cycle of flavins

To perform this type of reactions, the enzymes needs a reduced flavin cofactor. Some enzymes use FAD whereas others use FMN. Both molecules have the same catalytic cycle.
First NAD(P)H is used to convert oxidized flavin to reduced flavin. When reduced flavin is present it can react with molecular oxygen giving a peroxyflavin. This flavin species can be converted to a hydroperoxyflavin when a proton is taken up, or the peroxyflavin can perform a nucleophilic oxygenation with a substrate, leading to the product and hydroxyflavin. In the case of a hydroperoxyflavin an electrophilic oxygenation leads to the product and again hydroxyflavin. Normally a hydroperoxy compound is unstable, and in the absence of a substrate the hydroperoxy flavin will be converted to oxidized flavin and hydrogenperoxide. Flavoproteins can stabilize hydroperoxyflavins use it for catalysis.
The cycle is completed with the use of the reducing coenzyme NAD(P)H leading to reduced flavin to start another catalytic cycle. (Figure 1)
All flavin-dependent monooxygenase therefore need a flavin (FAD or FMN) and NAD(P)H cofactor and two main reaction happen: the reduction of the flavin the oxidation of the substrate and.

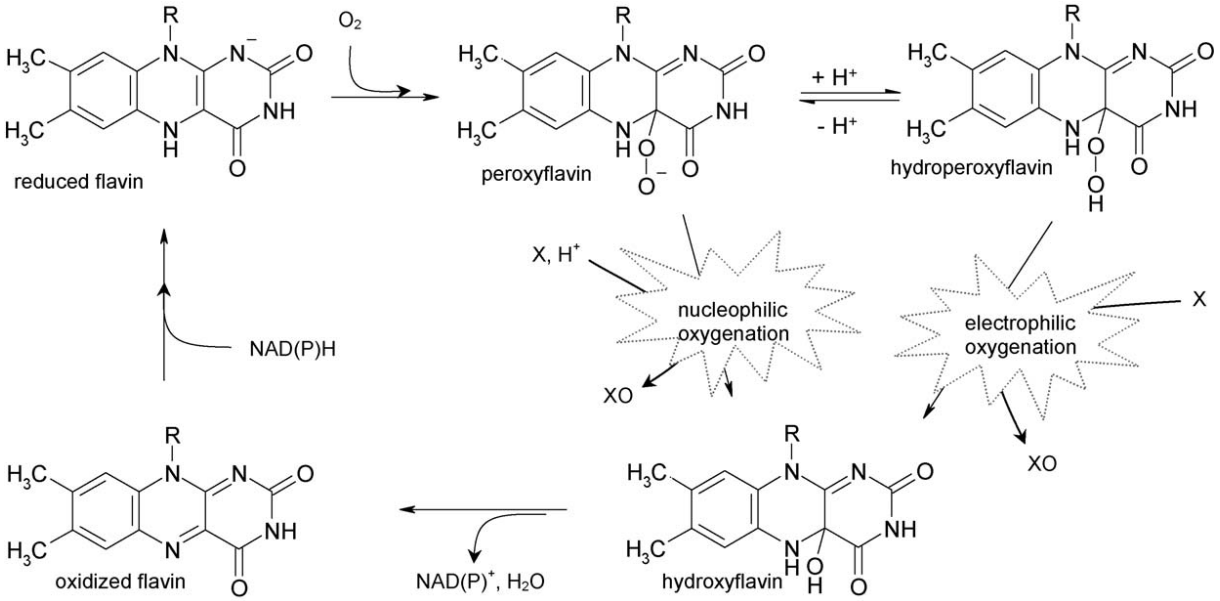

Figure 1. The catalytic cycle of flavins in external flavoproteins, taken from Van Berkel et al. 2006 [1].

N-hydroxylation in nature

As already mentioned, in addition to carbon atoms, heteroatoms like sulfur and nitrogen can also be oxidized. When nitrogen, in the form of an amine is oxidized, a hydroxyl amine is formed, this process is called N-hydroxylation. In maize, a P450 enzyme converts a lactone into a hydroxamic acid, which requires a N-hydroxylation [7]. These hydroxamic acids are used as chemical defense in many different cereal crops against bacteria, fungi and insects [8].

In animals, P450 enzymes perform N-hydroxylations as well. In this case, the reaction is less favorable for the organism. N-hydroxylation of some aromatic amines lead directly to mutagens [9].

In prokaryotes flavin-dependent N-hydroxylases (NMOs) from subclass B are more abundant. These one component enzymes are found mostly in the biosynthesis of siderophores of bacteria, but are also found in fungi [1].

Characteristics of NMOs

NMOs of subclass B of the flavoproteins are encoded by one single gene and often consist of about 440 amino acids. They use FAD and NADPH as cofactors and this last nucleotide is bound tightly during catalysis. FAD in contrast, is bound weakly which is hindering mechanistic studies [1]. NADH can be used to some extent [10], but FMN can not be used, nor it inhibits the activity [11].

Many NMOs show three conserved domains. There are two dinucleotide binding domains (Rossman folds) [1]: one N-terminal FAD binding domain and one towards the centre for binding NADPH [12]. One of the four characteristic fingerprints for nucleotide binding is the GXGXXG motif [13]. In four NMOs, the last glycine in the FAD-binding side has been replaced by a proline. This glycine to proline exchange in NMOs can be the cause of the low affinity for FAD.

Next to these two nucleotide binding motifs, there is a conserved hydrophobic region near the C-terminus of NMOs. This so called FATGY motif with the DXXXFATGYXXXXP sequence is supposed to be used for substrate binding [12,14]. In the genes *pvdA* and *sid1* which both encode a L-ornithine N^5-hydroxylase, this last proline is not observed.

The enzyme encoded by *iucD* is membrane bound, so next to the previous mentioned domain the enzyme also needs a hydrophobic domain which interacts with the membrane. Two binding sites have been revealed, one of them is a leader like sequence at the N-terminus of the protein [15,16].

Thirteen proteins belonging to the lysine N(6)-hydroxylase/L-ornithine N(5)-oxygenase family are known and are listed in table 2 [17].

Table 2. Members of the Lysine N(6)-hydroxylase/L-ornithine N(5)-oxygenase family.

Protein	Gene name	Origin	Number of amino acids
L-lysine 6-monooxygenase	*iucD or aerA*	*Escherichia coli*	425
L-ornithine 5-monooxygenase	*pvdA*	*Burkholderia cepacia*	444
L-ornithine 5-monooxygenase	*pvdA or pvd-1*	*Pseudomonas aeruginosa*	443
L-ornithine 5-monooxygenase	*sib2*	*Schizosaccharomyces pombe*	431
Alcaligin biosynthesis enzyme	*alcA*	*Bordetella parapertussis*	461
Alcaligin biosynthesis enzyme	*alcA*	*Bordetella pertussis*	461
Alcaligin biosynthesis enzyme	*alcA*	*Bordetella bronchiseptica*	461
L-lysine 6-monooxygenase mbtG	*mbtG*	*Mycobacterium tuberculosis*	431
L-lysine 6-monooxygenase mbtG	*mbtG*	*Mycobacterium bovis*	431
L-lysine 6-monooxygenase mbtG	*mbtG*	*Mycobacterium paratuberculosis*	428
Rhizobactin siderophore biosynthesis protein	*rhbE*	*Rhizobium meliloti*	454
L-ornithine 5-monooxygenase	*sid1*	*Ustilago maydis*	649
L-lysine 6-monooxygenase mbtG	*mbtG*	*Mycobacterium* sp (strain MCS)	430

All *alcA* or probably the same gene, only found in different species of *Bordetella.* The same is true for mbtG from *Mycobacterium tuberculosis* and *M. bovis.* Data are taken from the protein knowledgebase of Uniprot [17].

These proteins are found in bacteria and fungi and are all involved in the biosynthesis of siderophores (literally: iron carriers) where they hydroxylate amino groups which ultimately become hydroxamic acids which are important in iron binding. Recently, a NMO has been found in the synthesis of kutzneride 2, an antifungal and antibacterial compound. The enzyme hydroxylates a glutamine residue to make hydrazo bond formation possible.
In addition, a flavin dependent NMO has been found which does not belong to subclass B, but has more similarity to two component enzymes from subclass C.

NMOs in siderophore synthesis

As already mentioned, most known flavin dependent NMOs are part of siderophore synthesis in bacteria or fungi. Siderophores are iron-binding agents that bind Fe^{3+}, transport the ion into the cell were iron is released as Fe^{2+}.

Iron is essential to almost all forms of live. In soil, most iron is present as the insoluble Fe^{3+} ion. In marine waters iron is virtually undetectable and the same is true in animal tissues. The latter case is caused by iron scavenging systems. However, in soil, aquatic environments and in animal tissues bacteria are able to survive and grow. This is due to siderophores of which different types are known. A major class consists of molecules with a hydroxamate group (Figure 2). Organisms like *Escherichia coli* and *Salmonella typhimurium* make different, more complex, high affinity siderophores. These enterobactins are derived from the aromatic compound catechol. The last class consists of again a very different kind of molecules. These siderophores have roughly two parts: a hydrophobic tail keeps the siderophore linked to the membrane, the peptidic part binds iron ions. This peptidic part consists of modified amino acids. One important modification is the hydroxylation of the primary amine group lysine or ornithine residues. The oxygen atom incorporated is used to bind the iron ion [18]. This modification

is present in aerobactin, aquachelin and pyoverdine and is performed by NMOs. The structure of pyoverdine is shown figure 3 [19].

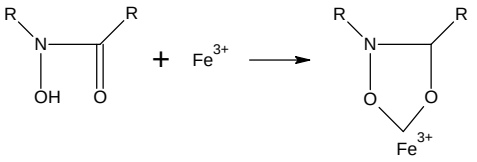

Figure 2. Iron binding by a hydroxamic acid [19].

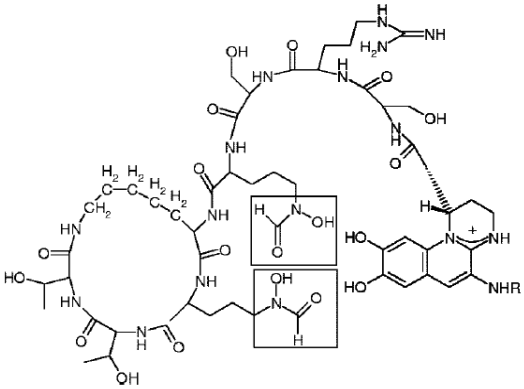

Figure 3 The structure of pyoverdine, a siderophore of *Pseudomonas aeruginosa.* The hydroxamic acid groups derived from l-ornithine are highled in boxes. Picture taken from Ge and Seah [10].

The most studied enzyme is L-lysine N^6-hydroxylase (IucD) of *Escherichia coli.* This enzyme is the first enzyme in the biocatalysis of the siderophore aerobactin. Like the name of the enzymes indicates, L-lysine N^6-hydroxylase catalyses the hydroxylation of the terminal amino group of L-lysine to N^6-hydroxylysine (Figure 4), being most active at a pH of 8.0 [20].
To do this conversion, the enzyme uses NADPH and FAD as cofactors. NADH instead of NADPH can also be used but the enzyme activity becomes twofold lower [10].

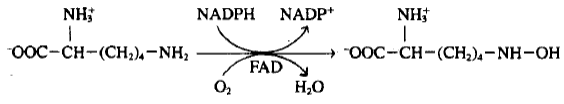

Figure 4. The reaction perfomed by L-lysine N^6-hydroxylase [21].

NAD(P)H is used to reduce FAD. When no substrate is present, this reduced FAD reacts with molecular oxygen which reoxidizes FAD and hydrogen peroxide is formed. Addition of substrate enhances the NADPH oxidase reaction by a factor of 10 and less hydrogen peroxide is formed. Instead FAD and molecular oxygen are used to yield the product; next to this water is formed.
The catalytic mechanism of the reaction is as followed. Hydroperoxiflavin is attacked by lone pair electrons of the terminal amino group of lysine (electrophilic oxigenation in figure 1). During catalysis, the lysine molecule is stabilized in the enzyme by ionic interactions, based on the facts that the amino and the carboxyl group in the backbone of lysine are necessary for catalysis. The positive charge on the two amino groups of lysine are stabilized and oriented by negatively charged amino acids of the protein, where as the negatively charged carboxyl group is stabilized by a positive charge on an amino acid of the protein [21].
Lysine N^6-hydroxylase cannot hydroxylate D-lysine and L-ornithine. The latter and some other lysine homologues (DL-2,3-diaminopropionic acid, N^6-acetyl lysine and DL-2,6-diaminopmelicacid) stimulate the oxidation of NADPH, but none of these compounds are hydroxylated [22]. The only known substrate next to L-lysine is (S)-2-aminoethyl-L-cystein [11].

Less studied than lysine N^6-hydroxylase is L-ornithine N^5-hydroxylase. Only recently it was possible to over express and purify this enzyme without obtaining inactive enzymes from inclusion bodies. The purified L-ornithine N^5-hydroxylase was originally found in *Pseudomonas aeruginosa* and is encoded by *pvdA*. The gen *sid1* from *Ustilago maydis* encodes for a L-ornithine N^5-hydroxylase as well [23].
Next to a part of the siderophore biosynthetic route in *Pseudomonas aeruginosa*, and *Ustilago maydis*, L-ornithine N^5-Hydroxylase is used in *Burkholderia cepacia* and *Aspergillus fumigatus* as well, also for siderphore synthesis.
Ornithine is a diamino acid like lysine, but its carbon chain is one atom shorter.
The enzymes Lysine N^6- and L-ornithine N^5-hydroxylase show a similarity of about 35 % but, L-ornithine N^5-hydroxylase cannot make use of lysine as a substrate. Lysine can however stimulate the oxidation of NADPH, leading to uncoupling and hydrogenperoxide

was produced instead of hydroxylamine. Like lysine N^6-hydroxylase, L-ornithine N^5-hydroxylase has its maximum activity at a pH of 8.0.
Two other, close structural homologues of L-ornithine (L-2,4-diaminobutyrate and 5-aminopentanoic acid) or no substrates for L-ornithine N^5-hydroxylase. These two homologues are able of binding to the active site, but cannot be hydroxylated. In contrast to L-lysine, these compounds show no stimulation of the NADPH oxidation.
This suggests the enzyme is quite specific for its physiological substrate. The length of the carbon chain with the terminal amino group as well as the presence of a second amino group are important for efficient hydride transfer between FAD and NADPH and therefore also important for substrate hydroxylation.
As already mentioned, L-ornithine N^5-hydroxylase uses FAD and NADPH as cofactor, NADH can not be used, whereas lysine N^6-hydroxylase could use it [10].
L-ornithine N^5-hydroxylase seems to use a, uptill now, unique kinetic mechanism for the conversion of ornithine. FAD is reduced by NADPH even in absence of substrate, which seems to be a waste of NADPH because FAD is oxidized readily back in the cell. However, like many other flavin monooxygenases this enzyme has a way of preserving the reduced FAD. It is hypothesized that L-ornithine N^5-hydroxylase uses a kind of hybrid of the methods for reaction coupling which is already known for related flavin enzymes like FMOs and cyclohexanone monooxygenase.
Substrate conversion by L-ornithine N^5-hydroxylase, just like lysine N^6-hydroxylase , takes place using a hydroperoxyflavin (see figure 1).

The protein AlcA from *Bordetella pertussis* is used in siderophore syntheses as well. This protein however does not use ornithine but its decarboxylated derivate putrescine. Although putrescine has to equevalent primary amino groups, only one is hydroxylated [24].

A NMO in kutzneride synthesis

Recently a NMO has also found in an entirely different synthetic route: KtzI plays a role in the biosynthesis of kutznerides, compounds isolated form *Kutzneria* sp. 744, a soil actinomycete. These compounds have an antifungal and antimicrobial function. Kutznerides are cyclic hexadepsipeptides, which mean they contain six residues and linkages between the residues are not made by a normal amide bond (CONH) but with a ester bond(COOR) [25]. All six residues used in kutznerides are abnormal amino acid residues used in proteins but are modified.
17 genes have been identified in the kutzneride biosynthesis, of which 8 likely catalyze oxidative transformations. One of these proteins, KtzI, has 35% identity with the PvdA protein discussed earlier. It is therefore proposed that the function of KtzI is either a Lysine or an Ornithine N-monooxynase. The enzyme is possibly necessary for the formation of the hydrazo linkage in kutzneride 2. In this kutzneride, a ring is formed using the two amino groups of a glutamine residue. The terminal amino group is first hydroxylated by the NMO KtzI. Via a nucleophilic attack of the α-amine the cyclic product is formed, displacing the hydroxyl group [26].

N-hydroxylation in valanimycin synthesis

The enzymes described above, all perfectly fit into class B of the flavoproteins. They consist of one subunit and use FAD. A protein which cannot be classified into one of the subclasses is Isobutylamine N-hydroxylase (IBAH or VlmH) from *Streptomyces viridifaciens.* This protein catalysis the reaction between isobutylamine (IBA) and isobutyl-hydroxylamine (IBHA), introducing a hydroxyl group on the primary amino group of the substrate [27].
This conversion is part of the synthesis route of the antibiotic valanimycin from L-valine. Valanimycin contains an azoxy bond, being formed from a hydrazo bond (N-N). In nature a wide variety of compounds contain a hydrazo linkage [28], but little is known about the bond formation. As already mentioned, kutzneride 2 contains a N-N linkage and this bond is formed from a hydroxylated amino group. Isobutylamine N-Hydroxylase has probably the same role. The hydroxylated amino group of IBHA reacts with L-serine to

form a hydrazo intermediate in the valanimycin synthesis.
Unlike KztI, or other NMOs, IBAH is dependent of a separate reductase (VlmR) which provides the oxidase component from reduced FMN [29] or FAD [30]. These characteristics distinct Isobutylamine N-Hydroxylase from other NMOs. The latter are one component systems, both reducing FAD and oxidizing the amine. Isobutylamine N-Hydroxylase however looks more like a subclass C enzyme, depending on a separate reductase and able to use FMN. However, no NMOs have been described in this subclass. Sequense similarities between Isobutylamine N-Hydroxylase and Sox/DszC from *Rhodococcus* sp. strain IGTS8 also indicate that Isobutylamine N-Hydroxylase should be assigned to subclass C. Sox/DszC catalysis the sulfoxidation of dibenzothiophene to the corresponding sulfone, using FMN and depending on a separate reductase which is typical for this class [31].

Recombinant NMOs

As already mentioned, IucD is a inner membrane bound protein. To obtain a soluble protein which can be purified for catalytic studies, several fusion proteins have been made. IucD456 and IucD439 contain next to the complete IucD sequence a N-terminal sequence of 30 and 13 residues of the soluble protein β-galactosidase. IucD398 is a truncated enzyme lacking the N-terminal 46 residues, and to this protein 19 residues of β-galactosidase have been fused. IucD439, the least modified enzyme keeps its activity. In vivo and in vitro, aerobactin is formed meaning the mutated NMO is active, although to slightly less extent[16]. IucD398 resembles the activity of IucD439 in substrate specificity and its use of the cofactors NADH and FAD. This suggests the truncated part of the enzyme is not essential for interactions of the enzyme with cofactors or the substrate [11]. Noteworthy is the fact that only one nucleotide binding side was identified, but the enzyme was still active. This means FAD and NADPH compete for the one binding site which is in the native enzyme only used by NADPH.
In the studies performed by Stehr and coworkers two nucleotide binding motifs are found [12]. This first binding site (residues 9-14) is not present in the truncated IucD. The unusual proline in the GXGXXP motif of the first nucleotide binding site has been studied. When proline was changed to the 'normal' glycine, the FAD binding ability decreased. The reaction of the hydroperoxyflavin with the amino group of the substrate decreased even more. Adjusting the pH and the substrate and cofactor concentrations resulted in an activity of the mutant enzyme which was quite comparable to that of the normal enzyme. This suggests the proline residue has an important structural function in FAD binding [22].

Several other recombinant enzymes are made. Two observations suggest cystein residues have a catalytic function in lysine N^6-hydroxylase. First the loss of activity of the enzyme due to storage (at -80°C or 4 °C) can be restored by adding thiol agents like DL-dithiothreito (DTT). Second, the enzyme can be inactivated by adding thiol modifying agents [32]. Therefore, several studies were performed with recombinant enzymes with cystein to alanine exchanges [32,33]. Lysine N^6-hydroxylase encoded by *iucD* of *E.coli* contains 5 cystein residues, on the possitions 31, 51, 146, 158, 166. Different mutations have been made, both with individual and with combined replacements. None of the recombinant enzymes showed a major change in their affinity for FAD or L-lysine, or their thermal stability. The k_{cat} of the monooxygenases was not affected significantly for most of the muteins, Cys146→Ala showed however a twofold decrease in k_{cat} whereas Cys51→Ala gave a twofold increase of the k_{cat}.

Pharmaceutical applications

NMOs can be of interest for the pharmaceutical industry for two reasons. They can be both used as drug targets and as biocatalysts.
The NMOs which are part of the biosynthesis route for siderophores like lysine N^6-hydroxylase and L-Ornithine N^5-hydroxylase can be targeted by drugs, trying to inhibit their action.
Iron is limited in animal tissues because iron scavenging systems take up free iron. For

iron can catalyze the Fenton reactions, forming oxygen radicals, free iron is dangerous to the cell [34]. Next to this, iron is needed for growth of most microorganisms and limiting iron will reduce this growth. Microorganisms however use siderophores to provide the organism of iron and therefore some siderophores are considered virulence factors [35].
Inhibiting siderophore production, expression or activity can there for be a solution to some diseases. Many siderophores contain hydroxamate groups for binding Fe^{3+}, these groups are formed by NMOs. The N-hydroxylation of ornithine in the production of pyoverdine, a siderophore from *Pseudomonas aeruginosa* strain PAO1 depends on the L-ornithine N^5-Hydroxylase encode by *pvdA* [36], for the production of the siderophores aerobactin (*Escherichia coli*), alcalignin (*Bordetella bronchiseptia*) the amine groups of lysine and putricine respectively are hydroxylated. This is an early step and important for iron binding, making the NMO which perform the reactions a good target for drugs. In addition, NMOs have not been described in human thus far giving them even more potential as a drug target.
In addition, the NMOs described have a very selective substrate range. L-ornithine N^5-hydroxylase for example can not convert L-lysine, which is very similar to L-ornithine. Because of this limited substrate range, compounds closely related to the physiological substrate can serve as inhibitors. But for lysine N^6-hydroxylase it has been shown the protein is very stringent in its substrate range and closely related compounds have no effect on the protein, neither as a substrate nor as an inhibitor [37]. This limits the amount of possible inhibitors and putative drugs.

On the other hand, NMOs can be used as tools to make drugs rather than targets for drugs. The hydroxylation of amines is difficult to achieve by chemical means giving NMOs a potential in synthesizing specific functional groups in medicines. Hydroxamic acids possess many biological activities. Anti bacterial, antifungal, anti-inflammatory, anti-asthmatic and they might serve as inhibitors of matrix metalloproteinases. These zinc dependent enzymes have a role in diseases like cancer, arthritis, and multiplesclerosis. Furthermore, enzymes are often enantioselective and regioselective. All chiral biological active parts of drugs have to be isolated and tested and drugs often have to be a single enantiomer [38]. Using enzymes therefore will circumvent several purification steps.
In addition, several compounds contain azoxy groups. Already mentioned is valanimycin, a antibiotic. Antifungal agents like maniwamycin A and B and azoxybaclin also contain azoxy groups [39.40]. Precursors of azoxy groups in nature are often hydrazo bonds, formed by the reaction of a hydroxylated amine and an amino group. NMOs can therefore play a role in the production of these and other azoxy-containing compounds with a pharmaceutical function.

Industrial applications

Some flavin dependent monooxygenases have shown industrial interesting properties. Baeyer-Villiger monooxygenases are of interest because of their broad substrate range and high enantioselectivity [1]. Cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoacicus* has been expressed in *E. coli.* Using racemic bicyclo[3.2.0]hept-2-en-6-oneas substrate, both regio-isomeric lactones could be obtained with a good yield in with an enantiomeric excess >98%. This could be done on kilogram scale in a 50 L fermentor [41]. Although for industrial use more scaling up has to done, this is already very promising. Other enzymes also can have applications in industry: subclass E enzymes can make enantiopure styrene oxides whereas dibenzothiophene monooxygenases from subclass C can be used in desulfurization of fossil fuels [42].
NMOs can also act as biocatalysts. But they have often a narrow substrate range, giving them a limited number of applications. Some possible applications have already been mentioned in the Pharmaceutical applications section. Introducing hydroxy groups to amines may also have applications in other parts of industry, replacing the Angeli-Rimini reaction for instance. This reaction to make hydroxamic acids however is quite good, giving high yields and the products are easy to purify [43]. NMOs therefore might have

potency, at this time they are not able to replace this synthesis route.
In general this is true for NMOs. Like other flavo proteins they might be powerful tools, up to date not enough is known about them.

Conclusion

A limited number of NMOs belonging to the flavoproteins have been described thus far. Most of them are involved in the first step of siderophore synthesis a bacteria and some fungi. The hydroxylated amino groups are further modified to hydroxamic acids which have a strong affinity for iron. Sequence similarities between known NMOs and a gene involved in kutzneride synthesis have revealed the existence of a putative NMO. This protein (KtzI) is involved in the synthesis of kutzneride 2. In this case the hydroxylation of the amino group has another purpose. Not a hydroxamic acid is formed, but the hydroxylated amine can form a hydrazo bond.
Next to this subclass B monooxygenase, there is at least one NMO which could belong to subclass C of the flavoproteins. The enzyme IBAH from *Streptomyces viridifaciens* is involved in the synthesis of the antibiotic valanimycin. Just like KtzI, the hydroxylamine formed by IBAH is used in a hydrazo bond formation. But unlike the NMOs in siderophore and kutzneride synthesis the flavin dependent IBAH is not a one component system. It is a two component system requiring a separate flavin reductase to provide the reduced flavin. Next to this, sequence similarity suggests this enzyme looks most like a subclass C enzyme.
In general it can be said NMOs have a limited substrate range. Only two good substrates are known for L-lysine N^6-hydroxylases: L-lysine and (S)-2-aminoethyl-L-cystein. L-ornithine N^5-hydroxylase cannot convert L-lysine and L-lysine N^6-hydroxylases cannot convert ornithine.
Mutation studies on lysine N^6-hydroxylase reveal the enzyme stays active even if the nucleotide binding site used for FAD binding has been deleted, indicating the NADPH pocket can bind both FAD and NADPH. This FAD binding pocket contains an unusual proline residue, but this residue has an improtant structural function and replacing it to the normal glysine residue changes the optimal substrate concentration and optimal pH for the enzyme.
Although other flavin dependent monooxygenases can have a good application in (pharmaceutical) industry, the applications of NMOs are quite limited. The best described NMOs have a small substrate range. When other substrates have to be converted the enzymes need to be modified, but successful studies have not been done yet.
On the other hand, NMO can be targets for the drug, making them interesting to pharmaceutical industry in another way. Since siderophores are essential for microorganisms to survive in the host, targeting proteins which make siderophores can prevent diseases. So NMOs can be most interesting for pharmaceutic industry as targets rather than as catalysts.

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