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The VAO/PCMH flavoprotein family

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Review article

The VAO/PCMH flavoprotein family

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ABSTRACT

The VAO/PCMH flavoprotein family consists of structurally homologous flavin-dependent enzymes that catalyze a wide range of chemical reactions. Family members share an architecture consisting of a conserved FAD-binding domain and a more variable substrate-binding domain, which enables varying interactions with a range of substrates while maintaining the same cofactor-binding fold. Here, we provide an overview of the current state of our knowledge on the members of the VAO/PCMH family. Based on a phylogenetic analysis, we divide the family into 11 subgroups. We discuss the properties of these subgroups, focusing on recent developments in terms of the discovery of new family members and mechanistic advances. We also highlight open questions that will provide challenges for future research.

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Abbreviations: 4PO, 4-phenol oxidizing; ADPS, alkyl-dihydroxyacetone phosphate synthase; AldO, alditol oxidase; ALO, D-arabinono-1,4-lactone oxidase; BBE, berberine bridge enzyme; ChoX, cholesterol oxidase; CKX, cytokinin dehydrogenase; D2HGDH, D-2-hydroxyglutarate dehydrogenase; DCPIP, 2,6-dichlorophenolindophenol; DHAP, dihydroxyacetone phosphate; DHCR24, 24-dehydrocholesterol reductase; DLDH, D-lactate dehydrogenase; DPA, decaprenylphosphoryl- β -D-arabinofuranose; DPR, decaprenylphosphoryl- β -D-ribofuranose; DPX, decaprenylphosphoryl-2-keto- β -D-erythro-pentofuranose; EUGH, eugenol hydroxylase; EUGO, eugenol oxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GALDH, L-galactono-1,4-lactone dehydrogenase; GALO, L-galactono-1,4-lactone oxidoreductase; GLO, D-glucono-1,4-lactone oxidase; GMC, glucose-methanol-choline; GUDH, L-gulonono-1,4-lactone dehydrogenase; GUO, L-gulonono-1,4-lactone oxidase; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PCMH, para-cresol methylhydroxylase; SSR, sterol side chain reductase; UDP-GlcNAc-EP, uridine diphosphate N-acetylglucosamine enolpyruvate; UDP-MurNAc, uridine diphosphate N-acetylmuramic acid; VAO, vanillyl alcohol oxidase.

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Flavoenzymes, which contain one of the vitamin B₂-derived cofactors flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN), are found in all domains of life and fulfill important roles in a variety of biological processes [1,2]. They utilize the reactive isoalloxazine ring of their cofactor to catalyze a diverse range of chemical reactions. Most flavoenzymes are oxidoreductases, using the electron transferring properties of the isoalloxazine ring system to catalyze the two electron oxidation or reduction of their substrates [3,4]. However, in recent years, a number of new chemical reactivities of the isoalloxazine ring have been unearthed, including some where it is used to catalyze non-redox reactions [5,6]. The VAO/PCMH (vanillyl alcohol oxidase/*para*-cresol methylhydroxylase) flavoprotein family contains flavoproteins that are characterized by the presence of a shared FAD-binding domain (pfam_FAD_binding_4). In addition to this conserved FAD-binding domain, which is responsible for binding the adenosine diphosphate and ribityl moieties of FAD, members of the VAO/PCMH family contain a more variable substrate-binding domain, which is positioned over the isoalloxazine ring of the cofactor (Fig. 1A). This architecture allows for great variation in the structure of the active sites and therefore the catalytic activity of these enzymes, while maintaining the conserved cofactor-binding fold. The VAO/PCMH family was originally identified in 1998 and at that time was found to encompass 13 enzymes of known function [7]. Almost a decade ago, the family was reviewed and found to encompass around 50 characterized enzymes [8]. Here, we provide an update on the state of our knowledge on the members of this flavoprotein family.

1. Phylogenetic analysis of the VAO/PCMH family

In order to identify members of the VAO/PCMH family, we performed a search in the structure and sequence databases. The Swissprot sequence database and Protein Data Bank were searched for annotated sequences and deposited structures respectively that

contain a domain belonging to the pfam_FAD_binding_4 family. In addition, we searched the primary literature for VAO/PCMH family members of known sequence and enzymatic function. This led to the identification of 115 sequences of VAO/PCMH family members with known enzymatic activity, as determined either by characterization of the purified protein or by genetic analysis. Of the 115 sequences, 35 encode proteins of which the three-dimensional structure has been determined. Most of the identified enzymes are classic flavin-dependent oxidoreductases that catalyze two electron reductions (known as reductases) or oxidations (known as oxidases if molecular oxygen is used as an electron acceptor and as dehydrogenases if another molecule is used as an electron acceptor). In addition, a small number of the identified enzymes display oxygenase or non-redox transferase activity (see below for details).

Almost all of the enzymes for which the presence of a cofactor has been studied contain FAD, as would be expected based on the presence of the conserved FAD-binding domain. The only exceptions are D-arabinono-1,4-lactone oxidase from *Trypanosoma brucei* and L-galactono-1,4-lactone dehydrogenase from *Brassica oleracea* (cauliflower), which have been reported to contain FMN, and L-gulonono-1,4-lactone dehydrogenase from *Mycobacterium tuberculosis*, in which no flavin could be identified [9–11]. However, two other VAO/PCMH-type enzymes that were originally reported to contain FMN, were later found to contain FAD (Dbv29 from *Nonomuraea* sp. ATCC 39727 and L-galactono-1,4-lactone oxidoreductase from *Trypanosoma cruzi*) with the original misassignment being attributed to the hydrolysis of FAD during analysis [12,13]. Therefore, further research is required to confirm the identity of the flavin cofactor in the above-mentioned enzymes.

A large number of VAO/PCMH family members contain a covalently bound flavin cofactor. In total, five different types of flavin binding mode can be distinguished within the family (Fig. 1B–F): non-covalent, monocovalent 8 α -N1-histidyl-FAD, monocovalent

Fig. 1. Structural architecture and cofactor binding modes of VAO/PCMH family members. (A) Cartoon representation of the three-dimensional structure of vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum* (PDB ID: 1VAO) showing the two-domain topology that is characteristic of members of the VAO/PCMH family. The FAD-binding domain, which is conserved throughout the family, is shown in green. The substrate-binding domain, which is more variable, is shown in red. The FAD cofactor, which is covalently bound to the enzyme via an 8 α -N3-histidyl-FAD bond, is shown as yellow sticks. (B–F) Stick representations of flavin binding modes found in members of the VAO/PCMH family. (B) Monocovalent 8 α -N1-histidyl-FAD of cholesterol oxidase (ChoX) from *Brevibacterium sterolicum* (PDB ID: 1H19). (C) Bicovalent 6-S-cysteinyl-8 α -N1-histidyl-FAD of glucooligosaccharide oxidase (GOOX) from *Sarocladium strictum* (PDB ID: 1ZR6). (D) Monocovalent 8 α -N3-histidyl-FAD of VAO. (E) 8 α -O-tyrosyl-FAD of *para*-cresol methylhydroxylase (PCMH) from *Pseudomonas putida* (PDB ID: 1WVF). (F) Non-covalent FAD of MurB from *Escherichia coli* (PDB ID: 2MBR).

8 α -N3-histidyl-FAD, monocovalent 8 α -O-tyrosyl-FAD and bicovalent 6-S-cysteinyl-8 α -N1-histidyl-FAD. Covalent flavinylation increases the redox potential of the cofactor and thus enhances the oxidative power of the enzymes [14]. A structure-based alignment of the identified sequences was performed using the PROMALS3D webserver and subsequently used to generate a phylogenetic tree using the phylogeny.fr webserver's BioNJ function. This phylogenetic tree is shown in Fig. 2. Using this tree, we divided the VAO/PCMH family into 11 subgroups of related enzymes based on sequence similarity (and in most cases) function. One sequence, glycolate oxidoreductase subunit E from *Escherichia coli*, did not belong to any of the identified subgroups. Below, we will discuss these subgroups in terms of their shared properties and functions and highlight newly discovered members and recent developments in our mechanistic understanding of the enzymes.

2. Subgroup 1: BBE-like

The subgroup with most characterized members is the berberine bridge enzyme (BBE)-like subgroup. This subgroup is named after berberine bridge enzyme, or reticuline oxidase (EC 1.21.3.3), which catalyzes the oxidative ring closure of (S)-reticuline to (S)-scoulerine, a key intermediate in the biosynthesis of protoberberine and benzophenanthridine alkaloids in plants [15]. The BBE-like subgroup is the most functionally diverse of the identified subgroups. Many of the members are involved in the biosynthesis of secondary metabolites in plants [16–18] or microorganisms [12,19–30]. The subgroup also contains a number of enzymes that catalyze the oxidation of the C1 hydroxyl groups of the aldose moieties of (oligo)saccharides [31–36]. Examples include glucosyligosaccharide oxidase from *Sariocladum strictum* (previously known as *Acremonium strictum*), chitooligosaccharide oxidase from *Fusarium graminearum* and the recently identified xylooligosaccharide oxidase from *Myceliophthora thermophila* C1. Many members of the BBE-like subgroup contain a bicovalently-linked 6-S-cysteinyl-8 α -N1-histidyl-FAD cofactor. The bicovalent linkage increases the redox potential of FAD and may be of importance in maintaining the correct position of the flavin cofactor in the active site, which is typically quite open in bicovalent flavoproteins.

An interesting recent development is the characterization of EncM, a BBE-like enzyme that utilizes a hitherto unknown flavin redox state, the flavin-N5-oxide, to catalyze the oxidative ring closure required to form the tricyclic core of the antibiotic enterocin in *Streptomyces maritimus* [27,37]. This occurs through a mechanism where the oxygen atom from the flavin-N5-oxide is first incorporated into the substrate at the C4 position, yielding hydroxylated substrate and oxidized FAD. Subsequent oxidation of the formed hydroxyl group, yielding a ketone and reduced flavin, triggers a Favorskii-type rearrangement reaction that leads to lactone formation, yielding the tricyclic enterocin core (Fig. 3). Subsequently, reduced flavin reacts with molecular oxygen, regenerating the flavin-N5-oxide. This has been proposed to occur via the initial formation of an N5-peroxide intermediate, which forms the flavin-N5-oxide after expelling water. However, a mechanism consisting of the formation and subsequent rearrangement of a C4a-peroxide intermediate, as typically found in flavin-dependent monooxygenases, is also possible. Elucidating the mechanism by which EncM enables the formation and stabilization of the flavin-N5-oxide will be an interesting challenge for future research. In addition to EncM, a flavin-N5-oxide was also found to be an intermediate in the catalytic cycle of the non-VAO/PCMH-type flavoenzyme DsZA [38]. It will be interesting to see whether more flavoenzymes that utilize this redox state will be identified in future. For a more comprehensive overview of the BBE-like subgroup, see the contribution of Macheroux and coworkers to this

issue [148].

3. Subgroup 2: cytokinin dehydrogenases

Cytokinin dehydrogenases (CKX, EC 1.5.99.12) are found in a wide variety of plants, where they are responsible for the breakdown of cytokinins, plant hormones that are involved in cell division and differentiation. Cytokinins are adenine derivatives with a substituent bound to the N6 atom, which can be an isoprenoid or aromatic functionality. CKX oxidizes the side chain amine of cytokinins to an imine, which is subsequently hydrolyzed, yielding adenine and an aldehyde [39] (Fig. 4). Following this, the FAD cofactor of CKX, which is covalently linked to the enzyme through an 8 α -N1-histidyl-FAD bond, is reoxidized. Various electron acceptors, such as quinones or 2,6-dichlorophenolindophenol (DCPIP) can be used by the enzyme *in vitro* [40]. For the *Zea mays* (maize) CKX1 (*ZmCKX1*) isoform, evidence exists that the physiological electron acceptor is a free radical formed by the oxidation of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, a compound found in maize phloem sap, by laccases or peroxidases [41]. Higher plants typically contain multiple genes encoding CKXs, with the best studied CKX families being those from *Arabidopsis thaliana* (7 isoforms) and *Z. mays* (13 isoforms). The various members of these families differ in terms of their tissue-specific expression patterns, (predicted) subcellular localization and substrate specificity, suggesting they have distinct functions *in vivo* [42–46]. The crystal structure of *ZmCKX1* revealed that the substrate binding site consists of an internal cavity, where the isoprenoid or aromatic side chain of the substrate binds, linked to the surface through a narrow pore [47]. The C–N bond that is cleaved occupies the pore, with the adenine ring structure bound to a funnel-shaped pocket on the surface of the protein. Based on the crystal structure, a mechanism for substrate oxidation was proposed where a conserved aspartate residue (Asp169 in *ZmCKX1*) plays a crucial role in catalysis. By interacting with the N10 atom of the cytokinin, Asp169 induces polarization of the substrate, facilitating the transfer of a hydride anion from the C11 atom to FAD. The importance of Asp169 for catalysis is supported by a recent site-directed mutagenesis study, which showed that the *ZmCKX1* D169N variant displays significantly lowered activity towards all tested substrates, while K_m values were not affected [48].

4. Subgroup 3: MurBs

UDP-N-acetylglucosamine enolpyruvate reductase, also known as MurB (EC 1.3.1.98), is involved in bacterial cell wall synthesis, where it catalyzes the second step in the biosynthesis of peptidoglycan, the reduction of UDP-N-acetylglucosamine enolpyruvate (UDP-GlcNAc-EP) to UDP-N-acetylmuramic acid (UDP-MurNAc) (Fig. 5). The enzyme achieves this by utilizing a non-covalently bound FAD cofactor to transfer a hydride equivalent from nicotinamide adenine dinucleotide phosphate (NADPH) to UDP-GlcNAc-EP [49].

Based on their three-dimensional structures, two different types of MurB can be distinguished. The two types share a similar overall fold, but differ in terms of their mechanism of substrate binding. Type I MurB, which is found in Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, is characterized by the presence of a loop region that is involved in substrate binding, closing the active site after binding [50,51]. The loop contains a tyrosine residue (Tyr190 in *E. coli* MurB) that forms an interaction with one of the phosphate groups of UDP-GlcNAc-EP or NADPH, which bind at a similar position. Type II MurBs are found in bacteria such as *Staphylococcus aureus* and *Thermus caldophilus* and lack this loop region as well as part of the substrate binding domain that

Fig. 2. Phylogenetic tree of the VAO/PCMH family showing the division into 11 subgroups. The subgroups are indicated by the colors of the branches. They are: berberine bridge enzyme-like (blue), cytokinin dehydrogenases (light red), MurBs (dark green), 4-phenol oxidizing (yellow), alkyl-dihydroxyacetone phosphate synthases (dark red), α -hydroxy acid dehydrogenases (grey) DprE1s (pink) sterol reductases (brown), aldonolactone oxidoreductases (purple), alditol oxidases (orange) and cholesterol oxidases (light green). Glycolate oxidoreductase subunit E from *Escherichia coli* (black) was not deemed to belong to any of the identified subgroups. The sequences in the tree are: Sc Pac11: Pac11 from *Streptomyces coeruleorubidus*, SI MCRA: mitomycin radical oxidase from *Streptomyces lavendulae*, Pn HDNO: 6-hydroxy-*N*-nicotine oxidase from *Paenarthrobacter nicotinovorans*, Sm EncM: EncM from *Streptomyces maritimus*, Fg ChitO: chitooligosaccharide oxidase from *Fusarium graminearum*, Ss GOOX: glucooigosaccharide oxidase from *Sarocladium strictum*, Mt XyLO: xylooligosaccharide oxidase from *Myceliophthora thermophila* C1, Mn LAO: lactose oxidase from *Microdochium nivale*, Cc HOX: hexose oxidase from *Chondrus crispus*, Sg GilR: GilR from *Streptomyces griseoflavus*, Ss SpnJ: SpnJ from *Saccharopolyspora spinosa*, Ac CrmK: CrmK from *Actinoboliteichus cyanogriseus* WH1-2216-6, Ssp ClmD2: ClmD2 from *Streptomyces* sp. CS40, Sg AknOx, aclacinomycin oxidoreductase from *Streptomyces galilaeus*, Nsp Dbv29: Dbv29 from *Nonomuraea* sp. ATCC 39727, Ssp TamL: TamL from *Streptomyces* sp. 307-9, Ec BBE1: berberine bridge enzyme from *Eschscholzia californica* (california poppy), Pp Phl p4: Phl p 4 from *Phleum pratense* (timothy-grass), Ha CHO: carbohydrate oxidase from *Helianthus annuus* (sunflower), Cs CBDAS: cannabidiolic acid synthase from *Cannabis sativa*, Cs THCAS: tetrahydrocannabinolic acid synthase from *Cannabis sativa*, At BBE-like 15: BBE-like protein 15 from *Arabidopsis thaliana*, At BBE-like 13: BBE-like protein 13 from *Arabidopsis thaliana*, Am STOX: (S)-tetrahydroprotoberberine oxidase from *Argemone mexicana*, Bw STOX: (S)-tetrahydroprotoberberine oxidase from *Berberis wilsoniae*, An YanF: YanF from *Aspergillus niger*, As SPS: solanapyrone synthase from *Alternaria solani*, Cg CHGG01242-2: CHGG_01242-2 from *Chaetomium globosum*, Ao IAAO: isoamyl alcohol oxidase from *Aspergillus oryzae*, Fg ZEB1: ZEB1 from *Fusarium graminearum*, Zm CKX: cytokinin dehydrogenase from *Zea mays* (maize, numbered by isoform), At CKX: cytokinin dehydrogenase from *Arabidopsis thaliana* (numbered by isoform), Tc MurB: MurB from *Thermus caldophilus*, Sp MurB: MurB from *Streptococcus pneumoniae*, Sa MurB: MurB from *Staphylococcus aureus*, Lm MurB: MurB from *Listeria monocytogenes*, Mt MurB: MurB from *Mycobacterium tuberculosis*, Pa MurB: MurB from *Pseudomonas aeruginosa*, Vc MurB: MurB from *Vibrio cholerae*, Ec MurB: MurB from *Escherichia coli*, Cc CndG: CndG from *Chondromyces crocatus* Cm c5, FeeG: FeeG from an unidentified soil microbe, Ps VAO: vanillyl alcohol oxidase from *Penicillium simplicissimum*, Rj EUGO: eugenol oxidase from

forms interactions with the uridine moiety of UDP-GlcNAc-EP in Type I MurBs [52,53]. Type II MurBs can be further divided into two groups based on the identity of an active site acid that protonates the carbanion intermediate formed after hydride transfer to UDP-GlcNAc-EP. In *S. aureus* MurB this role is performed by a serine residue (Type II-a), while in *T. caldophilus* MurB it is performed by a cysteine residue (Type II-b). All type I MurBs contain a serine residue that acts as the proton donor [54].

Recently, it was found that *Verrucomicrobium spinosum* contains a novel form of MurB, where it is fused to MurC, the enzyme that catalyzes the following step in peptidoglycan synthesis, the addition of L-alanine to the carboxylic acid group of UDP-MurNAc [55]. Expression of the fusion protein was found to complement the phenotype of *E. coli* *murB* and *murC* mutants. However, although MurC activity could be detected using the purified enzyme *in vitro*, MurB activity could not. Further characterization of this novel MurB will be an interesting future research line. MurB's role in bacterial cell wall synthesis makes it an interesting target for the development of novel antibiotics and various inhibitors have been identified and shown to possess antibacterial activity (see Ref. [56] for a review).

5. Subgroup 4: 4-phenol oxidizing

The 4-phenol oxidizing (4PO) subgroup contains enzymes that catalyze the oxidation of *para*-substituted phenols at the C α atom of the *para*-substituent. The subgroup contains both oxidases, such as vanillyl alcohol oxidase (VAO, EC 1.1.3.38) from *Penicillium simplicissimum*, and eugenol oxidase (EUGO) from *Rhodococcus jostii* RHA1, and dehydrogenases, such as *para*-cresol methylhydroxylase (PCMH, EC 1.17.99.1) from *Pseudomonas putida* and eugenol hydroxylase (EUGH) from *Pseudomonas* sp., that utilize a cytochrome *c* subunit as an electron acceptor [57–61]. Depending on the enzyme and type of substrate, these 4PO subgroup members can catalyze the oxidation of benzylic alcohols to aldehydes, the oxidative deamination of benzylic amines, the oxidative demethylation of 4-(methoxymethyl)phenol, the hydroxylation of 4-allylphenols, and the hydroxylation or dehydrogenation of 4-alkylphenols (Fig. 6A–C) [62–64]. Catalysis proceeds through the abstraction of a hydride from the C α atom of the side chain of an enzyme-bound phenolate form of the substrate, yielding a *para*-quinone methide intermediate that subsequently rearranges or reacts with water to give the reaction products [65,66]. *In vivo*, these enzymes are presumably involved in the metabolism of *para*-substituted phenolic compounds, possibly derived from the degradation of lignin [67].

These 4PO subgroup members are unique among the VAO/PCMH family members in that their FAD cofactor is covalently bound to a loop that is close to the C-terminus of the protein, rather than the more commonly found covalent linkages to a loop close to the N-terminus of the protein. This occurs through an 8 α -N3-histidyl-FAD bond in the case of VAO and EUGO and an 8 α -O-tyrosyl-FAD bond in the case of PCMH and (as predicted from its sequence) EUGH [68–70]. Recent progress in studying this group of enzymes includes the publication of the crystal structure of EUGO and a mutagenesis study that focused on understanding the molecular determinants and function of the differing oligomerization states of the oxidases VAO and EUGO [70,71]. Although the secondary and tertiary structure of these proteins is rather similar, they differ in terms of their quaternary structure. EUGO is exclusively dimeric in solution, whereas VAO forms both dimers and octamers, which can be described as tetramers of dimers. A loop that is positioned at the dimer-dimer interface of VAO, but is not present in EUGO, was shown to be responsible for this difference, with its deletion leading to the formation of a VAO variant that exclusively forms dimers without significantly affecting catalytic function. However, a recent report described the expression of a number of VAO homologues that displayed an octameric quaternary structure, despite not containing such a loop region [72]. Further research is required to understand the mechanism by which these novel VAO-like enzymes obtain their octameric oligomerisation state.

In addition to these well characterized enzymes, the 4PO subgroup contains two enzymes that catalyze the oxidative decarboxylation of *N*-substituted tyrosine derivatives: CndG from *Chondromyces crocatus* and FeeG from an unidentified soil microbe (Fig. 6D) [73,74]. These enzymes both contain three residues that are thought to be crucial for enabling the formation of the *para*-quinone methide intermediate in other 4PO subgroup members (Tyr108, Tyr503 and Arg504 in VAO). This suggests that the decarboxylation reaction proceeds through the initial formation of a *para*-quinone methide intermediate, which subsequently expels carbon dioxide. Unlike the other 4PO subgroup members, CndG contains a non-covalently bound FAD cofactor, in agreement with the fact that neither the histidine residue involved in covalent flavin binding in VAO nor the tyrosine residue involved in covalent flavin binding in PCMH are conserved in this enzyme.

6. Subgroup 5: alkyl-dihydroxyacetone phosphate synthases

Alkyl-dihydroxyacetone phosphate synthase (ADPS, EC 2.5.1.26) is involved in the biosynthesis of ether phospholipids, lipids which

Rhodococcus jostii RHA1, Pp PCMH: *para*-cresol methylhydroxylase from *Pseudomonas putida*, POPS1 EUGH: eugenol hydroxylase from *Pseudomonas* sp. OPS1, PHR199 EUGH: eugenol hydroxylase from *Pseudomonas* sp. HR199, Ec GlcE: glycolate oxidoreductase subunit E from *Escherichia coli*, Tb ADPS: alkyl-dihydroxyacetone phosphate synthase (ADPS) from *Trypanosoma brucei*, Dd ADPS: ADPS from *Dictyostelium discoideum*, Ce ADPS: ADPS from *Caenorhabditis elegans*, Cp ADPS: ADPS from *Cavia porcellus* (guinea pig), Hs ADPS, human ADPS, Ec DLDH: D-lactate dehydrogenase (DLDH) from *Escherichia coli*, Asp DLDH: DLDH from *Acidocella* sp. MX-AZ02, Aa DLDH: DLDH from *Acetobacter acetii*, Af DLDH: DLDH from *Archaeoglobus fulgidus*, Ec GlcD: glycolate oxidoreductase subunit D from *Escherichia coli*, St DLDH: DLDH from *Sulfolobus tokodaii* 7, At DLDH: DLDH from *Arabidopsis thaliana*, Kl DLDH: DLDH from *Kluyveromyces lactis*, Sc DLD1: DLD1 from *Saccharomyces cerevisiae*, Hs D2HGDH: human D-2-hydroxyglutarate dehydrogenase, At D2HGDH: D-2-hydroxyglutarate dehydrogenase from *Arabidopsis thaliana*, Sc DLD3: DLD3 from *Saccharomyces cerevisiae*, Sc DLD2: DLD2 from *Saccharomyces cerevisiae*, Ms DprE1: DprE1 from *Mycobacterium smegmatis*, Mt DprE1: DprE1 from *Mycobacterium tuberculosis*, Bm DHCR24: 24-dehydrocholesterol reductase (DHCR24) from *Bombyx mori* (silkworm), Mm DHCR24: DHCR24 from *Mus musculus* (mouse), Hs DHCR24: human DHCR24, Sl SSR2: sterol side chain reductase (SSR) 2 from *Solanum lycopersicum* (tomato), St SSR2: SSR2 from *Solanum tuberosum* (potato), Os DWF1: DWARF1 from *Oryza sativa* (rice), Zm DWF1: DWARF1 from *Zea mays* (maize), At DWF1: DWARF1 from *Arabidopsis thaliana*, Ar DWF1: DWARF1 from *Ajuga reptans*, St SSR1: SSR1 from *Solanum tuberosum* (potato), Sl SSR1: SSR1 from *Solanum lycopersicum* (tomato), Ac AldO: alditol oxidase from *Acidothermus cellulolyticus* 11B, Ssp SorB: sorbitol oxidase from *Streptomyces* sp. H-7775, Sc AldO: alditol oxidase from *Streptomyces coelicolor* A3(2), Ssp XylOx: xylitol oxidase from *Streptomyces* sp. IKD472, Pg GLO: D-glucono-1,4-lactone oxidase from *Penicillium griseoroseum*, Mt GUDH: L-gulonono-1,4-lactone dehydrogenase from *Mycobacterium tuberculosis*, Nt GALDH: L-galactono-1,4-lactone dehydrogenase (GALDH) from *Nicotiana tabacum* (tobacco), Ib GALDH: GALDH from *Ipomoea batatas* (sweet potato), At GALDH: GALDH from *Arabidopsis thaliana*, Bo GALDH: GALDH from *Brassica oleracea* (cauliflower), Sc ALO: D-arabinono-1,4-lactone oxidase (ALO) from *Saccharomyces cerevisiae*, Ca ALO: ALO from *Candida albicans*, Ld ALO: ALO from *Leishmania donovani*, Tc GALO: L-galactono-1,4-lactone oxidoreductase from *Trypanosoma cruzi*, Tb ALO: ALO from *Trypanosoma brucei*, St GUO: L-gulonono-1,4-lactone oxidase (GUO) from *Scyliorhinus torazame* (cloudy catshark), Ss GUO: GUO from *Sus scrofa* (pig), Rn GUO: GUO from *Rattus norvegicus* (rat), Mm GUO: GUO from *Mus musculus* (mouse), Bs ChoX: cholesterol oxidase (ChoX) from *Brevibacterium sterolicum*, Re ChoX: ChoX from *Rhodococcus erythropolis* CECT3014, Rr ChoX: ChoX from *Rhodococcus ruber* Chol-4, Pa ChoX: ChoX from *Pseudomonas aeruginosa* PA157, Bc ChoX: ChoX from *Burkholderia cepacia* ST-200, Csp ChoX: ChoX from *Chromobacterium* sp. DS-1. The scale bar indicates the branch length corresponding to 0.5 changes per position.

Fig. 3. Synthesis of the tricyclic core of the antibiotic enterocin catalyzed by EncM. EncM utilizes a novel flavin oxidation state, the flavin-N5-oxide, to catalyze a double oxidation of its substrate. The proposed physiological substrate, a dihydrooctaketide intermediate bound to the carrier protein EncC, is bound in the enzyme's active site with EncM's FAD cofactor in the N5-oxide form. Subsequently, the flavin-N5-oxide oxygenates the substrate at the C4 position, yielding an alcohol and oxidized FAD. Following this, oxidation of the formed alcohol to a ketone takes place, yielding fully reduced FAD. The product is then released from the enzyme and undergoes a Favorskii-type rearrangement to yield the tricyclic enterocin core. Meanwhile, the reduced flavin reacts with molecular oxygen, regenerating the flavin-N5-oxide.

Fig. 4. Oxidation of cytokinins as catalyzed by cytokinin dehydrogenase (CKX). The cytokinin is first oxidized at its side chain amine group, yielding an imine. The imine is subsequently hydrolyzed, yielding adenine and an aldehyde.

Fig. 5. Reduction of UDP-N-acetylglucosamine enolpyruvate to UDP-N-acetylmuramic acid catalyzed by MurB.

contain an ether-bond linked alkyl chain instead of one of the ester-bond linked acyl chains found in most phospholipids. Ether lipids form a major constituent of mammalian membranes and defects in their biosynthesis, in some cases caused by mutations in the gene

encoding ADPS, are linked to the genetic disorder rhizomelic chondrodysplasia punctata in humans [75]. Ether phospholipid biosynthesis starts in the peroxisomes with the synthesis of acyl-dihydroxyacetone phosphate (acyl-DHAP) from DHAP and acyl-coA, catalyzed by an acyltransferase. Subsequently, ADPS catalyzes the conversion of the acyl-DHAP to an alkyl-DHAP by the incorporation of a long-chain fatty alcohol, thus yielding the ether bond that is characteristic of ether phospholipids. ADPS is unique among VAO/PCMH family members in that it is not an oxidoreductase, but a transferase that uses a non-covalently bound FAD cofactor to catalyze a reaction that does not involve any change in the redox states of the substrates [76]. The reaction occurs through the formation of a covalent intermediate between acyl-DHAP and FAD and is proposed to proceed as follows (Fig. 7) [77,78]. First, acyl-DHAP is bound to the enzyme's active site in its enolate form. Tautomerization to the enolate may be stimulated by Tyr578

Fig. 6. Examples of reactions catalyzed by members of the 4-phenol oxidizing subgroup. (A) The oxidation of vanillyl alcohol to vanillin by eugenol oxidase (EUGO) or vanillyl alcohol oxidase (VAO). (B) The oxidation of *para*-cresol to 4-hydroxybenzyl alcohol by *para*-cresol methylhydroxylase (PCMH). (C) The oxidation of eugenol to coniferyl alcohol by eugenol hydroxylase (EUGH), EUGO or VAO. (D) The oxidative decarboxylation of *N*-substituted tyrosine derivatives catalyzed by CndG.

(numbering according to *Cavia porcellus* [guinea pig] ADPS), which could act as a proton acceptor, and His616 and His617, which could stabilize a negative charge on the DHAP O2 atom. These residues are conserved among all ADPS sequences included in our alignment and have been shown to be essential for activity by site-directed mutagenesis [76–78]. This tautomerization enables the subsequent nucleophilic attack of the C1 atom of DHAP on the N5 atom of FAD, yielding a covalent N5-acyl-DHAP adduct. Next, a fatty acid is expelled from the intermediate, yielding an N5-DHAP iminium intermediate. This intermediate breaks down through the attack of a fatty alcohol, yielding the alkyl-DHAP product and the oxidized FAD cofactor. In addition to the two domains typically found in VAO/PCMH family members, ADPS contains a third domain at its N-terminus [77,78]. This domain contains a gating helix that closes off the substrate-binding cavity. Recently, ADPS has gained interest as a potential anti-cancer drug target, as its inhibition leads to significant changes in the lipid composition of tumour cells and impairs their pathogenicity [79,80].

7. Subgroup 6: α -Hydroxy acid dehydrogenases

α -Hydroxy acid dehydrogenases catalyze the oxidation of the hydroxyl group of α -hydroxy acids to a carbonyl group, using electron acceptors other than molecular oxygen (Fig. 8). Depending on their substrate specificity and physiological function they are known either as D-lactate dehydrogenase (DLDH, EC 1.1.2.4), D-2-hydroxyglutarate dehydrogenase (D2HGDH, EC 1.1.99.39) or glycolate oxidoreductase. DLDH is found in bacteria, archaea, yeasts and plants, where it catalyzes the oxidation of D-lactate to pyruvate, the first step in its metabolism [81–85]. The crystal structure of *Escherichia coli* DLDH revealed that it contains a non-covalently bound FAD cofactor and that, in addition to the FAD-binding and substrate-binding domains typically found in VAO/PCMH family members, it contains a third domain that is responsible for association of the protein with the cytoplasmic membrane [86]. Similar to DLDH, D2HGDH catalyzes the oxidation of the hydroxyl groups of α -hydroxy acids, but it shows highest activity for the oxidation of D-2-hydroxyglutarate to α -ketoglutarate [87]. Mutations in the human D2HGDH gene cause the genetic disorder D-2-hydroxyglutaric aciduria, which is characterized by heightened levels of D-2-hydroxyglutarate in body fluids, with patients displaying severe developmental defects [88].

A recent study revealed that *Saccharomyces cerevisiae* contains three α -hydroxy acid dehydrogenases (named DLD1, DLD2 and DLD3). DLD1 functions as a conventional DLDH. However, DLD2 and

Fig. 7. Mechanism of the synthesis of alkyl-dihydroxyacetone phosphate (alkyl-DHAP) from acyl-DHAP by alkyl-dihydroxyacetone phosphate synthase (ADPS). Upon binding to the enzyme, acyl-DHAP is tautomerized to its enolate form. This enables the formation of a covalent adduct between the enzyme's FAD cofactor and acyl-DHAP. Subsequently, a fatty acid is released from this intermediate, yielding a covalent iminium intermediate. This subsequently reacts with a fatty alcohol, yielding the alkyl-DHAP product.

DLD3 do not only catalyze the oxidation of α -hydroxy acids using electron acceptors such as DCPIP, but can also transfer the electrons to α -keto acids, yielding new α -hydroxy acids [89]. Based on the fact that a *dld3Δ* mutant displayed increased D-2-hydroxyglutarate levels and decreased D-lactate levels, it was suggested that the

Fig. 8. Oxidation of α -hydroxy acids catalyzed by the α -hydroxy acid dehydrogenases glycolate oxidoreductase, D-lactate dehydrogenase (DLDH) and D-2-hydroxyglutarate dehydrogenase (D2HGDH).

enzyme may function as a transhydrogenase *in vivo*, converting D-2-hydroxyglutarate and pyruvate to α -ketoglutarate and D-lactate respectively. Glycolate oxidoreductase from *E. coli* catalyzes the first step of glycolate metabolism, its oxidation to glyoxylate [90]. Two genes encoding the putative VAO/PCMH-type oxidoreductases *glcD* and *glcE* have been shown to be required for glycolate oxidoreductase activity [91]. However, the function of the two gene products has not been studied in detail. It should be noted that the sequence of *glcE* is significantly shorter than that of the other α -hydroxy acid dehydrogenases and it is not closely related to the other sequences according to the phylogenetic tree. As such, we do not deem it to belong to the α -hydroxy acid dehydrogenase subgroup.

8. Subgroup 7: sterol reductases

Members of the sterol reductase family are responsible for the reduction of a carbon-carbon double bond during sterol biosynthesis. Their exact physiological function differs depending on the source organism. Sterol reductases from mammals are known as 24-dehydrocholesterol reductase (DHCR24, also known as seladin-1, EC 1.3.1.72) and catalyze the final step of cholesterol biosynthesis, the reduction of 24-dehydrocholesterol (desmosterol) to cholesterol (Fig. 9A) [92]. The reaction is NADPH dependent and stimulated by the presence of FAD, suggesting DHCR24 contains a non-covalently bound FAD cofactor and uses NADPH as an electron donor. Mutations in the human *DHCR24* gene cause the congenital disorder desmosterolosis, with patients displaying heightened plasma desmosterol levels and various congenital and developmental abnormalities [92]. In addition to mammals, DHCR24 has been identified in *Bombyx mori* (silkworm) [93]. This invertebrate cannot synthesize cholesterol *de novo*, but rather converts plant sterols to cholesterol via a process where they are first dealkylated to desmosterol, which is subsequently reduced by DHCR24.

Sterol reductases have also been identified in plants such as *Arabidopsis thaliana* and *Oryza sativa* (rice), where they are involved in the biosynthesis of C24-alkylated sterols such as campesterol or sitosterol [94,95]. These sterols are synthesized from their Δ 24-28 unsaturated derivatives, which are reduced by the sterol reductase DWARF1 (Fig. 9B). Typically, this reaction is believed to take place in two steps, with the Δ 24-28 unsaturated sterols first being isomerized to the Δ 24-25 unsaturated derivatives, which are subsequently reduced to the saturated products. Thus, like DHCR24, DWARF1 catalyzes the reduction of Δ 24-25 double bonds, with the main difference in the reactions lying in the prior isomerization step. Interestingly, *Ajuga reptans* DWARF1 was recently reported to catalyze direct reduction of Δ 24-28 double bonds without the prior isomerization step [96].

Cholesterol is generally only found in trace amounts in plants. However, plants from the genus *Solanum*, such as *Solanum tuberosum* (potato) and *Solanum lycopersicum* (tomato) rely on cholesterol as a precursor to steroidal glycoalkaloid toxins. Both *S. tuberosum* and *S. lycopersicum* contain two sterol reductase-encoding genes. One encodes a typical DWARF1-type reductase that catalyzes the Δ 24-28 double bond reduction required for the synthesis of plant sterols (SSR1), while the other encodes a reductase that only catalyzes the reduction of Δ 24-25 double bonds enabling it to convert desmosterol to cholesterol (SSR2) [97].

A thorough understanding of the molecular determinants of the differences in the reactions catalyzed by sterol reductases is hampered by a lack of structural data. Homology modelling of human DHCR24 did lead to a hypothesis regarding the cause of desmosterolosis [98]. Mutations found in patients may affect interactions with the FAD cofactor, the desmosterol substrate and the endoplasmic reticulum membrane, with which DHCR24 is

associated [99,100]. Further structural and biochemical studies are required to confirm these hypotheses.

9. Subgroup 8: DprE1s

The most recently identified subgroup of the VAO/PCMH family consists of DprE1 (EC 1.1.98.3). Together with a second enzyme, DprE2, DprE1 is responsible for the epimerization of decaprenylphosphoryl- β -D-ribofuranose (DPR) to decaprenylphosphoryl- β -D-arabinofuranose (DPA), the precursor of the arabinan moiety of the cell wall of mycobacteria such as *Mycobacterium tuberculosis*, the pathogen that causes tuberculosis [101]. The importance of this reaction is highlighted by the fact that the *dprE1* gene has been shown to be essential for growth in *Mycobacterium smegmatis* [102]. Epimerization of DPR to DPA occurs in a two-step process, where DPR is first oxidized to decaprenylphosphoryl-2-keto- β -D-erythro-pentofuranose (DPX) by DprE1, after which DPX is reduced to DPA by DprE2 (Fig. 10A) [103]. To achieve substrate oxidation, DprE1 uses a non-covalently bound FAD cofactor. Though DprE1 can use oxygen as an electron acceptor, artificial electron acceptors such as DCPIP lead to more efficient catalysis, suggesting the enzyme is a dehydrogenase rather than an oxidase. It has been suggested that membrane-embedded menaquinone may be the physiological electron acceptor [104].

Due to its crucial role in mycobacterial cell wall synthesis, DprE1 has gathered major interest as a potential antitubercular drug target in recent years. The DprE1 inhibitors identified so far can be classed into two groups: nitro-group containing suicide inhibitors and non-covalent competitive inhibitors [105]. The covalent inhibitors are first activated by reduction of their nitro group to a nitroso group, which only occurs after reduction of the enzyme's FAD cofactor by its substrate (Fig. 10B) [103,104,106,107]. The nitroso group subsequently reacts with the thiol group of an active site cysteine (Cys387 in *M. tuberculosis* DprE1), leading to the bulky compounds permanently blocking the active site and preventing catalysis. The identification of further DprE1 inhibitors and their application as antitubercular drugs will likely be an important line of research in the coming years.

10. Subgroup 9: aldonolactone oxidoreductases

Aldonolactone oxidoreductases are found in a wide variety of organisms where they catalyze the final step in the synthesis of L-ascorbic acid (vitamin C) or related antioxidant compounds. The exact reaction that is catalyzed depends on the host organism (Fig. 11) [108,109]. In animals, L-ascorbic acid is synthesized from L-gulonono-1,4-lactone by L-gulonono-1,4-lactone oxidase (GUO, EC 1.1.3.8) [110,111]. In plants, L-ascorbic acid is mainly synthesized from L-galactono-1,4-lactone by L-galactono-1,4-lactone dehydrogenase (GALDH, EC 1.3.2.3) [112–115]. Another enzyme that catalyzes the formation of L-ascorbic acid is L-gulonono-1,4-lactone dehydrogenase (GUDH) from *Mycobacterium tuberculosis* [10]. However, it is unclear what the physiological function of this enzyme is. Fungal aldonolactone oxidoreductases do not synthesize L-ascorbic acid, but rather the similar compounds D-erythorbic acid, which is synthesized from D-glucono-1,4-lactone by D-glucono-1,4-lactone oxidase (GLO) in *Penicillium griseoroseum* (also known as *Penicillium cyaneo-fulvum*), or dehydro-D-arabinono-1,4-lactone (also known as D-erythroascorbic acid), which is synthesized from D-arabinono-1,4-lactone by D-arabinono-1,4-lactone oxidase (ALO, EC 1.1.3.37) in yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* [116–119]. Aldonolactone oxidoreductases have also been identified in the protozoans *Trypanosoma brucei* (ALO), *Trypanosoma cruzi* (L-galactono-1,4-lactone oxidoreductase, GALO), and *Leishmania donovani* (ALO) [11,120,121]. These enzymes are named

Fig. 9. Reductions of sterols catalyzed by sterol reductases. (A) The mammalian enzyme DHCR24 reduces the Δ^{24-25} double bond of desmosterol, yielding cholesterol. (B) The plant enzyme DWARF1 catalyzes the isomerization of the Δ^{24-28} double bond of sterols such as 24-methylenecholesterol to a Δ^{24-25} double bond, followed by the reduction of this double bond, in this case yielding campesterol. *Ajuga reptans* DWARF1 can catalyze the direct reduction of Δ^{24-28} double bonds without the prior isomerization step.

Fig. 10. (A) Epimerization of decaprenylphosphoryl- β -D-ribofuranose (DPR) to decaprenylphosphoryl- β -D-arabinofuranose (DPA) as catalyzed by DprE1 and DprE2. DPR is first oxidized to decaprenylphosphoryl-2-keto- β -D-erythro-pentofuranose (DPX) by DprE1. DPX is subsequently reduced to DPA by the NADH-dependent reductase DprE2. (B) Mechanism of inhibition of DprE1 by the benzothiazine inhibitor BTZ043. In order for inhibition to occur, the enzyme's FAD cofactor must first be reduced by its substrate. Subsequently, the nitro group of BTZ043 is reduced to a nitroso group by reaction with the FAD cofactor. This nitroso group reacts with the thiol group of an active site cysteine residue (Cys387 in *Mycobacterium tuberculosis* DprE1), covalently modifying the enzyme and blocking the active site.

after the best substrate identified in *in vitro* experiments, however, the identity of their physiological substrate is unclear.

Despite the differences in substrate specificity, the basic reaction catalyzed by all aldonoaldehyde oxidoreductases is similar. The C2–C3 bond of the lactone substrate is oxidized to a double bond by transfer of two electrons to the enzyme's flavin cofactor. The subsequent reoxidation of the flavin cofactor can occur either by its reaction with molecular oxygen (GUO, yeast ALO, GLO) or electron

acceptors such as cytochrome *c* (GUDH, GALDH). *Arabidopsis thaliana* GALDH can be converted into an oxidase by a single alanine to glycine mutation [122]. A glycine or proline is found at this position in the oxidases GUO, yeast ALO and GLO and it is predicted to be located close to the C4a atom of FAD, the site of the reaction with oxygen. This suggests that in GALDH, this alanine residue acts as a gatekeeper, blocking access of oxygen to the flavin cofactor. The binding mode of the flavin cofactor also differs depending on the

Fig. 11. Oxidations catalyzed by aldolactone oxidoreductases. L-gulonono-1,4-lactone oxidase (GUO) and L-galactono-1,4-lactone dehydrogenase (GALDH) catalyze the formation of L-ascorbic acid from L-gulonono-1,4-lactone and L-galactono-1,4-lactone respectively. D-arabinono-1,4-lactone oxidase (ALO) from yeast catalyzes the oxidation of D-arabinono-1,4-lactone to dehydro-D-arabinono-1,4-lactone. D-Glucono-1,4-lactone oxidase (GLO) catalyzes the oxidation of D-glucono-1,4-lactone to D-erythroic acid.

enzyme, GUO, yeast ALO and GLO contain a covalently bound FAD cofactor, while GALDH from *A. thaliana* and GALO from *T. cruzi* contain non-covalently bound FAD [13,123–125]. A number of arabinonolactone oxidoreductases have been reported to contain non-covalently bound FMN (ALO from *T. brucei* and GALDH from *Brassica oleracea*) and no flavin was detected in GUDH [9–11]. However, as hydrolysis of FAD during analysis may lead to misassignment of the cofactor of flavoproteins as FMN instead of FAD, further investigations are required to confirm the identity of the flavin in these enzymes.

11. Subgroup 10: alditol oxidases

Members of the alditol oxidase subgroup (referred to as alditol oxidase [AldO], sorbitol oxidase or xylitol oxidase depending on their substrate specificity, EC 1.1.3.41) catalyze the oxidation of the primary hydroxyl groups of alditols (sugar alcohols), yielding the corresponding aldoses, with molecular oxygen acting as the electron acceptor (Fig. 12A). They have been identified in various *Streptomyces* species and in the thermophilic bacterium *Acidothermus cellulolyticus* 11B [126–129]. All characterized alditol oxidases contain a covalently bound FAD cofactor, which the crystal structure of the enzyme from *Streptomyces coelicolor* A3(2) revealed to be covalently bound to the enzyme via an 8 α -N1-histidyl-FAD bond [130]. The diffusion of oxygen into the active site of

S. coelicolor AldO was studied by a combination of molecular dynamics simulations and site-directed mutagenesis [131]. This revealed that there are multiple defined diffusion pathways through which oxygen reaches the active site of the enzyme. The majority of these pathways converge at a single point of entrance to the active site. The residue located at this site, Ala105, is homologous to the gatekeeper alanine residue identified in GALDH, suggesting this may be a crucial site in determining the oxygen reactivity of VAO/PCMH-type flavoenzymes.

In addition to the oxidation of alcohols, AldO from *S. coelicolor*, can catalyze the oxidation of thiol groups [132]. For example, L-dithiothreitol, which is identical to the sugar alcohol L-threitol except for the presence of thiol groups instead of hydroxyl groups at the terminal carbon atoms, is oxidized to the corresponding thioaldehyde, which subsequently cyclizes to a dithiohemiacetal (Fig. 12B). A number of other flavin-dependent alcohol oxidases from the structurally unrelated glucose-methanol-choline (GMC) oxidoreductase family were also shown to oxidize thiol analogues of their alcohol substrates, suggesting the ability to do so may be a general property of flavin-dependent alcohol oxidases.

12. Subgroup 11: cholesterol oxidases

Cholesterol oxidase (ChoX, EC 1.1.3.6) is found in bacteria that are capable of metabolizing cholesterol, where it catalyzes the first two steps in its metabolism. The first is the oxidation of the 3 β -hydroxyl group to a carbonyl, yielding cholest-5-en-3-one (Fig. 13). In most cases, this is subsequently isomerized to cholest-4-en-3-one [133]. Molecular oxygen is utilized as the electron acceptor for the first step of the reaction, being converted to hydrogen peroxide. Two types of ChoX exist, a VAO/PCMH-type that contains a covalently bound FAD cofactor linked to the protein through an 8 α -N1-histidyl-FAD bond and another type that belongs to the GMC oxidoreductase family and contains non-covalently bound FAD. VAO/PCMH-type ChoXs are found in Gram-positive bacteria such as *Brevibacterium sterolicum* and various species of *Rhodococcus* and Gram-negative bacteria such as *Chromobacterium* sp. DS-1, *Pseudomonas aeruginosa* and *Burkholderia cepacia* [134–139]. Unlike most ChoXs, the VAO/PCMH-type enzymes from Gram-negative bacteria do not catalyze the isomerization of cholest-5-en-3-one to cholest-4-en-3-one and the main product of the reaction is 6-hydroperoxycholest-4-en-3-one (Fig. 13) [140]. The exact mechanism by which 6-hydroperoxycholest-4-en-3-one is formed is unclear. It has been proposed that it may be the result of dioxygenase activity of the enzyme, but a mechanism where the oxidation proceeds through enzyme-initiated radical reactions is also a possibility [140,141]. The molecular determinants of which product is formed are unclear and would be an interesting topic for future studies, particularly considering that a crystal structure is available of both the cholest-4-en-3-one-forming VAO/PCMH-type ChoX from *B. sterolicum* and the 6-hydroperoxycholest-4-en-3-one-forming VAO/PCMH-type ChoX from *Chromobacterium* sp. DS-1 [142,143].

13. Concluding remarks

The past ten years have seen significant and surprising advances in our knowledge of enzymes of the VAO/PCMH family, including the characterization of the flavin-N5-oxide, a novel flavin redox state, and DprE1, a promising potential antitubercular drug target. As highlighted above, open questions regarding the biochemical and mechanistic characterization of these enzymes remain and will provide interesting challenges for future research.

Fig. 12. (A) Oxidation of xylitol to D-xylose catalyzed by alditol oxidase (AldO). (B) Oxidation of L-dithiothreitol to the corresponding thioaldehyde, which rearranges to a cyclic dithiohemiacetal, catalyzed by AldO.

Fig. 13. Oxidation of cholesterol as catalyzed by cholesterol oxidase (ChoX). Cholesterol is first oxidized to cholest-5-en-3-one. VAO/PCMH-type ChoX from Gram-positive bacteria, such as *Brevibacterium sterolicum* (Bs ChoX), subsequently catalyzes its isomerization to cholest-4-en-3-one. VAO/PCMH-type ChoX from Gram-negative bacteria, such as *Chromobacterium* sp. DS-1 (Csp ChoX), does not catalyze this isomerization step. Instead, oxygen is incorporated into the formed cholest-5-en-3-one, yielding 6-hydroperoxycholest-4-en-3-one as the product.

14. Methods

To perform the phylogenetic analysis of the VAO/PCMH family, a list of family members with known sequence and enzymatic function was compiled by searching the Uniprot and Protein Data Bank databases and the primary literature. A structure-based multiple sequence alignment of 112 obtained sequences was performed using the PROMALS3D webserver [144]. PDB files with the following PDB IDs were used for the alignment: 1FOX (*Escherichia coli* DLDH), 1HSK (*Staphylococcus aureus* MurB), 1119 (*Brevibacterium sterolicum* ChoX), 1VAO (*Penicillium simplicissimum* VAO), 1W1O (*Zea mays* CKX1), 1WVF (*Pseudomonas putida* PCMH), 1ZR6 (*Sarocladium strictum* glucooligosaccharide oxidase), 2BVF (*Paenarthrobacter nicotinovorans* 6-hydroxy-D-nicotine oxidase), 2EXR (*Arabidopsis thaliana* CKX7), 2GQT (*Thermus caldophilus* MurB), 2IPI (*Streptomyces galilaeus* aclacinomycin oxidoreductase), 2MBR (*Escherichia coli* MurB), 2UUU (*Dictyostelium discoideum* ADPS), 2VFR (*Streptomyces coelicolor* A3(2) AldO), 2Y08 (*Streptomyces* sp.

307-9 TamL), 3D2H (*Eschscholzia californica* BBE), 3JS8 (*Chromobacterium* sp. DS-1 ChoX), 3RJ8 (*Microdochium nivale* lactose oxidase) and 3TSH (*Phleum pratense* Phl p 4). Subsequently, three sequences, GilR from *Streptomyces griseoflavus*, CndG from *Chondromyces crocatus* and FeeG from an unknown source organism, were added to the alignment using the MAFFT version 7 webserver [145]. Subsequently, a phylogenetic tree was constructed using the phylogeny.fr webserver's BioNJ function [146]. The iTOL webserver was used for tree visualization [147].

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