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Review

The growing VAO flavoprotein family

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Abstract

The VAO flavoprotein family is a rapidly growing family of oxidoreductases that favor the covalent binding of the FAD cofactor. In this review we report on the catalytic properties of some newly discovered VAO family members and their mode of flavin binding. Covalent binding of the flavin is a self-catalytic post-translational modification primarily taking place in oxidases. Covalent flavinylation increases the redox potential of the cofactor and thus its oxidation power. Recent findings have revealed that some members of the VAO family anchor the flavin via a dual covalent linkage (6-*S*-cysteinyl-8α-*N*1-histidyl FAD). Some VAO-type aldonolactone oxidoreductases favor the non-covalent binding of the flavin cofactor. These enzymes act as dehydrogenases, using cytochrome *c* as electron acceptor.

Keywords: Alditol oxidase; Chitooligosaccharide oxidase; Covalent flavinylation; Eugenol oxidase; Flavoenzyme; L-Galactono-1,4-lactone dehydrogenase; Vanillyl-alcohol oxidase; VAO family; Vitamin C

The vanillyl-alcohol oxidase (VAO)[1] flavoprotein family comprises a group of enzymes that share a conserved FAD-binding domain [1]. VAO family members are involved in a wide variety of metabolic processes in all kingdoms of life. A remarkable feature of the FAD-binding module of this protein family is that it favors the covalent attachment of the flavin cofactor. The first VAO family members identified with such a covalent link concerned 6-hydroxy-D-nicotine oxidase (HDNO), involved in nicotine catabolism in *Arthrobacter nicotinovorans* [2,3] and *p*-cresol methylhydroxylase (PCMH), involved in the microbial detoxification of phenols [4]. Only recently, it was found that the isoalloxazine ring of the flavin cofactor can be tethered to the apoprotein via a dual covalent linkage [5]. All bi-covalent flavoenzymes characterized thus far share a VAO fold.

Covalent flavoenzymes are less widespread than their counterparts containing a dissociable flavin cofactor [6]. Several distinct types of covalent flavin binding have been recognized [6,7], but tethering of the 8α-methyl group of the flavin isoalloxazine ring to a histidine residue is most frequently observed. Enzymatic degradation to the level of the aminoacyl riboflavin moiety has been the conventional method to characterize the covalent protein–flavin link [8,9]. A more sophisticated approach involves the structural characterization of the isolated flavinylated peptide without submitting it to subsequent enzymatic degradation [10]. Alternatively, the mode of covalent flavinylation can be determined from the three-dimensional protein structure or predicted *in silico* [1]. The importance of the covalent link can be analyzed through the functional characterization of site-directed mutant proteins [11–14].

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[1] *Abbreviations used:* AldO, alditol oxidase; ADPS, alkyldihydroxy acetonephosphate synthase; AknOx, aclacinomycin oxidoreductase; BBE, *S*-reticuline oxidase (berberine bridge enzyme); ChitO, chitooligosaccharide oxidase; EUGO, eugenol oxidase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GALDH, L-galactono-1,4-lactone dehydrogenase; HDNO, 6-hydroxy-D-nicotine oxidase; MurB, *N*-acetylenolpyruvyl glucosamine reductase; PCMH, *p*-cresol methylhydroxylase; TbALO, *Trypanosoma brucei* arabinonolactone oxidase; TcGAL, *Trypanosoma cruzi* galactonolactone oxidase; VAO, vanillyl alcohol oxidase.

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Most evidence obtained thus far suggests that covalent flavinylation is a self-catalytic process, dependent on the primary folding of the polypeptide chain [7,15]. The ability of covalent incorporation of FAD by the apoprotein was first demonstrated for HDNO [16]. The formation of the flavin–protein linkage was promoted by the addition of small organic compounds, e.g. glycerol and glycerol-3-phosphate. For VAO it was shown that covalent flavinylation is not needed for effective binding of the cofactor [13]. This suggests that formation of the histidyl–FAD bond is preceded by non-covalent binding of the cofactor to the folded apoprotein (lock-and-key). It was also discovered that covalent binding of FAD to His422 in VAO requires the presence of an activating nucleophile, His61, in the FAD domain [17]. His422 replacements, prohibiting formation of the histidyl–FAD linkage, showed that covalent flavinylation increases the redox potential of VAO and thus its oxidation power [13]. For PCMH it was shown that binding of the cytochrome subunit is necessary for the generation of the 8α-*O*-tyrosyl FAD bond [18], and that the intermolecular subunit interactions induce small structural changes in the flavin-binding pocket that optimize the redox properties of the covalently bound FAD [19].

In this review, we report on the catalytic properties of some newly discovered VAO family members and their mode of flavin binding. Some new information about the aldonolactone oxidoreductase subfamily is presented as well.

New members of the VAO flavoprotein family

With the aid of the 3D-structure and sequence of VAO from *Penicillium simplicissimum* [20], about 50 different VAO homologs originally were identified [1]. Among these were several characterized flavoenzymes like 6-hydroxy-D-nicotine oxidase, *S*-reticuline oxidase, *p*-cresol methylhydroxylase, D-arabinono-1,4-lactone oxidase, D-lactate dehydrogenase, cholesterol oxidase, L-gulono-1,4-lactone oxidase, L-galactono-1,4-lactone dehydrogenase, hexose oxidase and alkyldihydroxyacetonephosphate synthase (ADPS) (Table 1). UDP-*N*-acetylenolpyruvyl glucosamine reductase (MurB), involved in the biosynthesis of the bacterial cell wall, was also identified as a family member, despite a low sequence similarity. During the last decade several new VAO family members have emerged. These include among others alditol oxidase, chitooligosaccharide oxidase, glucooligosaccharide oxidase, lactose oxidase, eugenol dehydrogenase, eugenol oxidase, cytokinin dehydrogenase, aclacinomycin oxidoreductase and Dbv29, a glycopeptide hexose oxidase. The mode of flavin binding of these enzymes is summarized in Table 1.

Histidyl-FAD enzymes

Analysis of VAO-type protein sequences has revealed that the target residue for covalent flavinylation is part of a conserved sequence region [1]. The linking histidine is typically found in the N-terminal part of the protein sequence downstream of three relatively small residues (e.g. xGGGHx sequence). Examples of such His-FAD containing proteins are HDNO [2,21], cholesterol oxidase [22], cytokinin dehydrogenase [23,24] and alditol oxidase [25]. The only exceptions to this rule are VAO and some close homologs (PCMH [26], and eugenol oxidase [27]). These enzymes contain a linking residue that is closer to the C-terminus. This suggests that during evolution, at least in two occasions the covalent flavin–protein linkage was formed at the two sequence regions (loops) that are in close contact with the dimethylbenzyl moiety of the flavin isoalloxazine ring in the VAO fold (Fig. 1). The N-terminal loop preferentially binds the flavin via the *N*1 atom of the histidine side chain (Table 1). In accordance with this, HDNO contains 8α-*N*1-histidyl FAD [19], instead of the originally identified 8α-*N*3-histidyl FAD [3]. Sequence alignments suggest that D-gluconolactone oxidase might also contain 8α-*N*1-histidyl FAD (Table 1). Contrarily, VAO contains an 8α-*N*3-histidyl FAD cofactor.

Recently discovered VAO members containing a histidyl-FAD include cytokinin dehydrogenase, alditol oxidase and eugenol oxidase. Cytokinin dehydrogenase (CKX) is involved in the enzymatic degradation of cytokinins (N^6-substituted purine derivatives), which play a major role in growth regulation in plants. The cytokinin dehydrogenase reaction involves the oxidation of the secondary amine group on the side-chain of the adenine ring resulting in cleavage of the side chain (Fig. 2A). The enzyme was initially classified as an oxidase but oxygen is only a poor substrate, a range of quinones have been identified as efficient electron acceptors [28]. Based on this, CKX has now been classified as a dehydrogenase. The crystal structure of maize CKX1 complexed with a cytokinin imine suggests that the product prevents the reduced flavin to react with molecular oxygen [23,29]. Typically, plant genomes contain a multitude of CKX genes.

Most VAO-type proteins containing a His-FAD cofactor have been found to act as an oxidase. This correlation has been used to discover novel covalent flavoprotein oxidases by genome database mining. An example of this approach of enzyme discovery is the recent identification of alditol oxidase (AldO) from *Streptomyces coelicolor* A3 [25]. AldO catalyzes the oxidation of the C1 hydroxyl group of preferably alditols into the corresponding aldehydes (Fig. 2B).

AldO is closely related to xylitol oxidase and sorbitol oxidase from other *Streptomyces* isolates [30,31] and all three oxidases display overlapping substrate specificities. AldO is most active with xylitol and sorbitol, which are converted into D-xylose and D-glucose, respectively [25]. The enzyme is an intracellular monomeric protein with a molecular mass of 45 kDa and could be expressed in impressive quantities (350 mg per liter culture) using *Escherichia coli* as expression host [25]. The recent elucidation of the crystal structure of AldO confirmed that the FAD

Table 1
(Predicted) FAD-binding mode of VAO family members

Enzyme	EC number	Flavin	Ref.
Histidyl-FAD enzymes			
Vanillyl-alcohol oxidase	1.1.3.38	8α-*N*3-histidyl FAD	[20,69]
Eugenol oxidase	1.1.3.x	8α-*N*3-histidyl FAD[a]	[27]
6-Hydroxy-D-nicotine oxidase	1.5.3.6	8α-*N*1-histidyl FAD[b]	[21]
Cholesterol oxidase	1.1.3.6	8α-*N*1-histidyl FAD	[22]
D-Arabinono-1,4-lactone oxidase	1.1.3.37	8α-*N*1-histidyl FAD	[70]
L-Gulono-1,4-lactone oxidase	1.1.3.8	8α-*N*1-histidyl FAD	[71]
D-Gluconolactone oxidase	1.1.3.x	8α-*N*3-histidyl FAD[c]	[72]
Alditol oxidase	1.1.3.x	8α-*N*1-histidyl FAD	[32]
Cytokinin dehydrogenase	1.5.99.12	8α-*N*1-histidyl FAD	[23,28]
Cysteinyl-histidyl FAD enzymes			
Glucooligosaccharide oxidase	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD	[5]
Chitooligosaccharide oxidase	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD[d]	[45]
Hexose oxidase	1.1.3.5	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD	[37]
S-Reticuline oxidase	1.21.3.3	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD	[38]
Aclacinomycin oxidoreductase	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FAD	[44]
Dbv29 (glycopeptide hexose oxidase)	1.1.3.x	6-*S*-cysteinyl, 8α-*N*1-histidyl FMN	[43]
Other covalent linkages			
p-Cresol methylhydroxylase	1.17.99.1	8α-*O*-tyrosyl FAD	[26]
Eugenol hydroxylase	1.17.99.x	8α-*O*-tyrosyl FAD[e]	[35]
Non-covalent flavoenzymes			
Alkyldihydroxyacetonephosphate synthase	2.5.1.26	FAD	[48]
D-Lactate dehydrogenase	1.1.1.28	FAD	[49]
L-Galactono-1,4-lactone dehydrogenase	1.3.2.3	FAD	[58]
UDP-*N*-acetylenolpyruvylglucosamine reductase	1.1.1.158	FAD	[50]

[a] Prediction from homology modeling with VAO [27].
[b] Originally identified as 8α-*N*3-histidyl FAD [3].
[c] Prediction from amino acid sequence gives 8α-*N*1-histidyl FAD.
[d] Prediction from homology modeling with GOOX [45].
[e] Prediction from amino acid sequence gives 8α-*O*-tyrosyl FAD.

cofactor is covalently tethered to a histidine residue (His46) via an 8α-*N*1-histidyl–FAD linkage [32]. The H46A mutant was expressed as the apoprotein in *E. coli* and found to be partially insoluble. This suggests that the covalent flavin–protein interaction is crucial for the structural integrity of AldO [25]. Crystal structures of AldO complexed with alditols have revealed the molecular basis for the selective oxidation reactions catalyzed by AldO. By an extensive hydrogen bond network, alditols are positioned with respect to the flavin cofactor in such a way that only one terminal hydroxyl group is oxidized. A detailed kinetic analysis has shown that AldO employs a ternary complex kinetic mechanism for the oxidation of xylitol ($K_m = 320\ \mu M$, $k_{cat} = 13\ s^{-1}$). Xylitol rapidly reduces the FAD cofactor ($k_{red} = 99\ s^{-1}$) upon which a binary complex is formed between reduced AldO and D-xylose. While complexed with the oxidation product, the flavin cofactor is able to utilize molecular oxygen as electron acceptor ($1.4 \times 10^5\ M^{-1}\ s^{-1}$). Besides xylitol and related alditols, AldO also converts other aliphatic alcohols. AldO shows considerable sequence and structural homology with cholesterol oxidase, but does not accept bulky hydrophobic substrates. This can be explained by the narrow-binding pocket near the flavin cofactor which determines the substrate specificity.

Another VAO-type histidyl-FAD containing oxidase that was recently discovered by utilizing genome sequence information is eugenol oxidase [27]. By analyzing the available bacterial genome sequences it was found that the proteome of the actinomycete *Rhodococcus* sp. RHA1 harbors a protein that shows 45% sequence identity to fungal VAO. The presence of a conserved histidine in its C-terminal region suggested that also in this protein, the FAD is covalently linked as in VAO. Indeed, heterologous expression in *E. coli* resulted in overproduction of flavinylated enzyme. Part of the recombinant protein was purified as the apoprotein. Addition of FAD to the purified protein resulted in fully flavinylated protein. This again confirms the autocatalytic mechanism of covalent FAD incorporation. Characterization of the enzyme revealed that it is most active as oxidase with eugenol ($K_m = 1.0\ \mu M$, $k_{cat} = 3.1\ s^{-1}$) and therefore it was named eugenol oxidase (EUGO) (Fig. 2C).

VAO and EUGO accept similar substrates, but with significantly different catalytic efficiencies. For example, while VAO is able to effectively hydroxylate 4-alkylphenols and to deaminate aromatic amines, EUGO is poorly active on these substrates. Another striking difference between VAO and EUGO is their oligomerization state: while VAO forms stable octamers, EUGO is a dimeric enzyme. Inspection of the VAO structure and sequence comparison

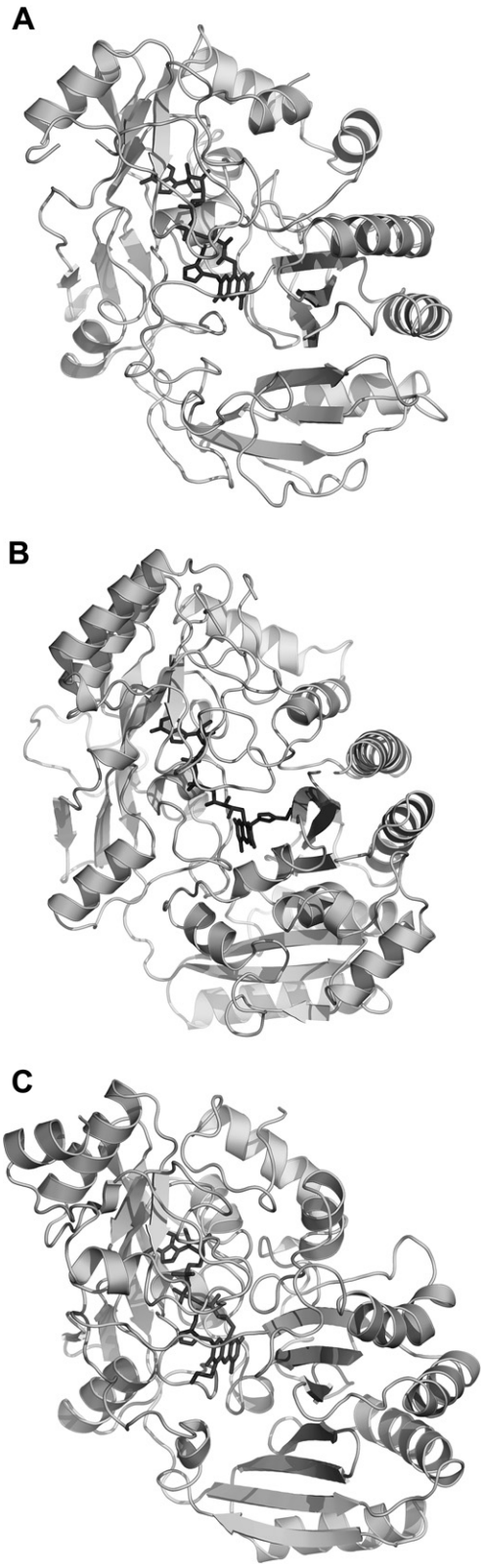

Fig. 1. Covalent flavin–protein linkages in the VAO fold. (A) Crystal structure of alditol oxidase with 8α-*N*1-histidyl FAD [32]. (B) 3D model of eugenol oxidase with 8α-*N*3-histidyl FAD [27]. (C) 3D model of chitooligosaccharide oxidase with 6-*S*-cysteinyl-8α-*N*1-histidyl FAD [45].

suggests that VAO has acquired a specific loop region that creates dimer–dimer interactions and thereby stabilizes octamers. This loop region is absent in the EUGO sequence. A nice feature, when compared with VAO, is the fact that EUGO is expressed in large quantities in *E. coli* facilitating production of this novel oxidase.

The substrate specificities of VAO and EUGO have some resemblance to that of the flavocytochromes PCMH [33] and eugenol dehydrogenase [34]. The latter enzyme is also referred to as eugenol hydroxylase [35]. Based on sequence comparisons [18,35,36] eugenol hydroxylase is predicted to contain 8α-*O*-tyrosyl FAD.

Histidyl–cysteinyl FAD enzymes

Only very recently it was discovered that in some flavoproteins the flavin cofactor is covalently attached to two amino acid residues. This bi-covalent linkage of FAD was first revealed in 2005 by elucidating the crystal structure of glucooligosaccharide oxidase [5]. Glucooligosaccharide oxidase preferably oxidizes cellooligosaccharides, that can reach the active site via an open carbohydrate-binding groove. In this fungal carbohydrate oxidase the FAD is tethered to Cys130 and His70 via, respectively, the 6- and 8α-position of the isoalloxazine ring (Fig. 3).

Based on this finding, it was recognized that also other established covalent flavoproteins may contain a bi-covalent FAD as cofactor. A dual covalent linkage was confirmed for hexose oxidase and *S*-reticuline oxidase (berberine bridge enzyme, BBE) [37,38] and probably is also the case for tetrahydrocannabinolic acid synthase, an enzyme involved in controlling marijuana psychoactivity [39]. A bi-covalent FAD cofactor is also predicted for two carbohydrate oxidases from sunflower and tobacco [40,41], and for the lactose oxidase from *Microdochium nivale* [42]. Apart from these known covalent flavoproteins, several new bi-covalent flavoproteins have been described recently: Dbv29, a glycopeptide hexose oxidase [43], aclacinomycin oxidoreductase (AknOx) [44] and chitooligosaccharide oxidase [45] (Table 1). All above-mentioned bi-covalent flavoproteins represent VAO-type enzymes, again showing that the VAO-fold is favorable for covalent protein–flavin interactions.

The reactions catalyzed by some bi-covalent flavoproteins demonstrate the unusual catalytic power of these enzymes. BBE for example, catalyzes the oxidative cyclization of the *N*-methyl group of *S*-reticuline in the benzophenanthridine alkaloid biosynthesis in plants [46]. This reaction involves two steps, the oxidation of the methylene iminium ion and the stereospecific ring closure forming the berberine bridge of *S*-scoulerine (Fig. 4A). Another unusual bi-covalent flavoprotein is AknOx, which catalyzes the last two steps in the biosynthesis of the polyketide antibiotic aclacinomycin in *Streptomyces* species. AknOx uses the same active site to catalyze these two consecutive FAD-dependent reactions but uses two distinct sets of catalytic residues to accomplish this. The first reaction involves the oxidation of the terminal sugar residue rhodi-

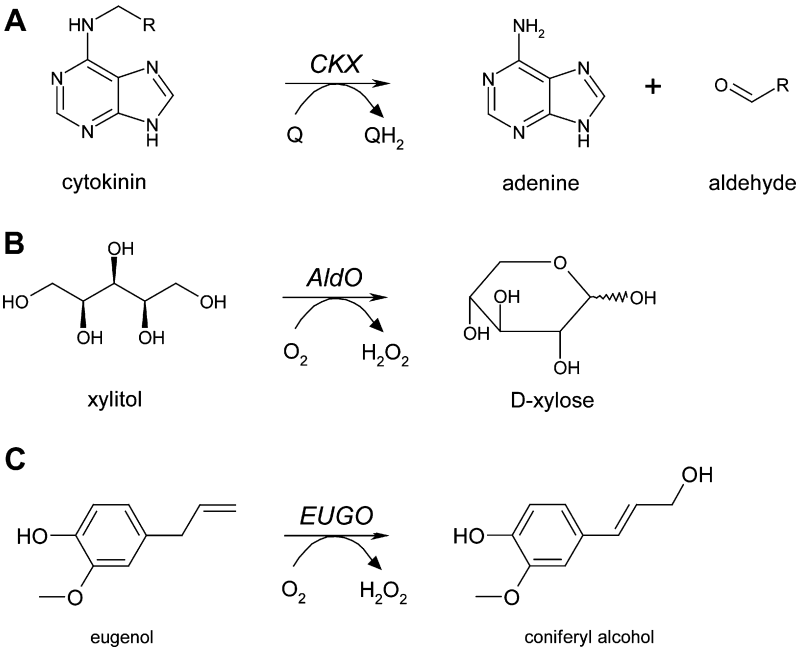

Fig. 2. Reactions catalyzed by the histidyl-FAD enzymes CKX (A), AldO (B) and EUGO (C).

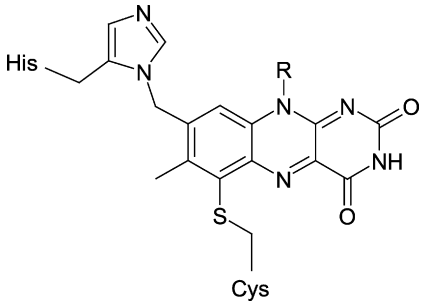

Fig. 3. Bi-covalent FAD linkage in glucooligosaccharide oxidase: 6-*S*-cysteinyl, 8α-*N*1-histidyl FAD [5].

nose into cinerulose A, which, in the second reaction, is converted to L-aculose by a desaturation step (Fig. 4B) [44].

The glycopeptide hexose oxidase Dbv29 is the first reported FMN-containing bi-covalent flavoprotein. Dbv29 catalyzes the four-electron oxidation of the *N*-acyl-glucosaminyl substituent to *N*-acylaminoglucuronic acid during the maturation of the glycopeptide A40926 antibiotic (Fig. 4C) [43].

As part of another genome database mining effort a chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum* was discovered and investigated [45]. In contrast to glucooligosaccharide oxidase, ChitO is highly active with *N*-acetylated mono- and oligosaccharides (Fig. 4D). Chitotetraose was found to be the best substrate ($K_m = 250$ μM, $k_{cat} = 6.3$ s^{-1}). ChitO also converts non-modified saccharides, e.g. glucose, cellobiose and lactose, but with a much lower catalytic efficiency. Based on the sequence similarity with glucooligosaccharide oxidase, a structural model of ChitO was constructed. This enabled the identification of residues that form the chitooligosaccharide-binding pocket. Based on this model, a specific glutamine residue was replaced by an arginine. As predicted, the engineered protein showed a somewhat lower affinity for *N*-acetylated substrates while the catalytic efficiency for non-acetylated carbohydrates improved ~20-fold [45]. This confirms that the respective residue is crucial for the recognition of specific classes of oligosaccharides. The availability of recombinant ChitO provides new opportunities to perform selective modifications of (oligo)saccharides.

So far, little is known about the biological significance of bi-covalent flavinylation. Single mutants of Dbv29 (H91A or C151A) showed comparable activity to wild type, while the double mutant retained only 10% activity [43]. From this it was argued that the bi-covalent linkage is required for a proper orientation of the flavin in the active site. The bi-covalent link in *S*-reticuline oxidase was also studied by mutagenic analysis [47]. The C166A variant showed an impaired flavin reduction rate of 370-fold and a decrease in redox potential, +53 mV vs. +132 mV for the wild-type enzyme. His104 protein variants could not be expressed in sufficient amounts for biochemical studies. From these data it was concluded that 6-*S*-cysteinylation of the flavin tunes the redox potential. In fact, the redox potential of wild-type *S*-reticuline oxidase is exceptionally high which is in line with the proposed rationale of covalent flavinylation, i.e. increasing the oxidative power of the respective enzyme.

Genomic data indicate that many new bi-covalent flavoenzymes still need to be discovered. Several of these hypothetical enzymes are found, for instance, in *Streptomyces* species [44] and in plants.

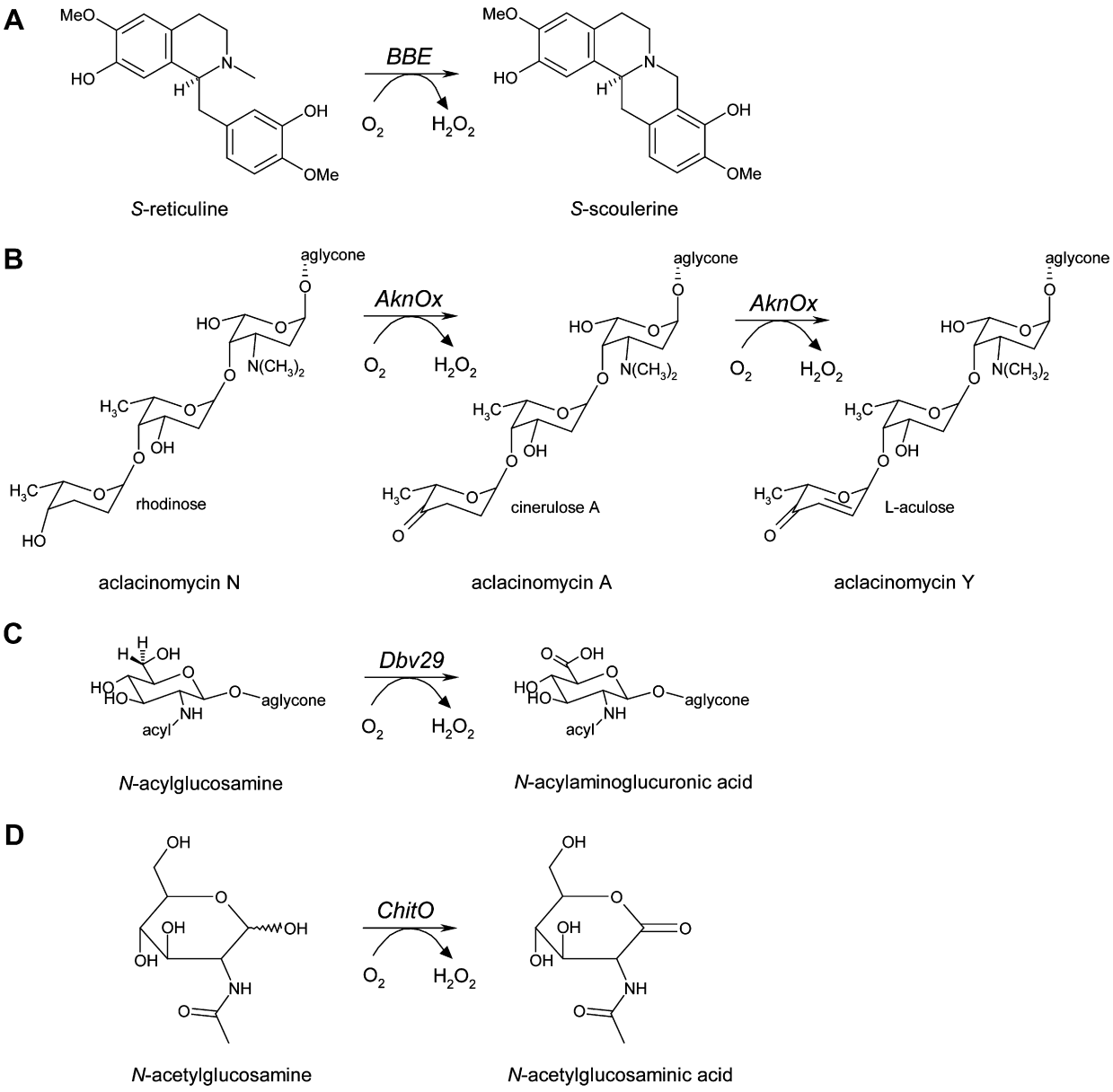

Fig. 4. Reactions catalyzed by the bi-covalent flavoproteins BBE (A), AknOx (B), Dbv29 (C) and ChitO (D).

Non-covalently bound FAD enzymes

Within the VAO family also members exist that bind their flavin cofactor non-covalently (Table 1). Generally the His residue in the conserved sequence region of the FAD-binding domain is lacking in these enzymes, and they do not react with molecular oxygen. For ADPS, D-lactate dehydrogenase, and MurB the crystal structures have been solved [48–50]. ADPS catalyzes ether bond formation in phospholipids, the constituents of eukaryotic cell membranes (Fig. 5A). The flavin cofactor is presumed to be involved in this non-redox reaction by trapping the dihydroxyacetonephosphate intermediate via covalent binding [48].

The peripheral membrane protein D-lactate dehydrogenase from *E. coli* catalyzes the oxidation of D-lactate to pyruvate, using quinone as electron acceptor. Besides the FAD-binding domain and the cap-domain, the enzyme also harbors a membrane-binding domain which interacts with the negatively charged phospholipid head groups of the membrane [49].

MurB is involved in peptidoglycan biosynthesis in bacteria, it catalyzes the reduction of enolpyruvyl-UDP-*N*-acetylglucosamine to UDP-*N*-acetylmuramic acid using NADPH as electron donor (Fig. 5B). The MurB structure consists of three domains, the typical VAO-type FAD-binding domain, and two additional domains comprising the substrate-binding site [50].

L-Galactono-1,4-lactone dehydrogenase (GALDH) is another VAO family member that binds the FAD in a non-covalent mode. This aldonolactone oxidoreductase is responsible for completing the biosynthesis of vitamin C in plants (Fig. 5C).

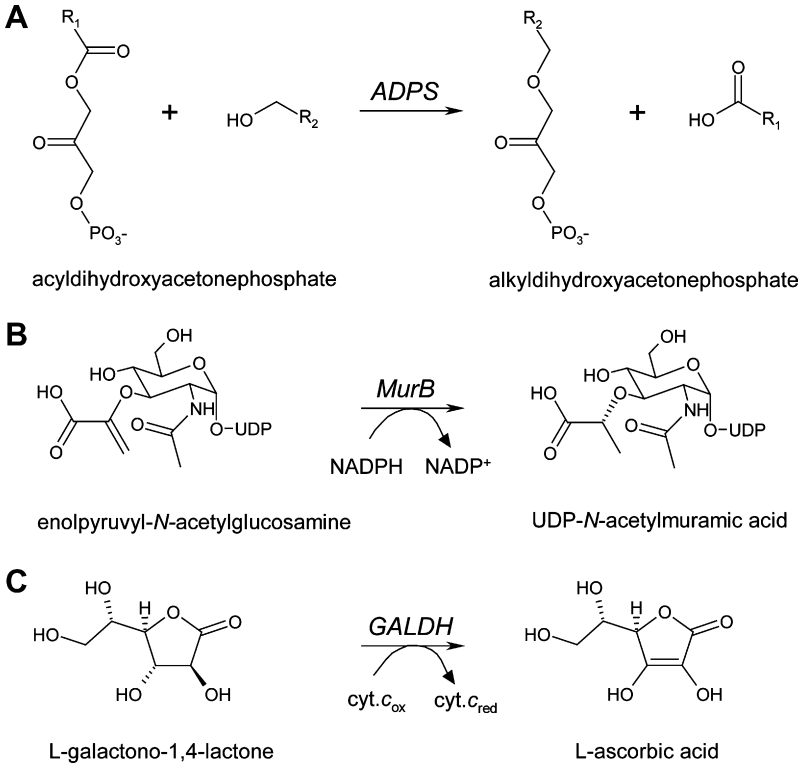

Fig. 5. Reactions catalyzed by the non-covalent VAO members ADPS (A), MurB (B) and GALDH (C).

GALDH has been isolated for the first time from cauliflower mitochondria by Mapson and Breslow in the 1950s [51]. Since then the enzyme was isolated from the mitochondria from a number of plants [52–55]. More recently, recombinant forms have become available for cauliflower, tobacco and *Arabidopsis thaliana* GALDH [58,56,57]. GALDH homologs in animals (L-gulono-1,4-lactone oxidase), yeast (D-arabinono-1,4-lactone oxidase) and fungi (D-gluconolactone oxidase), are involved in the synthesis of L-ascorbate or its analogs D-erythorbate and D-erythroascorbate [59–61]. In contrast to GALDH, these oxidases contain a covalently bound FAD (Table 1). Recently also a bacterial gulonolactone dehydrogenase [62] and two aldonolactone oxidases from trypanosome parasites [63,64] have been identified.

The aldonolactone oxidoreductases form a separate clade in the VAO family (Fig. 6). The plant GALDH enzymes share about 80–90% sequence identity but they have less than 25% of sequence identity with other aldonolactone oxidoreductases. The highest degree of sequence identity within this sub-family is found in the FAD-binding domain. No crystal structure is available for GALDH or its homologs and little is known about the nature of the active site and the catalytic mechanism. A recurrent feature within the group of aldonolactone oxidoreductases is the sensitivity towards thiol reactive compounds, suggesting the involvement of cysteine residues in catalysis [51,53,64–66].

GALDH is presumably localized in the mitochondrial intermembrane space associated with mitochondrial complex I [67] where it shuttles electrons into the electron transport chain via cytochrome *c* [68]. Mature GALDH from *A. thaliana* (AtGALDH) can be efficiently produced in *E. coli* when omitting the N-terminal mitochondrial targeting sequence [58]. The monomeric protein shows a high enantio-preference for L-galactono-1,4-lactone ($K_m = 0.17$ mM, $k_{cat} = 134\ s^{-1}$), though the L-gulono-1,4-lactone isomer is also oxidized at significant rate ($K_m = 13.1$ mM, $k_{cat} = 4\ s^{-1}$). Thus, a difference in orientation of the 3-hydroxyl group of the substrate is responsible for a 100-fold higher K_m and 2500-fold lower catalytic efficiency.

Most aldonolactone oxidoreductases use molecular oxygen as electron acceptor and contain a covalently bound histidyl-FAD (Table 1) [21,22,25]. Plant GALDH lacks this His-residue but contains a Leu instead. Replacement of Leu56 in AtGALDH by His did not result in covalent incorporation of FAD. Instead, FAD was more weakly bound in the mutant than in the wild-type protein [58]. Replacing Leu56 with Ala or Cys also yielded variants with loosely bound FAD. Variants L56I and L56F were similar to wild-type AtGALDH, and contained tightly bound FAD. The apo forms of the Leu56 variants could easily be reconstituted by the addition of FAD and their flavin absorption properties were nearly identical to that of the wild-type enzyme. The kinetic parameters of the Leu56 variants were determined and a rough correlation was found between the bulkiness of the residue at position 56 and the Michaelis-constant for L-galactono-1,4-lactone. The absence of a covalent flavin–protein link in the L56H variant might be explained by the absence of an acti-

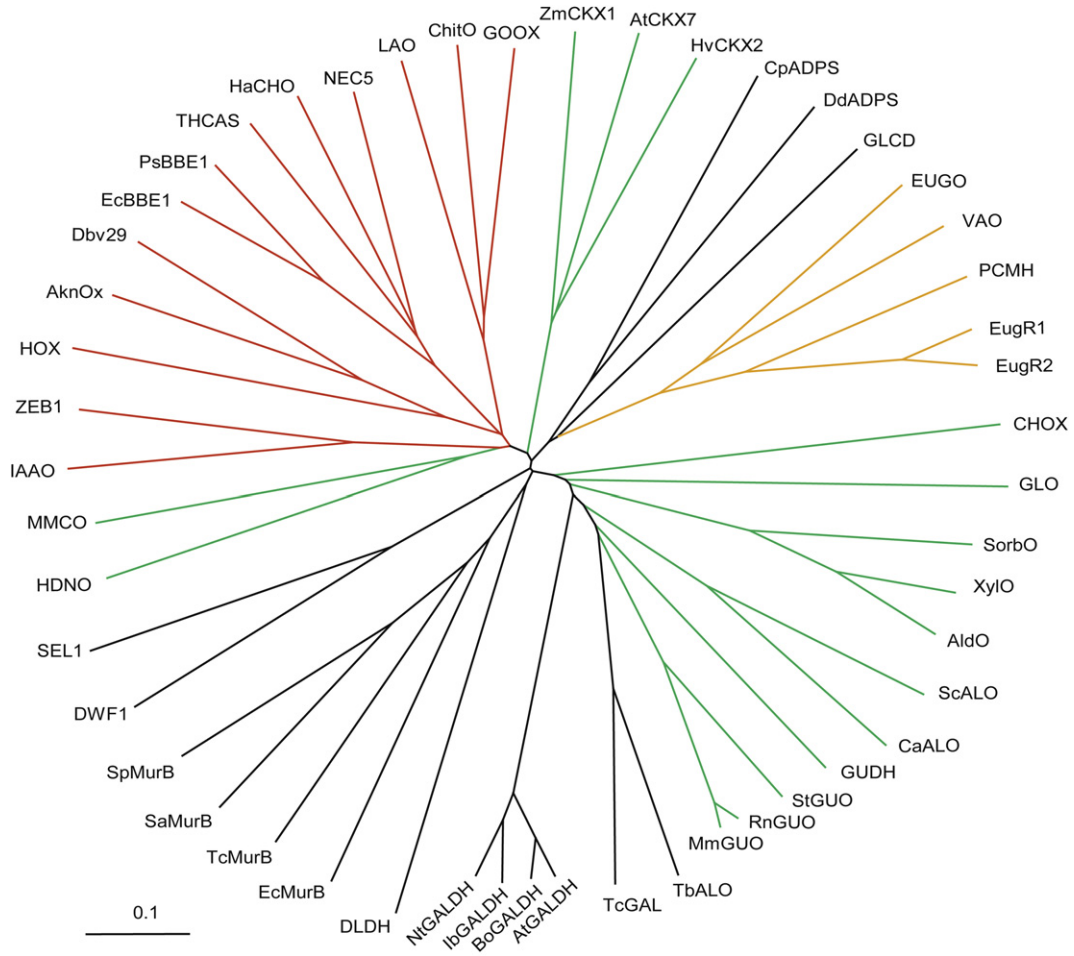

Fig. 6. Phylogenetic analysis of characterized VAO family members. The phylogenetic tree was constructed from a ClustalX multiple sequence alignment using the neighbor-joining method. (Predicted) covalent flavin linkages are highlighted. Linkages to the N-terminal loop are 8α-*N*1-histidyl in green and 6-*S*-cysteinyl-8α-*N*1-histidyl in red. Covalent linkages to the C-terminal loop are highlighted in orange. The accession numbers for the sequences used for phylogenetic analysis are: HDNO, P08159; AknOx, Q0PCD7; AldO, Q9ZBU1; *Dictyostelium discoideum* ADPS (DdADPS), O96759; *Cavia porcellus* ADPS (CpADPS), P97275; ChitO, XP_391174; cholesterol oxidase (CHOX), Q7SID9; *Zea mays* CKX 1 (ZmCKX1), Q9T0N8; *A. thaliana* CKX 7 (AtCKX7), Q9FUJ1; *Hordeum vulgare* CKX 2 (HvCKX2), Q8H6F6; *Saccharomyces cerevisiae* arabinonolactone oxidase (ScALO), P54783, *Candida albicans* ALO (CaALO), O93852; Dbv29, Q7WZ62; D-gluconolactone oxidase (GLO), Q671X8; D-lactate dehydrogenase (DLDH), P06149; DWARF1/DIMINUTO (DWF1), Q39085; Seladin-1 (24-dehydrocholesterol reductase, SEL1), Q15392; EUGO, Q9RDU1; *Pseudomonas* sp. HR199 eugenol hydroxylase flavoprotein subunit (EugR1), AAM21269; *Pseudomonas* sp. OPS1 eugenol hydroxylase flavoprotein subunit (EugR2), Q0SBK1; glucooligosaccharide oxidase (GOOX), Q6PW77; glycolate oxidase subunit D (GLCD), P0AEP9; gulonolactone dehydrogenase (GUDH), O06804; hexose oxidase (HOX), P93762; isoamyl alcohol oxidase (IAAO), Q9HGH9; lactose oxidase (LAO), CAI94231-2; AtGALDH, Q8GY16; *Brassica oleracea* GALDH (BoGALDH), O47881; *Ipomoea batatas* GALDH (IbGALDH), Q9ZWJ1; *Nicotiana tabacum* GALDH (NtGALDH), Q9SLW6; *Rattus norvegicus* gulonolactone oxidase (RnGUO), P10867; *Mus musculus* GUO (MmGUO), NP_848862; *Scyliorhinus torazame* GUO (StGUO), Q90YK3; mitomycin radical oxidase (MMCO), P43485; PCMH flavoprotein subunit, P09788; polyketide synthase (ZEB1), Q2VLJ1; *Eschscholzia californica* BBE (EcBBE1), P30986; *Papaver somniferum* BBE (PsBBE1), P93479; sorbitol oxidase (SorbO), P97011; sunflower carbohydrate oxidase (HaCHO), Q8SA59; tetrahydrocannabinolic acid synthase (THCAS), Q8GTB6; tobacco glucose oxidase (NEC5), Q84N20; TbALO, Q57ZU1; TcGAL, Q4DPZ5; *E. coli* MurB (EcMurB), P08373; *Staphylococcus aureus* MurB (SaMurB), P61431; *Streptococcus pneumoniae* MurB (SpMurB), P65466; *Thermus cadophilus* MurB (TcMurB), Q5SJC8; VAO, P56216; xylitol oxidase (XylO), Q9KX73. The bar indicates 10% divergence.

vating base similar to His61 in VAO [17], which is required for nucleophilic attack. The presence of such an activating residue is not easily predicted due to the lack of structural information for the aldonolactone oxidoreductase subfamily.

The mechanism of L-ascorbate production by AtGALDH involves two half-reactions. In the reductive half-reaction, the oxidized flavin cofactor is reduced to the hydroquinone state by the L-galactono-1,4-lactone substrate. The two-electron reduced enzyme is then re-oxidized in the oxidative half-reaction by cytochrome *c*. This oxidative half-reaction involves two one-electron steps and the transient formation of the red anionic flavin semiquinone [58]. The catalytic mechanism of AtGALDH was studied with the stopped-flow technique. The reduction of the flavin by L-galactono-1,4-lactone appears to be the rate limiting step in the catalytic cycle ($k_{red} = 750\ s^{-1}$). The re-oxidation by cytochrome *c* in the oxidative half-reaction

occurs relatively fast, with a bimolecular rate constant of $3.6 \times 10^6\ M^{-1}\ s^{-1}$, four orders of magnitude faster than the re-oxidation by molecular oxygen ($k_{ox} = 4.4 \times 10^2\ M^{-1}\ s^{-1}$).

Trypanosome parasites also contain an aldonolactone oxidoreductase that harbors a non-covalently bound flavin cofactor and uses cytochrome *c* as electron acceptor. The arabinonolactone oxidase from *Trypanosoma brucei* (TbALO) has a preference for substrates in which the C2 and C3 hydroxyl groups are arranged in the *trans*-configuration, besides L-galactono-1,4-lactone ($K_m = 154\ \mu M$, $k_{cat} = 21\ s^{-1}$) it can also oxidize D-arabinono-1,4-lactone ($K_m = 55\ \mu M$, $k_{cat} = 27\ s^{-1}$) [63]. Galactonolactone oxidase from *Trypanosoma cruzi* (TcGAL) also utilizes both L-galactono-1,4-lactone ($K_m = 161\ \mu M$, $k_{cat} = 673\ s^{-1}$) and D-arabinono-1,4-lactone ($K_m = 285\ \mu M$, $k_{cat} = 649\ s^{-1}$) [64]. Considering that L-galactono-1,4-lactone is the presumed physiological substrate [63,64] and that cytochrome *c* is employed as electron acceptor, TbALO and TcGAL should be re-named as galactonolactone dehydrogenases.

Both TcGAL and TbALO are proposed to possess a non-covalently bound FMN as cofactor [64]. This would be the first members (together with Dbv29 [43]) of the VAO-family that contain FMN rather than FAD as the prosthetic group. However, comparing the sequences of TcGAL and TbALO with other VAO family members reveals that the residues that normally interact with the pyrophosphate and adenine moiety of the FAD cofactor [1] are conserved in the trypanosomal enzymes. Therefore, more research is needed to confirm that indeed FMN is bound to these enzymes rather than FAD. Both TbALO and TcGAL lack the His residue in the FAD-binding domain involved in covalent binding of the cofactor, but contain a Lys residue instead [63,64]. Replacement of Lys55 of TcGAL by His or Leu yields mutants that are isolated as apoproteins [64]. It is not clear whether the isolated apoproteins can be reconstituted to the holo form, as is the case for the AtGALDH Leu56 mutants. Interestingly, a lysine residue in the C-terminal HWXK motif, conserved in all aldonolactone oxidoreductases and some related oxidases, is thought to be involved in catalysis of TcGAL. Replacement of this Lys by Gly (K450G) renders the protein completely inactive, though flavin is still bound [64].

Although there are no structural rules that enable prediction of whether or how a flavoenzyme reacts with oxygen [29], it is evident that structural data of the aldonolactone oxidoreductase subfamily would be beneficial in future research towards the elucidation of the molecular determinants of (covalent) flavin binding and oxygen reactivity.

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