Review

Elvira Romero and Giovanni Gadda*

Alcohol oxidation by flavoenzymes

Abstract: This review article describes the occurrence, general properties, and substrate specificity of the flavoenzymes belonging to the glucose-methanol-choline oxidoreductase superfamily and the L-α-hydroxyacid dehydrogenase family. Most of these enzymes catalyze the oxidations of hydroxyl groups, yielding carbonyl moieties. Over the years, carbanion, hydride transfer, and radical mechanisms have been discussed for these enzymes, and the main experimental evidences supporting these mechanisms are presented here. Regardless of the chemical nature of the organic substrate (i.e., activated and non-activated alcohols), a hydride transfer mechanism appears to be the most plausible for the flavoenzymes acting on CH-OH groups. The reaction of most of these enzymes likely starts with proton abstraction from the substrate hydroxyl group by a conserved active site histidine. Among the different approaches carried out to determine the chemical mechanisms with physiological substrates, primary substrate and solvent deuterium kinetic isotope effect studies have provided the most unambiguous evidences. It is expected that the numerous studies reported for these enzymes over the years will be instrumental in devising efficient industrial biocatalysts and drugs.

*Corresponding author: Giovanni Gadda, Department of Chemistry, Georgia State University, Atlanta, GA 30302-3965, USA; Department of Biology, Georgia State University, Atlanta, GA 30302-3965, USA; Center for Biotechnology and Drug Design, Georgia State University, Atlanta, GA 30302-3965, USA; and Center for Diagnostics and Therapeutics, Georgia State University, Atlanta, GA 30302-3965, USA, e-mail: ggadda@gsu.edu
Elvira Romero: Department of Chemistry, Georgia State University, Atlanta, GA 30302-3965, USA

List of abbreviations: AAO, aryl-alcohol oxidase; AO, alcohol oxidase; CDH, cellobiose dehydrogenase; CHD, choline dehydrogenase; CHO, choline oxidase; cyt c, cytochrome c; FAD, flavin adenine dinucleotide; FCB2, flavocytochrome b_2; FMN, flavin mononucleotide; GMC, glucose-methanol-choline; GO, glucose 1-oxidase; GOX, glycolate oxidase; HMFO, 5-hydroxymethylfurfural oxidase; HQ, hydroquinone; KIE, kinetic isotope effect; LCHAO, long-chain L-α-hydroxyacid oxidase; LHAD, L-α-hydroxyacid dehydrogenase; LMO, L-lactate 2-monooxygenase; LOX, lactate oxidase; MDH, L-mandelate dehydrogenase; NMR, nuclear magnetic resonance; PDH, pyranose dehydrogenase; PNOX, pyridoxine 4-oxidase; P2O, pyranose 2-oxidase; Q, quinone; QM/MM, quantum mechanics/molecular mechanics.

Introduction

Many flavoenzymes oxidize hydroxyl to carbonyl groups. These reactions involve the cleavage of both an OH and a CH bond, with the transfer of a hydride ion (i.e., two electrons and one proton) from the organic substrate to the flavin. After flavin reduction, the oxidized substrate can then dissociate from the enzyme active site either before or after flavin oxidation. The substrates harboring the hydroxyl group can be classified on the basis of their chemical nature in activated and non-activated substrates (1). Activated alcohols are those containing a carboxylate on the α-carbon atom that is dehydrogenated; non-activated alcohols lack such an electron-withdrawing group.

This review article will focus on flavin-dependent enzymes that oxidize alcohols belonging to either the glucose-methanol-choline (GMC) oxidoreductase superfamily or the L-α-hydroxyacid dehydrogenase (LHAD) family. Enzymes from these groups that catalyze other chemistries, such as hydroxynitrile lyase and formate oxidase, are not presented herein. Flavoenzymes belonging to the vanillyl alcohol oxidase superfamily have been recently discussed in Ref. (2) and will not be covered here.

GMC oxidoreductases contain either covalently or non-covalently bound FAD; present the p-hydroxybenzoate hydroxylase fold (Figure 1A–C) (3); and act on
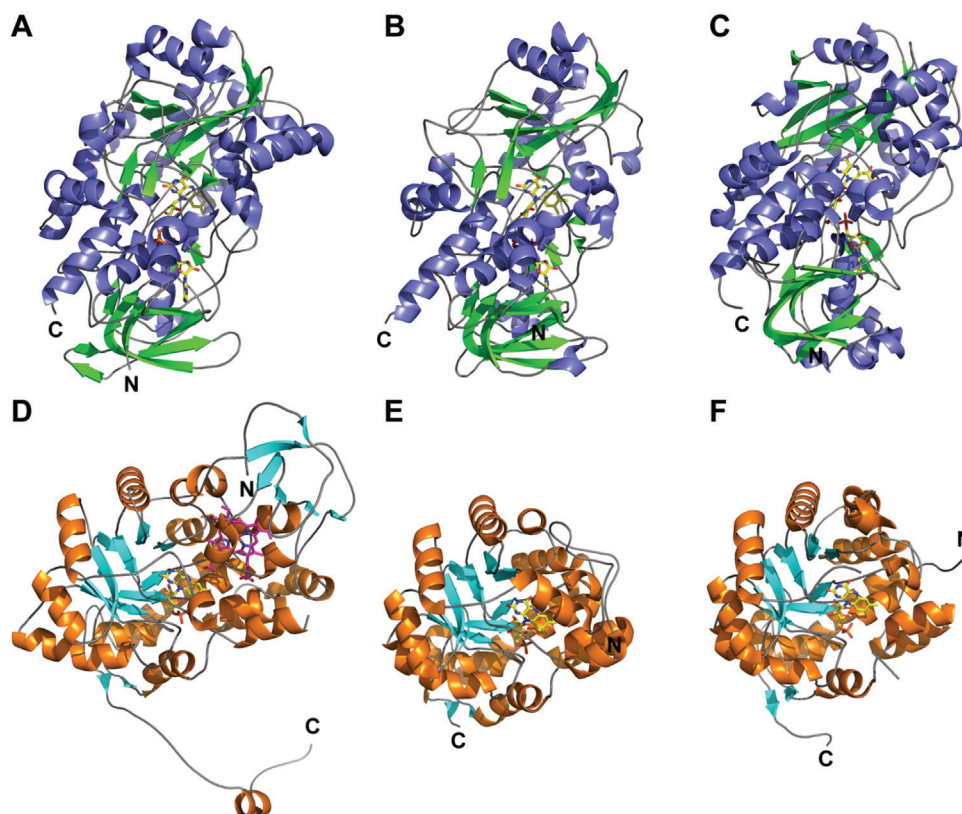
Figure 1 Structure of representative members of the GMC oxidoreductase superfamily (A–C) and the LHAD family (D–F). *Pe*AAO (A), *Ag*CHO (B), flavin-containing domain of *Pc*CDH (C), *Sc*FCB2 (D), *Hs*GOX (E), and *Av*LOX (F) (PDB codes: 3FIM, 4MJW, 1KDG, 1FCB, 2RDU, and 2NLI, respectively). The flavin (A–F) and the heme b_2 (D) are shown in stick representation (yellow and pink carbons, respectively).

hydroxyl groups of non-activated alcohols, carbohydrates, or sterols. LHAD enzymes contain non-covalently bound FMN; present the $\beta_8\alpha_8$-barrel fold, also known as TIM-barrel (Figure 1D–F) (4); and use L-α-hydroxyacids as substrates. Various GMC and LHAD enzymes are able to oxidize hydrated aldehydes to their corresponding carboxylic acids. Biochemical and structural information is available for many members of both classes, including site-directed mutagenesis and computational studies. Renewed interest has focused on the application of flavoprotein oxidases as biocatalysts for the synthesis of pharmaceutical compounds, as well as in biosensors, biofuel cells, and the food industry, which has been recently covered in Ref. (5).

In this review article, we present the general properties and substrate specificity of the enzymes acting on simple alcohols, carbohydrates, L-α-hydroxyacids, sterols, and the alcohol oxidases that oxidize aldehydes as well. We summarize the findings on the mechanisms for flavin reduction obtained with physiological substrates and non-physiological substrates, as well the most recent results on the mechanism for flavin oxidation. We provide

the functional roles established through site-directed mutagenesis and biochemical studies for a histidine conserved in the active site of all GMC and LHAD enzymes.

General properties and substrate specificity

A list of flavoenzymes that oxidize alcohols within the GMC oxidoreductase superfamily and the LHAD family is presented in Table 1. Although these enzymes perform the same type of chemistry, i.e., alcohol oxidation, they differ in their substrate specificity.

Flavoenzymes active on simple alcohols

Alcohol oxidase

Alcohol oxidase (AO; EC 1.1.3.13) is also widely known as methanol oxidase. Most of the AOs are homooctameric,

Table 1 GMC and LHAD enzymes that oxidize alcohols.

Enzyme	Name	PDB code[a]	Source[a]	Prosthetic group and attachment	Electron-donor substrate	Refs.
(A) GMC oxidoreductase superfamily						
Alcohol oxidase	AO		*Candida boidinii*	FAD, xylo-FAD	Alcohol	(91)
Aryl-alcohol oxidase	AAO	3FIM	*Pleurotus eryngii*	FAD	Alcohol	(8)
Cellobiose dehydrogenase	CDH	1KDG	*Phanerochaete chrysosporium*	FAD, 6-OH FAD, heme *b*	Carbohydrate	(21)
Cholesterol oxidase	CO	1B4V	*Streptomyces* sp.	FAD	Sterol	(63)
Choline dehydrogenase	CHD		*Halomonas elongata*		Alcohol	(17)
Choline oxidase	CHO	4MJW	*Arthrobacter globiformis*	8α-N3-histidyl FAD	Alcohol	(16)
5-Hydroxymethylfurfural oxidase	HMFO		*Methylovorus* sp.	FAD	Alcohol	(11)
Glucose oxidase	GO	1CF3	*Aspergillus niger*	FAD	Carbohydrate	(27)
Pyranose dehydrogenase	PDH	4H7U	*Agaricus meleagris*	8α-N3-histidyl FAD	Carbohydrate	(29)
Pyranose 2-oxidase	P2O	1TT0	*Trametes multicolor*	8α-N3-histidyl FAD	Carbohydrate	(131)
Pyridoxine 4-oxidase	PNOX	4HA6	*Mesorhizobium loti*	FAD	Alcohol	(18)
(B) LHAD family						
Flavocytochrome b_2	FCB2	1FCB	*Saccharomyces cerevisiae*	FMN, heme b_2	L-α-Hydroxyacid	(39)
Glycolate oxidase	GOX	2RDU	*Homo sapiens*	FMN	L-α-Hydroxyacid	(4)
Lactate oxidase	LOX	2NLI	*Aerococcus viridans*	FMN	L-α-Hydroxyacid	(54)
L-Lactate 2-monooxygenase	LMO		*Mycobacterium smegmatis*	FMN	L-α-Hydroxyacid	(132)
Long chain L-2-hydroxyacid oxidase	LCHAO	1TB3	*Rattus norvegicus*	FMN	L-α-Hydroxyacid	(57)
Mandelate dehydrogenase	MDH	1HUV	*Pseudomonas putida*	FMN	L-α-Hydroxyacid	(60)

[a]Only the main species and PDB codes referred in the text have been included.

with a non-covalently bound FAD molecule per monomer (6). The crystal structure of an AO is not available at this time; however, the analysis of the AO sequence and molecular model clearly showed that this enzyme belongs to the GMC oxidoreductase superfamily (7). The AO has been found in yeasts and filamentous fungi such as *Candida boidinii*, *Hansenula polymorpha*, and *Phanerochaete chrysosporium*. In all cases, the preferred substrate of AO is methanol, which is oxidized to formaldehyde and hydrogen peroxide (Scheme 1) (6). Other short primary
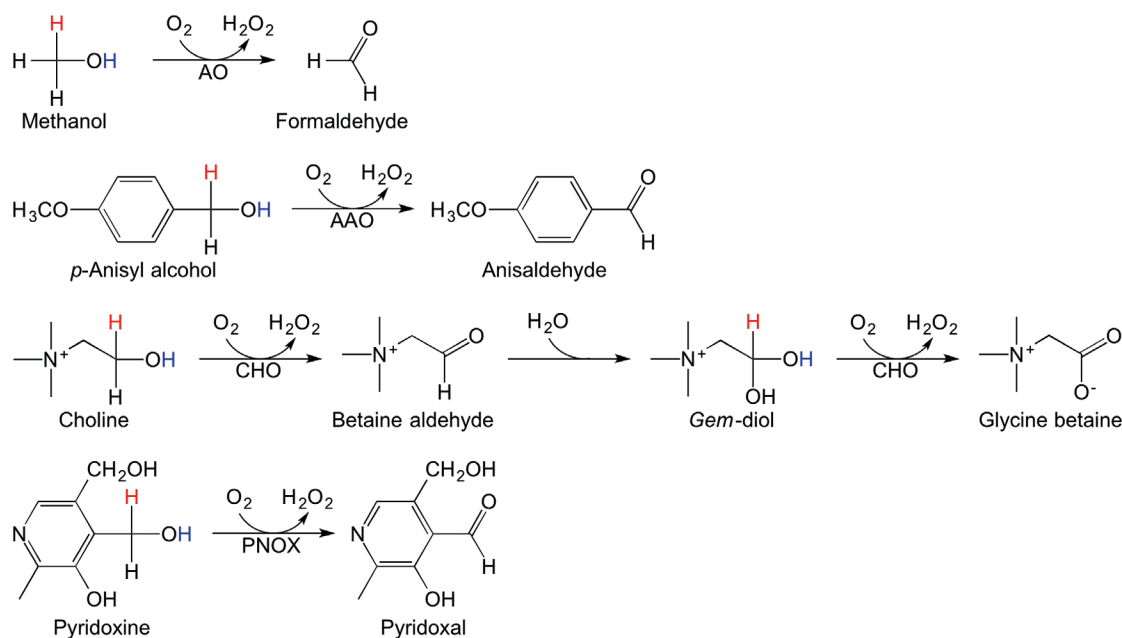
Scheme 1 Reactions catalyzed by GMC oxidoreductases acting on simple alcohols.

aliphatic alcohols with two to four carbon atoms are also substrates of the AOs (6).

Aryl-alcohol oxidase

Aryl-alcohol oxidase (AAO; EC 1.1.3.7) has been described in several fungi. The AAO from *Pleurotus eryngii* (*Pe*AAO) is the best characterized, and its crystal structure has been solved (8). These oxidases are monomeric proteins containing non-covalently bound FAD, and catalyze the oxidation of benzyl and cinnamyl alcohols and primary polyunsaturated aliphatic alcohols (Scheme 1). These reactions involve the formation of hydrogen peroxide at the expense of dioxygen. Another AAO, from *Bjerkandera adusta*, has been characterized (9). *Pe*AAO and *B. adusta* AAO exhibit different substrate selectivities (9, 10). Recently, a 5-hydroxymethylfurfural oxidase from *Methylovorus* sp. strain MP688 (*M*HMFO) has been characterized (11). This GMC enzyme efficiently oxidizes furan, benzyl, and cinnamyl alcohols and primary polyunsaturated aliphatic alcohols, including phenolic and non-phenolic compounds (11).

Choline oxidase

Choline oxidase (CHO; EC 1.1.3.17) has been described in the bacteria *Arthrobacter globiformis*, *Arthrobacter pascens*, *Arthrobacter nicotianae*, and *Alcaligenes* sp., as well as in the fungi *Cylindrocarpon didymum* M-1 and *Aspergillus fumigatus* [(12–14) and references therein]. The *A. globiformis* CHO (*Ag*CHO) has been extensively characterized (14), and its crystal structure solved in the presence of various ligands including the product of reaction (15, 16). This homodimeric enzyme contains a covalently bound FAD molecule per monomer, which is linked through the 8α-methyl group of the isoalloxazine ring to the N3 atom of His99. CHO catalyzes the four-electron oxidation of choline to glycine betaine through a betaine aldehyde intermediate (Scheme 1). The crystal structure of *Ag*CHO in complex with glycine betaine shows Glu312 at 4 Å from the positively charged trimethylammonium moiety of the ligand (16). The K_d value of the Glu312Gln mutant was ~500-fold higher than that of the wild-type *Ag*CHO, suggesting that a negative charge at the 312-position is important for choline binding through electrostatic interactions (15). Besides CHO, the membrane-associated choline dehydrogenase (CHD; EC 1.1.99.1) oxidizes both choline and betaine aldehyde (17). However, there is paucity of information about the biochemistry of CHD because of its instability after extraction from the membrane (17).

Pyridoxine 4-oxidase

Pyridoxine 4-oxidase (PNOX; EC 1.1.3.12) has been found in the bacteria *Pseudomonas* sp. MA-1, *Microbacterium luteolum*, and *Mesorhizobium loti* [(18) and references therein]. Recently, the crystal structure of *M. loti* PNOX (*Ml*PNOX) has been solved in the absence and presence of a ligand (18). This monomeric enzyme contains non-covalent FAD and catalyzes the oxidation of pyridoxine to pyridoxal (alcohol and aldehyde forms of vitamin B6, respectively; Scheme 1), whereas 2,6-dihydroxypyridine and 3-pyridinemethanol are poor substrates (19). Studies *in vitro* showed that dioxygen and 2,6-dichloroindophenol are good electron acceptors of *Ml*PNOX (19).

Flavoenzymes active on carbohydrates

Cellobiose dehydrogenase

Cellobiose dehydrogenases (CDH; EC 1.1.99.18) are secreted by fungi and have been grouped in different classes according to their sequence and catalytic properties (20). Most known CDHs are monomers, consisting of an N-terminal heme-containing domain (*b*-type) and a C-terminal flavin-containing domain, which are connected by 20–30 amino acids (21). The flavin is non-covalently associated with the protein. The crystal structure of the flavin-containing domain of *P. chrysosporium* CDH (*Pc*CDH) has been reported in free form and in complex with an inhibitor (21, 22). 6-Hydroxy FAD was found in the crystallized protein (21, 22).

Cellobiose is the preferred substrate of CDHs based on k_{cat}/K_m values (23). This disaccharide is oxidized by CHD at the C1-position to yield cellobiono-1,5-lactone (Scheme 2). Longer cellodextrins and lactose are also oxidized by CDHs to the corresponding lactones, whereas monosaccharides are poor or no substrates for these enzymes (23). Molecular modeling studies on *Pc*CDH suggested that the Glu279 forms a hydrogen bond with the second and third hydroxyl groups of the non-reducing end sugar moiety of the substrate (24). Accordingly, the Glu279Gln variant presented a 7-fold higher $K_{m(cellobiose)}$ value than the wild-type enzyme (24). In addition, this variant was essentially inactive toward lactose. CDHs reduce *in vitro* a wide variety of one- and two-electron acceptors, including quinones, metal ions, organic dyes, and cytochrome *c* (cyt *c*) (25). These enzymes are also able to transfer electrons to copper-dependent polysaccharide monooxygenases (26).
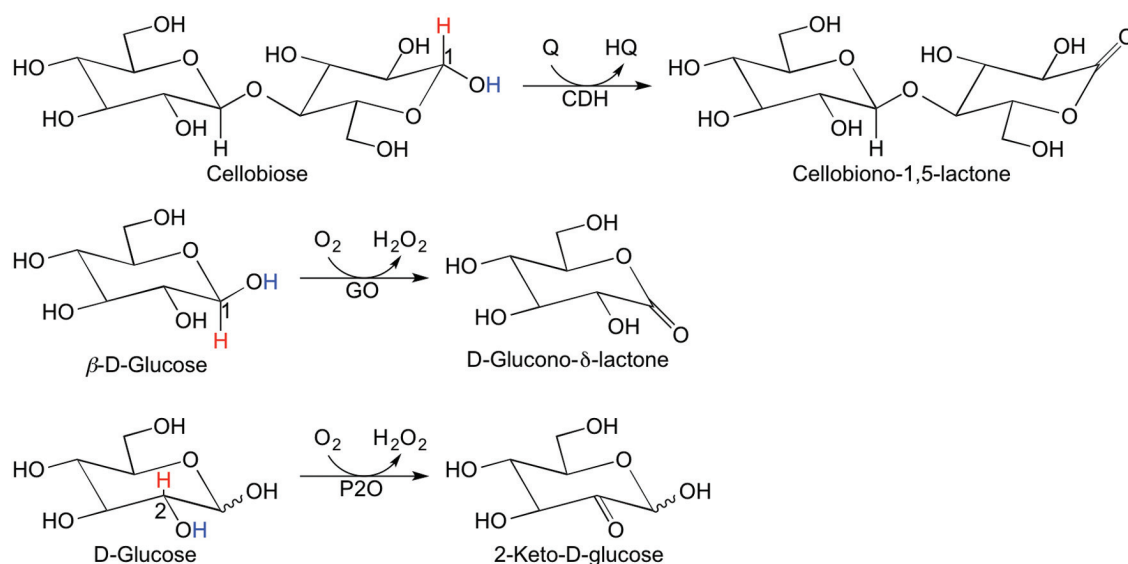
Scheme 2   Reactions catalyzed by GMC oxidoreductases acting on carbohydrates.

Glucose 1-oxidase

Glucose 1-oxidase (GO; EC 1.1.3.4) is found in fungi and insects. The GO from the fungi *Aspergillus niger* (*An*GO) and *Penicillium amagasakiense* are homodimers with non-covalent FAD, and their crystal structures were solved more than two decades ago (27). They catalyze the oxidation of β-D-glucose, exclusively at the C1-position, to form β-D-glucono-1,5-lactone (Scheme 2). Although GOs exhibit a marked preference for glucose, they have low activity on other sugars and glucose analogs such as 2-deoxy-D-glucose and glyceraldehyde (28). Besides dioxygen, quinones and one-electron acceptors are good oxidizing substrates for GOs *in vitro* (28).

Pyranose dehydrogenase

Pyranose dehydrogenases (PDH; EC 1.1.99.29) are monomeric fungal enzymes. The recently reported crystal structure of *Agaricus meleagris* PDH (*Am*PDH) reveals that the FAD is covalently linked through the 8α-methyl group of the isoalloxazine ring to the N3 atom of His103 (29). This enzyme is able to oxidize a wide variety of mono- and oligosaccharides, including L-arabinose, D-glucose, D-galactose, D-xylose, cellobiose, maltose, and maltotriose (29). Depending on the substrate and the enzyme source, PDH performs either monooxidations (at C1, C2, or C3) or dioxidations (at C1/3, C2/3, or C3/4) (30). With D-glucose, the oxidation of the hydroxyl moiety at the C2-position

is considerably faster than that at the C3-position. The electron acceptors of PDH *in vitro* are complexed metal ions and substituted quinones (e.g., ferricenium ion and 2,6-dichloroindophenol).
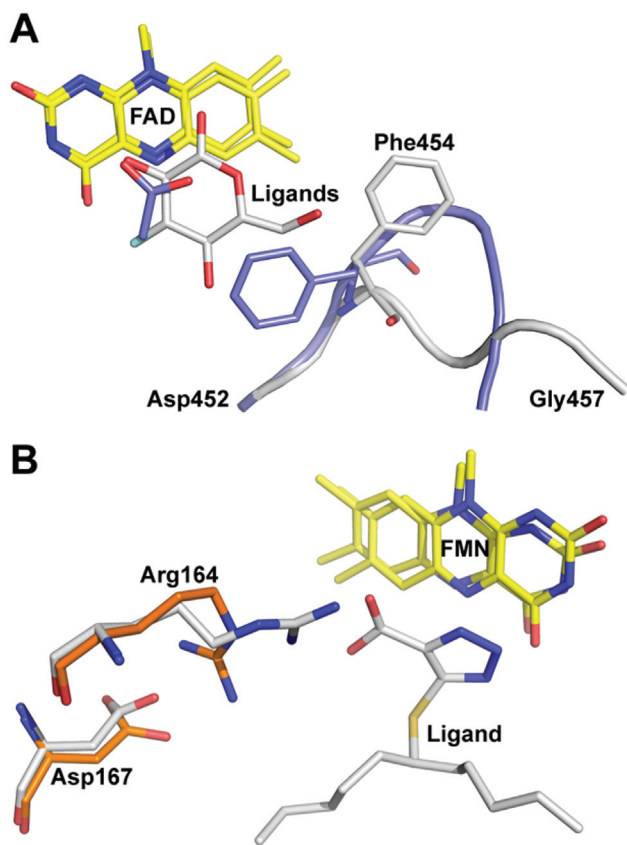
Pyranose 2-oxidase

Pyranose 2-oxidases (P2O; EC 1.1.3.10) are homotetrameric enzymes found in several fungi. The crystal structure of *Trametes multicolor* P2O (*Tm*P2O) has been determined in the absence and presence of various ligands (31), in addition to that of *Peniophora* sp. P2O without ligands (32). These P2Os contain 8α-N3-histidyl FAD and involve His167 in the binding of this cofactor. They catalyze the C2-oxidation of various aldopyranoses yielding 2-keto-aldoses. D-Glucose is the preferred substrate of P2O based on k_{cat}/K_m values (Scheme 2). Other carbohydrates such as D-xylose, D-galactose, and L-arabinose are also oxidized by P2O. In contrast to GO, P2O is not specific for the β-anomer of D-glucose (33), being able to oxidize substrates at the C3-position of the glycosyl moiety if there is no hydroxyl group at C2-position (34). The reduced P2O is efficiently reoxidized *in vitro* by dioxygen, as well as by various one- and two-electron acceptors such as quinones, organic radicals, and complexed metal ions (35). In *Tm*P2O, the loop segment containing the residues 452–457 is located over the active site entrance. A comparison of the wild-type *Tm*P2O crystal structure in complex with acetate and that of the His167Ala *Tm*P2O variant in complex with 3-fluoro-3-deoxy-D-glucose

suggested that the position of this loop may change to allow the substrate to access the active site, with relevance to substrate specificity (Figure 2A) (31).

Flavoenzymes active on L-α-hydroxyacids

Flavocytochrome b_2

Flavocytochrome b_2 (L-lactate dehydrogenase; FCB2; EC 1.1.2.3) is a mitochondrial enzyme that catalyzes the oxidative dehydrogenation of L-lactate to pyruvate in *Saccharomyces cerevisiae*, *Hansenula anomala*, and other yeasts (Scheme 3) (36). Although less efficiently,
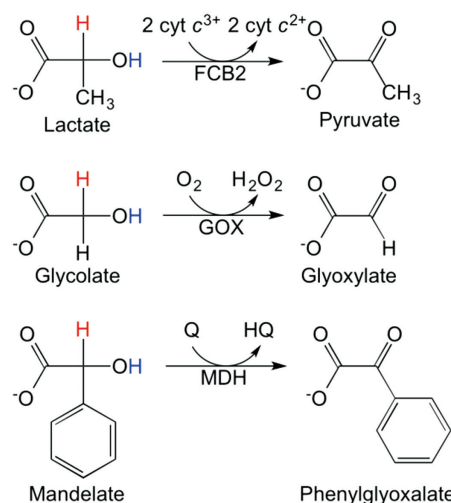
FCB2 oxidizes other L-α-hydroxyacids such as L-α-hydroxybutyrate (37, 38). Several crystal structures of *S. cerevisiae* FCB2 (*Sc*FCB2) have been reported (39). This homotetrameric enzyme carries a heme b_2 and an FMN per subunit (in the N- and C-terminal domains, respectively). Two electrons are transferred from L-lactate to FMN, and then they are transferred one at a time from the FMN to the heme b_2. The physiological electron acceptor of FCB2 is cyt c (40). The crystal structure of *Sc*FCB2 in complex with pyruvate shows that the ligand methyl group is in van der Waals contact with the side chains of both Leu230 and Ala198 (41). The double mutant Leu230Gly-Ala198Gly presented a 400-fold higher k_{cat} value than that of the wild-type enzyme using the bulky L-mandelate as substrate (37). Similar studies carried out with other LHAD enzymes also suggest that the size of the residues located in equivalent positions are important to determine the size of the substrate that the enzyme can oxidize efficiently (4, 42, 43).

Glycolate oxidase

The peroxisomal enzyme glycolate oxidase (GOX; EC 1.1.3.15) has been found in higher plants and vertebrates. Thus far, GOX from *Spinacia oleracea* (spinach; *So*GOX) and *Homo sapiens* (human; *Hs*GOX) are the best characterized. Several crystal structures of GOX are available in the presence and absence of ligands (4, 44). GOXs are homotetramers containing a non-covalently bound FMN per monomer. Both FMN and 6-OH-FMN were found in GOX from *Sus scrofa* (pig) liver (45). GOX preferentially oxidizes short-chain aliphatic L-α-hydroxyacids, with glycolate as the physiological substrate

Figure 2 Changes in the position of active site residues due to the presence of a ligand.
(A) Superimposition of the active site of wild-type *Tm*P2O in complex with acetate (ACT, purple carbons; PDB code: 1TT0) and the active site of the *Tm*P2O His167Ala variant containing 3-fluoro-3-deoxy-D-glucose (gray carbons; PDB code: 3PL8). The substrate-recognition loop (residues 452–457) is shown in cartoon representation.
(B) Superimposition of *So*GOX in the presence of 4-carboxy-5-(L-pentyl)hexylsulfanyl-1,2,3-triazole (gray carbons; PDB code: 1AL7) and the same enzyme in the absence of ligand (orange carbons; PDB code: 1GOX).

Scheme 3 Reactions catalyzed by LHAD enzymes.

(Scheme 3). In addition, *Hs*GOX can oxidize the long-chain acids α-hydroxyoctanoate and α-hydroxypalmitate (4, 46). A comparison of the *So*GOX structures in the presence and absence of the inhibitor 4-carboxy-5-(L-pentyl) hexylsulfanyl-1,2,3-triazole shows that Arg164 is a mobile residue (Figure 2B). This residue is oriented away from the catalytic site in one conformation whereas it points toward the catalytic site to interact with the ligand in the alternate conformation. This study, together with structural and mutagenesis results with other LHAD enzymes (47–52), suggests that the two fully conserved active site arginines are important for substrate binding.

Lactate oxidase

Lactate oxidase from the bacterium *Aerococcus viridans* (*Av*LOX; EC 1.1.3.15) catalyzes the oxidation of L-lactate using dioxygen as electron acceptor, yielding pyruvate and hydrogen peroxide. Similar enzymatic activity has been described in the ciliated protozoan *Tetrahymena pyriformis*, as well as in the bacteria *Streptococcus faecalis* and *Pediococcus* (53). *Av*LOX is highly specific for L-lactate but it presents low activity on α-hydroxybutyrate and related compounds (43). LOX is a homotetramer containing a non-covalently bound FMN in each monomer. The crystal structures of the enzyme with various ligands are available [(54) and references therein].

L-Lactate 2-monooxygenase

L-Lactate 2-monooxygenase (LMO; EC 1.13.12.4) has been described in *Mycobacterium* species including *Mycobacterium smegmatis* (*Ms*LMO) and *Mycobacterium phlei*. It is a homooctamer containing one FMN in each monomer. The crystal structure of LMO has not been solved; however, the catalytic mechanism has been extensively investigated. LMO catalyzes the oxidation of L-lactate to pyruvate, reducing dioxygen to hydrogen peroxide (55). The pyruvate is retained in the enzyme active site, where it reacts with hydrogen peroxide to form acetate and carbon dioxide.

Long-chain L-α-hydroxyacid oxidase

Long-chain L-α-hydroxyacid oxidase (LCHAO; isozyme B; EC 1.1.3.15) is a peroxysomal isozyme of GOX (isozyme A) (46, 56). The physiological substrate of LCHAO is unknown; however, L-α-hydroxyoctanoate and L-mandelate are better substrates than glycolate. Dioxygen is reduced to hydrogen peroxide by LCHAO (57). In addition, this enzyme is able to oxidize *in vitro* a variety of compounds, including thiol-glyoxylate adducts and creatol (58). The crystal structure of LCHAO from *Rattus norvegicus* (Norway rat) kidney (*Rn*LCHAO) has been solved in complex with ligands (57, 58). LCHAOs are homotetrameric enzymes containing a non-covalent FMN in each monomer.

L-Mandelate dehydrogenase

L-Mandelate dehydrogenase (MDH; EC 1.1.99.31) is present in several strains of *Pseudomonas*, including *Pseudomonas putida* ATCC 12633 (*Pp*MDH). The enzyme is associated to the membrane. The putative 39 residues involved in binding *Pp*MDH to the membrane were replaced with the equivalent segment in *So*GOX (20 residues), allowing the authors to obtain soluble protein (*Pp*MDH-GOX2) with similar properties to those of the wild-type *Pp*MDH (59). Subsequently, the crystal structure of *Pp*MDH-GOX2 was solved (60). MDH is a homotetramer carrying a non-covalent FMN bound to each monomer. It oxidizes L-mandelate to phenylglyoxalate using possibly a quinone (e.g., ubiquinone) as electron acceptor (Scheme 3). Small α-hydroxyacids such as glycolate and L-lactate are neither substrates nor inhibitors of MDH (61).

Flavoenzymes that oxidize sterols

Cholesterol oxidase

Cholesterol oxidase (CO; EC 1.1.3.6) is able to catalyze both the oxidation of the C3-OH group of cholesterol and the isomerization of the resulting cholest-5-en-3-one to yield cholest-4-en-3-one (Scheme 4). There are two types of COs, named I and II, that differ in their structure, kinetic properties, and redox properties (62). Type I CO belongs to the GMC oxidoreductase superfamily and contains non-covalently bound FAD, whereas type II CO is a member of the vanillyl alcohol oxidase superfamily and its FAD is covalently bound to the protein. The crystal structures of both *Streptomyces* sp. type I CO (*S*CO) and *Brevibacterium sterolicum* type II CO have been determined (63, 64). In addition, the crystal structure of *Rhodococcus equi* type I CO is available (65). Initially, the latter enzyme was misclassified as the non-covalent CO from *B. sterolicum* (66). All COs are monomeric proteins. In other bacteria (e.g., *Mycobacterium* sp.), the above reactions are catalyzed by
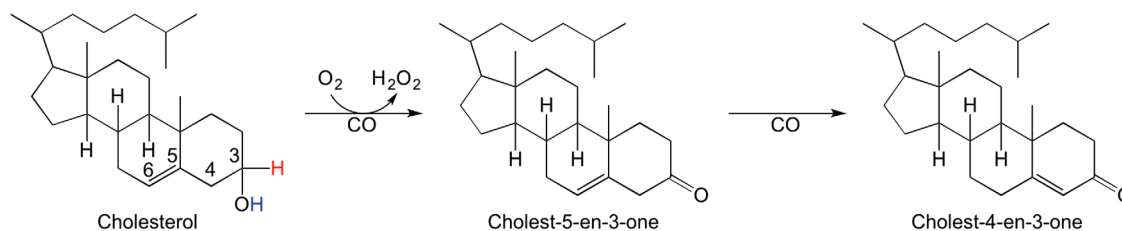
Scheme 4 Reactions catalyzed by CO.

an NAD(P)$^{+}$-dependent 3β-hydroxysteroid dehydrogenase belonging to a different superfamily (67). In *S*CO, the active site residue Glu361 is the catalytic base for the isomerization reaction catalyzed by the enzyme, as indicated by a 104-fold decrease in the specific activity of the Glu361Gln variant with respect to the wild-type enzyme (68).

Alcohol oxidases active on aldehydes

Water can spontaneously add to the carbonyl carbon of aldehydes yielding 1,1-diols, called *gem*-diols (Scheme 1). In aqueous solution, the relative fraction of aldehyde and *gem*-diol is thermodynamically governed by the chemical structure of the molecule (69). Thus, alcohol oxidases producing aldehydes that are highly hydrated in solution are expected to be able to oxidize aldehydes as well. The ability to oxidize hydrated aldehydes to their corresponding carboxylic acids, through an analogous mechanism to that employed for alcohol oxidation, has been described in *Ag*CHO, *Pe*AAO, *H. polymorpha* AO (*Hp*AO), *Halomonas elongata* CHD (*He*CHD), *M*HMFO, *Hs*GOX, *Ms*LMO, and *Rn*LCHAO (4, 70–74). Besides GMC and LHAD enzymes, *gem*-diol oxidase activity has been reported for alditol oxidase (EC 1.1.3.41), thiamine oxidase (EC 1.1.3.23), and nucleoside oxidase (EC 1.1.3.39) (75–77).

The reaction with hydrated aldehydes has been investigated in detail in *Ag*CHO, *Pe*AAO, and *M*HMFO. *Ag*CHO catalyzes two sequential reactions: (i) the oxidation of choline to betaine aldehyde and (ii) the oxidation of betaine aldehyde to glycine betaine (Scheme 1). More than 90% of the betaine aldehyde formed during the first reaction, in its *gem*-diol form, is oxidized to glycine betaine before dissociating from the *Ag*CHO active site (78). This was established by using betaine aldehyde and its isosteric analogue 3,3-dimethylbutyraldehyde with stopped-flow kinetics, nuclear magnetic resonance (NMR) spectroscopy, and colorimetric analysis of betaine aldehyde produced in the turnover of the enzyme with choline (79). In contrast, in CHO from *A. fumigatus* and *Pe*AAO, the aldehyde is released from the enzyme active site during the turnover with choline and a simple alcohol, respectively (13, 80). Similarly, it was shown in *Pe*AAO and *M*HMFO that the hydration of the aldehyde is required for the reaction to occur (74, 80). Mass spectrometry analysis of the *M*HMFO reactions in ^{18}O-labeled water confirmed that this enzyme oxidize the *gem*-diols to the corresponding carboxylic acids (74).

Mechanisms for flavin reduction

Over the years, three mechanisms have been considered for the dehydrogenation reactions in flavoenzymes that oxidize alcohols. The carbanion mechanism involves the abstraction of a proton from the α-carbon of the substrate by a strong active site base yielding a carbanion intermediate (Scheme 5A), which may nucleophilically attack the flavin N5 atom yielding a covalent adduct. The radical mechanism implicates a single-electron transfer from the substrate oxygen atom to the flavin concomitant to the abstraction of the hydroxyl proton by an active site base (Scheme 5B), yielding a flavin and a substrate radical from which the α-hydrogen atom is transferred to the flavin (22). The hydride transfer mechanism necessitates substrate activation through the abstraction of the hydroxyl proton to yield an alkoxide intermediate (or transition state) from which a hydride ion is transferred from the substrate α-carbon to the flavin (Scheme 5C).

The primary function of the protein is to modulate flavin reactivity to the extent that all, or at least most of, the unproductive reactions are abated in favor of the specific reaction that needs to be catalyzed. Owing to the extraordinary versatility of the flavin (81), flavoproteins may have different mechanisms of catalysis within enzyme complexes with physiological substrates and non-physiological substrates with different chemical structures (and sometimes reactivity). Thus, it is important to consider the mechanistic evidence available with physiological substrates and alternate, non-physiological, substrates distinctly.
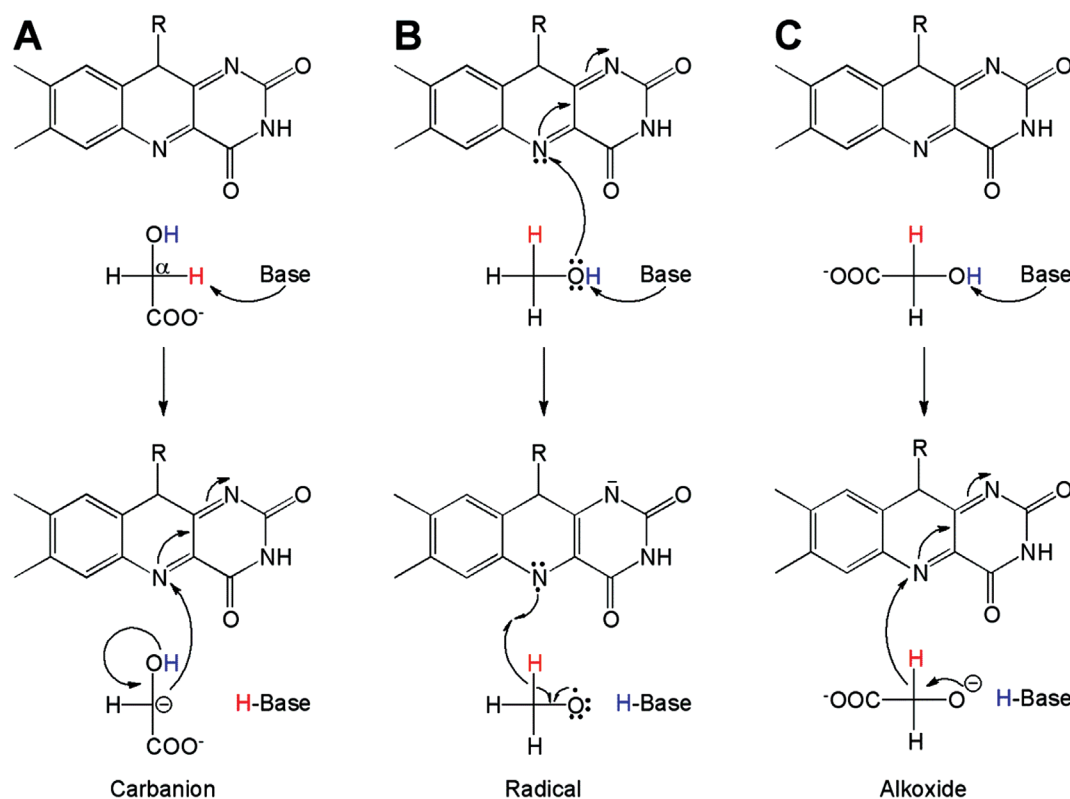
Scheme 5 Carbanion (A), radical (B), and hydride transfer (C) mechanisms proposed for flavoenzymes catalyzing dehydrogenation reactions.
Adapted from Refs. (22, 93). See the text for further explanation. Glycolate (A and C) and methanol (B) are depicted as examples of substrate. For FMN, -R, ribitol phosphate; and for FAD, -R, ribitol adenosine diphosphate.

Mechanisms with physiological substrates

The preponderance of studies with physiological substrates is consistent with a hydride transfer mechanism for alcohol oxidation in GMC and LHAD enzymes. The primary tools that have been used to study the mechanism for alcohol oxidation with physiological substrates are deuterium kinetic isotope effects (KIEs) and stopped-flow spectrophotometry. As two chemical bonds are cleaved in the oxidation reaction, primary substrate and solvent deuterium KIE can be used to probe the status of the CH and OH bonds, respectively, and multiple KIEs can provide information on the relative timing for their cleavage. This approach has established whether the reaction is stepwise, with the formation of an intermediate, or concerted, if such an intermediate is lacking. In concerted reactions, the relative timing for bond cleavage can be synchronous or asynchronous, depending on whether the cleavages of the bonds progress in the transition state of reaction to similar extents or not, respectively (82). While direct observation of a flavin radical or N5-flavin adduct with physiological substrates has not been reported, this is not sufficient to rule out radical and carbanion mechanisms because the intermediate may not accumulate to a detectable extent if the rate of its decay is faster than the rate of formation.

Both AgCHO and TmP2O exhibit a primary substrate deuterium KIE on the rate constant for anaerobic flavin reduction (k_{red}) of 9, suggesting that flavin reduction is rate limiting in the reductive half-reaction (83, 84). No solvent deuterium KIE was observed with these enzymes, suggesting that OH bond cleavage is rapid, i.e., not rate limiting, and it is decoupled from the CH bond cleavage (83, 85). These results are consistent with a stepwise mechanism. This mechanism was proposed also for AnGO and SCO based on primary substrate and solvent deuterium KIE studies. However, CH bond cleavage is not fully rate limiting in these enzymes, thereby hampering an unambiguous conclusion on the catalytic mechanism (86–88).

A large primary substrate deuterium KIE on k_{red} of ~9 was also determined for PeAAO, indicating that the cleavage of the CH bond is the rate-limiting step in the reductive half-reaction (89). The solvent deuterium KIE for $k_{cat}/K_{m(p\text{-anisylic})}$ using deuterated substrate was higher than

that using the non-deuterated substrate, with values of 1.7 and 1.2, respectively (89). In addition, the multiple KIEs on k_{red}, k_{cat}, or $k_{cat}/K_{m(p\text{-anisylic})}$ was not smaller than the product of the individual primary substrate and solvent deuterium KIE. These results are consistent with a catalytic mechanism in which OH and CH bond cleavages are concerted, which rules out carbanion and radical mechanisms. The quantum mechanics/molecular mechanics (QM/MM) energy profiles for the reaction of *Pe*AAO with the alcohol substrate indicated that OH bond is cleaved before the CH bond, consistent with a concerted asynchronous hydride transfer reaction, and that a stable reaction intermediate is not formed (90).

Quantitative structure-activity relation analyses were carried out for *C. boidinii* AO (*Cb*AO) (91). Using various β-substituted ethyl alcohols as substrates, a negative linear correlation was found between k_{cat}/K_m and σ_I, i.e., the electronic parameter reflecting the inductive effects of the substituent. Therefore, *Cb*AO presents the highest k_{cat}/K_m values using ethyl alcohols with substrates harboring an electron-donating substituent. This is consistent with an electron-deficient transition state, which is not in agreement with a carbanion intermediate. The primary substrate and solvent deuterium KIE determined for *Cb*AO showed that there is a change in the relative rate of the CH and OH bond cleavage, and thus in the rate-limiting step, depending on the alcohol that is used. In view of these results, it was concluded that *Cb*AO likely presents an asynchronous concerted hydride transfer mechanism (91).

Primary substrate and solvent deuterium KIEs were determined for *Sc*FCB2. This study suggested that the CH and OH bonds are not cleaved in the same step, based on $^D k_{red}$ and $^{D2O}k_{red}$ of ~5 and 1, respectively. Significant inverse solvent isotope effects of 0.90 and 0.87 were found on $k_{cat}/K_{m(L\text{-lactate})}$ using non-deuterated and deuterated organic substrate, respectively. These results suggest that the OH bond cleavage is coupled to an isomerization of the enzyme-substrate complex before the hydride transfer occurs. Similar experiments using the Tyr254Phe *Sc*FCB2 variant showed that the primary substrate and solvent deuterium KIEs arise from the same step in this mutant in accordance with a concerted hydride transfer mechanism for the cleavages of the OH and CH bonds (92). A drastic change in mechanism in the Tyr254Phe *Sc*FCB2 variant compared with the wild-type enzyme is highly unlikely because the two enzymes differ only by the hydroxyl group on residue 254, allowing the authors to conclude that a hydride transfer mechanism is operative in the wild-type enzyme as well. Computational studies provided independent support for a hydride transfer mechanism in this enzyme (93).

In *Pp*MDH, a reaction intermediate with absorbance in the 500–700 nm region was detected in the reductive half-reaction with mandelate (94). This intermediate was proposed to be a charge-transfer complex between the oxidized flavin and a species formed in turnover, based on primary substrate deuterium KIE of 3 and 2 on the rate constants defining the increase and decrease in absorbance at 560 nm (94). However, while the intermediate was still forming, there was already a significant reduction of the flavin, which did not permit the establishment of the relative fraction of flavin in the reduced state and in the charge-transfer complex. Partial cleavages of the OH and CH bonds do not rule out an alkoxide as the intermediate observed. Thus, either carbanion or hydride transfer mechanisms cannot be ruled out on the basis of these results.

In summary, as the most conclusive studies described above support a hydride transfer mechanism, and the active site residues are conserved among the members of each (super)family, it is likely that all enzymes described here present a hydride transfer mechanism with their physiological substrates.

Mechanisms with alternate, non-physiological substrates

The observation that various LHAD enzymes are able to catalyze halide elimination reactions was long considered evidence for a carbanion mechanism (Scheme 6A). *Ms*LMO is able to catalyze the elimination of HCl from β-Cl-lactate, producing pyruvate. Using [α-^{3}H]-β-Cl-lactate as substrate, 30% of the tritium was detected in the pyruvate product, suggesting that the elimination reaction occurs through the intramolecular transfer of the proton from the α-carbon to the β-carbon (95). Besides the halide elimination reaction, the *Ms*LMO carries out the oxidation of β-Cl-lactate, yielding Cl-acetate (Scheme 6A). As the ratio pyruvate/Cl-acetate in the reaction mixtures decreased with increasing dioxygen concentration, it was proposed that a carbanion is the intermediate for both the elimination reaction and the oxidative decarboxylation. *Sc*FCB2 and *Rn*LCHAO can catalyze the reduction of β-Br-pyruvate to β-Br-lactate using lactate as electron donor [(96) and references therein]. In these transhydrogenation reactions, some of the pyruvate was produced by Br elimination, as suggested by using isotope-labeled substrates. However, the halide elimination may occur from the reduced enzyme-product complex without the participation of a carbanion intermediate (Scheme 6B) (97, 98). A recent computational study supports a stepwise
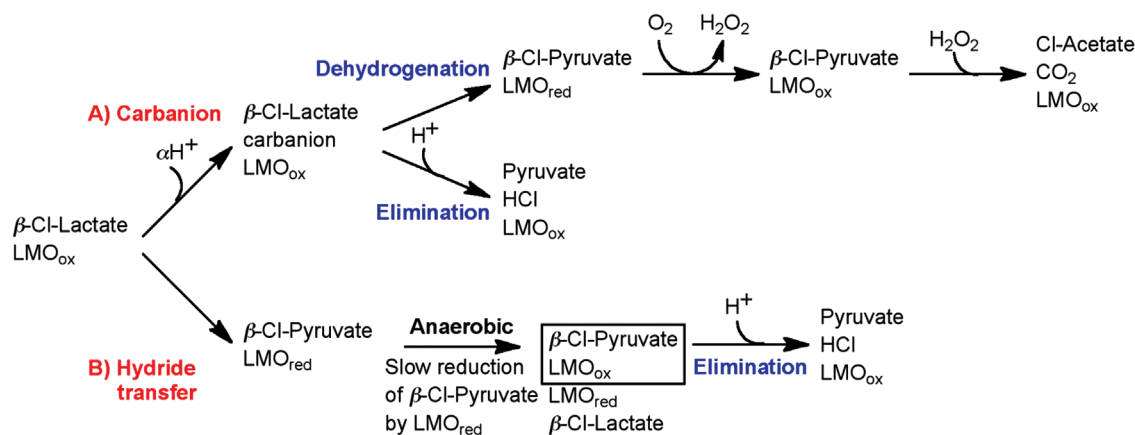
Scheme 6 Proposed pathways for halide elimination and oxidative dehydrogenation of β-Cl-lactate catalyzed by *Ms*LMO through carbanion (A) and hydride transfer (B) mechanisms.
LMO_{ox} and LMO_{red} refer to the oxidized and reduced enzyme, respectively.

hydride transfer mechanism for the reaction of *Rn*LCHAO with L-lactate (a poor substrate) (99).

Alternate, non-physiological substrates containing a cyclopropyl group have been used to distinguish between radical and hydride pathways (100). Cyclopropanol irreversibly inactivates the *Hp*AO through the formation of a cyclic N5-C4a-adduct, as was shown using NMR and infrared spectroscopies, and spectrophotometric experiments (101). In contrast, the enzyme reconstituted with 5-deaza-FAD does not form a covalent adduct with cyclopropanol. The 5-deaza-flavins show a high thermodynamic radical instability, being unable to participate in one-electron transfer reactions (102). In view of these results, the authors proposed that the covalent adduct in the *Hp*AO is formed through a radical mechanism involving the formation of flavin semiquinone and a cyclopropoxy radical (Scheme 7). However, *Hp*AO is able to oxidize cyclopropylmethanol, producing cyclopropylmethanal, using dioxygen as electron acceptor (101). During this reaction, ring-opened products were not detected, suggesting that radical intermediates are improbable in this reaction.

*S*CO was irreversibly inhibited after reacting with the alternate substrate 2α,3α-cyclopropano-5α-cholestan-3β-ol (103). On the basis of spectrophotometric, fluorescence, and mass spectrometric studies, it was suggested that this reaction yields a C6-alkylated flavin and either an N5 flavin adduct or a cyclic N5-C4a flavin adduct. The formation of the C6-flavin adduct may involve the anionic ring-opening and attack of the methylene anion on the C6 atom of the isoalloxazine ring. In the case of the N5-adduct, the anionic attack would occur at the N5 atom.

However, a radical mechanism involving the flavin semiquinone and a methylene radical was also considered.

*Ms*LMO is able to oxidize glycolate, although it is a poor alternate substrate compared with L-lactate. This reaction involves the formation of covalent glycolyl adducts at N5-position of the isoalloxazine ring (72, 104). It was proposed that the adduct is formed after abstracting the α-hydrogen as a proton from glycolate (carbanion mechanism) (104).

Quantum mechanical hydride tunneling

The contribution of quantum mechanical tunneling to hydride transfer reactions is being observed in a growing number of enzymes, including zinc-dependent (e.g., *Bacillus stearothermophilus* alcohol dehydrogenase) (105) and flavin-dependent alcohol oxidases. As the hydride transfer mechanism appears to be the most plausible for the GMC and LHAD enzymes, it is interesting to determine if hydride tunneling participates in the reactions of these enzymes. Competitive KIE experiments were carried out to assess hydride tunneling contributions to the reaction of wild-type *An*GO and a number of *An*GO variants presenting various extents of glycosylation or polyethylene glycol at their surface [(106) and references therein]. Native and deglycosylated *An*GO showed isotope effects on the Arrhenius prefactor (A_D/A_T) close to unity, whereas all *An*GO variants showed A_D/A_T values lower than unity. It was also found that the A_D/A_T decreased with increasing thermal melting temperature of the enzyme, suggesting that the *An*GO variants are less flexible than the wild-type enzyme
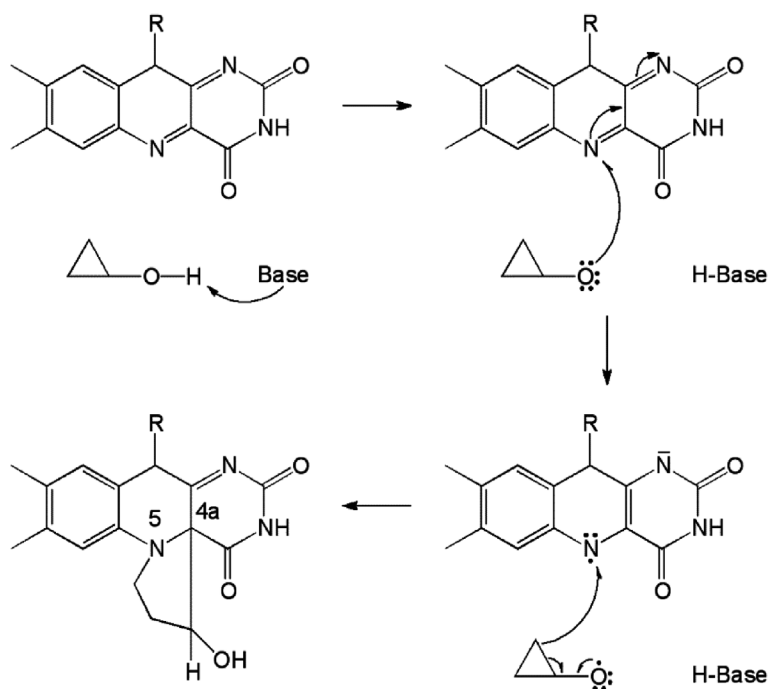
Scheme 7 Proposed mechanism of cyclopropyl ring cleavage catalyzed *Hp*AO through a radical mechanism. Adapted from Ref. (101).

and, thus, large-scale motions required to reach the active site configuration for efficient tunneling are impaired (107). As a consequence, the local motions play a more important role.

In *Ag*CHO, the effect of temperature on the primary substrate deuterium KIE was investigated at saturating dioxygen concentration (108). The $^D(k_{cat}/K_m)$ had a temperature-independent average value of ~10.6, the A_H/A_D was ~11, and the enthalpies of activation ($\Delta H^‡$) determined with choline and 1,2-$[^2H_4]$-choline were the same with a value of ~18 kJ/mol. These data are consistent with a highly preorganized enzyme-substrate complex in which minimal independent movements of the substrate and flavin are required other than those conducive to tunneling of the hydride ion in the reductive half-reactions. Preorganization could be disrupted in *Ag*CHO upon replacing Glu312, which binds the substrate trimethylammine, with aspartate or His99 with asparagine, which prevents the covalent attachment of the flavin to the protein (109, 110). In both *Ag*CHO mutants, the k_{red} and $^Dk_{red}$ values were temperature dependent and different $\Delta H^‡$ were determined for the cleavages of the substrate CH and CD bonds. Therefore, the optimal positioning of both the flavin and the substrate in the *Ag*CHO active site is important to ensure efficient hydride transfer through tunneling (109, 110).

In the case of *Pe*AAO, the combination of a significant secondary substrate deuterium KIE and a primary effect greater than the semiclassical limit was interpreted as suggesting hydride tunneling (111).

Mechanisms for flavin oxidation

Oxidases are able to efficiently reduce dioxygen, producing hydrogen peroxide. The reaction of flavoenzymes with dioxygen has been described in two recent review articles (112, 113), and only an overview and recent studies will be presented here. It is generally accepted that the initial step for dioxygen activation is a single-electron transfer from the flavin hydroquinone to dioxygen, yielding superoxide and flavin semiquinone radicals (112–114), which are stabilized in various flavoprotein oxidases by a positive charge (112, 113). Only with *Hs*GOX is there spectroscopic evidence for the transient formation of an intermediate resembling a flavin semiquinone in the reaction with dioxygen (115). In *Tm*P2O, a recent computational study suggests that the initial electron transfer is coupled to a proton transfer from an active site histidine (116). In *Ag*CHO, pH effects, solvent viscosity effects, KIE, and time-resolved absorbance spectroscopy are consistent with a mechanism for

dioxygen reduction in which the hydrogen atom from the flavin semiquinone and a proton from either the solvent or a solvent-exchangeable site in the protein are transferred to dioxygen in the same kinetic step without evidence for flavin-derived intermediates (117). This is in keeping with most of the flavoprotein oxidases, for which flavin-derived intermediates are typically not observed either because they do not form or because they decay faster than they form, with a notable exception being *Tm*P2O at pH <8 (118). A flavin C4a-adduct has been recently reported in the *Am*PDH crystal, an enzyme that poorly reacts with dioxygen; however, it is probably an artifact due to synchrotron radiation (29). In *Pe*AAO, computational studies suggested that a C4a-(hydro)peroxy flavin intermediate is not formed (119).

Conserved active site histidine

All GMC and LHAD enzymes contain a histidine located ≤5 Å from the flavin N5 atom (Figure 3). Evolutionary pressure has acted to conserve this histidine, suggesting important functional roles for this residue. Site-directed mutagenesis, kinetics, crystallography, and computational approaches have been variably used to investigate the conserved histidine in several enzymes of both classes. Depending on the GMC enzyme, the residue has been shown to either abstract the substrate hydroxyl proton, stabilize alkoxide intermediates, contribute to active site polarity, donate a proton to dioxygen, or stabilize C4a-(hydro)peroxy flavins or superoxide radicals. In the case of *Pe*AAO and *Tm*P2O, it has been unambiguously shown that an important role of the fully conserved histidine is the abstraction of the substrate hydroxyl proton during the reductive half-reaction, and the results with other GMC enzymes suggest the same conclusion. As these enzymes likely present the same mechanism, and their active site and structure is conserved, it seems plausible that the fully conserved histidine may act as a catalytic base in most GMC enzymes. The studies described below for the LHAD enzymes support the importance of the fully conserved histidine in the enzyme active site; however, further work is required to confirm the role of this residue as a catalytic base in the members of this family. On the basis of the available crystal structures in complex with a ligand, the substrate is located close to the fully conserved histidine (≤4 Å from the flavin N5 atom; Figure 3). These ligands were observed on the *re*-face of the flavin in the GMC enzymes, whereas they are located on the *si*-face in the members of the LHAD family (Figure 3).
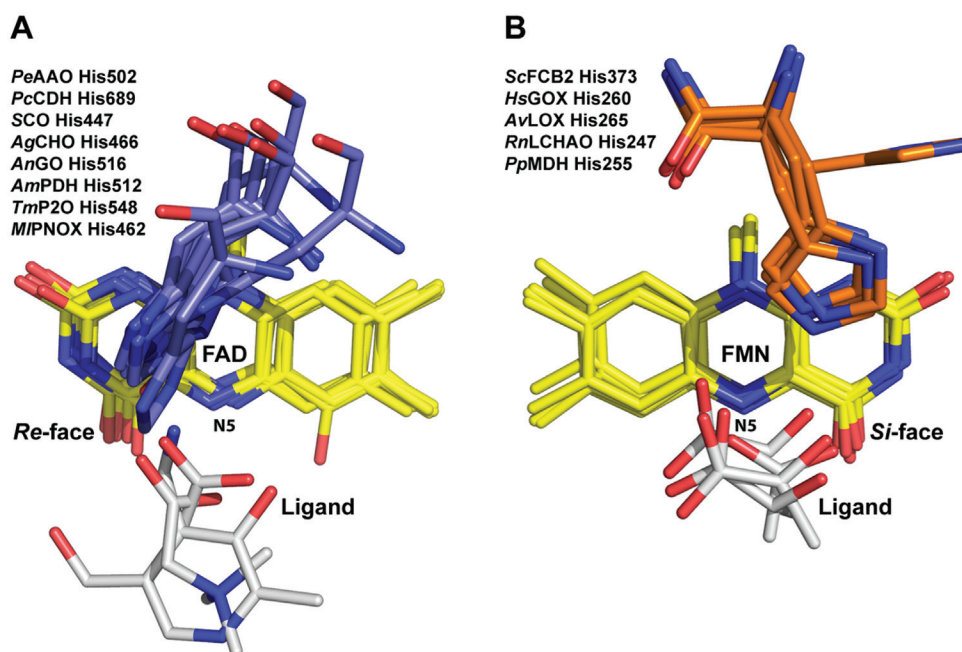
Figure 3 Superimposition of the active sites of representative members of the GMC oxidoreductase superfamily (A) and the LHAD family (B) showing the position of the fully conserved histidine and various ligands.
The figure was drawn by using the PDB files 3FIM, 1KDG, 1B4V, 4MJW, 1CF3, 4H7U, 1TT0, 4HA6, 1FCB, 2RDU, 2NLI, 1TB3, and 1HUV.

*Ag*CHO His466

The role of His466 in *Ag*CHO was investigated with mutagenesis, pH, and KIEs using steady-state kinetics, pH effects on imidazole rescuing of the enzymatic activity, and spectroscopic approaches (120). The His466Ala variant of *Ag*CHO exhibited k_{cat} and $k_{cat}/K_{m(choline)}$ values ~60- and 1000-fold lower than those of the wild-type enzyme (120). The $k_{cat}/K_{m(choline)}$ and the k_{cat} values increased with increasing pH, reaching limiting values at high pH but with significantly higher pK_a values than the wild-type enzyme (i.e., 9.0 vs. 7.6 for $k_{cat}/K_{m(choline)}$) (120, 121). This result suggested a contribution of His466 to the active site polarity (120). Primary substrate and solvent deuterium KIEs on the steady-state parameters k_{cat} and $k_{cat}/K_{m(choline)}$ in the His466Ala enzyme were consistent with a concerted mechanism for CH and OH bond cleavage (120), in contrast to the wild-type *Ag*CHO where OH bond cleavage precedes CH bond cleavage (83). These results indicated that His466 is important for stabilization of the alkoxide intermediate of reaction. The turnover number k_{cat} of the His466Ala *Ag*CHO variant could be partially rescued in the presence of imidazolium, but not imidazole (120), consistent with the side chain of His466 being protonated in the rate-limiting step for overall enzyme turnover. These results are also consistent with His466 acting to stabilize the alkoxide intermediate of the reaction. As in the wild-type enzyme alkoxide formation by the action of a base is fast and the subsequent hydride transfer to the flavin is the slowest kinetic step in catalysis, these data do not rule out *a priori* that His466 may act as catalytic base. Indeed, both the crystal structure of *Ag*CHO in complex with the reaction product of choline oxidation, glycine betaine (Figure 3) (16), and computational studies using QM/MM simulations (90) are consistent with His466 acting as the base that deprotonates choline. However, biochemical evidence to further support this conclusion is not available yet.

*Pe*AAO His502

Mutagenesis and pH effects using both steady-state and rapid kinetics and computational studies revealed the roles of His502 in *Pe*AAO (119). The k_{cat} and k_{red} values of the His502Ala/Ser variants were considerably lower than those of the wild-type enzyme (~1200–2800-fold). The QM/MM energy profiles for the reaction of *Pe*AAO with the alcohol substrate indicated that the His502 is not protonated at the ε-position and thus may act as the catalytic base (119). The k_{cat} and $k_{cat}/K_{m(p\text{-anisylic})}$ values for the wild-type *Pe*AAO were pH independent (89). The effect of pH

on $k_{cat}/K_{m(p\text{-anisylic})}$ for the His502 variants could not be determined; however, a pK_a value of ~8.5 was determined in the pH profile of the k_{cat} value for these variants (90). The pH profile on $k_{cat}/K_{m(p\text{-anisylic})}$ for the His546Ala/Ser *Pe*AAO variants (a residue located 3.7 Å from the His502) was consistent with the involvement of an unprotonated group in catalysis with a pK_a value of ~3.8, whereas pK_a values of ~3.0 and 9.0 were determined in the pH profile of the k_{cat} value for the latter variants (90). The acidic pK_a value was assigned to His502, as it was not observed in the pH profiles of the His502 variants (90). The $k_{cat}/K_{m(dioxygen)}$ for the wild-type *Pe*AAO was pH independent in the range ~5–8 (90). However, a slight decrease of the $k_{cat}/K_{m(dioxygen)}$ value was observed at pH 9.0 and a pK_a value of 8.9 was determined. Computational studies suggested that the His502 may donate a proton to dioxygen during the oxidative half-reaction (90).

*Tm*P2O His548

Rapid reaction kinetics, pH effects, and mutagenesis were used to study the role of His548 in *Tm*P2O (122). The His548Arg *Tm*P2O variant contained both covalently and non-covalently bound FAD. To obtain homogenous populations, the His167 that provides the covalent attachment of the flavin to the protein was replaced with alanine. The His167Ala-His548Arg double mutant could be reduced anaerobically by D-glucose. However, the reduction rate constant was ~220 times lower than that of the single His-167Ala mutant. The rate constant for flavin reduction of wild-type *Tm*P2O was pH independent, whereas the reaction of the His167Ala-His548Arg double mutant demonstrated the participation of a base with a pK_a value >10.1 (122). This result indicated that the observed pK_a value corresponds to the arginine, and suggested that, for the wild-type *Tm*P2O, the His548 residue is unprotonated in the assayed pH range (pK_a<5.5). It was concluded that the *Tm*P2O His548 likely acts as a base that deprotonates D-glucose. During flavin oxidation, His548 may transfer a proton to dioxygen, coupled to the initial electron transfer, on the basis of computational and experimental studies (116, 118). In addition, this residue may stabilize the C4a-hydroperoxy flavin intermediate in the oxidative half-reaction catalyzed by the enzyme (116, 118).

Other studies in GMC enzymes

In the case of *An*GO, it was reported that a protonated group facilitates the reaction of the reduced flavin with

dioxygen based on the pH dependence of the $k_{cat}/K_{m(dioxygen)}$ value (pK_a ~8) (123). As the His516Ala AnGO variant exhibited considerably lower $k_{cat}/K_{m(dioxygen)}$ values at low pH than those of the wild-type enzyme (~250-fold), it was concluded that the protonated His516 stabilizes the superoxide anion during the oxidative half-reaction (123).

The role of the fully conserved histidine in SCO (His447) was also investigated. The k_{cat} value of the wild-type SCO was ~120- and 4400-fold higher than that of the His447Gln and His447Asn variants, respectively (87). Structural data suggested the presence of a hydrogen-bonding network involving His447, Glu361, Asn485, and Trp541, which is required to position the substrate and coordinate general base and electrophilic catalysis. This network is perturbed in the His447Asn variant, in contrast to the case of the variant with the highest oxidation activity, i.e., the His447Gln enzyme. It was concluded that, although His447 is important for substrate activation, the other components of the network are also required (87). Atomic resolution crystallography studies (0.92–0.99 Å) suggested that the His477 Nε2 atom in the oxidized enzyme is protonated in the pH range 4.5–7.3, whereas it is deprotonated at pH 9.0 (124).

In PcCDH, the role of His689 was studied using site-directed mutagenesis and kinetics (125). The k_{cat} value for the His689 variants was >1000-fold lower than that of the wild-type PcCDH, indicating an important role of His689 in catalysis (125). Recently, the crystal structures of AmPDH and MlPNOX have been reported (18, 29). Molecular dynamics simulations suggested that His512 in AmPDH can act as a catalytic base, mainly in C3-oxidations of the substrate (126). The k_{cat} value of the His467Ala MHMFO variant was 4400 times lower than that of the wild-type enzyme, whereas the $K_{m(vanillylic)}$ value was only slightly affected by the mutation (11).

Studies in LHAD enzymes

The MsLMO His290 and ScFCB2 His373 were replaced with glutamine, yielding mutant enzymes with rate constants for anaerobic flavin reduction with L-lactate ≥3500-fold lower than the wild-type enzymes (127, 128). However, the crystal structure of the ScFCB2 His373Gln variant revealed that the position of several active site residues changes due to the mutation (128). The PpMDH His274 (PpMDH-GOX2 His255) was replaced with various residues, resulting in enzyme variants at least 10^5-fold less active against L-mandelate than wild-type PpMDH (129). The HsGOX His-260Gln/Ala variants were expressed and purified (130). However, the characterization of these mutants could not be carried out because the mutant proteins did not contain flavin (130). Crystallographic data suggested that the equivalent histidine (His265) in AvLOX is placed close to the substrate binding site at pH 7.5 and 8.0, whereas it flips away at pH 4.5 (54).

Expert opinion

The flavoenzymes belonging to the GMC oxidoreductase superfamily and the LHAD family are widely distributed in all kingdoms of living organisms where they play a wide variety of roles. This is mainly due to the high versatility of the isoalloxazine ring, together with the fine-tuning of the enzyme active site. Important efforts have been made in recent decades to determine the crystal structures of these enzymes, understand their kinetic mechanisms, and identify residues involved in catalysis.

All flavoenzymes discussed here catalyze the oxidation of a CH-OH group to form a carbonyl moiety. In addition, some of these enzymes are able to oxidize the gem-diol form of aldehydes, although less efficiently. These dehydrogenation reactions involve the cleavage of the substrate CH and OH bonds, and the transfer of two electrons and one proton to the flavin. Although carbanion, radical, and hydride transfer mechanisms have been considered for the dehydrogenation reactions in flavoenzymes, the most recent experimental evidences with physiological substrates support the latter mechanism for both the GMC oxidoreductases and the LHAD enzymes. Among the variety of approaches used to determine the mechanisms of these enzymes over the years, deuterium KIE studies have provided the most unambiguous conclusions with physiological substrates. Site-directed mutagenesis, and kinetic, crystallographic, and computational studies have been insightful to determine the roles of the fully conserved active site histidine proximal to the flavin cofactor.

Outlook

The flavoenzymes belonging to either the GMC oxidoreductase superfamily or the LHAD family have been extensively investigated during the past decade. Studies have mainly focused on the oxidases, providing valuable biochemical, mechanistic, and structural data. This information may help improve the properties of these enzymes by protein engineering, yielding more powerful and stable biocatalysts. A number of unsolved questions and challenges will need to be addressed in the future. These are as follows: (i)

to understand at a molecular level the reaction, or the lack thereof, of the reduced flavin with dioxygen; (ii) to improve the stability of the enzymes under conditions of extreme temperature and pH, high hydrogen peroxide concentrations, organic solvents, etc., for biocatalytic conversions; (iii) to recognize the importance of enzyme isomerizations in catalysis; (iv) to elucidate the flavinylation process, and to improve the expression of recombinant enzymes with increasing levels of flavin incorporation; (v) to develop methods to efficiently express and purify membrane-associated enzymes; (vi) to resolve the structures of enzymes in complex with their physiological substrate; and (vii) to understand how substrate access to and product release from the active site can be improved in those enzymes in which these processes limit enzymatic turnover.

Highlights

– Most of the flavoenzymes belonging to the GMC oxidoreductase superfamily and the LHAD family catalyze the oxidation of a CH-OH bond, yielding a carbonyl moiety.
– The ability to oxidize the *gem*-diol form of aldehydes has been described in several GMC oxidoreductases and LHAD enzymes.
– Deuterium KIE studies in general support a hydride transfer mechanism for the GMC oxidoreductases and LHAD enzymes, with cleavage of the substrate OH and CH bonds occurring in stepwise or concerted fashions.
– GMC oxidoreductases and LHAD enzymes have a fully conserved active site histidine proximal to the flavin that acts as a catalytic base in most of these enzymes.
– Although a C4a-hydroperoxyflavin intermediate is not common in the reaction of flavin-dependent oxidases, it has been observed in the catalytic pathway of *Tm*P2O.
– Hydride tunneling and enzyme motions are important features of alcohol oxidation by flavoenzymes.

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Elvira Romero studied Biology at the University of Alcalá in Spain. She performed her PhD work on the flavin-dependent enzyme aryl-alcohol oxidase from *Bjerkandera adusta* at the Center of Biological Research of the Spanish National Research Council. She subsequently moved to Virginia Tech University in the USA to study the flavin-dependent monooxygenase siderophore A from *Aspergillus fumigatus*. Currently, she is a Postdoctoral Associate in the Department of Chemistry at Georgia State University, Atlanta, where she studies enzymes requiring flavins, hemes, or iron-sulfur clusters as cofactors. She is interested in exploring the reaction mechanisms, structures, and dynamics of redox enzymes.

Giovanni Gadda earned a Laurea in Biological Sciences and a PhD in Biochemistry at the Università degli Studi di Milano, Italy, working on the flavin-dependent enzymes ferredoxin NADP$^+$ reductase, D-amino acid oxidase and cholesterol oxidase. He then trained as a Postdoctoral Research Associate at Konstanz University, Germany, and Texas A&M University in the USA, working on cholesterol oxidase and nitroalkane oxidase. In 2000 he joined the faculty at the Departments of Chemistry and Biology of Georgia State University, Atlanta, where he is currently a Distinguished University Professor. He is the founder and organizer of the Annual Southeast Enzyme Conference and has served as the Director of the Chemistry Graduate Programs since 2007. His research interests are in mechanistic enzymology with emphasis on understanding the structure-function relationships that govern how redox enzymes, and flavoenzymes in particular, influence the energetics of reaction intermediates and transition states.