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REVIEW

Covalent attachment of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to enzymes: The current state of affairs

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Abstract

The first identified covalent flavoprotein, a component of mammalian succinate dehydrogenase, was reported 42 years ago. Since that time, more than 20 covalent flavoenzymes have been described, each possessing one of five modes of FAD or FMN linkage to protein. Despite the early identification of covalent flavoproteins, the mechanisms of covalent bond formation and the roles of the covalent links are only recently being appreciated. The main focus of this review is, therefore, one of mechanism and function, in addition to surveying the types of linkage observed and the methods employed for their identification. Case studies are presented for a variety of covalent flavoenzymes, from which general findings are beginning to emerge.

Keywords: covalent flavoproteins; flavin adenine dinucleotide; flavin mononucleotide; flavinylation; redox cofactors

Cofactors are recruited by protein molecules to extend the chemistry available within the active sites of enzymes. The chemistries displayed are variable and the range of protein-specific cofactors available bears testimony to the types of biological reaction achieved by many enzyme molecules. Cofactors may be present in the freely dissociable form, but others, such as lipoic acid and biotin, are permanently linked to the host protein. Other cofactors (e.g., heme and pyridoxyl phosphate) fall into both categories in that they can be attached covalently to the protein or operate in the freely dissociable form. The covalent addition of specific cofactors to unique residues within a polypeptide chain is a fundamental problem in studies of biological molecular recognition. In many cases, this specificity is purchased at the expense of recruiting modification enzymes that direct the covalent addition of a cofactor to the host protein. For example, the additions of biotin and lipoic acid to the ϵ-amino groups of specific Lys residues in carboxyltransferases and the 2-oxoacid dehydrogenases are directed by a biotin holoenzyme synthase (Sweetman et al., 1985; Takai et al., 1987) and lipoate–protein ligases (Brookfield et al., 1991; Morris et al., 1994), respectively. In a similar fashion, a heme cytochrome *c* lyase (Steiner et al., 1996) is responsible for the covalent addition of heme to two Cys residues via thioether linkages. In some enzymes, an existing side chain is modified to yield what is in effect a covalently attached cofactor, although no pre-existing cofactor molecule is incorporated into the enzyme. Several examples of this type of modification are provided in the next paragraph.

For histidine decarboxylase of *Lactobacillus* 30A (Recsei & Snell, 1984), a pyruvoyl group is generated from an existing residue, Ser 82. The enzyme is synthesized as a proenzyme, which, in a subsequent self-catalytic process, is cleaved between Ser 81 and Ser 82, producing α and β chains. Ser 82, at the newly produced N-terminus of the α chain, is deaminated, yielding the catalytic pyruvoyl group. Specific Ser residues in histidine ammonia-lyase and phenylalanine ammonia-lyase are self-catalytically converted to a dehydroalanine cofactor (Langer et al., 1994). In methylamine dehydrogenase from various methylotrophic bacteria, the tryptophan tryptophylquinone (TTQ) cofactor is generated by the cross-linking of two Trp residues (McIntire et al., 1991). It is thought that TTQ cofactor synthesis is an enzyme-catalyzed process, although the identity and location of the necessary auxiliary enzymes has yet to be described. For bacterial and eukaryotic copper-containing monoamine and diamine oxidases, a Tyr side group is

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converted to catalytically competent topa quinone (TPQ) cofactor in a self-catalytic process involving the bound copper, O_2, and water. After substrate reduction of TPQ, the electrons are passed to the same bound copper, where O_2 is reduced to H_2O_2 (Nakamura et al., 1996; Ruggiero et al., 1997). The green fluorescent protein, in Pacific jellyfish and similar animals, is another striking example of the use of amino acids to self-catalytically generate a useful protein-bound group. The fluorophore of the *Escherichia coli*-produced protein results from cyclization through condensation of the backbone carbonyl carbon of Ser 65 with the backbone amide nitrogen of Gly 67, and oxidation of the C_α-C_β bond of Tyr 66 (Yang et al., 1996). A final interesting example of a redox-active, modified aminoacyl group is provided by NADH peroxidase of *Enterococcus faecalis* 10C1. This FAD-containing enzyme also harbors a cysteinylsulfenic acid group, which participates in the catalytic redox reaction (Yeh et al., 1996). From these choice examples, apparently, there are numerous and varied ways aminoacyl groups can be transmogrified into useful entities in proteins and enzymes. In the future, we will likely encounter and marvel at other unusual structures. Other examples of aminoacyl groups recruited as cofactors can be found elsewhere (Rucker & Wold, 1988; Rucker & McGee, 1993; McIntire, 1994).

The covalent attachment of flavins to proteins has been recognized for about 40 years following the work of Singer and coworkers (Kearney & Singer, 1955a, 1955b; Singer et al., 1956; Kearney et al., 1971) on mammalian succinate dehydrogenase. Since then, many covalent flavoproteins have been identified. Each contains one of five modes of linkage to the host protein. A major question arising from these observations is the role of the covalent link and the mechanism by which it is formed. Is the role of covalent attachment to saturate the active site with the cofactor? Given that flavins are usually bound at active sites with high affinity (ensuring saturation even in the absence of a covalent link), this seems an unlikely hypothesis. Alternatively, a linked flavin may acquire characteristics lacking in a noncovalently bound counterpart, e.g., modified redox properties. The oxidation–reduction potentials of aminoacyl flavins are about 50–60 mV more positive than for unmodified forms (Edmondson & De Francisco, 1992), but similar increases in potential can also be induced by noncovalent interactions with protein. Studies on the effects of pH on the oxidation–reduction potentials of 8α-*N*-imidazolylflavins (pK_a 6.02) indicate that, as the pH increases, the redox potential decreases (Williamson & Edmondson, 1985). The data suggest that modulation by the protein environment of the histidyl group pK_a in histidyl flavins could exert some control over the oxidation–reduction potential of the flavin. Flavin is a versatile cofactor that has the potential to display many chemistries (Ghisla & Massey, 1989), and covalent incorporation may selectively enhance a reaction pathway and suppress unwanted side reactions. Within the context of this hypothesis, one needs to accommodate the finding that the same enzyme can be isolated as a covalent or noncovalent flavoprotein. Although catalyzing identical reactions, cholesterol oxidase from *Brevibacterium sterolicum* contains a noncovalently bound FAD (Li et al., 1993), whereas a second cholesterol oxidase from this organism (Croteau & Vrielink, 1996) and one from *Schizophyllum commune* are covalent flavoenzymes (Kenney et al., 1979b). Interestingly, monomeric sarcosine oxidases from *Arthrobacter*, *Streptomyces*, and *Bacillus* species (Nishiya & Imanaka, 1996), and rabbit kidney microsomes (Reuber et al., 1997) contain 1 mol/mol of covalently bound FAD, whereas the heterotetrameric sarcosine oxidase from *Corynebacterium sp. P-1* contains 1 mol/mol each of noncovalently bound FAD and covalently bound FMN (Willie et al., 1996).

For some enzymes, one is tempted to conclude that there is strong, but as yet undefined, evolutionary pressure to maintain the covalent flavin–protein link. In cases where careful analysis was undertaken, it was determined that membrane-bound eukaryotic and prokaryotic succinate dehydrogenases and related membrane-bound bacterial fumarate reductases from diverse organisms all harbor 8α-*N*3-histidyl-FAD (Ackrell et al., 1990). The same can be said for mitochondrial membrane-bound monoamine oxidases A and B, which have an 8α-*S*-cysteinyl-FAD linkage (Kearney et al., 1971; Yu, 1981; Hsu et al., 1989; Weyler et al., 1990; Chen et al., 1994a). On the other hand, there is a soluble fumarate reductase (Pealing et al., 1992) and a soluble monoamine oxidase (Schilling & Lerch, 1995) that are very similar to the membrane-associated forms, but the soluble forms contain noncovalently bound FAD. Perhaps the covalent bond prevents loss of flavin from a membrane protein, because rebinding of flavin would be much less likely in the membrane environment. Similarly, it might be advantageous to have covalently linked flavin in some bacterial enzymes located in the periplasm or to have eukaryotic enzymes localized in organelles where low levels of free FAD/FMN may exist. Alternatively, the existence of a covalent bond may have a structural basis. The portion of the polypeptide chain tethered to flavin would be held in place by the strong noncovalent forces that anchor the isoalloxazine ring. Such forces are evident in proteins containing noncovalently bound flavin. The provision of a covalent link to flavin would be much the same as a disulfide bond. However, although these types of crosslinks are important and often conserved during evolution, they may not necessarily be essential.

Although the existence of covalent flavoproteins has been known for many years, only recently has the role of covalent flavins in catalysis and their mechanisms of attachment to proteins been appreciated. This review focuses on the types of linkage found in covalent flavoproteins, the mechanisms by which these links are formed, and the potential roles of the links in catalysis. Substantial advances have been made in recent years in understanding the roles and mechanisms of formation of the covalent links to flavin. Consequently, in this review, we are able to address some of the questions posed in earlier reviews on this subject (e.g., see Singer & McIntire, 1984; Decker & Brandsch, 1991; Edmondson & De Francisco, 1992; Decker, 1993).

The covalent flavoproteins: Occurrence and identification

The covalent flavoproteins (comprising approximately 25 different enzymes) fall into two categories: those in which the flavin is attached to the protein at the 8α-methyl group and those where linkage is to the C6 atom of the flavin isoalloxazine ring (Table 1). The former category can be subdivided depending on the identity of the amino acid side chain linked to the flavin. Whereas Tyr, His, and Cys are able to link to the 8α-methyl of FMN or FAD, linkage to the C6 position of the flavin is restricted to Cys and FMN.

Covalent flavoproteins are generally identified by means of acid precipitation followed by proteolytic fragmentation of the polypeptide. Flavin-linked peptides are subsequently identified by fluorescence emission at acid pH (~3) before or after oxidation with performic acid. Alternatively, in a simpler procedure, the protein is electrophoresed in a polyacrylamide gel under denaturing conditions to separate noncovalently bound flavins and, following electrophoresis, flavin-type fluorescence is observed at acid pH before

Table 1. *Known flavoproteins containing covalently linked flavin redox centers*[a]

Enzyme	Sequence	Reference
6-*S*-Cysteinyl FMN		
Dimethylamine dehydrogenase	CNGAGTNSPGMN	Yang et al. (1995)
Trimethylamine dehydrogenase	CIGAGSDKPGFQ	Kenney et al. (1978)
		Boyd et al. (1992)
8α-*N*1-Histidyl FAD		
Cholesterol oxidase		Kenney et al. (1979b)
Cyclopiazonate oxidocyclase		Edmondson and Kenney (1976)
L-Galactonolactone oxidase		Kenney et al. (1979a)
L-Gulonolactone oxidase	VGGGHSPS	Koshizaka et al. (1988)
Thiamin dehydrogenase		Kenney et al. (1976)
8α-*N*3-Histidyl FAD		
Choline oxidase	DNPN(HSR)	Ohishi and Yagi (1979)
		Ohta-Fukuyami et al. (1980)
Dimethylglycine dehydrogenase	SELTAGSTWHAAGLTTYFMPGINLK	Cook et al. (1985)
		Lang et al. (1991)
Fumarate reductase	PMRSHTVAAEGGSAAVAED	Cole (1982)
6-Hydroxy-D-nicotine oxidase	SGGHNPNGYA	Bruhmuller and Decker (1973)
		Brandsch et al. (1987a)
Sarcosine dehydrogenase		Wittwer and Wagner (1981)
Succinate dehydrogenase	SHTVAA(E/Q)GGI(D/N)LAGG	Walker and Singer (1970)
		Robinson and Lemire (1992)
D-Gluconolactone oxidase		Harada et al. (1979)
2-Keto-D-gluconate dehydrogenase		McIntire et al. (1985)
D-Gluconate dehydrogenase		Singer and McIntire (1984)
Vanillyl-alcohol oxidase		de Jong et al. (1992)
8α-*N*3-Histidyl FMN		
Sarcosine oxidase	GIAKHDHVAWAFA	Chlumsky et al. (1993)
		Willie et al. (1996)
8α-*O*-Tyrosyl FAD		
p-Cresol methylhydroxylase	YNWRGGGSM	McIntire et al. (1981)
		Kim et al. (1994)
4-Ethylphenol methylenehydroxylase		Reeve et al. (1989)
8α-*S*-Cysteinyl FAD		
Flavocytochrome *c* sulfide dehydrogenase	YTCY	Kenney and Singer (1977)
		Dolata et al. (1993)
Flavocytochrome *c* sifite dehydrogenase	VTCPFSN	Kenney et al. (1977)
Monoamine oxidase A	SGGCY	Nagy and Salach (1981)
Monoamine oxidase B	SGGCY	Kearney et al. (1971)
		Walker et al. (1971)
		Nagy and Salach (1981)
Unknown linkage		
Berberine bridge enzyme	SGGHSYEGLS	Dittrich and Kutchan (1991)
		Kutchan and Dittrich (1995)
L-Pipecolic acid oxidase		Mihalik et al. (1991)
D-Sorbitol dehydrogenase		Singer and McIntire (1984)
Mitomycin resistance protein	ATGHPS	August et al. (1994)
D-Sorbitol dehydrogenase		Shinagawa and Ameyama (1982)
D-Fructose dehydrogenase		Ameyama and Adachi (1982)
Hexose oxidase		Groen et al. (1997)

[a]Amino acid residues shown underlined are those forming the covalent link to flavin. References to sequence information refer to either peptide or gene sequences or both. The mitomycin resistance protein is proposed to be a covalent flavoprotein, but formal proof is lacking.

or after spraying the gel with performic acid (necessary to overcome the internal quenching in thioether-linked flavins).

Determination of the identity of the chemical link to flavin in flavinylated peptides is not necessarily straightforward. For example, physical-chemical properties such as fluorescence and pK_a values of flavin titratable groups in flavinylated peptides may overlap and may be altered by the chemical composition of the peptide. Various approaches have been used to identify the nature of the

peptide-flavin link, ranging from simple chemical analysis/synthesis and UV-visible/fluorescence spectroscopic methods to NMR techniques. Mention of some of these methods can be found in the references displayed in Table 1. However, a general approach to the identification of the covalent link has been described by Singer and McIntire (1984), in which differential chemical reactivities and spectroscopic properties of the known flavin derivatives are exploited. The general scheme allows comparison of the behavior of an unidentified flavinylated amino acid with the behavior of the known flavin derivatives, thus enabling rapid identification of the unknown flavin.

In short, the general procedure for aminoacyl flavin identification involves proteolytic digestion of the flavoenzyme to generate a flavinylated peptide, followed by purification and dephosphorylation of the flavinylated peptide by treatment with nucleotide pyrophosphatase and alkaline phosphatase. The purified peptide is digested with aminopeptidase M to yield the flavin-linked amino acid, then the aminoacyl flavin is purified further by, e.g., ion exchange chromatography, reverse-phase HPLC, or thin-layer chromatography (TLC). A comparison with synthetic standards may aid identification at this stage. 6-*S*-Cysteinyl flavins are readily identified by virtue of their unusual flavin absorption spectra. A preliminary identification of 8α-substituted flavins is made by comparing the quantum yield of fluorescence at acid pH (<3.5) with that at neutral pH, and the enhancement of fluorescence after performic acid oxidation. 8α-*O*-Tyrosyl flavin becomes fluorescent only after reductive cleavage with dithionite, which yields free Tyr and free flavin (McIntire et al., 1981). The product of performic acid oxidation of 8α-*S*-cysteinyl flavin, 8α-*S*-cysteinylsulfonyl flavin, produces free cysteine sulfinic acid and free flavin on reduction. Released Tyr or cysteine sulfinic acid can be measured and quantitated by amino acid analysis. Additional tests (Singer & McIntire, 1984) for the identity of the aminoacyl flavins can also be made: for example, 8α-*N*1- and 8α-*N*3-histidyl riboflavins can be identified by high-voltage electrophoresis following sequential treatment with sodium borohydride, methyl iodide, and prolonged exposure to acid to yield *N*1- and *N*3-methylhistidine, respectively. *N*1- and *N*3-histidyl riboflavin also can be distinguished by paper electrophoresis at pH 5.0 or isoelectric focusing (McIntire et al., 1985). Prolonged acid treatment of 8α-*S*-cysteinyl riboflavin yields free Cys (detected with chloroplatinic acid) and, following dansylation of 8α-*O*-tyrosyl riboflavin and acid treatment, the liberated *N*-dansyltyrosine can be readily analyzed. Interestingly, 6-*S*-cysteinyl riboflavin is more light sensitive than riboflavin or other aminoacyl flavins. At low pH, 6-*S*-cysteinyl riboflavin gives rise to a blue fluorescent photoproduct and, at high pH, a nonfluorescent photoproduct. Because routes to the synthesis of 8α-aminoacyl flavins (Edmondson et al., 1978; Singer & Edmondson, 1980; McIntire et al., 1981) and 6-*S*-cysteinyl riboflavin (Ghisla et al., 1980) are known, unequivocal identification is made by comparing the mobility of the unknown sample with synthetic standards during, e.g., TLC, HPLC, or by mass analysis.

Covalent attachment of flavins: Protein-mediated or self-catalytic?

Early studies have concentrated on the determination and comparison of the amino acid sequences around the flavin linkage residue in attempts to identify sequence elements required for the specific recognition of a putative flavinylating enzyme. Proteolytic fragmentation of covalent flavoproteins has allowed the isolation of

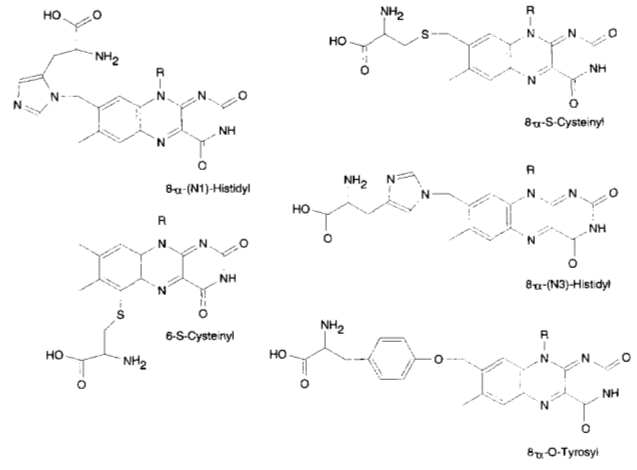

Fig. 1. Structures of the five flavin amino acids found in flavoenzymes.

flavinylated peptides in pure form, which, in turn, has enabled the determination of peptide sequence around the linkage residue. Using this approach, in many cases the sequences of flavinylated peptides from several covalent flavoproteins were determined before gene sequencing studies revealed the entire sequences of the enzymes. The flavinylated peptide studies revealed relatively little sequence homology around the flavinylation site among those enzymes containing the same chemical linkage to the flavin (Table 1). The only exceptions are for mammalian succinate dehydrogenase and bacterial fumarate reductase (which catalyze similar reactions), and bacterial choline oxidase and 6-hydroxy-D-nicotine oxidase. The former pair of enzymes share 11 identical amino acid residues in a stretch of 15 flanking the 8α-*N*3-histidyl linkage (mainly C-terminal to the covalent link to flavin), whereas the latter pair have 4 identical amino acids immediately on the N-terminal side of the covalent link to flavin. Other similarities in sequence around the site of attachment are restricted to enzymes that are related throughout the whole of their primary sequences, e.g., tri- and dimethylamine dehydrogenases (Boyd et al., 1992; Yang et al., 1995).

It has been observed that several covalent flavoproteins (succinate dehydrogenase, fumarate reductase, dimethylglycine dehydrogenase, monoamine oxidase, sarcosine oxidase) (Reuber et al., 1997), and flavocytochrome *c*:sulfide dehydrogenase (Lang et al., 1991), and the putative covalent flavoprotein fructosyl amino acid oxidase have Gly-X-Gly-X-X-Gly- dinucleotide binding-fold consensus sequences very close to the N-termini, which can be quite distant in the primary structure from the flavin attachment site. This motif is also found in enzymes with noncovalently bound flavin (van Driessche et al., 1996); however, L-gulono-γ-lactone oxidase, *p*-cresol methylhydroxylase, and 6-hydroxy-D-nicotine oxidase are covalent flavoproteins that do not have this so-called "fingerprint" (Brandsch, 1993; Kim et al., 1994). Additionally, the regions very near the flavin attachment sites in numerous covalent flavoproteins (see Table 1) display either -Gly-Gly- (succinate dehydrogenase, fumarate reductase, 6-hydroxy-D-nicotine oxidase, monoamine oxidase, berberin bridge enzyme), -Gly-Gly-Gly- (L-gulono-γ-lactone oxidase), -Gly-Gly-Gly-Gly- (*p*-cresol methylhydroxylase), or -Gly-Ala-Gly- (dimethyl- and trimethylamine dehydrogenases) motifs. Because the glycyl residues are least bulky, they could allow for flexibility (Yan & Sun, 1996) or accommo-

dation at the active (flavin) sites. Because the isoalloxazine, ribityl, phosphoryl, diphosphoryl, and adenyl moieties are quite large, the Gly groups surrounding the flavin may allow for necessary close approach of other bulkier amino acyl residues required for flavin attachment and/or catalysis.

Although similarities may exist in the short run, the overall poor conservation of longer, contiguous sequences around the residues that form the covalent links to flavin brings into question the idea of there being a common modifying enzyme responsible for flavin attachment, because specific recognition of the reactive residue is required to prevent covalent addition of the flavin to other identical residues in the polypeptide chain. Indirectly, the sequence studies suggested that the process of flavinylation is self-catalytic, and that specificity for the linkage residue is acquired after folding of the polypeptide chain. After folding, a recognition site for the flavin is formed in much the same way as for those flavoproteins that assemble noncovalently with flavin. For the covalent flavoproteins, however, the folded enzyme presents "catalytic" flavinylation residues at the active site that mediate formation of the covalent link to flavin. Until recently, little was known about the mechanisms by which FMN or FAD are linked covalently to the apoenzyme. In part, the disparate mechanisms proposed for covalent linkage may stem from the fact that the nature of the flavin cofactor linkage is variable, although some types are more prevalent than others. It is not surprising, therefore, that only a few common aspects have been defined within the family as a whole. Of the mechanisms that have been advanced for the covalent addition of flavin to protein, supportive evidence has been presented in only a few notable cases. In the following section, those covalent flavoproteins for which mechanisms have been proposed are considered and common features of these mechanisms are discussed. The potential or proven role of the covalent link to flavin is also discussed, where known.

p-Cresol methylhydroxylase (8α-*O*-tyrosyl FAD)

The enzyme *p*-cresol methylhydroxylase (PCMH) from *Pseudomonas putida* catalyzes the oxidation of *p*-cresol (4-methylphenol) to 4-hydroxybenzyl alcohol, which is subsequently oxidized to 4-hydroxybenzaldehyde by PCMH. The enzyme comprises two *c*-type cytochrome subunits and two flavoprotein subunits, each possessing a single FAD cofactor covalently associated at its 8α-carbon via an ether linkage with the phenolic oxygen of Tyr 384 (McIntire et al., 1981). The same link is found in the related enzyme 4-ethylphenol methylenehydroxylase (Reeve et al., 1989). The genes for the two component subunits of PCMH have been expressed in the heterologous host *E. coli*, singly and together (Kim et al., 1994). In either case, the heme is found to be covalently associated with the cytochrome subunit. In contrast, expression of the flavoprotein subunit alone leads to essentially deflavo enzyme even when expressed in the presence of excess FAD (Kim et al., 1995). However, when the flavoprotein and heme-containing subunits are co-expressed, or, alternatively, if cell extracts containing separately expressed subunits are mixed, flavin is found covalently associated with the flavoprotein subunit. Analysis has indicated that only FAD (as opposed to FMN and riboflavin) is linked, and attempts to "catalyze" covalent linkage with other *c*-type cytochromes were unsuccessful. In light of these investigations, FAD is thought to bind noncovalently to the apo-flavoprotein, but covalent linkage occurs only when the *c*-type cytochrome binds to the flavoprotein subunit. The model is supported further by the observation that the apo-flavoprotein subunit does not interact with the cytochrome subunit; in other words, the noncovalent binding of FAD triggers a conformational change in the flavoprotein subunit, enabling formation of the heterocomplex. These findings also suggest that, before FAD binding, the α2 flavoprotein is more or less in the properly folded (native) form, less the flavin. It is not known if native apo-flavoprotein assembly requires an external agent, e.g., a chaperon.

Formation of the 8α-*O*-tyrosyl link requires a two-electron reduction of the isoalloxazine ring, and reoxidation is therefore mediated by long-range electron transfer to the associated *c*-type cytochrome and then to other redox acceptors. The proposed mechanism for flavinylation of PCMH does not invoke the use of accessory enzymes/proteins (other than the *c*-type cytochrome subunit of the enzyme) for formation of the bond; the process is therefore thought to be "self-catalytic" (Fig. 2). The reaction involves the base-assisted removal of an 8α-proton from FAD, but, despite the availability of a structure for the enzyme (Mathews et al., 1991; Kim et al., 1995), there is no structural evidence to suggest the identity of the base (a carboxylate oxygen of Asp 440 is 5.5 Å from an 8α-hydrogen of FAD; Fig. 3). It is possible that structural changes after formation of the covalent link might disguise the identity of the base. Alternatively, the phenolate-form of Tyr 384 (the linkage residue) may itself abstract the proton, before donating

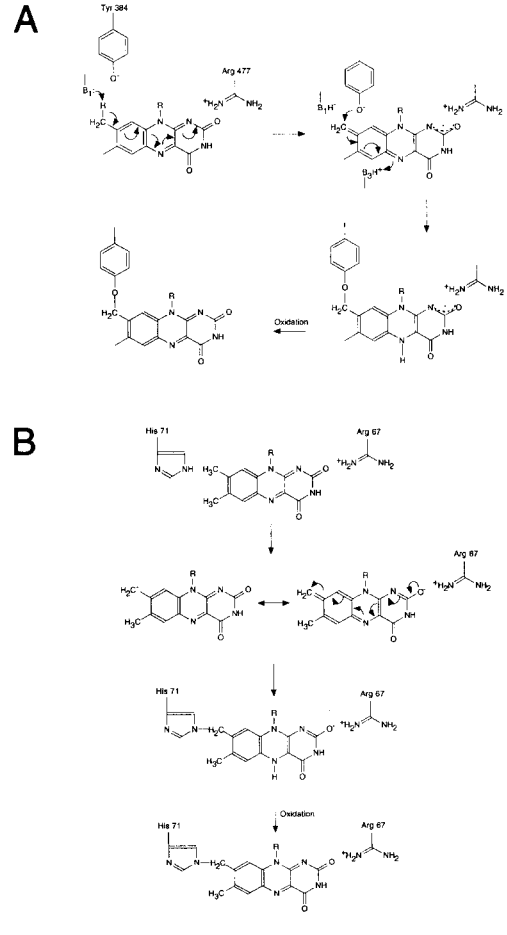

Fig. 2. Proposed reaction schemes for flavinylation of *p*-cresol methylhydroxylase (**A**) adapted from Kim et al. (1995); and 6-hydroxy-D-nicotine oxidase (**B**) adapted from Brandsch and Bichler (1991).

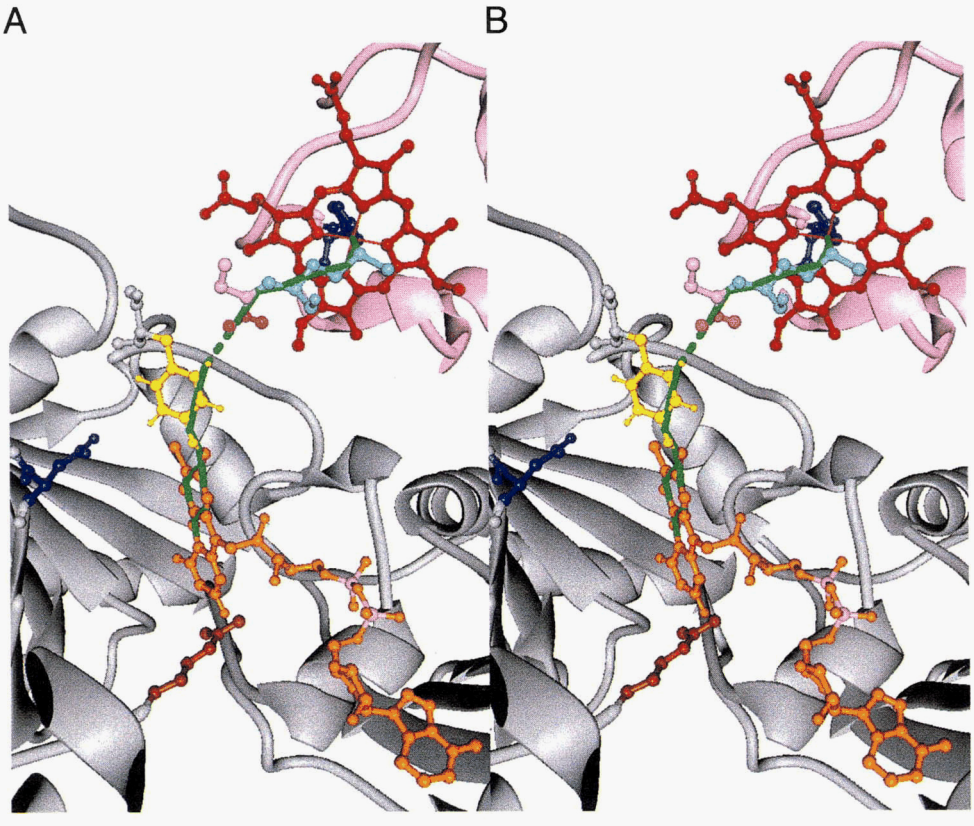

Fig. 3. Molecular graphics representation of a close-up stereo (wall-eye) view of the 8α-*O*-tyrosyl FAD and heme-binding sites of *p*-cresol methylhydroxylase. This is a cut-away view, for maximum clarity. Pink and grey portions are ribbon representations of the polypeptide backbones of the cytochrome and flavoprotein subunits, respectively. Color codes: gold, FAD (note that the adenine moiety is pointing to the lower right corner); yellow, Tyr 384, which is attached via its phenolic oxygen to the 8α-carbon of FAD; very dark blue, middle left, Asp 440; dark orange, below flavin, Arg 477; bright red, heme group (note that the covalent thioether links of the heme to the protein are not shown); cyan, below heme, Met 50 of the cytochrome subunit, which is axially bonded via its sulfur to Fe(III) of the heme; dark blue, above heme, His 19, the second axial ligand of Fe(III); dark pink, lone pair of the backbone carbonyl oxygen of Ala 49. It was proposed (Kim et al., 1995) that electrons tunnel from FAD to heme along the following path: 8α-carbon of FAD $\rightarrow$ Tyr 384 phenolic oxygen $\rightarrow$ several bonds of phenyl ring to the upper right hydrogen (small black sphere) of Tyr 384 $\rightarrow$ 2.96 Å jump from hydrogen to cytochrome via an Ala 49 backbone oxygen lone pair (dark pink) $\rightarrow$ through cytochrome backbone bonds to Met 50 $\rightarrow$ through bonds of the Met 50 side group to the sulfur ligand of heme Fe(III). The predicted pathway is illustrated in green.

it to another residue. Proton abstraction would convert the FAD into an iminoquinone methide form, with the 8α position being susceptible to attack by the phenolate oxygen of Tyr 384. This step would be facilitated by the presence of a positively charged residue in the N1 and C2 region of the flavin as seen for other attachment mechanisms (see below). This residue would serve to stabilize the negative charge as it develops at the N1/C2 region during the formation of the iminoquinone methide flavin. Arg 477 is a likely candidate for this role, because it is only 3.3 Å and 2.5 Å from the N1 and C2 atoms, respectively (Fig. 2). Either Tyr 172 or water are thought to donate a proton to the flavin N5 atom during linkage. In support of the mechanism, it is known that 2-electron reduction of protein-free 8α-*O*-tyrosyl riboflavin and 8α-*S*-cysteinylsulfonyl riboflavin causes expulsion of the aminoacyl group, producing oxidized riboflavin (McIntire et al., 1981; Edmondson & De Francisco, 1992). The principle of microscopic reversibility suggests that the reverse reaction occurs within the enzyme, without intervention of a flavinylating enzyme. The proposed self-catalytic mechanism has been in the literature for some time (Walsh, 1980, 1982; Edmondson & De Francisco, 1992) and a similar mechanism has been advanced for the nonenzymic base-catalyzed nucleophilic attack at the 8α position of riboflavin analogues in organic solvents (Frost & Rastetter, 1980). A refinement of the X-ray crystallographic structure and studies of flavinylation of PCMH by in vitro mutagenesis and related techniques should enable the proposed mechanism to be tested in future work.

Interestingly, the cleavage of the tyrosyl-FAD bond does not occur to any appreciable extent when FAD is reduced in intact flavocytochrome or intact cytochrome-free flavoprotein, although reductive cleavage is facile in denatured protein or isolated flavin peptides. There is probably stereochemical stabilization of this bond in the native flavoprotein; the dihedral angle associated with this bond does not allow for the proper arrangement of atomic orbitals required for Tyr elimination.

Until recently, the unusual PCMH FAD-binding fold was considered to be unique among other known structures. Recently, it was recognized that *Mur* B (UDP-*N*-acetylenolpyruvylglucosamine reductase), an enzyme required for bacterial cell wall biosynthesis (Bensen et al., 1995), has an FAD-binding fold very similar to that of PCMH, but *Mur* B contains noncovalently bound FAD. Several

other flavoproteins have moderate homology to the ADP-binding domain sequence of PCMH (Murzin, 1996).

A role for the function of the 8α-*O*-tyrosyl FAD of PCMH has been proposed recently (Kim et al., 1995). Inspection of the crystallographic structure of PCMH reveals that the covalent link is located within a probable electron transfer pathway between the flavin and the *c*-type cytochrome (Fig. 3). Analysis using the GREENPATH algorithm (Beratan & Onuchic, 1996) indicates that the optimal path for electron transfer from the N5 of the flavin to the iron of the heme passes through the phenolic moiety of Tyr 384, which is the residue that forms the 8α-*O*-tyrosyl FAD link. By having a covalent link at this position, electronic coupling to the heme will be improved because a "through-space-jump" in PCMH without the 8α-*O*-tyrosyl FAD would reduce the efficiency of wave function propagation during electron transfer. However, these arguments cannot be extended to another flavocytochrome that contains a covalent link to flavin: in flavocytochrome *c* sulfide dehydrogenase, electron transfer to heme is via the dihydrouracil portion of the flavin isoalloxazine ring, whereas the covalent link (8α-*S*-cysteinyl FAD) is attached to the dimethylbenzene moiety that is not implicated in electron transfer (Chen et al., 1994b; Mathews & Durley, 1996).

Vanillyl alcohol oxidase (VAO) from *Penicillium simplicissum* catalyzes the same reaction as PCMH, that is, oxidation of a *p*-hyroxybenzyl alcohol derivative to the corresponding aldehyde. The preliminary report of the X-ray analysis of the crystals of this enzyme appeared recently (Mattevi et al., 1997). It will be interesting to compare the active sites of VAO and PCMH, because the former enzyme has FAD linked via an 8α-(N3)-His linkage, but the latter has an 8α-*O*-Tyr bond to FAD. In addition, VAO is an oxidase, whereas PCMH is a dehydrogenase/electron transferase.

Monoamine oxidase (8α-*S*-cysteinyl FAD)

Monoamine oxidase (MAO) is an integral protein of the outer mitochondrial membrane and is a major amine-degrading enzyme in mammalian tissues (Singer & Ramsay, 1995). Two forms (A and B) have been identified, distinguishable by their substrate preference, and encoded by separate genes (Bach et al., 1988; Powell et al., 1989). Both MAO A and B genes map to human chromosome Xp11.23 (Lan et al., 1989). Each contains an FAD molecule covalently linked by an 8α-*S*-cysteinyl bond (Kearney et al., 1971; Walker et al., 1971; Nagy & Salach, 1981; Yu, 1981); the cofactor is linked to Cys 406 and Cys 397 in the A and B forms, respectively. Site-directed mutagenesis studies on MAO B have shown that removal of the covalent link by replacing Cys 397 with Ser or His eliminates activity (Gottowik et al., 1993; Wu et al., 1993). Likewise, replacement of Cys 406 in the A form results in complete loss of catalytic activity (Wu et al., 1993). The linked cofactor is therefore critical for biological activity.

Although there is no structural information available for the enzymes, mutagenesis experiments have been conducted on the basis of homology studies with other flavoproteins (Kwan et al., 1995; Zhou et al., 1995a). The homology studies indicate that the FAD is bound in a "dinucleotide binding domain" (DNBD). This domain comprises a β-sheet, the conserved sequence Gly-X-Gly-X-X-Gly, an α-helix, and a second β-sheet ending with a Glu residue. The Glu residue is thought to interact with the ribose of the AMP moiety of the FAD via a γ-COOH hydrogen bond. In MAO A and B, this residue is Glu 43 and Glu 34, respectively, and mutagenesis studies have shown that Glu 34 of MAO B is required for catalytic activity (Kwan et al., 1995). An additional residue (Tyr 44) has been identified as being important for catalysis and the incorporation of FAD into MAO B (Zhou et al., 1995a). This residue is thought to interact with the flavin isoalloxazine ring through its aromatic side chain; when Tyr 44 is exchanged for Phe, FAD is incorporated and full biological activity is retained. However, a less conservative exchange (e.g., to Ser or Ala) leads to poor FAD incorporation and low levels of biological activity.

Flavinylation of MAO B has been investigated by expressing the protein in COS cells devoid of riboflavin (Zhou et al., 1995b). When expressed under these conditions, inactive deflavo apoenzyme is produced that nevertheless correctly inserts into the outer mitochondrial membrane. The covalent attachment of FAD is therefore not required for correct targeting or membrane insertion. Expression of the protein in COS cells to which riboflavin has been added enables the purification of enzyme with approximately 75% wild-type activity. On the other hand, purified apo-MAO B is refractory to reconstitution by FAD. On the basis of these observations, the formation of the covalent link is thought to occur co-translationally. The authors favor a mechanism in which the FAD is activated chemically by phosphorylation or pyrophosphorylation prior to formation of the covalent link. They argue that phosphorylation or pyrophosphorylation of the 8α position provides a good leaving group to be displaced by the incoming thiolate of Cys 397. However, no enzyme activities have yet been reported that catalyze such chemical activation of the flavin, and, in principal, base-assisted attack of unmodified FAD might occur in much the same way as proposed for PCMH (see above). In support of the "nonactivated" mechanism, the synthetic "activated flavin," 8α-hydroxy-FAD, the hypothetical intermediate that is activated by phosphorylation, is linked no more readily than conventional FAD (Zhou et al., 1995b), although, in ^{14}C incorporation assays, it is impossible to dissect out contributions due to poor recognition of the analogue and those due to bond formation. To date, no evidence concerning the necessity of the covalent link in MAO B has been forwarded, but several possibilities have been discussed viz contribution to the stability or integrity of the enzyme, a role in substrate stereo-selectivity, modification of redox potential, or the need for cofactor economy.

The cDNA encoding the gene for MAO A from human liver has been expressed in *Saccharomyces cerevisiae* and the heterologously expressed enzyme assembles covalently with FAD and exhibits kinetic behavior similar to the human placental enzyme (Weyler et al., 1990). These observations are suggestive of a self-catalytic process for flavinylation, because the heterologous host is expected to be devoid of activating or linking enzymes. Mutation of the reactive Cys 406 to Ala in MAO A produces a mutant form that assembles noncovalently with FAD when expressed in *S. cerevisiae* (Hiro et al., 1996). Partial catalytic activity was initially retained, but lost with increasing time. The cofactor is lost rapidly during turnover of the mutant and the apo-form cannot be reconstituted by the addition of FAD. The authors suggest that the covalent link between FAD and MAO A is not required for catalytic ability per se, but rather provides a structural role in the maintenance of the active site of the enzyme.

A recent report provides details concerning the production of rat liver MAO B in the heterologous host *E. coli* (Hirashiski et al., 1995). Although the largest quantity of protein in the cell was MAO in inclusion bodies, some active enzyme was located in the inner membrane, and the latter contained covalently bound FAD. The properties of the active MAO were similar to that of enzyme

isolated from liver. Similar results were reported by another group for human MAO B produced in *E. coli* (Lu et al., 1996). Because it is very unlikely that *E. coli* produces an enzyme anything like MAO, these results suggest a self-catalytic mechanism for FAD attachment to the oxidase.

6-Hydroxy-D-nicotine oxidase (8α-*N*3-histidyl FAD)

Arguably, as regards flavinylation, the most extensively studied covalent flavoprotein is the enzyme 6-hydroxy-D-nicotine oxidase (6HDNO), originally isolated from *Arthrobacter oxidans*. The enzyme is a 48-kDa monomer that is involved in the degradation of nicotine. Peptide analysis of the enzyme revealed that it contains 8α-*N*3-histidyl FAD (Bruhmuller & Decker, 1973). Expression of the gene encoding 6HDNO in *E. coli* leads to the production of catalytically active enzyme that contains covalently linked FAD, as judged by the incorporation of ^{14}C-labeled riboflavin (Brandsch & Bichler, 1985). This observation argues strongly against the involvement of a flavinylating enzyme. Consequently, a self-catalytic model for flavinylation has been proposed, in which the adoption of a specific configuration by the nascent polypeptide facilitates binding and the attachment of FAD to the "reactive" His.

Expression of the gene encoding 6HDNO in an *E. coli*-based in vitro transcription/translation system leads to the production of apoenzyme that is refractory to reconstitution by FAD (Brandsch & Bichler, 1986). The authors conclude that flavinylation is likely to be co-translational. Before the gene sequence was determined (Brandsch et al., 1987b), the co-translational model was investigated by creating a series of genes truncated in the C-terminal and N-terminal regions (Brandsch et al., 1987a). Deletions of up to 40% in the C-terminal region had little effect on flavin incorporation, but flavinylation was found to be impaired in mutants truncated by more than 30 residues from the N-terminus. These investigations indicated that the FAD-binding His mapped approximately 70 residues from the N-terminus. Taken together, these findings were judged to support a co-translational model for flavinylation. On the other hand, these results raise serious questions regarding a self-catalytic flavinylation mechanism. How is it that C-terminal truncated proteins, with as much as 40% of the sequence missing, are able to fold into native, apo-enzymes with properly formed FAD-binding sites? Is it possible that the N-terminal and C-terminal portions fold independently into separate domains?

Following hyperexpression of the 6HDNO gene in *E. coli*, more than 50% of the expressed enzyme is found in the apo-form (Brandsch & Bichler, 1987). The apoenzyme can be reconstituted using FAD and an "energy-generating" system, consisting of ATP, phosphoenolpyruvate, and pyruvate kinase. On the basis of these experiments, the authors erroneously suggested that flavinylation was, after all, mediated by a specific flavinylating enzyme and an energy-dependent process. However, this model was rapidly superseded by a self-catalytic model following the discovery that only the phosphoenolpyruvate component of the "energy-generating" system was required to reconstitute the apoform of 6HDNO (Nagursky et al., 1988). In later studies, other 3-carbon compounds (e.g., 3-phosphoglycerate, glyceraldehyde-3-phosphate, and glycerol-3-phosphate) were found to mediate flavinylation, and flavinylation was found to protect the enzyme from proteolysis (Brandsch et al., 1989).

The expression of 6HDNO as a fusion protein has enabled purification of the apoenzyme. Using this material in ^{14}C FAD incorporation assays, flavinylation was shown to be self-catalytic in vitro (Brandsch & Bichler, 1991). Reconstitution with FAD requires the presence of the 3-carbon mediating factors, which are thought to act as allosteric effectors, stabilizing a protein conformation favorable for flavinylation. Other work has focused on the isolation of site-directed mutants of 6HDNO in which the "reactive" His was targeted (Mauch et al., 1989). None of the mutants (incorporating either Tyr, Cys, or Ser for His) assembled covalently with FAD, and only two mutants (Ser and Cys) bound FAD noncovalently. In the absence of a structure for the enzyme, it is difficult to propose a precise mechanism for the self-catalytic flavinylation of 6HDNO. However, limited mutagenesis has been performed in the region of the FAD attachment site. Arg 67 was identified as being critical for the flavinylation process (Mauch et al., 1990) and mutagenesis of this residue demonstrated the requirement for positive charge at this position. Additionally, attempts to reconstitute the apo-form of 6HDNO with various flavin analogues were unsuccessful. The failure of the enzyme to covalently incorporate 5′-deaza-FAD was taken to indicate that the quinone methide form of the FAD was a likely intermediate in the flavinylation mechanism (Brandsch & Bichler, 1991). In light of these observations, a reaction mechanism for the formation of the 8α-*N*3-histidyl FAD bond has been proposed (Fig. 2).

Recent studies have focused on the requirement for flavinylation in the mitochondrial import of 6HDNO-dimethylglycine dehydrogenase fusions (Stoltz et al., 1995), the interaction of 6HDNO with the chaperone protein GroE (Brandsch et al., 1992), and the covalent incorporation of flavin analogues (modified in the adenine moiety of FAD) into the active site of the enzyme (Stoltz et al., 1996a, 1996b). In oxidase, where the FAD-linking His residue was replaced with Cys, noncovalently bound FAD could be displaced rapidly by added 8-methylsulfonyl-FAD or 8-chloro-FAD (Stolz et al., 1996b). Either becomes covalently attached to the substitute Cys to produce the covalent 8-*S*-cysteinyl-FAD, not 8α-*S*-cysteinyl-FAD as in the normal enzyme. [Note: The 8-*S*-cysteinyl-FAD bound to the mutant form of 6HDNO was misidentified as 8-*N*-acetylcysteinyl-FAD by Stoltz et al. (1996a). The latter is the synthetic model compound used for comparison of physical properties (see Massey et al., 1979, not Moore et al., 1979, as quoted by Stoltz et al.). Additionally, the model compound should more appropriately be called 8-*S*-(*N*-acetylcysteinyl)-FAD.] This strategy allows incorporation of FAD analogues with varying properties into this oxidase, and the method should be useful with other covalent flavoproteins.

Succinate dehydrogenase/fumarate reductase (8α-*N*3-histidyl FAD)

Succinate dehydrogenase (SDH), the first identified covalent flavoprotein, contains an 8α-*N*3-histidyl FAD cofactor (Walker & Singer, 1970; Walker et al., 1972). In bacteria, the enzyme is a cell membrane-bound complex, whereas, in eukaryotes, the complex is located in the matrix side of the inner mitochondrial membrane. The enzyme catalyzes the oxidation of succinate to fumarate in aerobic organisms and eukaryotes. The reverse reaction occurs in anaerobic organisms through the action of the genetically related fumarate reductase complex (FRD), which also contains the same covalently linked FAD. Both complexes usually comprise four nonidentical subunits (Robinson & Lemire, 1995); the flavoprotein subunit (about 70 kDa), the iron–sulfur protein (about 27 kDa, containing three iron–sulfur clusters), and two hydrophobic membrane-anchoring subunits (about 13 and 16 kDa). The flavo-

protein domains of SDH and FRD contain the active sites of the enzymes, and are highly conserved at the amino acid level around the sites of flavin attachment. Initially, this was taken to indicate that formation of the covalent linkage with FAD is mediated by an auxiliary enzyme specific for this potential recognition sequence, but, in light of recent work, this now seems improbable.

Four mutant strains of *Bacillus subtilis* that lack SDH activity have been isolated by Hederstedt (1983). The mutants assemble correctly in the membrane, possess intact Fe-S clusters, but are totally devoid of FAD. Initially, it was thought that the mutants were defective for the notional flavinylating enzyme, or that the structural gene for the flavoprotein subunit carried a lesion at the site of FAD attachment. Sequencing revealed that all the flavoprotein subunits possessed mutations, although none were located at the codon for His 40, by then identified as the linkage residue (Maguire et al., 1986). One strain possessed a mutation at Gly 47, part of the conserved sequence around His 40. However, other strains carried mutations up to 100 residues beyond His 40 in the primary structure. The data were taken to indicate that the correct formation of a binding site for FAD by residues throughout the peptide chain is necessary for flavinylation to proceed. A requirement for cell-specific factors for FAD attachment was demonstrated by the expression of the *B. subtilis sdh* operon in *E. coli* (Hederstedt et al., 1987). Heterologous expression in *E. coli* leads to the flavoprotein subunit lacking covalently bound FAD, and being incorrectly processed at the N-terminus.

Recently, the covalent attachment of FAD to SDH from *S. cerevisiae* has been investigated (Robinson et al., 1994; Robinson & Lemire, 1995, 1996). In this species, the flavin is attached to His 90 of the flavoprotein subunit. Using antiserum specific for flavin, flavinylation was studied in vivo and in vitro. The data revealed that flavinylation takes place within the mitochondrial matrix, after the import of the flavoprotein subunit and the cleavage of a leader peptide sequence. The presence of citric acid cycle intermediates, such as succinate and malate, stimulate flavinylation, but are not absolutely required for the process. In this regard, the flavinylation of SDH is analogous to the requirement for three carbon molecules for the flavinylation of 6HDNO (see above). Again, it is suggested that the binding of these molecules to the active site may stabilize a protein conformation that allows flavinylation to proceed. SDH also interacts with the mitochondrial chaperone protein hsp60 and flavinylation is enhanced in the presence of hsp60. C-terminal deletion mutants of *S. cerevisiae* SDH with up to 90 residues removed (far from the postulated FAD binding site) do not recognize FAD, despite being correctly imported and processed (Robinson & Lemire, 1996). These data were taken to indicate that a precise structural configuration of the flavoprotein subunit is required for flavinylation. A mutant flavoprotein subunit in which His 90 is exchanged for Ser is processed correctly and translocated into the mitochondria for assembly into the SDH complex; the mutant binds FAD noncovalently (Robinson et al., 1994). Interestingly, this complex is catalytically inactive with succinate, but is still able to reduce fumarate. In corresponding studies of the *E. coli* FRD complex, the residue involved in the formation of the covalent link, His 44, has been exchanged for either Arg, Cys, Ser, or Tyr, and the flavoproteins were expressed in an *E. coli* strain lacking the gene for the wild-type protein (Blaut et al., 1989; Ackrell et al., 1990). The mutant complexes assemble correctly, and contain stoichiometric quantities of noncovalently associated FAD. With the exception of the H44R flavoprotein subunit, these complexes catalyze the reduction of fumarate, albeit in a reduced capacity. However, none of the complexes oxidize succinate, indicating a requirement for the 8α-*N*3-histidyl covalent bond.

The above observations have been rationalized on the basis of flavin redox potential (Blaut et al., 1989). The midpoint potential of succinate is +30 mV, whereas that for menaquinol (the natural electron donor for fumarate reduction) is −74 mV. The reduced activity of the mutant complexes with fumarate and the inability to oxidize succinate might reflect an altered flavin redox potential, as a consequence of the lack of the covalent linkage and/or changes to the active site environment. Measurement of the redox potentials of the flavins in the mutant complexes should address this hypothesis.

Dimethylglycine dehydrogenase (8α-*N*3-histidyl-FAD)

Dimethylglycine is converted to sarcosine by dimethylglycine dehydrogenase and this enzyme, and the related sarcosine dehydrogenase, are located in the mitochondrial matrix. Using rabbit reticulocyte lysate, rat cells, and stably transfected HepG2 cells, it was demonstrated that the presence of higher than normal FAD levels accelerated the formation of soluble holo-enzyme in the mitochondrial matrix (Otto et al., 1996), and that the flavinylation did not require external factors. Reduced levels of riboflavin in tissue culture medium led to reduced amounts of precursor and mature forms (the precursor minus a signal sequence) of the dehydrogenase in the mitochondria. Interestingly, the precursor and cytoplasmically produced mature forms could be transported efficiently into the matrix in the HepG2 cells. However, only the precursor form was imported to the matrix in isolated rat cells.

Sarcosine oxidase (8α-*N*3-histidyl-FMN)

Some bacteria can be grown on sarcosine as the sole source of carbon. The induced sarcosine oxidase (SOX) is responsible for the oxidative demethylation of sarcosine, to produce 5,10-methylenetetrahydrofolate, glycine, and H_2O_2. The structural genes for the subunits of SOX from *Corynebacterium sp. P-1* were isolated and expressed in *E. coli*. As in the normal enzyme, the isolated recombinant heterotetramer contained 1 mol/mol each of noncovalently bound FAD, covalently bound FMN, and tightly bound NAD^+ (Chlumsky et al., 1993; Willie & Schuman-Jorns, 1995; Willie et al., 1996). Although the cofactor content is identical for normal and *E. coli*-produced enzyme, the physical-chemical and biochemical properties are quite different for the two forms. Unlike the normal SOX, about 50% of the FAD in the recombinant enzyme exists as a reversible 4a-adduct with a Cys group.

Sarcosine oxidase (8α-*N*3-histidyl-FAD)

Monomeric SOX, containing one molecule of covalently bound FAD per subunit, and its structural gene was cloned from a *Bacillus* species and sequenced. Normal enzyme was expressed in *E. coli*, and it was found that FAD added to crude extracts increased the yield of active enzyme threefold, indicating that the bulk of the recombinant oxidase was manufactured in the apo-form (Koyama et al., 1991). The gene for SOX from *Streptomyces* sp. KB210-8SY was cloned and overexpressed in *Streptomyces lividans* (Suzuki et al., 1992), and genes for SOX from another *Bacillus* species and an *Arthrobactor* species have also been cloned and sequenced (see Reuber et al., 1997).

This year, the gene for a monomeric rabbit liver peroxisomal SOX was cloned and functionally expressed in *E. coli* (Reuber et al., 1997). This liver enzyme is probably the same as L-pipecolic acid oxidase, an enzyme thought to be involved in Zellweger syndrome, and possibly other human maladies. Although the rabbit enzyme shows 25–28% identity with monomeric SOX from a variety of microorganisms, it has not been demonstrated that the peroxisomal enzyme contains 8α-*N*3-histidyl-FAD (as do the oxidases from microorganisms) or 8α-*N*3-histidyl FMN. With all the work that has been done on SOX from microorganisms and from mammals, the site of attachment in the polypeptide is still unknown. Comparison of the amino acid sequences of these monomeric oxidases with that of the FMN-binding protein of the heterotetrameric SOX (see below) is of no aid in this regard, because there are no sequence homologies. On the other hand, His 49 of rabbit SOX aligns with the FAD-binding His 84 of dimethylglycine dehydrogenase (Reuber et al., 1997).

Cholesterol oxidase (8α-*N*1-histidyl FAD)

This bacterial enzyme oxidizes the 3β-hydroxyl group of steroids to the corresponding ketones. The enzyme can also isomerize a double bond at the steroid Δ^5 position to one at the Δ^4 position (Croteau & Vrielink, 1996). As mentioned earlier, *Brevibacterium sterolicum* produces one cholesterol oxidase containing noncovalently bound FAD, and another with FAD covalently attached to a His residue. A His 6-fusion protein of the second form of this oxidase was produced in *E. coli*. The resulting purified soluble protein contained covalently bound FAD, implying a self-catalytic process for flavinylation. The oxidase has been crystallized (Croteau & Vrielink, 1996). Currently, the crystal structures are known for PCMH (8α-*O*-tyrosyl-FAD), TMADH (6-*S*-cysteinyl-FMN; see below), and flavocytochrome *c*552 (8α-*S*-cysteinyl-FAD), and the analysis of the crystal structure of vanillyl oxidase (8α-*N*1-histidyl-FAD) is in progress (Mattevi et al., 1997). Hopefully, the structure of the cholesterol oxidase will soon be solved. Because it contains an 8α-*N*1-histidyl-FAD moiety, the structures of enzymes with five different types of covalent linkages will be available.

L-Gulono-γ-lactone oxidase (8α-*N*1-histidyl FAD)

Native rat liver microsome L-gulono-γ-lactone oxidase containing covalently bound FAD was produced in a *Baculovirus*/insect cell expression system. When the cells were grown under riboflavin-deficient conditions, mostly native apoenzyme was produced. Addition of FAD to a cell extract resulted in a significant increase in enzyme activity. It was demonstrated that all the flavin was bound noncovalently to the apoenzyme, although the specific activity was about the same as enzyme containing covalently bound FAD (Nishikimi et al., 1994b).

The gene for the nonfunctional human oxidase was mapped to chromosomal location 8p21.1 (Nishikimi et al., 1994a). This oxidase catalyzes the last step in the biosynthesis of L-ascorbic acid in higher animals. Thus, the L-gulono-γ-lactone oxidase deficiency is responsible for the human body's inability to manufacture vitamin C.

Trimethylamine dehydrogenase (6-*S*-cysteinyl FMN)

Trimethylamine dehydrogenase (TMADH) and dimethylamine dehydrogenase (DMADH) are the only proteins known to contain covalent linkage via the C6 atom of the flavin. These enzymes catalyze the oxidative demethylation of trimethylamine and dimethylamine, respectively, to form formaldehyde for anabolism in methylotrophic bacteria (Steenkamp & Mallinson, 1976; Meiberg & Harder, 1979). Each enzyme comprises a 4Fe-4S center and FMN covalently linked to the protein as a 6-*S*-cysteinyl FMN. They are the first and only enzymes discovered to contain covalently bound flavin with substitution in other than the 8α position. Evidence for substitution at the C6 position was gained from UV-visible spectroscopy of a flavinylated peptide (Steenkamp & Singer, 1976; Steenkamp et al., 1978), NMR data, comparison of spectroscopic data with 6-hydroxy flavins and reaction of *N*-ethylmaleimide with the product of anaerobic photolysis of the coenzyme (Steenkamp et al., 1978), and comparison with synthetic 6-*S*-cysteinyl-riboflavin (Ghisla et al., 1980).

The mechanism of formation of the covalent link has been studied with TMADH. Expression of the cloned gene (Boyd et al., 1992) in the heterologous host *E. coli* leads to partial flavinylation of the enzyme (Scrutton et al., 1994; Packman et al., 1995), suggesting that formation of the 6-*S*-cysteinyl FMN link is self-catalytic. On this basis, a flavinylation mechanism was proposed (Fig. 4), which is supported by studies of selected mutant enzymes by spectroscopic methods and investigations by electrospray ionization mass spectrometry (Packman et al., 1995; Mewies et al., 1996). The exchange of residue Arg 222 for Val or Glu leads to the production of deflavo enzyme, whereas replacement by Lys (retention of positive charge) compromises, but does not abolish, flavinylation. These results are consistent with the need for a positively charged residue in the region of the N1 atom and C2 carbonyl of the flavin, as predicted by the mechanism. The exchange of His 29 for Gln severely compromises, but does not abolish flavinylation, consistent with the proposed role for the imidazole side chain of His 29 in stabilizing the thiolate anion (the reactive nucleophile) of Cys 30. Recent crystallographic studies (Mathews et al., 1996) indicate that competition for the ribityl phosphate-binding site between FMN and an inorganic anion (probably inorganic phosphate or sulfate) is a possible cause of partial flavinylation of the wild-type enzyme expressed in *E. coli*. Additionally, the expression level of the wild-type protein in *E. coli* is

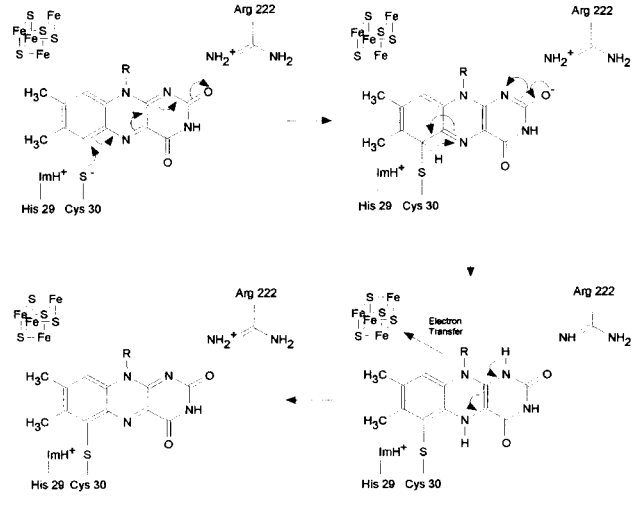

Fig. 4. Proposed mechanism for formation of the 6-*S*-cysteinyl FMN bond in trimethylamine dehydrogenase as proposed by Scrutton et al. (1994).

much higher than in the natural host, *Methylophilus methylotrophus*. Because flavin biosynthesis in *E. coli* is constitutive, the poor supply of FMN to the enzyme during folding may contribute to the production of deflavo TMADH (Packman et al., 1995; Mewies et al., 1996). Fully folded deflavo enzyme cannot be reconstituted by FMN, which may reflect difficulty in expelling the inorganic anion from the ribityl phosphate-binding site (Mathews et al., 1996).

The role of the 6-*S*-cysteinyl FMN in TMADH has been investigated by isolating a C30A mutant in which the 6-*S* cysteinyl FMN bond cannot form. The mutant assembles noncovalently with FMN and is catalytically active (Scrutton et al., 1994). Stopped-flow studies revealed that the reductive half-reaction of the mutant enzyme is not substantially compromised (Huang et al., 1996). However, multiple turnovers of the C30A mutant lead to progressive inactivation of the enzyme due to conversion of the enzyme-bound FMN to 6-hydroxy FMN. A mechanism for this inactivation was proposed, which involves a substrate–flavin N5 adduct formed during the reductive half-reaction of the enzyme. Recent work, however, on a W355L mutant (and to a much lesser extent, the wild-type enzyme) has demonstrated that the enzyme is purified with 6-hydroxy FMN, i.e., modification of the flavin does not require the presence of substrate (Mewies et al., 1997). In light of these data, it seems likely that modification of the flavin occurs through an electrophilic iminoquinone methide intermediate, similar to that proposed for the flavinylation mechanisms of PCMH and 6HDNO (Fig. 2). The same mechanism may also occur for the C30A mutant, with the role of substrate being to induce minor structural change around the flavin to favor hydroxylation at C6. The C6 atom is expected to be more electrophilic when the flavin is in the iminoquinone methide form. Inactivation of the flavin by solvent-derived hydroxide demonstrates that the C6 position of the flavin is susceptible to attack by nucleophiles. In this regard, the proposed inactivation chemistry supports the mechanism for flavinylation (which may also occur via a flavin methide intermediate, cf. Fig. 2), because the latter also invokes a nucleophilic attack by Cys 30 at the C6 position of FMN. As discussed recently, flavinylation at C6 in TMADH has probably evolved to prevent modification of the flavin during catalysis (Mewies et al., 1997). Also, linkage at the 8α-methyl would prevent formation of an iminoquinone methide in other covalent flavoproteins (Mewies et al., 1997). Consequently, it is tempting to speculate that covalent flavoproteins might have evolved in general to suppress hydroxylation at C6 in those cases where the electronic structure of the isoalloxazine ring, as dictated by its protein environment, and accessibility to the C6 atom of the flavin, renders the C6 position susceptible to nucleophilic attack. Small amounts of 6-hydroxy flavin have been found in *Peptostreptococcus elsdenii* electron-transferring flavoprotein and pig liver glycolate oxidase (Mayhew et al., 1974). Glycolate oxidase containing the derivatized flavin is inactive and the properties of the electron-transferring flavoprotein are perturbed substantially when bound to 6-hydroxy FAD. The very small quantities of 6-hydroxy flavin in these proteins indicates that the hydroxylation chemistry is inefficient, and this is likely due to poor accessibility of the C6 atom to water and/or inability to stabilize the flavin iminoquinone methide tautomer.

Concluding remarks

In recent years, substantial advances have been made in understanding the mechanisms of attachment of flavin to protein. Progress in the field has moved from an essentially taxonomic phase, in which the different types of covalent link were identified, to a mechanistic phase that has led to an appreciation of the chemistry of covalent attachment. With a few enzymes, notably PCMH and TMADH, the role of the covalent link in the biological function of the enzyme is beginning to emerge. This more recent work dawns yet another transition in covalent flavoenzyme research—that of understanding the functional consequences of covalent linkage.

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