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Forum Review

The Covalent FAD of Monoamine Oxidase: Structural and Functional Role and Mechanism of the Flavinylation Reaction

DALE E. EDMONDSON and PAIGE NEWTON-VINSON

ABSTRACT

The family of flavoenzymes in which the flavin coenzyme redox cofactor is covalently attached to the protein through an amino acid side chain is covered in this review. Flavin-protein covalent linkages have been shown to exist through each of five known linkages: (a) 8α-*N*(3)-histidyl, (b) 8α-*N*(1)-histidyl, (c) 8α-*S*-cysteinyl, (d) 8α-*O*-tyrosyl, or (e) 6-*S*-cysteinyl with the flavin existing at either the flavin mononucleotide or flavin adenine dinucleotide (FAD) levels. This class of enzymes is widely distributed in diverse biological systems and catalyzes a variety of enzymatic reactions. Current knowledge on the mechanism of covalent flavin attachment is discussed based on studies on the 8α-*S*-cysteinylFAD of monoamine oxidases A and B, as well as studies on other flavoenzymes. The evidence supports an autocatalytic quinone-methide mechanism of protein flavinylation. Proposals to explain the structural and mechanistic advantages of a covalent flavin linkage in flavoenzymes are presented. It is concluded that multiple factors are involved and include: (a) stabilization of the apoenzyme structure, (b) steric alignment of the cofactor in the active site to facilitate catalysis, and (c) modulation of the redox potential of the covalent flavin through electronic effects of 8α-substitution. Antioxid. Redox Signal. 3, 789–806.

INTRODUCTION TO COVALENT FLAVINS IN BIOLOGICAL SYSTEMS

FLAVIN-CONTAINING ENZYMES catalyze a variety of biological redox reactions and are ubiquitous to all organisms. The aromatic, heterocyclic flavin ring [shown as its flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) forms, Fig. 1] can function in either one- or two-electron transfer reactions either as the free form or as bound to its partner apoenzyme. The biological function of the flavin ring is modulated by its binding to and specific interactions with its protein environment. The molecular elucidation of how these interactions contribute to the altered chemistry of the flavin ring system has been and continues to be an area of active research. For an insight into the history as well as current work in the field, the reader is referred to a series of symposium volumes that are published approximately every 3 years in conjunction with international symposia on Flavins and Flavoproteins since the inception of this series in 1965 (78) to the present (27).

Although most of the known flavoenzymes bind their flavin coenzymes through noncovalent forces, there is a subset of enzymes in which the flavin moiety is bound to the protein by a covalent linkage to an amino acid side chain. The first known example of covalent flavins occurring in a biological system was

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322.

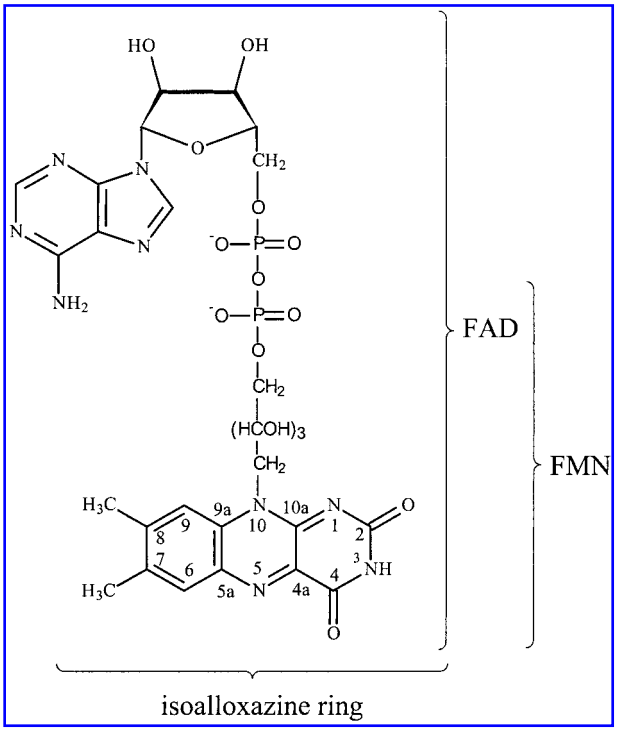

FIG. 1. Structure of the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) levels of the flavin cofactor. The conventional numbering scheme of the isoalloxazine is shown.

that of the inner mitochondrial enzyme succinate dehydrogenase (76, 77). Through a series of structural and physical studies as well as confirmation by chemical synthesis (26, 86, 89), the structure of the covalent flavin in succinate dehydrogenase was determined to be 8α-*N*(3)-histidylFAD (Fig 2). Subsequent structural studies on the covalent flavin found in the outer mitochondrial membrane-bound enzyme monoamine oxidase (MAO) demonstrated it to be 8α-*S*-cysteinylFAD (38, 39, 87, 88) (Fig. 2). Since the early 1970s, other forms of covalent flavin coenzymes from a variety of purified flavoenzymes have been determined. They include 8α-*N*(1)-histidylFAD (l-gulono-γ-lactone oxidase) (40), 8α-*O*-tyrosylFAD (*p*-cresol methylhydroxylase) (51, 52), and 6-*S*-cysteinylFMN (trimethylamine dehydrogenase) (41, 82) (Fig 2). It is of interest to note that only one known example exists for either 8α-*O*-tyrosylFAD or 6-*S*-cysteinylFMN linkages, with both being found in bacterial enzymes. Presumably these types of covalent flavins also occur in other flavoenzymes from other sources, and it remains for future work to identify them. Since these initial structural determinations, a number of flavoenzymes from mammalian, bacterial, and plant sources have been demonstrated to contain covalent flavin coenzymes. A recent review of the field (53) lists those known at this time, and the reader is referred to that article for further details. In addition, Table 1 lists those enzymes containing covalent flavin for which the three-dimensional structure has been solved by x-ray crystallography.

The occurrence of these covalent flavins in flavoenzymes leads to several questions that will be considered in this review. One question is what mechanistic and/or structural advantage, if any, is imparted to an enzyme that contains a covalent flavin rather than a conventional noncovalent flavin? Additionally, the question of the mechanism responsible for covalent flavin incorporation into proteins has received considerable attention and is important to our understanding of posttranslational modifications of key metabolic enzymes and their bioactivation. To address these questions, we will summarize studies from our laboratory that have used the outer-membrane mitochondrial enzymes MAO A or MAO B. Whenever possible, similar studies of other enzymes containing other 8α-amino acid substitutions will be cited and compared. The MAOs are of physiological relevance because their natural substrates include neurotransmitters such as serotonin and dopamine. Furthermore, there is a vast literature on the inhibition of these enzymes by both irreversible and reversible inhibitors and their present and potential clinical use (28, 37, 83, 92).

Detection and occurrence of covalent flavins

The classical procedure used to detect the presence of covalent flavin coenzymes consists of purifying the enzyme of interest and determining if the flavin moiety can be released from the protein on acid precipitation. The observation of acid-precipitable flavin constitutes good evidence for a covalent linkage.

A useful and general method to identify the presence of covalently bound flavins in flavoproteins is to perform western blot analysis on cell lysates subjected to denaturing gel elec-

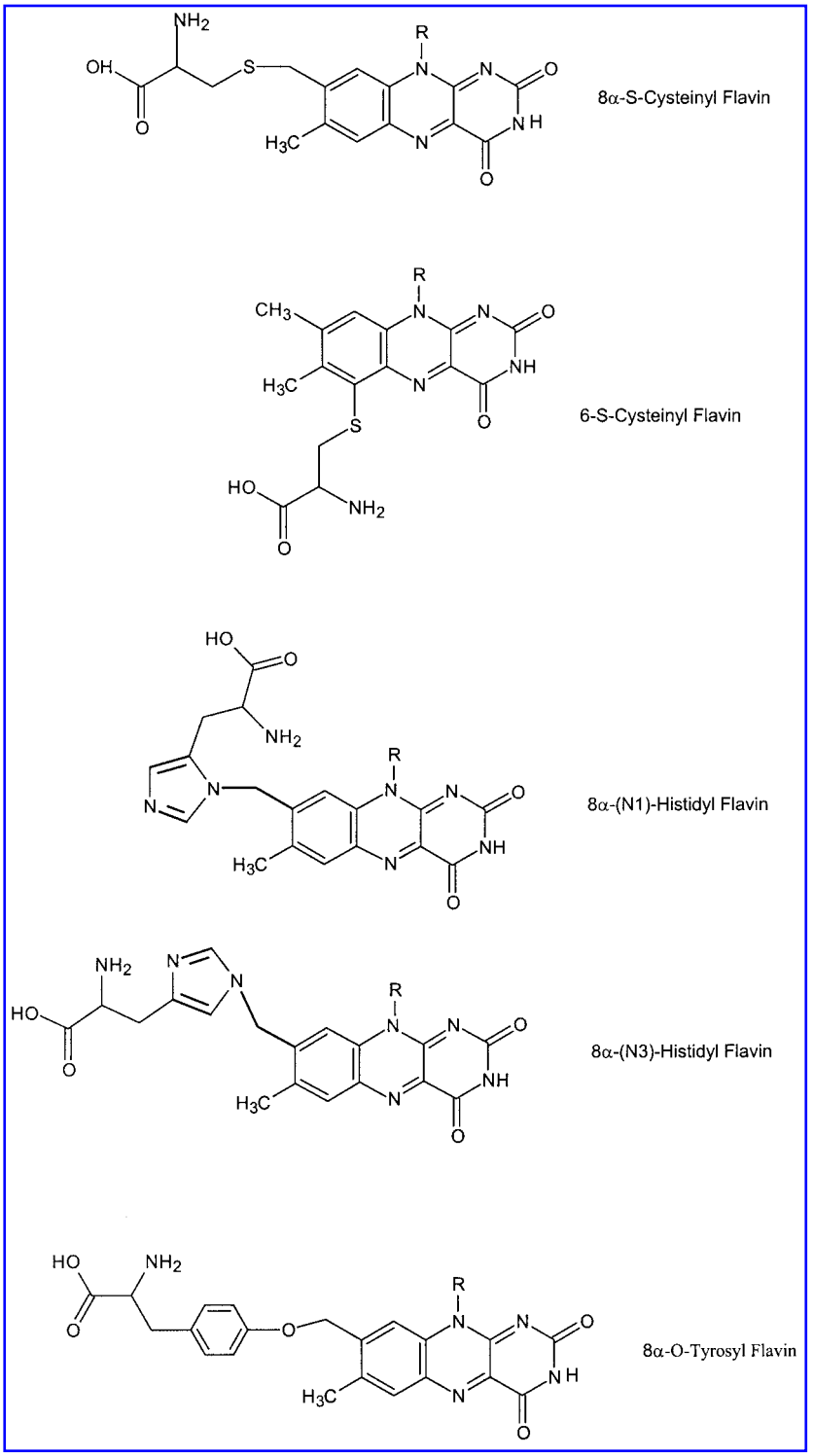

FIG. 2. Structures of the five known covalent flavin linkages to amino acids.

trophoresis. Under these conditions, noncovalent flavins are released from their respective flavoproteins and will not be retained with the protein in the gel. Only covalent flavin will remain protein-bound and be detected at the position corresponding to the molecular weight of its associated enzyme. This approach is based on the method published by Barber *et al.* (1) in which FAD is covalently linked (via the adenosyl moiety) to bovine serum albumin and rabbit polyclonal antiserum is raised against this antigen. The antiserum reacts with the

Table 1. Flavoproteins Containing Covalent Flavin Cofactors with Known Three-Dimensional Structures

Flavoprotein	*PDB identifier*	*Reference*
Monomeric sarcosine oxidase	1B3M	(84)
p-Cresol methylhydroxylase	1DII	(11)
Trimethylamine dehydrogenase	2TMD	(2)
Fumarate reductase	1FUM and 1QLA	(35, 45)
Flavocytochrome *c* sulfide dehydrogenase	1FCD	(10)
Vanillyl-alcohol oxidase	1VAO	(47, 48)

known covalent flavins and exhibits the highest sensitivity to the covalent 8α-histidylflavin-containing proteins. Our laboratory has found that the antiserum reacts with either the FAD or FMN form of covalent flavins as well as a wide variety of flavin analogues that have been covalently incorporated into MAO A or MAO B as 8α-*S*-cysteinylflavins (54). The *N*(5)flavocyanine adduct of MAO A or B formed by acetylenic inhibitors such as pargyline is also detected by using this method. Therefore, this approach to detect covalent flavins, in various samples offers the advantage of sensitivity (the limit of detection is ~10 pmol) and a broad selectivity, and can readily be performed with standard laboratory equipment.

The determination of the nature and sequence location of the flavin linkage requires proteolytic digestion of the protein and purification of the flavin peptide. 8α-Histidyl linkages can be readily determined by pH-fluorescence titrations of the flavin peptide because deprotonation of the imidazole ring leads to extensive quenching of the flavin fluorescence (89). 8α-*S*-Cysteinylflavin, 6-*S*-cysteinylflavin, and 8α-tyrosylflavin peptides differ in that the flavin fluorescence is almost totally quenched with these substitutions at all pH values. In these cases, identification of the linkage requires a more detailed analysis. Comparison of spectral properties, including visible absorption and electron paramagnetic resonance spectra, with model compounds has been a typical approach in determining the covalent linkage present (14).

With the advent of sensitive and high-resolution mass spectrometry techniques in the analysis of proteins, our laboratory has used both matrix-assisted laser desorption ionization and electrospray mass spectrometry in the study of covalent flavins. The electrospray technique offers high-resolution data of intact proteins (70, 85) and therefore can be used to determine whether covalent flavin is present if the amino acid sequence of the protein is known and no other posttranslational modifications exist. On a more precise level, proteolytic digests of the protein can be specifically analyzed for flavin peptides by using precursor ion-scanning techniques. In this method, only ions producing the metaphosphate ion (PO_3^-, 79 amu) upon fragmentation are detected (9, 34) (Fig 3). Flavin peptides can therefore be specifically detected because all of them are expected to be at either the FMN or FAD levels. Once detected, sequence analysis can readily be done by Edman or mass spectral techniques. The only probable interference would arise from the presence of phosphoamino acids in the protein under investigation. This approach offers sensitivity (low picomole levels) and selectivity, but requires access to sophisticated instrumentation.

Flavin nucleotide level

Initially, all of the covalent flavins identified were determined to be at the FAD, rather than the FMN level. The conclusion from these observations was that all covalent flavins are at the FAD level. Since the late 1970s, two examples of enzymes containing covalently linked FMN have been described. The first was the 6-*S*-cysteinylFMN in a bacterial trimethylamine dehydrogenase (80, 81). An 8α-*N*(3)-histidyl flavin in bacterial sarcosine oxidase was determined to be at the FMN rather than the expected FAD level by adenosine analysis and by ^{31}P nuclear magnetic resonance (NMR) spectroscopy of the intact form and after proteolytic digests of the purified enzyme (91). These data demonstrate the presence of covalent 8α-

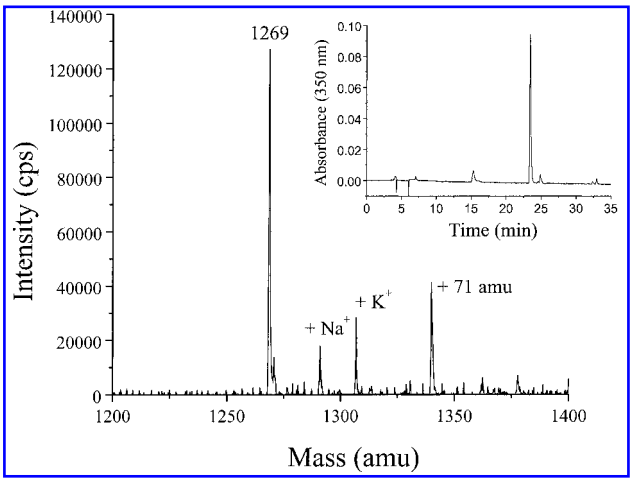

FIG. 3. Example of precursor ion scanning mass spectrometry in the determination of flavin peptide identity. The **inset** graph shows the HPLC profile of a tryptic/chymotryptic digest of purified recombinant human liver MAOB. The absorbing peak was isolated and subjected to collision-induced fragmentation, and the subsequent loss of phosphate was detected. The deconvoluted mass spectrum in the main panel shows the species that lost a phosphate group upon fragmentation. The peak at 1,269 amu corresponds to the pentapeptide Ser-Gly-Gly-Cys^{397}FAD-Tyr. The corresponding Na^+ and K^+ adducts are also labeled. The origin of the fourth peak is not known, but thought to be an artifactual adduct arising from trichloroacetic acid precipitation of the protein prior to proteolysis.

aminoacylFMN as well as FAD in proteins. Therefore, the coenzyme level in flavoenzymes containing covalently bound flavin is likely determined by the nature of the flavin binding site in the apoenzyme as occurs in those flavoproteins containing noncovalent flavins. As will be discussed below, this conclusion is important in contributing to the current view of the mechanism of covalent flavin incorporation.

PROPOSED MECHANISMS OF COVALENT FLAVIN ATTACHMENT

To consider possible mechanisms that may be operable in the process of forming a covalent bond between a nucleophilic amino acid side chain and the 8-methyl group of the flavin ring, it is instructive to consider existing information on the covalent attachment of other cofactors in biological systems. The covalent attachment of the heme to cytochrome *c* involves the formation of two thioether links between the heme and two cysteinyl residues in the protein (65, 66). This process requires the membrane localization of the apocytochrome and the action of a heme lyase (an ATP-dependent reaction) that is located in the inner mitochondrial membrane (13, 67). Therefore, this process requires energy, an enzyme catalyst, and the localization of the reactants near the site of the lyase. The covalent linkage of lipoic acid involves the formation of an amide linkage between the carboxyl group of the cofactor and the ϵ-amino group of the transacetylase (46). Two ligases, lipoyl-protein ligase A (LplA) and lipoyl-protein ligase B (LipB), independently catalyze this reaction (58, 59). LplA operates in an ATP-dependent fashion (59) and primarily uses exogenous lipoic acid. LipB transfers the lipoyl group from endogenously generated lipoyl-acyl carrier protein from the fatty acid biosynthetic pathway (36). Examples of an autocatalytic mechanism are found from studies on formation of the 2,4,5-trihydroxyphenylalanine cofactor found in quinoprotein amine oxidases in both mammalian and bacterial sources. The formation of this cofactor is the autocatalytic oxidation of a tyrosyl residue on the incorporation of Cu^{2+} into the enzyme in the presence of O_2 (62, 72). The detailed mechanism is still under investigation; however, presumably, oxidation of tyrosine to a catechol is the initial step followed by further hydroxylation of the ring position ortho to the side chain.

One possible mechanism for covalent flavin incorporation is that an activated form of the coenzyme is necessary for the process to occur. Therefore, in the biosynthetic formation of FAD, either the aminoacyl riboflavin would need to be converted to the FAD level by a flavokinase/FAD synthetase or "activation" of FAD itself by a putative activating enzyme prior to covalent incorporation into an apoenzyme would need to occur. Evidence against aminoacyl flavin phosphorylation and adenylation comes from studies both in our laboratory and in others that have shown that aminoacylriboflavin analogues are not converted to their respective FMN or FAD forms by either mammalian or bacterial FAD synthetases. Despite extensive efforts by a number

of laboratories, no evidence has been found for any free 8α-modified flavins (at the FAD or FMN level) that could possibly function as an intermediate in the covalent flavinylation reaction.

Evidence that riboflavin is the precursor for flavin incorporation came from early studies by Lambooy's laboratory, which demonstrated that dietary riboflavin and riboflavin analogues could be covalently incorporated into succinate dehydrogenase and into MAO in rats that had become riboflavin-deficient (12, 44). Similar results with succinate dehydrogenase were demonstrated in a riboflavin-auxotrophic strain of *Saccharomyces cerevisiae* (69). These results identified the source of FAD and FMN that is covalently incorporated, but provided no information on the mechanism.

From studies on a bacterial 6-hydroxy-D-nicotine oxidase (from *Arthrobacter oxidans*), several lines of evidence pointed to the flavinylation reaction as a cotranslational event requiring energy, special effectors such as phosphoenolpyruvate, and the formation of a peptidyl tRNA intermediate (4–6, 30, 61). However, work on the purified apoenzyme demonstrated that FAD could be covalently incorporated without the presence of any of these factors, and therefore the reaction now is viewed as an autocatalytic process (7).

With the advent of cloning and expression of enzymes containing covalent flavins, the ability to perform site-directed mutagenesis studies has provided additional insights into the flavinylation reaction, and these studies suggest a posttranslational, rather than a cotranslational, process. In 6-hydroxy-D-nicotine oxidase, mutation of Arg^{67} with Ala prevents covalent flavinylation at His^{71}, as well as substantially reducing catalytic activity (50). The same study also showed that substitution of Ala for Ser^{68} or Lys for Arg^{67} did not prevent covalent flavinylation. However, the latter conservative mutation resulted in a 65% decrease in catalytic activity. Studies with a transfected COS cell system by Abell's group failed to show any advantage of using 8α-hydroxyFAD (a possible "activated" form of FAD) as compared with normal FAD on expressed MAO B activity and covalent flavinylation (93). Taken together, these early studies provided no direct support for an enzyme-mediated covalent incorporation of FAD or FMN into the apoenzyme. These results do indicate that specific interactions of FAD with residues in addition to the site of covalent attachment are necessary for covalent attachment to occur, but that these interactions are not necessarily required for catalytic activity.

The experimental support for an autocatalytic mechanism for covalent flavin incorporation into proteins came from the model system studies of Frost and Raststetter (23), who demonstrated the covalent addition of nitrogen-containing heterocyclic ring systems (*e.g.*, imidazole) to the 8α-position of flavin *N*(5)-oxide analogues. This reaction was interpreted to occur via a quinone-methide tautomeric form of the flavin (90). This work provided the first demonstration of the addition of nucleophiles to the 8α position of flavins, although previous studies suggested the formation of a quinone-methide tautomeric form of oxidized flavin. ^{1}H-NMR studies by Bullock and Jardetsky (8) showed the facile exchange of 8-methyl protons of FMN when heated in aqueous phosphate buffer, and Hemmerich's laboratory observed the facile dimerization of flavin analogues when heated in aqueous pyridine solutions with the covalent linkage forming via the 8-methyl groups (32). In both cases, no similar reactions were observed to occur via the 7-methyl position of the flavin and the ability of the flavin, to form a tautomeric quinone 8-methide was suggested to be responsible for this reactivity.

Other studies have shown the ability of certain 8α-substituted flavins to reductively eliminate the 8α-substituent also proposed to occur through a quinone-methide intermediate. Edmondson and Singer (16) demonstrated the reductive release of cysteine sulfinic acid on two-electron reduction of the sulfone form of 8α-S-cysteinylriboflavin (Fig 4). Later work also demonstrated this elimination to occur on reduction of synthetic 8α-*O*-tyrosylriboflavin or flavin peptides derived from *p*-cresol methylhydroxylase (14, 52). These studies showed the reactivity of the 8α position of the flavin ring to either addition, H-exchange, or elimination reactions. Therefore, the proposal that covalent flavin incorporation occurs via an

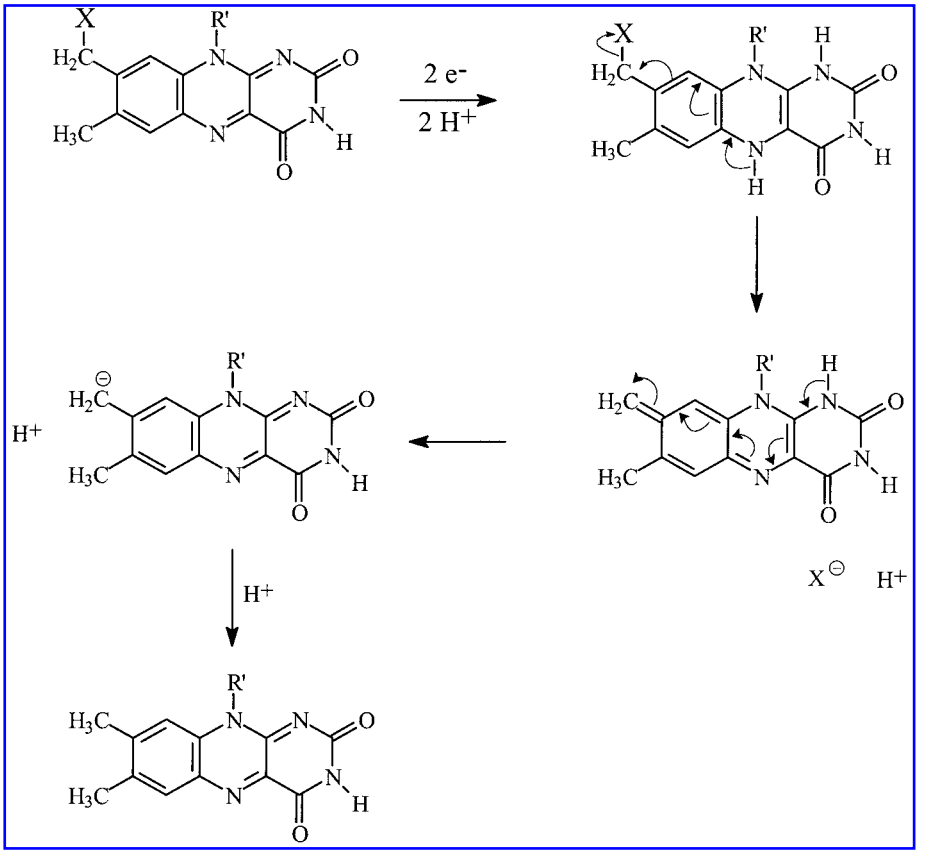

FIG. 4. Mechanism of 8α substituent elimination. X can be either a cysteine sulfinate or tyrosyl group.

autocatalytic quinone-methide addition reaction appears reasonably secure from data on flavin model systems. Further evidence supporting this mechanism in enzyme systems required more sophisticated approaches.

SUPPORT FOR AN AUTOCATALYTIC QUINONE-METHIDE MECHANISM OF COVALENT FLAVIN INCORPORATION

Providing a system that would be amenable to *in vitro* studies of flavin incorporation required an expression system in either bacteria or yeast that could be manipulated to the desired experimental conditions. Studies on covalent flavinylation in an *in vitro* system were initially performed on 6-hydroxy-D-nicotine oxidase (30). Subsequent work on the expressed subunits (one flavin-containing and one heme-containing) of *p*-cresol methylhydroxylase showed the requirement for both in the flavinylation reaction (42, 43). When the genes encoding both subunits were expressed together in *E. coli*, the expressed enzyme contained heme in the heme subunit and covalent 8α-*O*-tyrosylFAD in the flavin subunit. However, when the flavin subunit was individually expressed, the protein formed did not contain covalent FAD. FAD was covalently incorporated *in vitro* on the addition of the heme-containing subunit to a mixture of apo-flavin subunit and FAD. These data provided direct evidence for an autocatalytic covalent flavinylation reaction and, as this reaction occurred with purified component proteins, no other auxiliary enzyme(s) or other cofactors are required. The molecular basis for the heme subunit requirement for the reaction is not known in detail. The apo-flavin subunit is capable of noncovalently binding FAD with high affinity in the absence of the heme subunit, forming an enzyme that cannot be reduced by *p*-cresol but can be reduced by the intermediate 4-hydroxybenzyl alcohol (19). Therefore, subunit association is not required for FAD–enzyme asso-

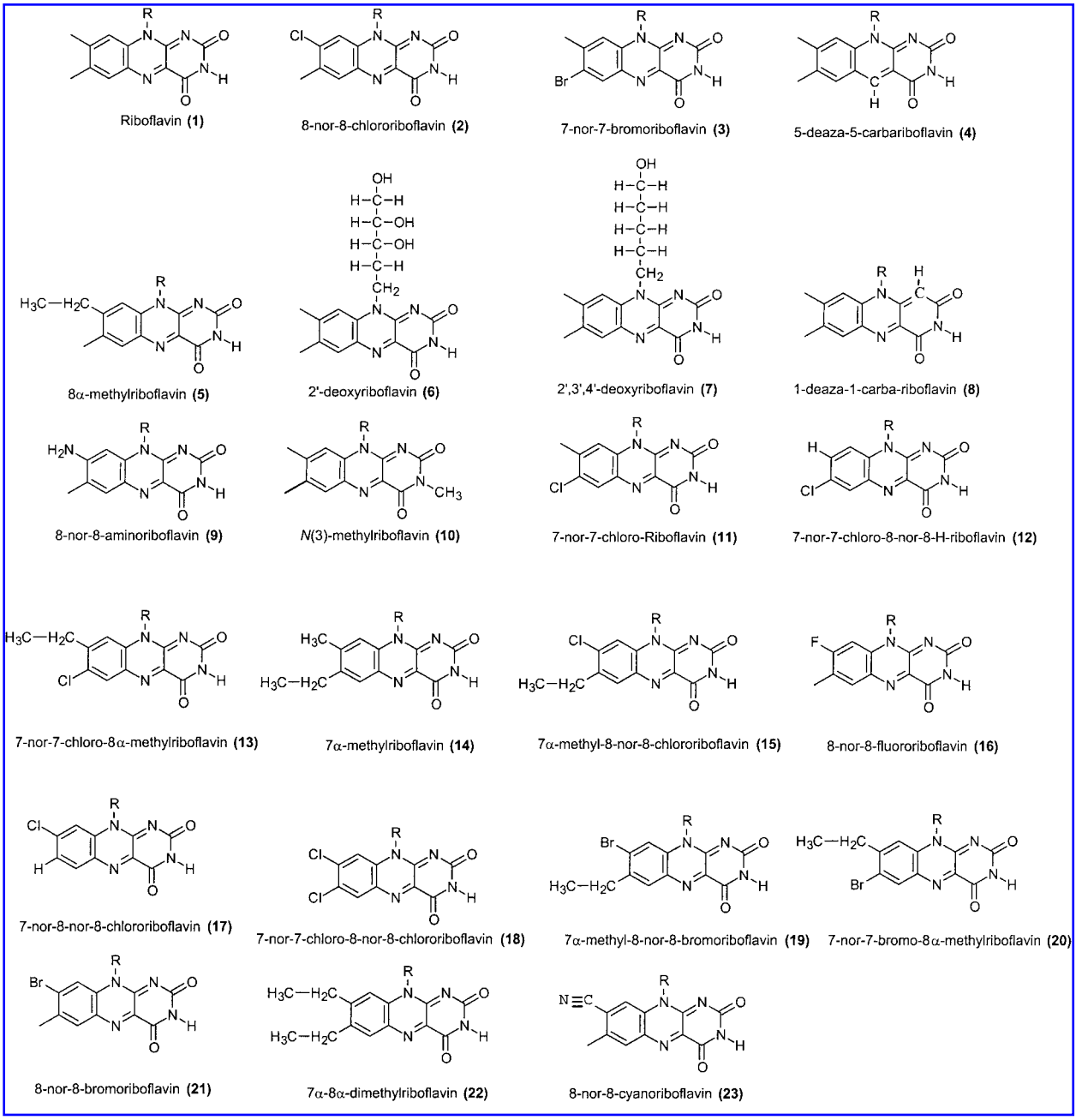

FIG. 5. Chemical structures of the flavin analogues used in MAO studies. R denotes the ribityl side chain. In the text, these flavin analogues are also referred to as their respective FAD analogues (see Fig. 1).

ciation but appears to be required for covalent binding. One possibility for the role of the heme subunit in the covalent flavinylation reaction is that it functions as an efficient electron acceptor for the reduced tyrosylFAD product that is formed upon nucleophilic attack on the quinone-methide, thereby completing the reaction sequence.

In an effort to provide additional insight into the flavinylation reaction with enzymes containing an 8α-*S*-cysteinylFAD, Miller and Edmondson (54) developed a yeast strain auxotrophic for riboflavin by a targeted gene disruption of the *rib5* gene encoding the enzyme catalyzing the terminal step in riboflavin biosynthesis. Expression of MAO A or B in this strain of yeast in the presence of different riboflavin analogues (Fig. 5) provided a system to investigate the influence of flavin structure on the covalent incorporation of FAD into each

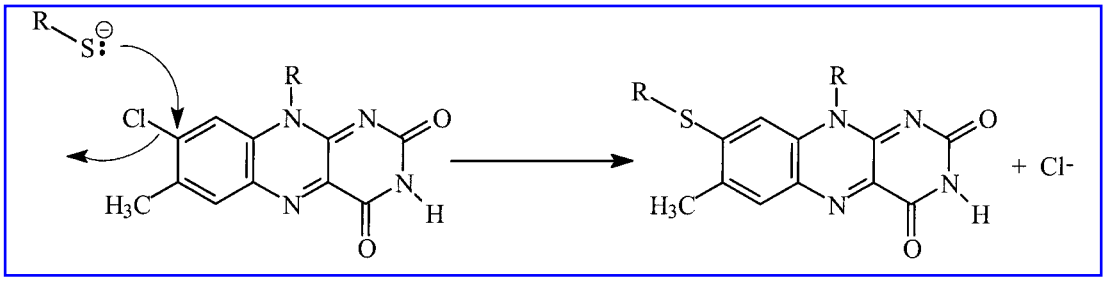

FIG. 6. Mechanism for nucleophilic displacement of 8-halogen substituent of flavin ring as demonstrated in model systems (32) and in MAO A (90).

of these proteins. Direct evidence for a nucleophilic addition of Cys^{406} (MAO A) to 8-nor-8-chloroFAD (**2,** Fig. 5) was demonstrated by covalent incorporation of this flavin analogue into the protein and by the characteristic absorption spectrum that occurs on nucleophilic displacement of the 8-halogen substituent of the flavin by a thiol group (Fig. 6) (57). This 8-thioflavin MAO A (or B) exhibited levels of catalytic activity similar to that observed with a riboflavin control (99% for MAO A and 83% for MAO B relative to control activity). Similar studies with 7-nor-7-bromoflavin (**3,** Fig. 5) showed that covalent linkage occurred at the 8α-methyl position because the 7-bromo substituent was still present in the isolated flavin peptide as detected by mass spectrometry. Enzyme resulting from the covalent attachment of this derivative possessed catalytic activity greater than the riboflavin control (185% for MAO A and 140% for MAO B). From a survey of 19 different flavin analogues (Fig. 5), the following conclusions can be made. An alkyl or halogen group must be present at the 8 position of the flavin for covalent linkage to occur. No covalent flavinylation is observed when 8-nor-8-H-flavin analogues are tested. The flavin analogue must be autooxidizable for covalent incorporation to occur because 5-deaza-5-carbaFAD (**4,** Fig. 5) is not covalently incorporated into the enzyme. From these data, it is predicted that the site for covalent binding to the protein using 8α-methylFAD (**5,** Fig. 5) is to the α rather than to the β carbon of the ethyl group at the 8 position (this prediction has not yet been verified experimentally).

Evidence for a functional role of the hydroxyl groups of the ribityl side chain in flavin coenzyme binding and in the catalytic mechanisms of several flavoenzymes containing noncovalent flavin has been found (20, 60). Previous site-directed mutagenesis results on MAO B led Abell and co-workers (94) to conclude that a specific interaction between Asp^{227} and the 3′-ribityl-OH of FAD was required for the covalent flavinylation reaction (formation of the 8α-thioether linkage with Cys^{397}) but not catalytic activity. Given the inherent uncertainties of mutagenesis results with a structurally ill-defined enzyme system, Miller *et al.* (55) used the rib5$^-$ yeast expression system to test for the requirement of flavin side-chain hydroxyls in the flavinylation reaction. The results demonstrated that *N*(10)ω-hydroxylpentylisoalloxazine (2′,3′,4′-deoxyriboflavin, **7,** Fig. 5) is covalently incorporated into MAO A and MAO B. Both reconstituted enzymes are catalytically functional as they exhibit similar or higher levels of activity as control samples using riboflavin (134% for MAO A and 228% for MAO B).

Taken together, the data on the covalent flavinylation of MAO A or MAO B with flavin analogues support the view that the mechanism for covalent flavin incorporation occurs via a quinone-methide mechanism (Fig. 7). Binding of the flavin coenzyme to the apoprotein (presumably folded to a native-like structure) is expected to facilitate the quinone-methide tautomeric form of the flavin ring. By molecular interactions still undefined, the nucleophilic amino acid is sterically positioned for efficient nucleophilic attack resulting in the 8α-covalent reduced flavin. Oxidation of the reduced flavin would complete the cycle to form the posttranslationally modified flavoenzyme.

This proposed mechanism poses some questions that have not been addressed in detail. If this mechanism is operative, it should be pos-

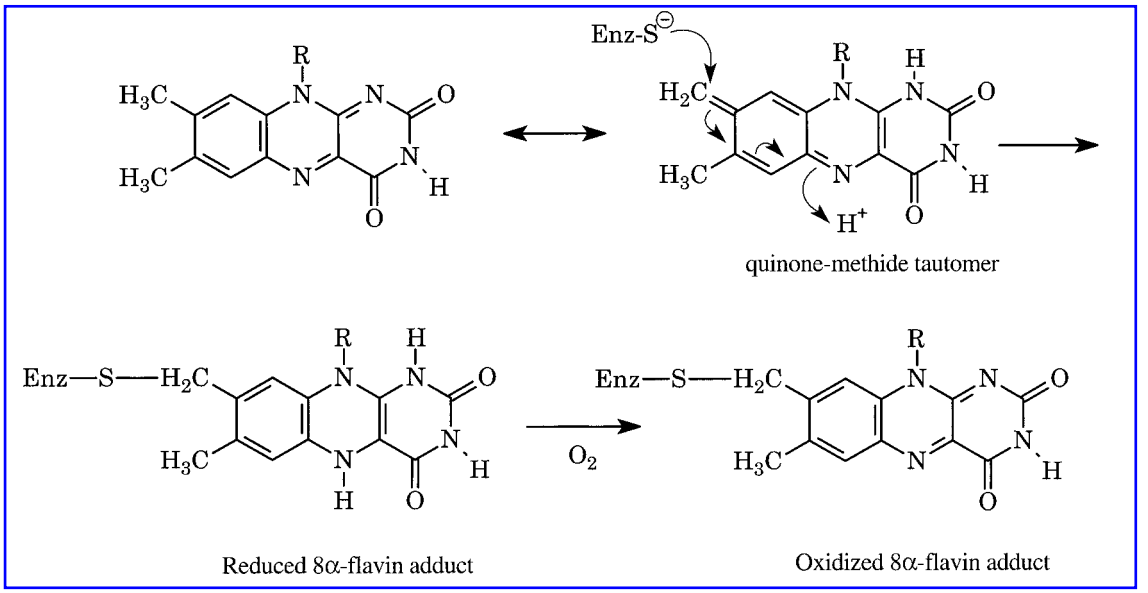

FIG. 7. The quinone-methide mechanism of covalent flavinylation. After loss of a proton in the 8α-position, a quinone-methide tautomer is formed. A nucleophilic amino acid residue can then attack the 8α-methide. The reduced 8α-flavin adduct will then oxidize to produce active flavin. This mechanism may be adapted to account for attachment at the C(6) position.

sible to generate an apoenzyme, add FAD, and demonstrate autocatalytic FAD incorporation. This process has been shown with the flavin subunit of apo-*p*-cresol methylhydroxylase in the presence of the heme subunit (93). Expression of l-gulono-γ-lactone oxidase in a riboflavin-deficient insect cell culture resulted in the formation of an apoprotein that could be reactivated on the addition of FAD, but no covalent attachment was detected (68). The apoenzyme of l-gulono-γ-lactone oxidase apparently folds to a conformation that binds FAD to form a catalytically active complex, but without covalent incorporation into the enzyme. These observations contrast with the "simple" view of an autocatalytic mechanism of covalent flavin incorporation in which the apoprotein folds to a conformation that binds FAD with ensuing nucleophilic attack of the flavin quinone-methide tautomer. There are presumably a number of factors involved in initial flavin binding. These factors may include the orientation of FAD such that nucleophilic attack by the appropriate amino acid is possible and protein–flavin interactions that would promote formation of the flavin quinone-methide tautomer required for the flavinylation reaction.

One may ask if replacement of one nucleophile for another (through mutagenesis) would result in a mutant protein with a different type of covalent flavin (*e.g.*, replacement of a cysteinyl residue by a histidyl or tyrosyl residue). Attempts to achieve the formation of altered covalent flavins have been unsuccessful when tested with MAO A or B (29) (as judged from the absence of reconstituted catalytic activity). Similar experiments have been attempted with fumarate reductase (63), 6-hydroxy-D-nicotine oxidase (49), and vanillyl-alcohol oxidase (22) without success in formation of an altered covalent flavin coenzyme. These negative results do not invalidate the mechanism shown in Fig. 7, but reflect the importance of the proper steric alignment of the nucleophilic amino acid, which may be altered on amino acid replacement. A second question regarding the mechanism in Fig. 7 is that, if the reaction follows the principle of microreversibility, then reduction of flavoenzymes should result in the release of "normal" flavin coenzyme. To the authors' knowledge, this experiment has not been attempted in any detailed manner. For the reverse reductive elimination reaction to occur in an observable manner, the rate of elimination should be observable. One requirement for the reductive elimination reaction to proceed is for the orbitals of the leaving group (amino acid nucleophile) to overlap with the orbitals of the

benzenoid portion of the flavin ring. Conformational constraints (especially for tyrosyl or imidazole substituents) in the protein milieu may reduce the mobility of the 8α-substituent such that the reductive elimination reaction rate would occur too slowly to be readily observable.

CURRENT VIEWS ON THE STRUCTURAL OR MECHANISTIC ADVANTAGE FOR COVALENT FLAVIN BINDING TO SELECT FLAVOENZYMES

The reason(s) for the existence of covalent flavins in flavoenzyme is not clear. It does not relate to function as there are numerous examples of flavoenzymes isolated from different sources that catalyze similar reactions even though one contains a covalent flavin and the other contains a flavin coenzyme bound by noncovalent forces. Examples include the cholesterol oxidases containing an 8α-*N*(1)-hisitidyl-FAD (enzymes isolated from *Schizophyllum commune*) and those containing noncovalent FAD (*Streptomyces hygroscopicus*). Other examples are the mammalian amine oxidases (8α-*S*-cysteinylFAD) and a MAO from *Aspergillus niger* (containing noncovalent FAD) (73). Other examples also exist that demonstrate that the reaction catalyzed by the enzyme does not seem to correlate with the requirement for covalent flavin binding. Although high-resolution structural data on flavoenzymes containing covalent and noncovalent flavins are available (Table 1), these data have not provided the anticipated insights into this question.

One possible explanation for covalent binding is the requirement to keep the flavin coenzyme from being released from its binding site on the enzyme. The implication here is that nature did not provide a flavin binding site in the enzyme sufficient to keep the flavin coenzyme in a tightly bound form and, therefore, to correct this situation, covalent flavin binding evolved to protect the flavin coenzyme from being released. To address this possibility, several laboratories have mutagenized the site for covalent flavin linkage and tested whether flavin coenzymes could still bind. This has been attempted for at least six different enzyme systems representing each type of covalently bound flavin: succinate dehydrogenase (71), trimethylamine dehydrogenase (75), 6-hydroxy-D-nicotine oxidase (49), vanillyl-alcohol oxidase (22), MAO A (33, 63), and fumarate reductase (3). In all cases, the enzyme formed in the expression system contained noncovalently bound flavin and exhibited lower levels of catalytic activity. For succinate dehydrogenase, the presence of covalent flavin was found to be necessary for succinate dehydrogenase activity, but not for the reverse reaction the enzyme catalyzes, which is fumarate reductase activity. Therefore, it appears that flavoenzymes that normally have covalent flavin are still capable of binding the cofactor without the covalent attachment.

In the case of human liver MAO A, the C406A mutant was expressed in a riboflavin-deficient strain of *S. cerevisiae* (63) in the presence of riboflavin. When mitochondria were isolated and tested for catalytic activity, ~30% activity was observed with the mutant as compared with wild-type enzyme (containing covalent FAD). In the absence of riboflavin in the growth medium, no activity was observed in isolated mitochondria; however, activity could be restored on the addition of FAD (but not riboflavin or FMN). These data show that the expressed enzyme is targeted to the mitochondrial outer membrane and folds to a native conformation in the absence of bound flavin. This finding provides direct evidence for the view that protein folding occurs prior to flavin binding. The flavin binding site was found to be specific for the FAD coenzyme level, and the dissociation constant was determined to be ~60 n*M* (Table 2), which is within the range of K_d values found for other flavoenzymes that contain noncovalent flavins. The conclusion from these results is that the apoprotein is capable of tightly binding the flavin coenzyme by noncovalent interactions, and therefore the suggestion that a covalent linkage is required to compensate for weak binding is not supported by the available experimental data.

One major difference between MAO A containing a covalent flavin and the C406A mutant is the relative stability of the enzyme when extracted from the mitochondrial outer membrane. Release of the native enzyme from the membrane requires both phospholipase digestion and detergent extraction, which demon-

Table 2. Activities of Reconstituted apoC406A MAO A in the Presence of Different FAD Analogues and the Binding Constants of the FAD Analogues for apoC406A MAOA

Flavin analogue	*Percent specific activity of FAD control*	K_d (*n*M)
(**1**)* FAD	100	62 ± 5
(**11**) 7-Nor-7-chloroFAD	475 ± 10	44 ± 2
(**2**) 8-Nor-8-chloroFAD	694 ± 14	62 ± 5
(**5**) 8α-MethylFAD	<5	90 ± 12†
(**4**) 5-Deaza-5-carbaFAD	<5	69 ± 5†
(**8**) 1-Deaza-1-carbaFAD	<5	377 ± 32†
(**9**) 8-Nor-8-aminoFAD	<5	240 ± 64†
(**10**) N(3)-MethylFAD	<5	920 ± 108†
(**18**) 7-Nor-7-chloro-8-nor-8-chloroFAD	317 ± 4	42 ± 2
(**14**) 7α-MethylFAD	47 ± 3	30 ± 8
(**19**) 7α-Methyl-8-nor-8-bromoFAD	323 ± 13	46 ± 6
(**15**) 7α-Methyl-8-nor-8-chloroFAD	383 ± 10	42 ± 4
(**23**) 8-Nor-8-cyanoFAD	437 ± 38	61 ± 17

Data were taken from ref. 63.
*Numbers refer to the corresponding structures in Fig. 5.
†Determined by competitive binding experiment with 7-nor-7-chloroFAD.

strates its tight association with the membrane. Upon solubilization, the catalytic activity of the enzyme containing covalent flavin (wild type) is relatively stable in the presence of competitive inhibitors, thiol reagents, and cold temperatures. In contrast, the activity of the C406A mutant is unstable to solubilization under conditions in which the native form is stable. The mutant form is relatively stable as long as it remains in its membrane environment. These data suggest that the presence of the covalent FAD functions to stabilize the protein structure in a native conformation in MAO A (at least to conditions of detergent solubilization). Whether this is a common trait among enzymes containing covalent flavins remains for future investigations. The apoenzyme of vanillyl-alcohol oxidase appears to be stable to reconstitution, and the three-dimensional structure is very similar to that of the wild-type holoprotein (22). One difference between MAO A and vanillyl-alcohol oxidase is that the former is membrane-bound, whereas the latter enzyme is soluble, a property that may impact the stabilization effect of covalent flavinylation. Unpublished results in this laboratory have shown that the catalytic activity of the C397A mutant of MAO B is stable to detergent solubilization from its membrane environment. Detailed comparative stability studies with wild-type MAO B have yet to be performed.

The use of flavin analogues as probes of the flavin binding site in flavoenzymes is an established approach (25), which yields important structural insights into the flavin binding site in the protein in the absence of crystallographic information. In those cases where structural data from crystallography are available, the conclusions from flavin analogue data compare favorably (21). The binding affinities of 13 different FAD analogues to apo-C406A-MAO A (covalent linkage abolished) were determined and are shown in Table 2. The data show that the enzyme is able to accommodate a number of modifications in the flavin ring without compromising the binding affinity (K_d values ranging from 30 to 90 n*M*). Only three analogues exhibited reduced binding: 1-deaza-1-carbaFAD, 8-nor-8-aminoFAD, and *N*(3)-methylFAD (structures **8, 9,** and **10** in Fig. 5). Only the *N*(3)-substituted FAD exhibits a substantially weaker binding to the enzyme, which suggests specific flavin–protein interactions at that position as is often encountered with other flavoenzymes. Of interest is the finding that tight binding of the FAD analogue does not necessarily result in catalytic activity. 8α-MethylFAD is bound tightly to the enzyme (K_d = 90 n*M*) and is expected to exhibit a redox potential similar to FAD. However, compared with FAD, no detectable activity is recovered using this analogue upon reconsti-

tution. In contrast, when the same analogue (8α-methylFAD) is covalently bound to C406 (presumably through the 8α-carbon rather than the β-carbon), the activity levels are similar (69%) to that observed with covalently bound FAD (54). These results suggest that replacement of the 8-methyl group on the FAD with an ethyl group results in steric alterations of the flavin in its binding site such that it no longer can efficiently function in catalysis. Thus, comparison of the relative activities of MAO A upon disruption of the 8α-covalent linkage demonstrates an interesting difference between FAD and 8α-methylFAD. Assuming no major changes in K_m values, loss of covalent binding of FAD results in ~70% decrease in activity. Loss of the covalent linkage for 8α-methylFAD results in a disproportionate decrease in catalytic activity (from 69% of control for covalent linkage to a nondetectable quantity for noncovalent binding). These data suggest that one role for a covalent thioether linkage is to maintain the flavin coenzyme in the correct conformational orientation for catalysis. This conclusion may also be applicable to other covalent flavoenzymes and remains to be tested experimentally.

As stated above, the level of catalytic activity observed with C406A MAO A is ~30% that of wild-type enzyme. When FAD analogues exhibiting higher oxidation–reduction potentials were tested (*e.g.*, 8-cyanoFAD, $E_{m,7} = -50$ mV or 8-chloroFAD, $E_{m,7} = -150$ mV), the reconstituted activity levels were found to be four- to fivefold higher than that observed with FAD ($E_{m,7} = -200$ mV; $E_{m,7}$ values quoted are of the free flavin analogue in neutral, aqueous solution). Although there is no linear correlation of activity with the redox potential of the free flavin analogue, the trend is that the activity is increased when the potential is increased. The oxidation–reduction potentials of 8α-substituted flavins have been known since the early 1970s (16) when it was shown that substituents in this position increased the $E_{m,7}$ values ~50 mV relative to the unsubstituted flavin. Therefore, one conclusion that can be made is that the formation of an 8α-substituted flavin results in an increase in the potential of the flavin ring, which can be further modulated by the protein environment.

An effect on the redox potential of the flavin cofactor by covalent attachment in flavoproteins has also been observed. It has been noted in the literature (22) that for flavoenzymes for which the redox potential has been determined, those containing covalently bound flavin possess a higher redox potential than those containing noncovalently bound flavin. Table 3 contains examples for which this property has been tested for the same enzyme either through mutation at the site of covalent linkage (vanillyl-alcohol oxidase and *p*-cresol methylhydroxylase) or by comparing two flavoenzymes from different sources that share the same function (cholesterol oxidase). For vanillyl-alcohol oxidase, it was shown that the covalent flavin form exhibits a potential 120 mV higher than the H422A mutant containing noncovalent FAD (22). In the case of two types of cholesterol oxidase, one from *Streptomyces hygroscopicus*, which contains noncovalently bound FAD, and another from *Brevibacterium sterolicum* containing covalent FAD, the latter possesses a mid-

Table 3. Oxidation–Reduction Potentials of Representative Flavoproteins Containing Covalent and Noncovalent Flavin

Enzyme	*Flavin coenzyme*	E_m, *mV (vs. SHE)*	*Reference*
Vanillyl-alcohol oxidase	Covalent FAD (8α-*N*3-histidyl)	+55	(22)
Vanillyl-alcohol oxidase H422A mutant	Noncovalent FAD	−65	(22)
Cholesterol oxidase (*Brevibacterium sterolicum*)	Covalent FAD (His-linkage)	−101	(24)
Cholesterol oxidase (*Streptomyces hygroscopicus*)	Noncovalent FAD	−217	(24)
p-Cresol methylhydroxylase	Covalent FAD	+84	W. McIntire, personal communication
p-Cresol methylhydroxylase[Y384F]	Noncovalent FAD	+47	W. McIntire, personal communication

point redox potential 100 mV higher than that of the form possessing noncovalent FAD (24). For clarity, Table 3 only compares the redox potentials for flavoenzymes that can be compared directly, but this observation seems to apply to flavoenzymes in general (for a more complete listing of flavoenzyme redox potentials, see ref. 79). For MAO B, recent studies show that the one-electron potentials for both the Ox/Sq and Sq/Hq couples to be ~+40 mV and therefore the two-electron potential also to be +40 mV (64), a finding similar to those of other flavoenzymes containing covalently bound cofactor.

A number of studies have commented on a direct Hammett relationship between the electron-withdrawing or electron-donating properties of the 7- and 8-substituents in flavin analogues (31, 74). Analysis of the accumulated data on a large number of flavin analogues by Edmondson and Ghisla (15) has shown that this relationship can be summarized by the following equation:

$$E_{m,7} = 202\ (\pm 11)\ \sigma_p + 140\ (\pm 22)\ \sigma_m - 181\ (\pm 4)\ \text{mV}$$

where $E_{m,7}$ is the midpoint potential of the flavin analogue and σ_p and σ_m are the Hammett σ values for the groups at the 8 and 7 positions, respectively. This equation shows that the electron withdrawing/donating properties of the 8α-substituent influence the redox potential of the flavin in that a group with a higher σ value (reflecting higher electron-withdrawing properties) correlates with a higher redox potential. Therefore, covalent binding of groups that influence the electron density of the flavin cofactor contributes to its effective potential, which is expected to be important in catalysis. Although the effect of flavin modification by an amino acid residue is more complex than can be described in these terms, and other flavin–protein interactions certainly also contribute to flavoenzyme redox potentials, model relationships such as this can aid in the understanding of what is occurring in an enzyme system.

The influence of electronic effects of 8α-substitution on the flavin coenzyme properties may also be exhibited in other redox forms, such as the semiquinone form of the flavin. Unfortunately, little direct information is currently available on the known enzyme systems. Previous flash photolysis studies (17) have demonstrated that the pK_a of 8α-substituted flavin semiquinones are decreased ~1 pK_a unit (pK_a = 7.1) relative to that exhibited by unsubstituted flavins (pK_a = 8.3). Although the protein environment is known to modulate the pK_a of flavin semiquinones in flavoproteins, it is of potential mechanistic importance for radical reactions involving covalent flavins if 8α-substitution modulates the acid–base properties of the bound flavin. In this respect, it is of interest to note that the flavoenzymes tested containing 8α-covalent flavins all exhibit anionic flavin semiquinones as opposed to neutral semiquinones (18).

Thus, the available data point to several factors that contribute to the reason for the existence of 8α-covalent flavin coenzymes in selected flavoenzyme systems: (a) stabilization of apoenzyme structure; (b) steric orientation of flavin coenzyme in its binding site suitable for catalytic function; and (c) contribution to an increase in the oxidation–reduction potential of the flavin, which permits it to be a more electron-deficient moiety in catalysis. Further work is required to identify other additional factors and/or more thoroughly describe the molecular basis of those factors described above.

SUMMARY

Due to the application of molecular biological approaches and the application of expression systems to flavoenzymes containing covalent flavins, our insights into the basis for covalent flavin linkage in this subclass has increased substantially during the past several years. The authors predict that these insights will increase over the next few years when more systems are examined and more structural information becomes available. Due to space limitations, this review is not intended to be comprehensive, and the authors regret not being able to include all the contributions to this area. As stated in the introduction, the review concentrates on results obtained on MAO over the past several years, which may or may not be representative of data obtained with

other enzymes containing covalent flavins. It is hoped that the ideas and conclusions presented will encourage additional investigations by others in this area.

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ABBREVIATIONS

FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; LipB, lipoyl-protein ligase B; LplA, lipoyl-protein ligase A; MAO, monoamine oxidase; NMR, nuclear magnetic resonance.

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Address reprint requests to:
Dr. Dale E. Edmondson
Department of Biochemistry
Emory University School of Medicine
Rollins Research Center
1510 Clifton Road
Atlanta, GA 30322

E-mail: dedmond@bimcore.emory.edu

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