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BIOSYNTHESIS AND FUNCTION OF ENZYMES WITH COVALENTLY BOUND FLAVIN

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Proteins with the flavin coenzyme covalently linked to an amino acid side-chain (henceforth called covalent flavoproteins) have interested flavinologists for the last 25 years. The subject has been reviewed in some detail recently (12, 14). This review emphasizes the mechanism of synthesis of the covalent bond, but a brief outline of the occurrence and functions of the presently known covalent flavoproteins is also given.

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COVALENT COFACTOR-APOENZYME ATTACHMENT

General Features of Coenzyme Attachment

Most enzymes require cofactors for their activity. Cations are the most common; they are usually involved in the stabilization of transition states. Coenzymes, which are of organic nature, generally occur in a freely dissociable form, but some are known as covalently bound cofactors only (Table 1). Several cofactors, however, occur both in free and covalently attached form. The flavoproteins belong to the latter group.

Discovery and Occurrence of Covalently Bound Coenzymes in Flavoproteins

Covalent attachment of flavin coenzyme and apoprotein was first observed in 1955 by Singer and associates, who were studying succinate dehydrogenase of beef heart mitochondria (55). Until this time, it was thought that all the flavoproteins contained their cofactor, FMN or FAD, only noncovalently attached to the polypeptide structure; in many instances the reversible removal of the coenzyme was experimentally demonstrated.

Table 1 Free and bound forms of coenzymes[a]

Vitamins	Coenzymes	Covalently bound in	Noncovalently attached to
Biocytin	Biotin	Acetyl-CoA carboxylase	—
—	α-Lipoic acid	Dihydrolipoyl transacetylase	—
Retinol	13-*cis*-Retinal	Rhodopsin	
Pantothenic acid	4-P-Pantetheine	Acyl carrier protein	
	Coenzyme A	—	Pyruvate dehydrogenase
—	Heme	Cytochrome *c*	Cytochrome *b*
Pyridoxine	Pyridoxal-P	Phosphorylase	Alanine aminotransferase
Riboflavin	FAD	Succinate dehydrogenase	D-Amino acid oxidase
	FMN	Trimethylamine dehydrogenase	NADH-Q reductase

[a] Coenzymes derived from the following vitamins were always found in noncovalent attachment: cobalamin, folic acid, niacin, phylloquinone and thiamin.

After the discovery of the first covalent flavoprotein, it was soon recognized that more than one type of bond can be formed between flavin and apoprotein (Figure 1): Histidyl residues can be attached through N-atom 1 or 3 to the 8α-methyl group of the isoalloxazine ring forming a secondary amine bond; cysteinyl groups of a polypeptide chain may form a thioether linkage with either the 8α-methyl group or with carbon atom 6 of the xylene ring of the flavin molecule; a fifth binding type is represented by the tyrosyl(O)-8α-flavin bond. The physicochemical properties of the differently bound flavin derivatives and the methods used to identify the bond type have been presented recently in great detail (17).

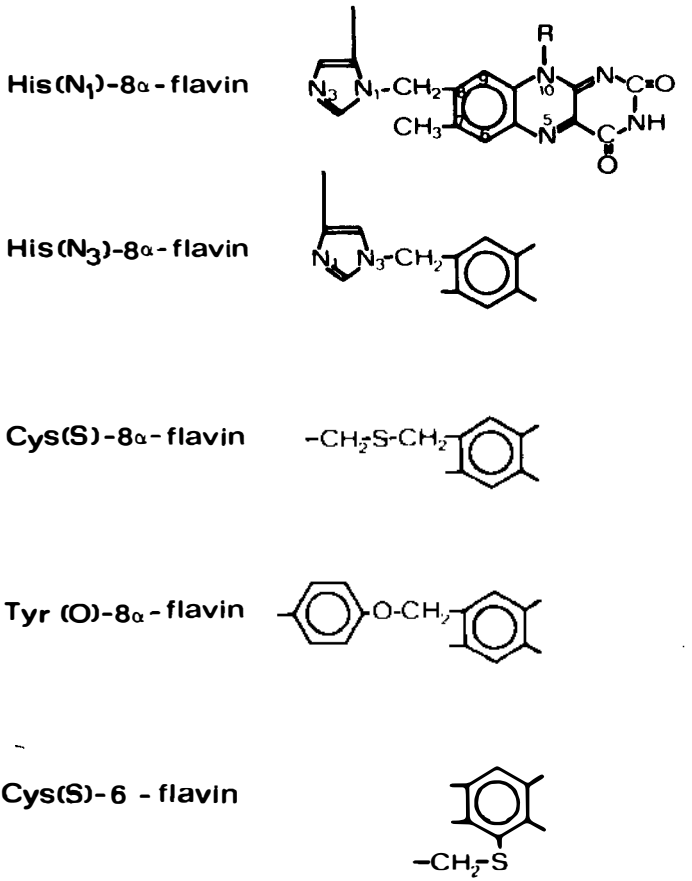

Figure 1 Aminoacyl-flavin bonds observed in flavoproteins. Reprinted from *BioFactors* 3:71 (1991) with permission of Oxford University Press and from *Chemistry and Biochemistry of Flavoenzymes* 2:347 (1991), copyright CRC Press, Inc. Boca Raton, Florida.

During the past 35 years, some 20 flavoproteins (Table 2) have been identified as covalent flavoproteins. This list does not reveal an obvious relation between bond type and either the mechanism of the catalyzed reaction or the source of the enzyme. The His(N3)-8α-FAD linkage appears to be the most abundant in nature. The simultaneous existence, often within the same organism, of covalently and noncovalently linked flavoproteins raises the question of the significance of covalency. Presently, the covalent flavoenzymes appear to be the minority. Inspection of Table 2, however, reveals that many covalent flavoproteins are engaged in the dissimilation of organic material. The potential of many bacterial species to degrade oxidatively a wide variety of natural and manmade organic compounds is almost without limit. One

Table 2 The presently known covalent flavoproteins[a]

Enzyme	Source
Histidyl(N3)-8α-FAD	
Succinate dehydrogenase	Mitochondria, yeast, *B. subtilis*
Fumarate reductase	*W. succinogenes*, *E. coli* (anaerobic)
6-Hydroxy-D-nicotine oxidase	*Arthrobacter oxidans*[b]
Choline oxidase	*Arthrobacter globiformis*
Dimethylglycine dehydrogenase	Liver mitochondria
Sarcosine dehydrogenase	Liver mitochondria, *Pseudomonas*
Sarcosine oxidase	*Corynebacterium* sp. U-96
D-Gluconolactone oxidase	*Penicillium cyaneofulvum*
Histidyl(N1)-8α-FAD	
Thiamin dehydrogenase	Soil bacterium (ATCC 25589)
Cyclopiazonate oxidocyclase	*Penicillium cyclopium*
Cholesterol oxidase	*Schizophyllum commune*
L-Galactonolactone oxidase	Yeast
L-Gulonolactone oxidase	Liver microsomes
Cysteinyl(S)-8α-FAD	
Monoamine oxidase	Liver mitochondria
Flavocytochrome c_{552}	*Chromatium*
Flavocytochrome c_{553}	*Chlorobium thiosulfatophilum*
Cysteinyl(S)-6-FMN	
Trimethylamine dehydrogenase	Bacterium sp. $W_3A_4$1
Dimethylamine dehydrogenase	*Hyphomicrobium X*
Tyrosyl(O)-8α-FAD	
p-Cresol methylhydroxylase	*Pseudomonas putida*

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[b] Recently, *Arthrobacter oxidans* has been renamed *Arthrobacter nicotinovorans* (Kodama, Y., Yamamoto, H., Amano, N., Amachi, T. 1992. Reclassification of two strains of *Arthrobacter oxydans* and proposal of *Arthrobacter nicotinovorans* sp. nov. *Int. J. System. Bacteriol.* 42:234–39).

might expect, therefore, to find in oxidative catabolisms additional covalent flavoproteins. In cells of higher organisms, particularly in mammalian species, the number of covalent flavoproteins is limited. Four covalent flavoproteins were distinguished in rat liver mitochondria (1, 53): succinate dehydrogenase, monoamine oxidase, sarcosine dehydrogenase, and dimethylglycine dehydrogenase. Another covalent flavoprotein, L-gulonolactone oxidase, was discovered in liver microsomes (32).

BIOSYNTHESIS OF THE COVALENT FLAVIN-PROTEIN BOND

Covalent bond formation requires the expenditure of free enthalpy; in the case of biotinylation it is provided by ATP. Mechanisms involving ATP can also be formulated for covalent flavoprotein formation; they would necessitate either the activation of the coenzyme, e.g. by hydroxylation of the 8α-CH_3 group followed by (pyro)phosphorylation, or the formation of a highly reactive N-derivative of the imidazole moiety of the receiving amino acid, e.g. phosphohistidyl-apoprotein. These mechanisms would certainly require enzymatic catalysis. However, activation processes not involving ATP (or similar kinds of metabolic energy) or even enzymes can be envisaged (11, 21, 60).

Insight into the details of the synthesis of the covalent apoenzyme-flavin bond has been gained recently for the histidyl(N3)-8α-FAD linkage in 6-hydroxy-D-nicotine oxidase. This flavoprotein is involved in the oxidative degradation of D-nicotine in the soil bacterium *Arthrobacter oxidans* (13). Several of the question raised above can now be answered for that particular enzyme. Whether or not the same principle of synthesis is valid for other enzymes of the same type or even for other covalent cofactor-apoprotein linkages is still unknown.

Synthesis of the Histidyl(N3)-8α-Flavin Linkage

COVALENT ATTACHMENT IN VITRO Covalent FAD attachment to the growing peptide chain of the apoenzyme in an extract of *A. oxidans* was demonstrated in 1980 (25). Substantial progress was made when the gene of 6-hydroxy-D-nicotine oxidase was identified on a plasmid (pAO1) (7), cloned, and sequenced. It could be expressed in a cell-free coupled transcription/translation system from *Escherichia coli* (3). The synthesis of a flavin-free apoprotein was achieved in UV-irradiated *E. coli* extracts by immunoprecipitation and by SDS gel electrophoresis (2). Addition of FAD to this system led to the formation of the enzymatically active protein with covalently bound cofactor. A highly purified apo-6-hydroxy-D-nicotine oxidase was obtained either from cell-free extracts of diphenyl iodonium (DPI)-treated *E. coli* cells carrying the

6-hydroxy-D-nicotine oxidase gene or through the formation of a fusion protein with β-galactosidase (15).

The apoprotein of 6-hydroxy-D-nicotine oxidase could be flavinylated and converted into the enzymatically active form in the presence of an ATP-regenerating system and FAD (8-demethyl-FAD could not replace FAD) without further additions, thereby suggesting at first the participation of an ATP-dependent enzymatic process.

Eventually it was observed that the ATP-regenerating system can be replaced by phosphoenolpyruvate alone and even by other phosphorylated three-carbon compounds such as glycerol 3-phosphate, glyceraldehyde 3-phosphate, or glycerate 3-phosphate (4). The K_m value for FAD in this reaction is 3 μM; that for phosphoenolpyruvate is about 1 mM. The latter value is in the range reported for the phosphotransferase system of bacterial carbohydrate metabolism (52). Similar concentrations of C_3-compounds are found in cell lysates.

The effector molecules do not participate in the reaction; neither ^{14}C- nor ^{32}P-phosphoenolpyruvate are bound to the protein or cleaved during the flavinylation. The high efficiency of glycerol 3-phosphate also excludes an energy-providing function. Evidently, these phosphate esters serve as allosteric modulators of the protein. The presence of 45% glycerol allows a conformation of the apoprotein that facilitates the access of FAD to its binding site and the spontaneous covalent attachment. Still, glycerol 3-phosphate increases the rate of holoenzyme formation even in the presence of glycerol. The same observation was made in the presence of 20% saccharose (5).

The flavinylation of the apo-6-hydroxy-D-nicotine oxidase in vitro does not require an additional enzyme; the most highly purified preparations of the apoprotein are flavinylated and converted to the active enzyme at the same rate as the apoenzyme of crude extracts.

Two flavin derivatives, 8-Cl-FAD and 5-deaza-FAD, were tested as possible substitutes for FAD. In 8-Cl-FAD the $-CH_3$ group is replaced by a reactive, electronegative group (5). 5-Deaza-FAD cannot adopt the quinomethide form of FAD that has been proposed as intermediate in the mechanism of FAD binding (Figure 2). Neither derivative is able to bind covalently to the apoenzyme or to restore the enzymatic activity of apo-6-hydroxy-D-nicotine oxidases. The strong influence of the methylene bridge between the isoalloxazine and the histidine rings on the enzymatic activity is remarkable.

SITE-DIRECTED MUTAGENESIS Knowledge of the primary structure of 6-hydroxy-D-nicotine oxidase made it possible to elucidate the specificity and importance of individual amino acids for covalent binding of the coenzyme as well as for the catalytic process. Replacement of His_{71} by uncharged residues such as alanine or hydroxyamino acids (serine, tyrosine) allowed neither binding nor enzymatic activity (43). Cysteine acts as covalent acceptor

of the 8-methyl group of flavin in a number of flavoproteins (Table 2). Substituting His_{71} in 6-hydroxy-D-nicotine oxidase with cysteine did not lead to covalent FAD binding under a variety of conditions; however, a substantial amount of enzyme activity was obtained with the mutated apoenzyme in the presence of FAD and substrate. This surprising finding suggests that covalent attachment per se is not a necessary prerequisite of activity; rather, the correct topology at the active center is important and can be best accomplished in some enzymes by covalent fixation of the cofactor.

One can also expect that the environment of the active center, i.e. amino acid residues near the binding or catalytic site of the enzyme, influences the enzymatic activity. As an example, the role of Arg_{67} was studied in some detail (41). Replacement of Arg with Ala abolished covalent FAD attachment. Covalent flavinylation of apo-6-hydroxy-D-nicotine oxidase was reestablished by Lys in position 67. However, incorporation of FAD into the Lys_{67}-polypeptide was dependent to a much higher degree on the presence of the allosteric effector glycerol 3-phosphate than the incorporation into the wild-type protein. This finding underlines the importance of a certain conformational state of the apoenzyme for the covalent binding of the cofactor.

SH-group blockers were found to be potent inhibitors of holoenzyme formation from apoenzyme and FAD. The inhibitory effect of dithiodinitrobenzoic (DTNB) on the flavinylation reaction could be prevented by mercaptoethanol. Interestingly, iodacetamide, a compound that reacts with the thiolate ion, had no effect on holoenzyme formation at concentrations up to 1 mM. The conformation of the 6-hydroxy-D-nicotine oxidase is not stabilized by a disulfide bridge. Apparently, the inhibitory effect of SH-blockers on holoenzyme formation and enzyme activity is not so much an effect on the conformation of the enzyme but rather one that involves an interaction with an SH group in the reaction centers essential for FAD binding and enzymatic activity.

MECHANISM OF COVALENT FLAVIN ATTACHMENT The proposed nonenzymatic mechanism of holo-6-hydroxy-D-nicotine oxidase synthesis (Figure 2) requires a particularly high conformational specificity of the binding region. The incoming flavin cofactor could then be arranged and held for a finite time in a position that would put the 8α-C in exact and close proximity to the binding atom of the proper amino acid residue; furthermore, a base would have to be in a position to facilitate proton abstraction. The endergonic condensation reaction would be thermodynamically compensated by the reoxidation of the intermediately formed $FADH_2$. Thus, this mechanism requires an oxidant capable of taking electrons from the reduced flavin. In contrast to the process involving a (mono-oxygenase-type) hydroxylation, however, oxygen is not

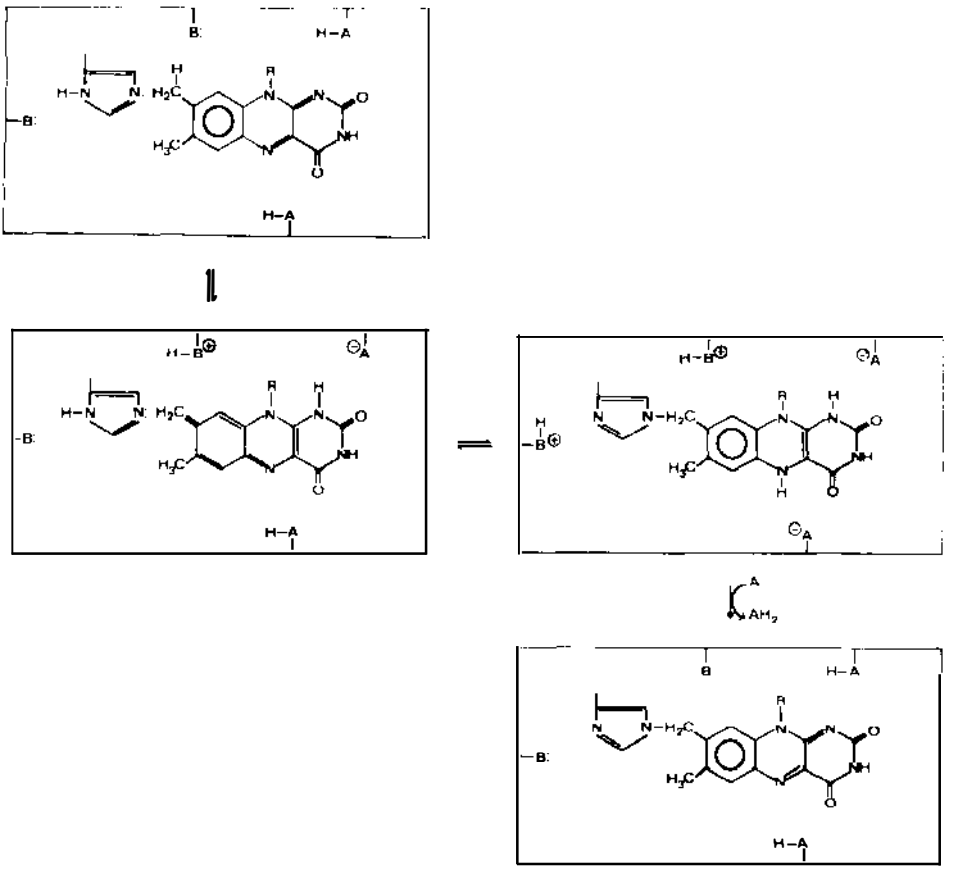


Figure 2 Mechanism of covalent flavinylation of a N3-histidyl residue.

necessarily required. This mechanistic difference can be used to elucidate the coupling reaction.

Reoxidation of the reduced adduct of FAD and the apoenzyme was proposed as the thermodynamic driving force in the establishment of a stable covalent flavoprotein (11). This mechanism (Figure 2) implies that covalent incorporation of FAD into the apoenzyme can take place in the absence of oxygen, provided that an electron acceptor is present to dehydrogenate the fully reduced flavin. Recent experiments (5) indicate that holoenzyme formation takes place in the absence of molecular oxygen, but a requirement of an external electron acceptor could not be demonstrated. Thus, the details of the mechanism,

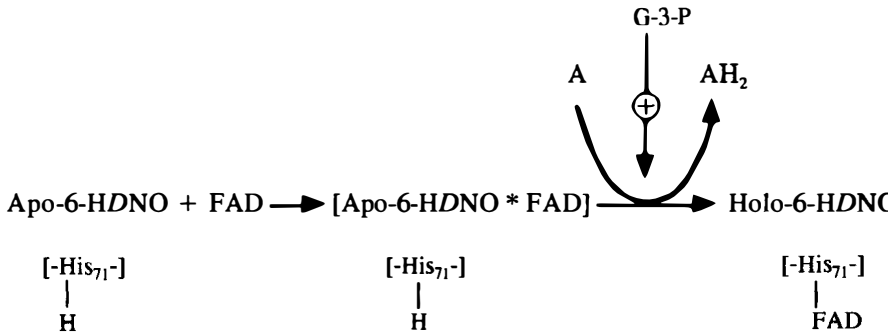


Figure 3 Autocatalytic holoenzyme formation: The flavinylation of 6-hydroxy-D-nicotine oxidase.

including the possibility of an internal electron acceptor, remain to be elucidated.

The covalent flavinylation represents a synthetic chemical reaction that requires the presence of a protein and a cofactor and displays high specificity for both components (Figure 3). The "enzymatic" character of the reaction is further stressed by the absolute requirement for an allosteric effector with certain structural features.

The cell-free flavinylation of the flavoprotein subunit of succinate dehydrogenase and fumarate reductase overexpressed in *E. coli* also seem to need allosteric effectors; in this case it is a dicarboxylic acid, e.g. citrate, isocitrate, succinate or fumarate (4), again indicating a certain degree of specificity of the effector molecule. The "enzymatic" process of flavinylation differs from any other enzymatic reaction in that the "enzyme" is also the substrate and the catalytic cycle operates only once!

Specific Features of Covalent Flavoproteins

CLEAVAGE OF THE COVALENT BOND The mechanism of biological cleavage of the aminoacyl-flavin bond is yet to be discovered. The turnover of all natural products requires that every molecule that is biosynthesized must also be degraded at a similar rate. Therefore, every type of bond encountered in covalent flavoproteins has to be cleaved by a biological process—not necessarily, however, in the same organism. Both 8α-S-cysteinyl- and

N-histidyl-flavins have been found to be partly metabolized in and excreted from rats. The fact that such covalent flavins cannot replace riboflavin as vitamins indicates that little or no rupture of an 8α-linkage occurs that would lead to regeneration of the original 8-methyl within the mammal (8a). Until now, no instance has been reported of a biological reaction breaking one of the known protein-flavin bonds. The 7α- and 8α-hydroxyflavins found in mammalian tissues and fluids (50) resemble a first stage in the metabolism of free riboflavin rather than a product of the cleavage of a covalent flavin-protein adduct.

IS COVALENT FLAVIN ATTACHMENT NECESSARY OR ADVANTAGEOUS? Although a rationale for the existence of covalent flavin-apoprotein binding cannot be presented yet, the possibility of an advantage of covalency in special circumstances should not be dismissed out of hand. Further investigations are needed to consider the energy profiles of the transition states and the redox potentials of reactions catalyzed by covalent flavoproteins as compared to flavoenzymes bearing noncovalently attached cofactors. Particular attention should be given to the metabolic stabilities of the different types of flavoproteins. It will be interesting to learn more about the proteolytic inactivation and the turnover of covalent vs noncovalent flavoproteins in various cell types. Covalent bonding might also be a means of cofactor economy, particularly in cells where the supply of riboflavin is likely to become a limiting metabolic factor.

At present it is difficult to assign specific qualities to the covalent versus the noncovalent flavoproteins and to correlate the binding mode with the functions of the enzymes. Neither the observed differences in the fluorescence properties nor those found in NMR studies of histidyl-flavins allow such assignments. Structurally, the presently known amino acid sequences adjacent to the binding group do not reveal a common denominator. Possibly, there exist certain common features of the tertiary structure that characterize the FAD-binding domains of some covalent flavoproteins but are not evident from the short stretches of available amino acid sequences.

Similar considerations apply to the transition states in flavin catalysis. The firm attachment of the aromatic ring to the polypeptide backbone is likely to influence the mobility of the molecular structure of the coenzyme. Such an effect would be expected to reveal itself in several physical properties of the enzymes, most conspicuously in the redox potentials of the flavin/semiquinone and semiquinone/dihydroflavin couples. Measurements (16, 20, 54) of some histidyl(N)- and cysteinyl(S)-flavins as well as of the free forms (riboflavin and FAD) indicate that 8α-substituted flavins have a more positive (ca 25 mV) redox potential than the free flavins. A significant difference between the various binding types does not exist. In view of the wide range of redox

potentials of flavoproteins, it is difficult to attribute functional significance to the differences between covalently and noncovalently bound flavins, especially as the measurements were taken not on flavoproteins of comparable function but on model compounds.

The primary structure of 6-hydroxy-D-nicotine oxidase as derived from the genomic DNA lacks the characteristic nucleotide-binding region, -x-x-Gly-z-Gly-z-z-Gly-x- (where x stands for a hydrophobic amino acid such as Leu or Val and z for an unspecified amino acid). This motif has been identified in flavoproteins both with (e.g. succinate dehydrogenase) and without (e.g. 6-hydroxyl-L-nicotine oxidase) covalent flavin attachment. It is thought to be instrumental in the initial attachment of the coenzyme.

Recently, a cDNA for the dimethylglycine dehydrogenase from rat liver mitochondria has been isolated and sequenced (39). Upstream of the flavinylated histidine residue, the deduced amino acid sequence exhibits a motif resembling the dinucleotide-binding site characteristic of the flavoprotein subunit of succinate dehydrogenase and fumarate reductase. The same combination of a site for covalent binding of FAD and the dinucleotide-binding domain is also found in monoamine oxidase of the outer mitochondrial membrane (51). These features suggest a primary interaction of FAD with the dinucleotide-binding domain. The ensuing folding of the polypeptide chain might bring a reactive amino acid side-chain in close proximity to the isoalloxazine ring and thus determine the chance of covalent flavinylation.

Is covalent flavin binding to the apoenzyme correlated to the type of the catalyzed reaction? An unequivocal answer to that question cannot yet be given; in two instances only, succinate dehydrogenase and choline oxidase, is it obvious at present that the enzyme from many sources contains covalent FAD and apparently always of the same binding type; the similarity of the bond type even crosses the prokaryote/eukaryote border. It may be that flavoproteins catalyzing a given reaction have the same kind of coenzyme attachment irrespective of the species. This relationship, however, could reflect a genetic inheritance as well as a requirement of the reaction mechanism.

The 6-hydroxynicotine oxidases from *Arthrobacter oxidans* that catalyze the same type of reaction in the same organism but on the enantiomeric (D- and L-)substrates only are genetically unrelated and use free and covalently bound FAD, respectively. Thus, the catalyzed reaction per se cannot be a decisive factor in the choice of coenzyme binding. It is questionable in that case whether the stereospecificity of the reaction requires a specific type of cofactor binding. The apoenzyme of 6-hydroxy-D-nicotine oxidase is catalytically inactive and cannot regain substantial enzymatic activity by the sole addition of (free) FAD. A succinate dehydrogenase-deficient mutant of *Bacillus subtilis* characterized by the lack of covalently bound FAD in the

flavoprotein subunit (26) is still able to integrate the latter into the membrane-associated enzyme (27); enzymatic activity of this mutated succinate dehydrogenase could not be restored by addition of (free) FAD. Nevertheless, an intrinsic correlation between covalent FAD attachment and certain functions, e.g. stereospecificity or assembly of integrated structures, cannot be deduced from these findings.

One aspect of covalency is the potential difference in metabolic stability between covalent and noncovalent flavoproteins. The apoenzyme of 6-hydroxy-D-nicotine oxidase is much more susceptible to proteolytic degradation than the holoenzyme (6). The faster destruction of the apoproteins can be seen as part of an ordered intracellular turnover.

Thus, the question whether covalent attachment of the cofactor in a holoenzyme has prevailed throughout the evolutionary screening process because of biological significance or whether it is a chance event of neutral selective value (11) is still unresolved. However, the fact that at least in two instances a requirement exists for a rather specific allosteric effector would argue against the latter assumption.

NUTRITIONAL ASPECTS The well-known features of riboflavin deficiency need not be elaborated here; but we must ask whether covalent attachment requires specific levels or ways of vitamin availability. This discussion is seriously hampered by the fact that most data on covalent flavoproteins and on the relation between their synthesis and the cofactor supply come from studies with prokaryotic organisms and with eukaryotes that are able to synthesize riboflavin de novo. One has to be very careful in transposing such data to the vitamin-requiring human or animal.

The riboflavin-requiring mutant strain of *A. oxidans* (24) provided some clues regarding the vitamin dependence of the syntheses of covalent and noncovalent flavoproteins. While the synthetic capacity for the latter was fully engaged at an extracellular riboflavin concentration of 2 μM, the maximal production of the covalent flavoprotein, 6-hydroxy-D-nicotine oxidase, required a concentration of 8 μM. The decisive value, of course, is that of the intracellular precursor pool, free FAD. In the mutant bacteria, this pool increased proportionally up to an extracellular riboflavin concentration of 12 μM. At 15 μM it was 6 times the FAD level found at 2 μM; concomitantly, the activity of 6-hydroxy-D-nicotine oxidase increased 5.3-fold (29). The half-maximal rate of enzyme synthesis required 5 μM free FAD while the average half-saturation of the noncovalent flavoproteins was attained at < 1 μM. At full saturation of covalent flavoprotein synthesis, the intracellular concentration of free FAD was 43 μM, the total FAD content 66 nmol per gram wet weight (30). In comparison, the FAD content of rat liver is about 35 nmol per gram wet weight corresponding to a cytosolic concentration of ca 50 μM. Apparently, the riboflavin supply of a well-fed rat is very near the

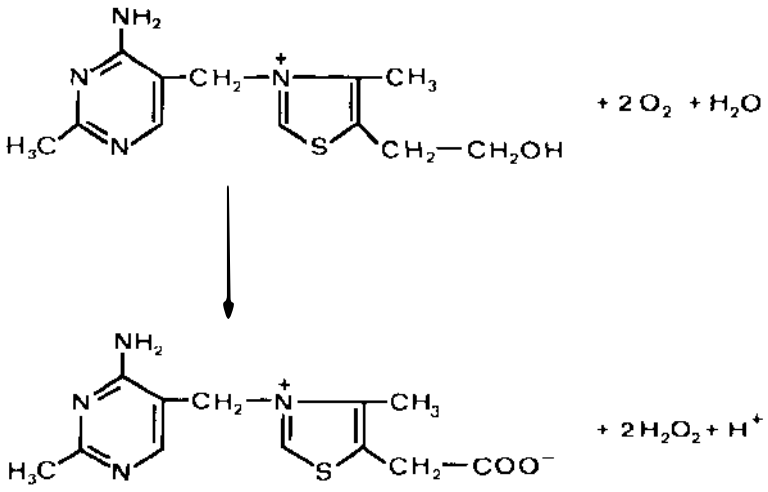
saturation level of covalent flavoprotein synthesis but quite in excess of the level necessary for the production of noncovalent flavoproteins—if these syntheses proceed under conditions comparable to those in the mutant bacteria. In this case, reduced availability of the vitamin may primarily affect the synthesis of covalent flavoproteins both for lack of sufficient cofactor and as a result of an increased rate of apoenzyme proteolysis (see above).

Table 2 clearly shows that important reactions of the energy-providing pathway (succinate dehydrogenase), the regulation of hormone and neurotransmitter levels (monoamine oxidase, enzymes of choline degradation), and the homeostasis of the phospholipid spectrum of membranes (dimethylglycine and sarcosine dehydrogenases) are afflicted by impaired activity of covalent flavoproteins. Ongoing studies of the mechanism of synthesis of the latter enzymes (R. Brandsch, personal communication) may soon provide us with data about the effects of riboflavin and cofactor availability on covalent flavoprotein function in mammals.

PRESENTLY KNOWN FLAVOPROTEINS WITH COVALENTLY BOUND FLAVIN COFACTORS (FAD OR FMN)

Flavoproteins with a Histidyl(N1)-8α-Flavin Linkage

THIAMIN OXIDASE The bacterial enzyme (23, 46) consists of a single peptide chain of M_r = 50,000 containing one mole of covalently bound FAD. It catalyzes the sequential dehydrogenations of the alcoholic form to the carboxylate form, yielding under anaerobic conditions 1 mol of fully reduced $FADH_2$ after addition of 0.5 mol thiamin; oxygen is reduced to H_2O_2 (Equation 1).

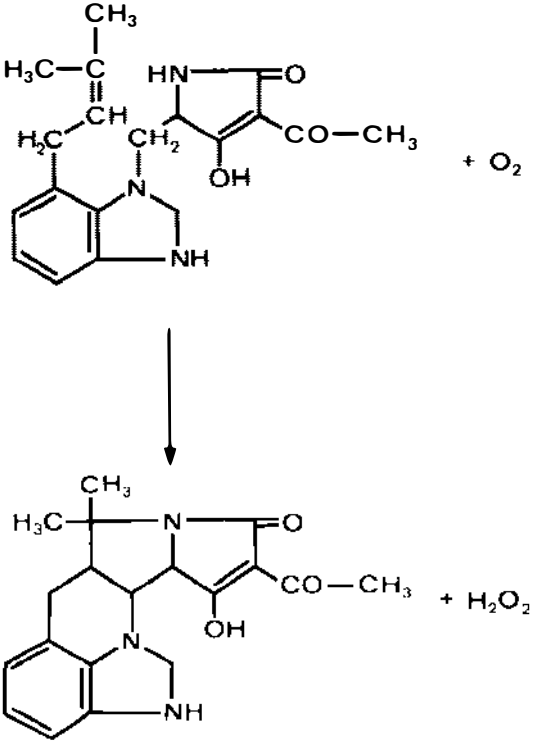


1.

Thus it is an oxidase with redox potentials of the two couples (oxidized/semiquinone and semiquinone/reduced form of FAD) of + 80 and + 30 mV at pH 7.2, respectively. The type of covalent binding between FAD and the apo-thiamin oxidase was established (18, 31) as His(N1)-8α-FAD using amino acid analysis as well as spectrophotometric, fluorescence, NMR, and EPR measurements.

Oxythiamin and pyrithiamin that are sometimes used as "antivitamins" react with thiamin oxidase, the former as substrate, the latter as irreversible inhibitor (23, 46). The coenzyme form of thiamin, thiamin diphosphate, is neither a substrate nor an inhibitor of the enzyme.

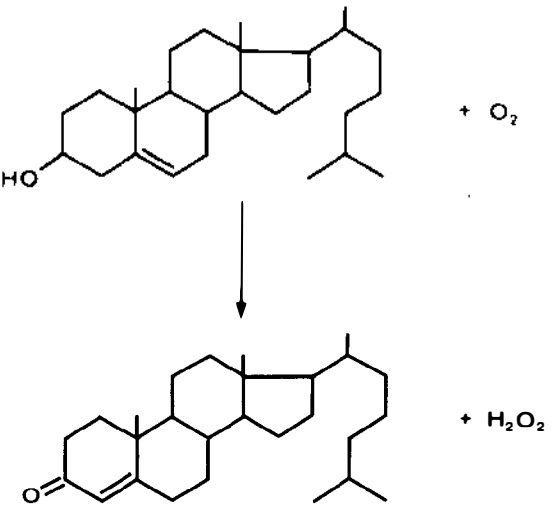
CYCLOPIAZONATE OXIDOCYCLASE An enzyme from *Penicillium cyclopium* has been obtained in homogeneous form that catalyzes the dehydrogenation and cyclization of β-cyclopiazonate to α-cyclopiazonate (Equation 2).



2.

The enzyme contains FAD covalently attached via its 8α-methyl group to the N1 of a histidyl residue (19) as shown by NMR measurements, spectroscopy and fluorescence quenching ($pK_a \simeq 5.2$), as well as by its ability to be reduced by borohydride and to form 8-formyl-riboflavin upon storage.

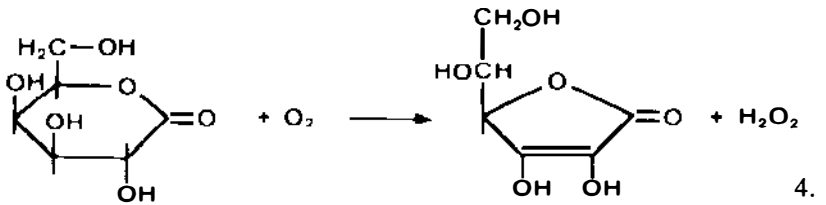
CHOLESTEROL OXIDASE *Schizophyllum commune*, a soil organism, is the source of cholesterol oxidase that converts free cholesterol into cholest-4-en-3-one with the concomitant production of H_2O_2 (Equation 3); coupled with peroxidase and a chromogenic substrate, this reaction is widely used for the determination of cholesterol in biological samples.



3.

Cholesterol oxidase is an auto-oxidizable, monomeric protein of M_r = 53,000 containing one mole of covalently bound FAD (22). The covalent linkage between the peptide and the FAD was identified as His(N1)-8α-riboflavin by its absorption spectrum and the typical pH-dependent fluorescence quenching (pK_a = 5.8; 5.05 after acid hydrolysis to the aminoacyl-flavin) of His(N1)-8α-riboflavin derivatives.

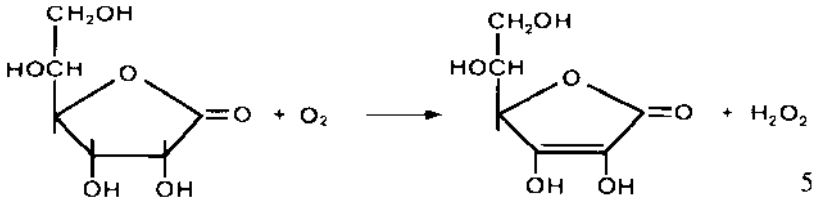
L-GALACTONOLACTONE OXIDASE The last steps of L-ascorbic acid synthesis in yeast cells are the formation of 2-keto-L-galactono-γ-lactone from L-galactonolactone followed by isomerization to L-ascorbate (Equation 4).



4.

The cofactor FAD is covalently bound in L-galactonolactone oxidase to the apoenzyme by a histidyl(N1)-8α-flavin linkage (33).

L-GULONO-γ-LACTONE OXIDASE This enzyme catalyzes the last enzymatic step in the biosynthesis of L-ascorbic acid in some animal tissues. It converts L-gulonolactone to 2-keto-gulono-γ-lactone and thence by isomerization to L-ascorbate (Equation 5).

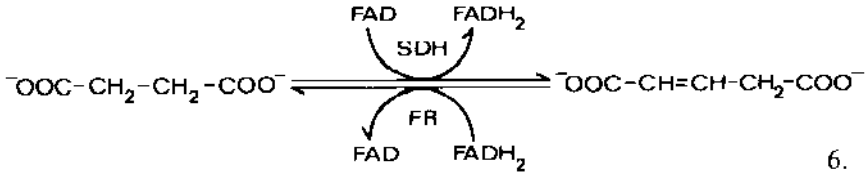


5.

The lack of L-gulonolactone oxidase in humans, primates, and guinea pigs renders these species dependent on an external supply of L-ascorbate (vitamin C). The structure of the oxidase is highly conserved throughout the animal kingdom as demonstrated by immunological methods (47). L-Gulonolactone oxidase from rat liver possesses a M_r of 50,605 as shown by sequence analysis of a cDNA (37). The covalent flavin of this enzyme was identified as His(N1)-8α-riboflavin by the methodology used to analyze the cofactor of thiamin dehydrogenase.

Flavoproteins with a Histidyl(N3)-8α-Flavin Linkage

SUCCINATE DEHYDROGENASE AND FUMARATE REDUCTASE The flavoprotein most widely distributed among aerobic organisms is succinate dehydrogenase (SDH). The corresponding enzyme of the anaerobic world is fumarate reductase (FR). Both enzymes share not only the same chemical reaction (Equation 6) (though physiologically operating in opposite directions) and the binding type of the apoenzyme but also a common genetic ancestry.



6.

Succinate dehydrogenase of eukaryotic organisms is a multienzyme complex located on the matrix side of the inner mitochondrial membrane. It forms complex 2 of the respiratory chain. The electrons of its $FADH_2$ are transferred directly to ubiquinone. Aerobically grown bacteria make use of a similar succinate dehydrogenase complex present in the cytoplasmic membrane.

The basic structure of the complexes of succinate dehydrogenase and of fumarate reductase has been highly conserved during evolution. They all consist of two hydrophilic subunits forming the catalytic part of the complex that is anchored to the mitochondrial and cytoplasmic membranes, respectively, by one or two hydrophobic polypeptides (28). The larger (about 70 kDa) of the hydrophilic subunits (Sdh A and Fr A, respectively) carry both covalently bound FAD and iron-sulfur clusters as cofactors. The same type of cofactor binding is present in the mitochondrial succinate dehydrogenase of beef heart (55), in *Saccharomyces cerevisiae* (48), and in succinate dehydrogenase and fumarate reductase of bacterial origin (34).

In anaerobic phosphorylation-coupled electron transport, fumarate reductase catalyzes the reverse reaction (Equation 6), the hydrogenation of fumarate to succinate. This constitutes the terminal step in some fermentations, e.g. in *Wollinella succinogenes* (40).

By site-directed mutagenesis of the *E. coli frd* operon, the FAD-carrying histidine$_{44}$ of fumarate reductase was replaced by Cys, Ser, Tyr, and Arg (1a). FAD was bound firmly but noncovalently in these fumarate reductase mutants. They retained significant fumarate reductase activity when assayed by reduction of benzyl viologen but were unable to oxidize succinate (1). Apparently, covalent FAD binding to the enzyme is required for the oxidation of succinate (E_m = +30 mV) but not for fumarate reduction by menaquinol (E_m = –74 mV), the physiological electron donor.

6-HYDROXY-D-NICOTINE OXIDASE 6-Hydroxy-D-nicotine oxidase, a flavoprotein of *Arthrobacter oxidans* with FAD covalently attached through a His(N3)-8α-riboflavin bond (45), acts specifically on the D-isomer of 6-hydroxy(nor)nicotine. This enzyme is instrumental in the catabolism of D-nicotine and D-nornicotine (Figure 4); while D-nicotine has not yet been detected in natural sources, D-nornicotine has been found in tobacco plants.

6-Hydroxy-D-nicotine oxidase catalyzes the stoichiometric conversion of 6-hydroxy-D-nicotine to 6-hydroxy-N-methylmyosmine and the hydrolysis of the latter to [6-hydroxypyridyl(3)](N-methylaminopropyl)-ketone ("ketone") with the simultaneous reduction of molecular oxygen to H_2O_2. The enzyme consists of a single peptide chain of M_r = 47,077, as derived from the gene structure, including 1 mol of covalently bound FAD (8). Some of its properties are listed in comparison with those of 6-hydroxy-L-nicotine oxidase in Table 3.

The elucidation of the synthesis of 6-hydroxy-D-nicotine oxidase and its regulation were greatly facilitated by the use of a riboflavin-requiring (rf) mutant of *A. oxidans* (24). Quantitative riboflavin incorporation experiments and the appearance of 6-hydroxy-D-nicotine-oxidase-specific mRNA in the

Figure 4 Initial steps in the bacterial degradation of nicotine.

Table 3 Properties of 6-hydroxy-D-nicotine oxidase (HDNO) and 6-hydroxy-L-nicotine oxidase (HLNO)[a]

Property	HDNO	HLNO
Relative mol mass (M_r)	49,077	106,600
Polypeptide chains/mol	1	2
FAD (mol/mol enzyme)	1	2
Binding of FAD	Covalent	Noncovalent
N-terminal amino acids	V-S-S-K-L-	-M-Y-D-A-I
C-terminal amino acids	N-L-Q-S-A	-A-H-I-S-L
Turnover number (mol/min/mol, 30°C)	1,320 (pH 9.2)	4,160 (pH 7.5)
K_m (K_i) (mM)		
6-hydroxy-D-nicotine	0.05	(0.1)
6-hydroxy-L-nicotine	(1.5)	0.02
λ_{max} vis (nm)	355, 450, 475(S)	370, 443, 463(S)
Reactivity towards		
oxygen	Yes	Yes
1 e-acceptors	No	No
2 e-acceptors	Yes	No
Intermediate flavin radical		
with $S_2O_3^{2-}$	Anionic (red)	
with $h\nu$ + EDTA		Anionic (red)

[a] Reprinted from *BioFactors* 3:73 (1991) with permission of Oxford University Press and from *Chemistry and Biochemistry of Flavoenzymes* 2:351 (1991), copyright CRC Press, Inc. Boca Raton, Florida

stationary phase of growth (42) indicate that about 50% of the total covalently bound flavin resides in 6-hydroxy-D-nicotine oxidase, while the other half belongs to constitutive proteins including succinate dehydrogenase.

CHOLINE OXIDASE The metabolic degradation of choline has been observed in animal as well as in microbial cells. Two major pathways emerged from these studies: the first one operates mainly in animal cells and employs two separate enzymes, choline dehydrogenase forming betaine aldehyde and betaine dehydrogenase converting the aldehyde to betaine. Choline dehydrogenase from rat liver might also contain a modified flavin as the prosthetic group; its structure has yet to be identified. The second route requires only one enzyme for both processes: choline oxidase converts choline to betaine with the reduction of oxygen to hydrogen peroxide (Equation 7).

$$(CH_3)_3N^+\text{-}CH_2\text{-}CH_2OH + 2\ O_2 + H_2O \longrightarrow (CH_3)_3N^+\text{-}CH_2\text{-}COO^- + H^+ + 2\ H_2O_2 \qquad 7.$$

This oxidase activity is present in several microorganisms. The coenzyme of choline oxidase is FAD covalently bound to N3 of a histidyl residue by its 8α-C (49).

DIMETHYLGLYCINE DEHYDROGENASE AND SARCOSINE DEHYDROGENASE Betaine can be further metabolized to dimethylglycine, sarcosine (monomethylglycine), and glycine. In animal tissues this reaction sequence is catalyzed by dehydrogenase-type flavoproteins. In bacteria, oxidases seem to convert sarcosine to glycine. The overall process consists in the stepwise oxidative removal of both methyl groups of dimethylgycine (Equations 8 and 9):

$$(CH_3)_2N\text{-}CH_2\text{-}COOH + A_{ox} + THF \longrightarrow CH_3NH\text{-}CH_2\text{-}COOH + A_{red} + 5,10\text{-methylene THF} \quad 8.$$

$$CH_3NH\text{-}CH_2\text{-}COOH + A_{ox} + THF \longrightarrow H_2N\text{-}CH_2\text{-}COOH + A_{red} + 5,10\text{-methylene THF.} \quad 9.$$

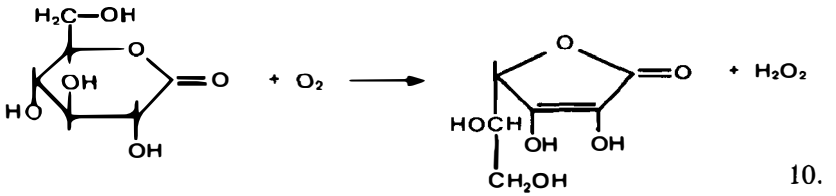
Tetrahydrofolate (THF) is the cofactor noncovalently bound to the dehydrogenases that accepts the methyl groups at the oxidation level of formaldehyde as 5,10-methylenetetrahydrofolate.

The animal dehydrogenases involved in the conversion of dimethylglycine to glycine are present exclusively in liver mitochondria (9); they are structurally related but clearly distinguishable entities.

The sarcosine oxidases isolated from bacterial sources contain 1 mol each of noncovalently and covalently [histidyl(N3)-8α-] bound FAD per mole of enzyme. The two types of FAD cofactors may serve different functions in enzymatic catalysis. It appears that the noncovalent FAD participates in the dehydrogenase function and transfers its electrons to the covalent flavin. The covalent $FADH_2$ then reduces oxygen to hydrogen peroxide, thus acting as an oxidase flavin (38). The sarcosine oxidase of *Corynebacterium sp. P-1* is also unusual because its subunit composition contains four dissimilar subunits (38).

D-GLUCONOLACTONE OXIDASE (DEHYDROGENASE) Analogous to the synthesis of L-ascorbic acid is the production by *Penicillium cyaneo-fulvum* of D-erythorbic (D-araboascorbic) acid (Equation 10); while the former pathway involves the oxidation of L-gulono-γ-lactone in animals or galactono-γ-lactone in plants, the latter requires the action of D-glucono-γ-lactone oxidase.

Oxygen is the most efficient electron acceptor. Hydrogen peroxide is formed in stoichiometric amounts during erythorbic acid production (58); the enzyme appears to be an oxidase rather than a dehydrogenase.



10.

Flavoproteins with Flavin Bound to a Cysteinyl Residue

This group of covalent flavoproteins includes two different binding types: polypeptides bound through cysteinyl residues to the 8α position of the flavin and those where the thioether linkage is between a cysteinyl sulfur and the carbon atom 6 of the isoalloxazine ring.

MONOAMINE OXIDASE The most intensively studied representative of the Cys-S-8α type is monoamine oxidase (MAO) (59). The enzyme is widely distributed in animal tissues where it resides in mitochondria. It is instrumental in the inactivation of various neurotransmitters, hormones, and drugs. The oxidation of the amine results in the formation of the corresponding aldehyde and hydrogen peroxide (Equation 11)

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + NH_3 + H_2O_2. \qquad 11.$$

This enzyme of M_r = 52,000 contains one mole of covalent FAD as the prosthetic group. Phenylhydrazine and some amines with an acetylenic structure, e.g. pargyline, are able to attach covalently to the flavin of MAO. These "suicide inhibitors" lead to an irreversible inactivation of the enzyme.

FLAVOCYTOCHROMES C Some bacterial species, e.g. *Chromatium* and *Chlorobium*, contain flavocytochromes c in which both the heme and the flavin component are covalently attached to different subunits of the enzyme (10). Heme and flavin exist in a 2:1 ratio. In both the purple and the green phototrophic sulfur bacteria, the FAD is bound by a thioether bridge between its 8α-carbon and a cysteinyl residue of the respective subunit (35, 36). These flavocytochromes c apparently act as electron acceptors in the oxidation of sulfide to elemental sulfur. Another flavocytochrome c catalyzing the hydroxylation of *p*-cresol in *Pseudomonas* has a differently bound flavin and is discussed below.

TRIMETHYLAMINE DEHYDROGENASE Trimethylamine dehydrogenase is unique among the flavoproteins with covalent cofactor attachment: the flavin component is FMN rather than FAD, and the bond between the apoenzyme and the coenzyme is a thioether involving cysteine and the 6-position of the

isoalloxazine ring of FMN (57). The evidence for this type of linkage came mainly from NMR spectroscopy, sulfur chemistry, lack of the fluorescence typical for 8α-substituted flavins, and the reactions with thiol reagents such as iodoacetamide.

Trimethylamine dehydrogenase is induced in a variety of obligate and facultative methylotrophic bacteria growing on trimethylamine as sole carbon source. The enzyme is a dimer of two identical subunits of M_r 83,000 each; it contains one iron-sulfur cluster of the 4 Fe-4 S^{2+} type and one covalent flavin per subunit. The enzyme catalyzes the oxidative demethylation of trimethylamine yielding dimethylamine and formaldehyde; in the cell, the electrons are transferred to a FAD-containing electron transfer protein. Using an artificial electron acceptor the reaction (Equation 12) is

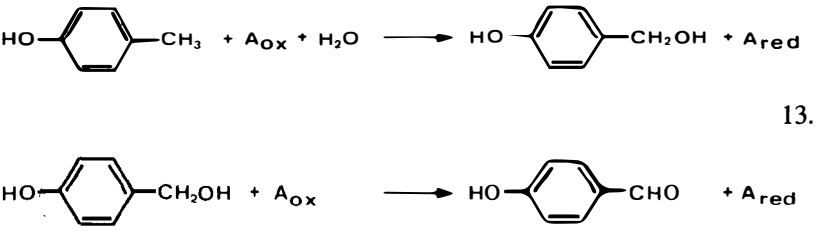
$$(CH_3)_3N + H_2O + A_{ox} \longrightarrow (CH_3)_2NH + CH_2O + A_{red}. \qquad 12.$$

The enzyme does not oxidize monoamines; however, the "suicide inhibitors" of monoamine oxidase are also effective inhibitors of trimethylamine dehydrogenase.

DIMETHYLAMINE DEHYDROGENASE Dimethylamine dehydrogenase converts the product of the trimethylamine dehydrogenase reaction, dimethylamine, to methylamine, formaldehyde, and hydrogen peroxide in an analogous fashion (Equation 12). The spectral and structural properties of the enzyme are similar to those of trimethylamine dehydrogenase although they are different proteins. The dimer (M_r 138,000) contains a similar iron-sulfur cluster and 6-S-cysteinyl-FMN as the flavin prosthetic group (56).

Flavoprotein with a Tyrosyl(O)-8α-Flavin Linkage

p-CRESOL METHYLHYDROXYLASE *Pseudomonas putida* and some other members of this family initiate the anaerobic degradation of *p*-cresol by dehydrogenation/hydration of its methyl group followed by the reversible dehydrogenation of the intermediate *p*-hydroxybenzaldehyde to *p*-hydroxybenzyl alcohol (Equation 13).



A constitutive isozyme and an inducible isozyme of *p*-cresol methylhydroxylase, both with similar enzymologic properties, have been observed on a plasmid of the pseudomonad [see (10)]. The enzyme consists of two flavin- and two heme-carrying subunits. *p*-Cresol methylhydroxylase is the sole flavoprotein known so far to have the prosthetic group FAD attached through the 8α-carbon of the isoalloxazine ring to the phenolic O- of a tyrosyl residue of the respective subunit (44).

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