What's in a covalent bond? On the role and formation of covalently bound flavin cofactors
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Introduction

Enzymes can be divided into two groups: (a) enzymes that perform catalysis without the use of cofactors; and (b) enzymes that require one or more cofactors. Examples of the first group are hydrolases, which carry out catalysis by employing the amino acids present in the polypeptide chain. Cofactor-dependent enzymes usually make use of nonprotein groups. These cofactors may be inorganic in nature, e.g. Cu$^+$ or Fe–S clusters, but organic molecules are also employed, e.g. NADP$^+$ or pyridoxal phosphate. Enzymes may harbor a combination of cofactors, such as mitochondrial complex II (succinate dehydrogenase), which contains heme, flavin, and three Fe–S clusters. Cofactors are often noncovalently linked, and dissociate from the enzyme during catalysis and thereby act as coenzymes, e.g. NADP$^+$, coenzyme A, or ubiquinone. Alternatively, the cofactor is noncovalently bound but dissociation from the enzyme is not required for catalysis. In fact, avid binding ensures that the cofactor does not dissociate easily, and this may only occur if the protein is denatured. In contrast, some specific cofactors, e.g. lipoic acid and biotin, are exclusively bound covalently to the polypeptide chain. The covalent lipoyl–lysine and biotinyl–lysine bonds function as swinging arms complex II (succinate dehydrogenase), which contains heme, flavin, and three Fe–S clusters. Cofactors are often noncovalently linked, and dissociate from the enzyme during catalysis and thereby act as coenzymes, e.g. NADP$^+$, coenzyme A, or ubiquinone. Alternatively, the cofactor is noncovalently bound but dissociation from the enzyme is not required for catalysis. In fact, avid binding ensures that the cofactor does not dissociate easily, and this may only occur if the protein is denatured. In contrast, some specific cofactors, e.g. lipoic acid and biotin, are exclusively bound covalently to the polypeptide chain. The covalent lipoyl–lysine and biotinyl–lysine bonds function as swinging arms

Abbreviations

6-HDNO, 6-hydroxy-D-nicotine oxidase; BBE, berberine bridge enzyme; ChitO, chito-oligosaccharide oxidase; CholO, cholesterol oxidase type II; DAAO, D-amino acid oxidase; GMC, glucose oxidase/methanol oxidase/cholesterol oxidase; GOOX, gluco-oligosaccharide oxidase; LaspO, L-aspartate oxidase; MAO, monoamine oxidase; MSOX, monomeric sarcosine oxidase; Na$^+$-NQR, Na$^+$-translocating NADH-quinone reductase; P2Ox, pyranose 2-oxidase; PCMH, p-cresol methylhydroxylase; PuO, putrescine oxidase; TMADH, trimethylamine dehydrogenase; VAO, vanillyl-alcohol oxidase.
that shuttle intermediate compounds between the active sites of the respective enzyme complexes [1]. In some enzymes, amino acyl groups act as covalent cofactors, e.g. in disulfide reductases [2], and in other proteins, redox cofactors are formed \textit{in situ} from amino acyl groups [3], e.g. topaquinoine in serum amine oxidase, tryptophan tryptophylquinone in bacterial methylamine dehydrogenase, and cysteine tryptophylquinone in bacterial quino-cytochrome amine dehydrogenases. Topaquinoine is made without an external catalyst, whereas the formation of tryptophan tryptophylquinone and cysteine tryptophylquinone does require external enzymes [4,5].

Heme and flavin cofactors are the only examples that can be either covalently or noncovalently bound to enzymes. Most flavoproteins contain a tightly but noncovalently bound flavin. Nevertheless, it is estimated that about 10% of all flavoproteins contain a covalently bound flavin. Several types of covalent flavin–protein linkages that have been discovered are described in detail in the next section.

**Types and occurrence of covalent flavin–protein bonds**

The first experimental data to suggest the existence of covalent flavoproteins were published in the 1950s [6–8]. Verification of this atypical flavin binding mode was obtained upon isolation of succinate dehydrogenase [9–11]. The flavin–protein bond was identified as an 8-$\alpha$-$N^1$-histidyl–FAD linkage [12]. The seven known types of covalent flavin binding are 8-$\alpha$-$N^1$-histidyl–FAD/FMN, 8-$\alpha$-$N^1$-histidyl–FAD/FMN, 8-$\alpha$-$O$-tyrosyl–FAD, 8-$\alpha$-$S$-cysteinyln–FAD, 6-$\alpha$-cysteinyln–FMN, 8-$\alpha$-$N^1$-histidyl-6-$\alpha$-cysteinyln–FAD/FMN, and phosphoester-threonyln–FMN (Fig. 1). The most abundant type of covalent flavin attachment is the one in which FAD is bound to a histidine (Table 1). Cysteinyln–FAD and cysteinyln–FMN linkages are less widespread, and the tyrosyl–FAD linkage has been found only in $p$-cresol methylhydroxylase (PCMH) and its close relative 4-ethylphenol methylene hydroxylase [13].

Most of the above-mentioned covalent flavin–protein binding types have been known for some time [14]. However, a novel kind of covalent FAD linkage was discovered recently on inspection of the crystal structure of gluco-oligosaccharide oxidase (GOOX) from the fungus \textit{Acremonium strictum} [15]. For each enzyme molecule, there is one FAD molecule that is covalently tethered via two bonds: an 8-$\alpha$-$N^1$-histidyl–FAD linkage, and a 6-$\alpha$-cysteinyln–FAD linkage. This was the first report of a bicovalent flavoenzyme and, soon after, it was established that several other covalent flavoenzymes also contain a flavin bound in the same manner. These include aclacinomycin oxidoreductase [16], berberine bridge enzyme (BBE) [17], hexose oxidase [18], hexose glycopeptide oxidase dbv29 [19], $\Delta$-tetrahydrocannabinolic acid synthase [20], cannabinolic acid synthase [20], and chito-oligosaccharide oxidase (ChitO) [21].

Another novel type of covalent flavin binding has been described for the NqrB and NqrC subunits of the Na$^+$-translocating NADH-quinone reductase (Na$^+$-NQR) from \textit{Vibrio alginolyticus}. In this case, FMN is covalently linked to a threonine residue via a phosphoester bond [22]. Consequently, it represents the only covalent flavin–protein bond that does not involve a linkage via the isoalloxazine moiety of the flavin. Besides the covalently linked FMN cofactors, the Na$^+$-NQR complex (NqrABCDDEF), which is an integral membrane enzyme, also contains a noncovalently bound FAD in subunit NqrF and riboflavin as cofactor [23]. Thereby, it represents the first reported enzyme to utilize riboflavin as a cofactor. The observation that the covalent FMN linkage in NqrC from \textit{V. cholerae} does not occur when the protein is expressed in \textit{Escherichia coli} suggests that a specific ancillary enzyme is needed for covalent FMN incorporation [24]. As the biochemical data on this unusual type of covalent FMN binding are scarce, the mechanism of covalent threonyln–FMN linkage formation and the functional role of the covalent FMN–protein linkage in NqrB-type and NqrC-type flavoproteins remain unknown.

Two of the largest flavoprotein families are the glucose oxidase/methanol oxidase/cholesterol oxidase (GMC) family and the vanillyl-alcohol oxidase (VAO) family. Each family has its own distinct protein fold for binding of FAD. The VAO family of flavoproteins includes a relatively large number of covalent flavoproteins [25,26]. Inspection of the genome database has revealed that, based on the presence of a conserved histidine, roughly one out of four VAO-type protein sequences represents a histidyl–FAD-containing flavoprotein. Additionally, members of this family have been shown to accommodate four types of covalent attachment (8-$\alpha$-$N^1$-histidyl–FAD, 8-$\alpha$-$N^1$-histidyl–FAD, 8-$\alpha$-$O$-tyrosyl–FAD, and 8-$\alpha$-$N^1$-histidyl-6-$\alpha$-cysteinyln–FAD). This suggests a correlation between protein fold and the ability to form a covalent flavin–protein linkage. Strikingly, although the VAO-type covalent flavoproteins share a similar structural fold, the residue that covalently tethers the FAD cofactor via the 8-methyl moiety is not conserved. The 8-$\alpha$-$N^1$-histidyl–FAD-containing homologs form an FAD linkage via a histidine close to the N-terminus, which is located in the FAD-binding
Fig. 1. (A) All known types of covalent flavin–protein linkages. FMN is shown in black, FAD in black and gray, and known linking amino acids in green. Sites of covalent attachment are indicated by arrows. The numbering of some isoalloxazine atoms is indicated. (B) Types of covalent flavin–protein linkages in some known covalent flavoprotein structures. FAD is shown as sticks (yellow) together with the linking amino acid (green). As no threonyl–FMN-containing flavoprotein structure is known, only a peptidyl-linked threonyl–FMN is shown. The images were generated with PYMOL [90].
Table 1. Covalent flavoproteins and their modes of covalent FAD or FMN binding. The family to which each flavoprotein belongs to is indicated according to the following codes and PFAM ordering: pyridine nucleotide-disulfide oxidoreductase (PF07992); TMD (trimethylamine dehydrogenase domain), Oxidored_FMN (PF00724); VAO, FAD_binding_4 (PF01565); GMC, GMC_oxidred_N (PF00732); succinate dehydrogenase, FAD_binding_2 (PF00890); AMO, Amino_oxidase (PF01593); MSOX, DAAO (PF01266); BDR (reductase FAD-binding domain of reductase), FAD_binding_6 (PF00970); NQR, NQR2_RnfD_RnfE (PF03116).

<table>
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<tr>
<th>Flavin–protein bond</th>
<th>Enzyme</th>
<th>$N^1$-Histidyl or $N^3$-histidyl</th>
<th>Origin</th>
<th>Family</th>
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<td>2IPI</td>
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<td>DAAO</td>
<td>3DJD</td>
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<td>2OLN</td>
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<td>Plant</td>
<td>VAO</td>
<td>–</td>
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<td>Unknown</td>
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<td>Bacteria</td>
<td>BDR</td>
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domain (Fig. 1B). In contrast, the residues that form the $8\alpha$-N$^1$-histidyl–FAD and $8\alpha$-O-tyrosyl–FAD linkages are located at two different positions in the cap domain (Fig. 1B). The $8\alpha$-N$^1$-histidyl–FAD linkage type appears to be prevalent in VAO-type covalent flavoproteins (Table 1) and, in some cases, is accompanied by a 6-S-cysteinyl–FAD linkage. In addition to the GMC-type and VAO-type flavoprotein folds, other folds have been shown to facilitate covalent flavin binding (Table 1).

There seems to be no relationship between a specific covalent bond type and a class of organisms (Table 1). $8\alpha$-S-Cysteinyl-FAD and the most abundant type of monocovalent flavin binding, $8\alpha$-histidyl–FAD, are found in all kingdoms of life. The rare covalent flavin–protein linkages, 6-S-cysteinyl–FMN, threonyl–FMN, and $8\alpha$-O-tyrosyl-FAD, have so far only been found in bacterial proteins. Also, the variety of substrates transformed by the different flavin-containing enzymes shows that a covalent flavin is not required to convert a specific class of substrates. This is nicely exemplified by a number of cases where the same substrate can be converted by a covalent flavoenzyme as well as by a noncovalent flavoenzyme. This is the case for hexose oxidase, which contains a bicovalent FAD cofactor [18], and glucose oxidase, which contains noncovalent FAD [27]. Both enzymes catalyze the oxidation of the C1 hydroxyl moiety on glucose, yielding the corresponding lactone as product. Similarly, cholesterol oxidases with covalent FAD and noncovalent FAD provide another case of structurally unrelated enzymes catalyzing the same reaction (convergent evolution) [28,29]. One exception seems to be membrane-bound succinate dehydrogenase (and the closely related fumarate reductase), which is found in both prokaryotes and eukaryotes, and contains the same covalent FAD.

Fig. 2. General mechanism for covalent $8\alpha$-histidyl–flavin, tyrosyl–flavin or cysteinyl–flavin formation. B1–B3 represent active site bases potentially involved in covalent flavinylation, and L stands for the ligand amino acid (histidine, tyrosine, or cysteine) that covalently binds to the flavin. Extracted from [38,45,48,51,83].

Table 1. (Continued).

<table>
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<tr>
<th>Flavin-protein bond</th>
<th>Enzyme</th>
<th>$N^1$-Histidyl or $N^3$-histidyl</th>
<th>Origin</th>
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<td>–</td>
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<td>Bacteria</td>
<td>DAAO</td>
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<td>$N^1$</td>
<td>Archaea</td>
<td>Pyridine nucleotide-disulfide oxidoreductase</td>
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<tr>
<td>6-S-Cysteinyl</td>
<td>TMADH [168]</td>
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<td>Bacteria</td>
<td>TMD</td>
<td>2TMD</td>
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<td>TMD</td>
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<td>–</td>
<td>–</td>
<td>Bacteria</td>
<td>NQR</td>
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* Sequence homology with BBE suggests an $8\alpha$-histidyl-6-S-cysteinyl–FAD linkage.
binding in all cases. This indicates that, during evolution, there has been some benefit in acquiring and retaining this specific type of covalent FAD–protein bond.

From the list of covalent flavoproteins in Table 1, it is clear that most of these enzymes are involved in oxidative processes. In fact, it is striking that most covalent flavoproteins are oxidases, and only a few reductases and dehydrogenases are known that contain a covalent flavin. This is probably because covalent flavinylation usually significantly increases the redox potential (see below), thereby limiting the type of electron-accepting redox partners to high-potential partners.

Formation of covalent flavin–protein bonds

For enzymes containing covalent heme or biotin, the covalent attachment is catalyzed by a holocytic heme c-lyase and a biotin-holo-carboxylase synthetase, respectively [30,31]. For covalent flavin incorporation, no ancillary enzymes that aid in forming the covalent cofactor–protein bond have been described so far, although it is believed that such enzymes are needed for the phosphoester-threonyl–FMN linkage (see above). Despite the growing number of known covalent flavoproteins, no unique protein sequence motif has been found that can predict whether a flavoprotein will contain a covalently bound flavin. Recent studies on the mechanism of covalent flavinylation strongly suggest that it represents a post-translational self-catalytic protein modification. In fact, the chemistry underlying covalent flavinylation (Fig. 2) has been proposed by numerous investigators since the discovery of covalent flavoproteins in the 1950s. A full mechanistic scheme was first published by Walsh [32,33], although Bullock & Jardetzkey [34] proposed that the flavin iminoquinone methide isomer (formed in step 1 of Fig. 2) formed during the aerobic oxidation of the 8α-hydrogens with solvent deuterium at high temperature in D₂O. This intermediate is also involved in the base-catalyzed formation of 8α-N-morpholino-2',3',4',5'-tetraisobutyrylriboflavin and 8α-N¹-imidazolyl-2',3',4',5'-tetraisobutyrylriboflavin, and a dimer of this flavin linked via the 8α-carbons of each flavin unit [35]. The best-studied enzymes with regard to the mechanism of covalent flavinylation are monomeric sarcosine oxidase (MSOX), PCMH, 6-hydroxy-D-nicotine oxidase (6-HDNO), VAO, and trimethylamine dehydrogenase (TMADH). In the next paragraphs, details on covalent flavinylation of these flavoenzymes are presented.

MSOX

Bacterial monomeric MSOX catalyzes the oxidative demethylation of sarcosine to yield glycine, formaldehyde, and hydrogen peroxide. MSOX contains one covalent FAD per enzyme molecule, and the FAD is linked via the 8α-methyl group of the isoalloxazine moiety to Cys315 [36]. To study the covalent incorporation of FAD, an elegant method was applied in order to obtain apo-MSOX: the enzyme was produced using a riboflavin-dependent E. coli strain [37]. With this approach, the apo-protein could be overexpressed and purified. A time-dependent reduction of FAD under anaerobic conditions was observed upon incubation of apo-MSOX with FAD. The covalent coupling of FAD to apo-MSOX resulted in an increase in catalytic activity. During the aerobic coupling reaction, stoichiometric amounts of hydrogen peroxide were produced, implying the presence of a reduced flavin intermediate during covalent coupling, which is reoxidized by molecular oxygen. These data suggest that covalent coupling of FAD occurs in a self-catalytic manner. Further evidence for the mechanism of covalent coupling was obtained by conducting experiments where FAD analogs were incubated with apo-MSOX. Covalent FAD binding was not observed with the analogs 1-deaza-FAD and 5-deaza-FAD. This is explained by a lower redox potential than that of free, unmodified FAD, which could cause the decrease in acidity of the C8-methyl protons of the FAD analogs (Fig. 2) through decreased electrophilicity of the flavin ring system [37].

PCMH

Bacterial PCMH catalyzes the oxidation of p-cresol to 4-hydroxybenzyl alcohol. The αβ tetramer consists of two flavoprotein subunits, each containing one covalent FAD (PchF or α), and two c-type cytochrome subunits (PchC or β), each containing one covalent heme cofactor. For PCMH, the covalent 8α-O-tyrosyl–FAD is also proposed to be formed self-catalytically [38]. However, the covalent link does not form when the apo α-subunit and FAD are incubated together. Covalent binding occurs only when FAD is incubated with PchF and PchC: FAD first binds noncovalently to the α-subunit, and when PchC binds to the holocytochrome PchF or α, a conformational change is induced in the latter that leads to covalent flavinylation and further structural changes [39]. When the 8α-O-tyrosyl–FAD covalent bond forms, the isoalloxazine moiety of FAD becomes reduced, which in turn, reduces the β-subunits, as occurs during normal catalytic oxidation of the substrate [38]. Interestingly, whereas 5-deaza-FAD
does not bind covalently to MSOX, it does bind covalently to PCMH [40].

6-HDNO

The second step in the bacterial degradation of nicotine is catalyzed by 6-HDNO, which was one of the first discovered covalent flavoproteins and has been extensively studied [41–43]. By incubating the apo form of 6-HDNO with \(^{14}C\) FAD, it was shown that in vitro covalent flavinylation is a self-catalytic process [44]. Covalent flavinylation could be enhanced by the addition of compounds such as glycerol 3-phosphate, glycerol, and sucrose. Recently, the crystal structure of 6-HDNO was solved, and this revealed that FAD is covalently bound via an \(8\alpha-N^1\)-histidyl linkage [45], not the previously proposed \(8\alpha-N^3\)-histidyl linkage [46].

VAO

For VAO, which oxidizes a range of phenolic compounds, the covalent histidyl–FAD linkage is not essential for folding, FAD binding, and activity. In VAO, His422 covalently binds FAD. The H422A mutant was expressed as a noncovalent flavinylated protein. Studies also revealed that covalent flavinylation can occur after folding of the polypeptide chain: the apo-proteins can tightly bind FAD upon its addition. This has also been shown for the VAO H61T mutant, which lacks a covalently linked FAD but is able to bind FAD tightly but noncovalently, and is also able to perform catalysis. The apo and holo forms of this VAO mutant display highly similar crystal structures, indicating that, prior to self-catalytic covalent flavinylation, FAD binding occurs via a lock-and-key mechanism [47]. Recently, the apo form of wild-type VAO was produced and used for a study of FAD binding [48]. It was shown that, as observed for MSOX [37] and dimethylglycine dehydrogenase [49], the apoprotein readily binds and covalently incorporates FAD by a relatively slow process (0.13 min\(^{-1}\) for VAO) that involves reduction of the cofactor.

TMADH

Bacterial TMADH catalyzes the oxidative N-demethylation of trimethylamine to yield dimethylamine and formaldehyde. For TMADH, which contains 6-S-cysteinyl–FMN, a self-catalytic mechanism was proposed in which the cysteinyl thiolate attacks the C6 of the isoalloxazine moiety, after which the reduced covalent complex is reoxidized by transfer of two electrons to the enzyme's Fe–S complex (Fig. 3) [50]. Alternatively, the iminoquinone methide may also form as in Fig. 2, and the cysteinyl–thiolate attacks its electrophilic 6-position to give covalently tethered reduced FMN. For all the enzymes mentioned above, with the possible exception of TMADH, similar mechanisms for covalent coupling of the flavin at the C8\(\alpha\) position have been proposed (Fig. 2) [32,33,38,45,51,52]. Owing to the increasing number of covalent flavoprotein crystal structures available, the proposed mechanisms of covalent flavinylation can be validated by comparing active site residues that may be important for the formation of these covalent bonds. The amino acids that are involved in specific interactions with the flavin ring system and may facilitate formation of the covalent protein–flavin bond are indicated in Table 2 [51].
Table 2. Distances between the covalent flavin factor and structural elements and amino acids putatively involved in covalent flavinylation. Protein Data Bank files used: CholO, 1I19; 6-HDNO, 2BVFA; GOOX, 1ZR6; VAO, 1VAO; alditol oxidase, 2VFR; aclacinomycin oxidase, 2IPI; cytokinin dehydrogenase, 1W1Q; PCMH, 1WVE; succinate dehydrogenase, 1ZOY; MAO, 1O5W; TMADH, 2TMD; flavocytochrome c552/c553, 1FCD.

<table>
<thead>
<tr>
<th>Protein</th>
<th>N1–C2 = O2 locus (Å)</th>
<th>N5 (Å)</th>
<th>Flavin C8α or C6 atom (Å)</th>
<th>Protein ligand atom (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alditol oxidase</td>
<td>His372 O2 (2.8)</td>
<td>Ser106 (3.0)</td>
<td>Trp9 NE1–C8α (5.8)</td>
<td>Trp9 NE1–His46 ND1 (4.8)</td>
</tr>
<tr>
<td>VAO</td>
<td>Arg504 O2</td>
<td>Asp170 (3.4)</td>
<td>Trp80 NE1–C8α (4.8)</td>
<td>Trp80 NE1–His131 ND1 (4.6)</td>
</tr>
<tr>
<td>Choline oxidase</td>
<td>His202 O2 (3.9)</td>
<td>Pro188 amide (4.7)</td>
<td>Tyr107 OH–C8α (5.7)</td>
<td>Tyr107 OH–His105 ND1 (5.0)</td>
</tr>
<tr>
<td>Cytokinin dehydrogenase</td>
<td>Tyr491 O2 (2.5)</td>
<td>Asp169 (5.2)</td>
<td>Gln132 OE1–C8α (6.0)</td>
<td>Gln132 OE1–His70 ND1 (4.6)</td>
</tr>
<tr>
<td>Aclacinomycin oxidase</td>
<td>His138 N1 (3.9)</td>
<td>Cys130 amide (4.0)</td>
<td>Cys130 amide–C6 (4.4)</td>
<td>Cys130 amide–Cys130 SG (3.0)</td>
</tr>
<tr>
<td>GOOX</td>
<td>Tyr426 O2 (2.7)</td>
<td>Thr129 (4.2)</td>
<td>Proton relay system</td>
<td>Thr129 OG1–C6 (5.2)</td>
</tr>
<tr>
<td>6-HDNO</td>
<td>Asn413 O2 (3.3)</td>
<td>His130 amide (4.6)</td>
<td>Proton relay system</td>
<td>Thr129 OG1–Cys130 SG (3.8)</td>
</tr>
<tr>
<td>PCMH</td>
<td>Arg474 O2 (3.0)</td>
<td>Glu380 (3.8)</td>
<td>Asp440 OD1–C8α</td>
<td>Asp440 OD1–Tyr384 OH (5.3)</td>
</tr>
<tr>
<td>MSOXα</td>
<td>Lys348–O2 (2.8)</td>
<td>Tyr254 (4.5)</td>
<td>His45 ND1–C8α</td>
<td>His45 ND1–Cys315 SG (4.7)</td>
</tr>
<tr>
<td>Flavocytochrome c552/c553a</td>
<td>Helix dipole</td>
<td>Glu167 (4.8)</td>
<td>Arg168 NH1–C8α</td>
<td>Arg168 NH1–Cys42 SG (5.1)</td>
</tr>
<tr>
<td>TMADHα</td>
<td>Arg222 O2 (2.7)</td>
<td>Cys30 amide (2.9)</td>
<td>His29 ND1–C8α</td>
<td>His29 ND1–Cys30 SG (5.6)</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>Helix dipole</td>
<td>Gin62 amide (3.4)</td>
<td>His365 ND1–C8α</td>
<td>FMN phosphate–His57 ND1 (5.2)</td>
</tr>
<tr>
<td>MAO Aα</td>
<td>Helix dipole</td>
<td>Tyr444 (7.2)</td>
<td>Trp397 NE1–C8α</td>
<td>Arg51 NH1–Cys406 SG (6.2)</td>
</tr>
</tbody>
</table>

* The data presented for these enzymes were abstracted from Trickey et al. [51]. b Complex with inhibitor covalent bound at the N5 position of FAD.

The first step of the proposed mechanisms for covalent flavinylation of the C8α position involves abstraction of a proton from the C8 methyl group. It is possible that the amino acyl residue that will covalently couple to the flavin fulfills this purpose, but, in any case, the abstracted proton also needs to be removed from this region of the protein. In the cases presented in Table 2, there are potential bases near the residues that tether the flavin (4.2–5.6 Å). Following deprotonation of C8α, or in the case of a thiolate attack at the C6 position (Fig. 3), stabilization of the negative charge at the N1–C2 = O2 locus of the isoalloxazine moiety is required. A positive charge near this locus can be supplied by histidine, lysine (e.g. MSOX [51]), arginine (e.g. PCMH [52] and VAO (Fraaije, unpublished results)), an internal positive electrostatic field, or a helix dipole (e.g. monoamine oxidase; Fig. 4). For cytokinin dehydrogenase and GOOX, the nearest amino acyl side chain is that of a tyrosine at 2.5 and 2.7 Å, respectively. For 6-HDNO, an asparagine residue is present at 3.3 Å. In these cases, the nearest amino acyl side chains are polar but uncharged. It might be for these enzymes that the tyrosine and asparagine serve as proton donors to stabilize the negative charge on the N1 position or create an effective microenvironment by amide backbones. Following proton abstraction from the C8 methyl group, the histidyl–imidazolyl, tyrosyl–phenolate or
cysteinyl–thiolate attacks at the C8α, thereby forming a covalent bond between the polypeptide chain and the reduced flavin.

Covalent flavinylation via the C8α or C6 position results in a negative charge at the N5 position on the reduced isoalloxazine ring system. This may be subsequently protonated by a nearby amino acid side chain, a proton relay system formed by water molecules, or peptide backbone amides. The importance of a proton-donating residue near N5 was demonstrated in the case of replacing Asp170 in VAO. Most of the analyzed Asp170 mutants suffered from incomplete FAD binding [53]. Finally, reoxidation of the reduced flavin occurs by transferring two electrons to oxygen, heme, or an Fe–S cluster.

The bicovalently linked FAD cofactor provides a new lead for investigating the covalent flavinylation mechanism. The proposed mechanisms for covalent flavinylation via the C8α or C6 position of the isoalloxazine ring system could also be valid for the formation of the bicovalent flavin–protein bond. However, it is difficult to predict in which order these steps take place, i.e. whether covalent flavinylation occurs first via the C8α or the C6 position. The observation that mutants of BBE, ChitO and GOOX with only one of the two covalent linkages can be produced suggests that formation of each covalent bond is independent of each other.

Whereas the mechanistic features of covalent flavinylation have been largely elucidated, there is little known about the degradation of flavin–peptides. This appears to be a relevant process, as flavin–peptides are associated with allergic reactions [54,55] and heart disease-associated autoimmune responses [56].

Roles of covalent flavinylation

For many years, the role of covalent flavin binding was not clear. However, in recent years, a number of studies on individual enzymes have provided insights into the function of covalent flavin attachment in several cases, as discussed below in more detail.

Redox potential

That the redox potential of flavins can be influenced by chemical modifications or varying environments (e.g. in a protein) has been known for some time. On comparison of redox potentials that have been determined for noncovalent, monocovalent and bicovalent flavoproteins, a clear trend becomes apparent: covalent coupling of a flavin increases the midpoint potential significantly (Fig. 5). A similar effect has been observed with chemically modified flavins such as 8α-N-imidazolylriboflavin, which displays a midpoint potential of −154 mV at pH 7.0, as compared to −200 mV for free riboflavin [57]. The E_m values for other modified flavins at pH 7.0 are as follows: 8α-N-histidylriboflavin, −160 mV; 8α-N2-histidylriboflavin, −165 mV; 8α-O-tyrosylriboflavin, −169 mV; 8α-S-cysteinylriboflavin, −169 mV; and 6-S-cysteinylriboflavin, −154 mV [58–60]. A detailed analysis of a large

Fig. 5. Redox potentials of noncovalently, monocovalently and bicovalently bound flavoproteins. The arrows indicate redox potentials of flavoproteins in which one of the covalent bonds has been disrupted by site-directed mutagenesis (see Table 3). Noncovalent: −1 mV [91], −21 mV [92], −23 mV [93], −26 mV [94], −58 mV [95], −65 mV [96], −77 mV [97], −79 mV [98], −85 mV [99], −90 mV [100], −92 mV [101], −97 mV [102], −108 mV [103], −114 mV [104], −118 mV [105], −129 mV [106], −132 mV [107], −145 mV [98], −149 mV [108], −152 mV [109], −159 mV [110], −170 mV, −255 mV, −172.5 mV, −245 mV [111], −190 mV [112], −200 mV [113], −205 mV [114], −207 mV (FAD), −212 mV [115], −216 mV [116], −217 mV [28], −325 mV [117], −228 mV [118], −230 mV [119], −233 mV [120], −237 mV, −243 mV, −227 mV [121], −251 mV [122], −255 mV [123], −268 mV [124], −271 mV [125], −277 mV [126], −277 mV [127], −280 mV [128], −290 mV [129], −340 mV [130], −344 mV [131], −367 mV [132]. Monocovalent: +160 mV [133], +164 mV [63], +70 mV [134], +55 mV [62], +50 mV [135], +40 mV [136], +8 mV [137], −2 mV [138], −3 mV [139], −50 mV [67], −101 mV [29], −109 mV [71], −105 mV [66]. Bicovalent: +132 mV [68], +131 mV [70], +126 mV [140]. SHE, standard hydrogen electrode.
number of flavin analogs has revealed a Hammett relationship between the electron-donating or electron-withdrawing properties of substituents at positions 7 and 8 on the isoalloxazine ring and the redox potential of the respective flavin [61]. Although the redox potential can be modulated by other flavin–protein interactions, it is clear that electron-withdrawing substituents at position 8 increase the flavin redox potential substantially [61]. The increase in redox potential would allow an enzyme to oxidize the substrate more efficiently, although the redox potential change of the flavin alone will not necessarily give an accurate estimate of relative activities; e.g., PCMH (+93 mV) versus PchF (+62 mV), where the former is more than 50 times more active ($k_{cat}$ value) than the latter [52] (see below). Similarly, it has been observed that two sequence-unrelated cholesterol oxidases from one bacterium, one with covalent FAD and the other with noncovalent FAD, exhibit similar $k_{cat}$ values while exhibiting significantly different redox midpoint potentials (~101 and ~217 mV, respectively) [28,29]. Additionally, a higher redox potential results in a more restricted selection of electron acceptors that can be used, often leaving molecular oxygen as the only suitable electron acceptor. This may explain why most covalent flavoproteins exhibit oxidase activity, in contrast to noncovalent flavoproteins which most often are dehydrogenases/reductases. An exception is PCMH, which uses a high-potential c-type heme (+230 mV) as the electron acceptor [52].

The redox potentials of several covalently and noncovalently bound flavins in mutant forms of the respective proteins have been determined (Table 3). In all of these cases, the redox potential is drastically lowered upon removal of the covalent link between the flavin and the polypeptide chain. The first systematic study on the effect of covalent flavinylation on the redox potential, kinetic behavior and protein structural integrity was performed with VAO [62], where FAD is covalently attached via an 8α-$N_3$-His422 linkage. His422 was mutated to alanine, serine, and cysteine. All altered forms of VAO contained tightly but noncovalently bound FAD, and the crystal structure of the H422A mutant is nearly identical to the structure of wild-type VAO [62]. This indicates that covalent binding does not involve drastic conformational changes in the three-dimensional structure of the enzyme, and that the covalent histidyl–FAD link is not required to keep FAD bound to the enzyme. Redox potential measurements of wild-type and H422A VAO showed that the loss of the covalent linkage resulted in a significant decrease of the redox potential from +55 mV for wild-type VAO to −65 mV for the H422A mutant. In addition, for the H422A mutant, the observed rate of reduction by substrate was one order of magnitude lower than with wild-type VAO (0.3 s$^{-1}$ versus 3.3 s$^{-1}$, respectively). Clearly, there is a relationship between the redox potential and the oxidative power of the enzyme, which is reflected in the reduced observed rate of reduction [62]. This finding is supported by studies on another VAO mutant. When His61, which was expected to be involved in activating His422 for covalent flavinylation, was mutated to a threonine, covalent binding of FAD no longer occurred [47]. Instead, FAD was noncovalently bound, and the crystal structure of the H61T mutant revealed no major structural variations as compared with wild-type VAO [47]. The mutation resulted in a similar effect on the catalytic efficiency, a 10-fold decrease in $k_{cat}$, as was found for the H422A mutant. These data clearly indicate that the covalent histidyl–FAD bond induces an increase of the redox potential, which enhances the oxidative power and facilitates efficient catalysis.

With PCMH, it was also shown that after the tyrosine normally covalently bound to FAD was mutated to phenylalanine, the enzyme could still tightly bind the flavin noncovalently. Moreover, the mutant

Table 3. Redox potentials of covalent flavoproteins and their corresponding mutants containing noncovalently bound flavin.

<table>
<thead>
<tr>
<th>Wild-type protein</th>
<th>Midpoint potential (mV)</th>
<th>Mutation</th>
<th>Midpoint potential (mV)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAO</td>
<td>+55</td>
<td>H422A</td>
<td>−65</td>
<td>[62]</td>
</tr>
<tr>
<td>PCMH</td>
<td>+84</td>
<td>Y384F</td>
<td>+47</td>
<td>[52,63]</td>
</tr>
<tr>
<td>CholO</td>
<td>−101</td>
<td>H69A</td>
<td>−204</td>
<td>[29]</td>
</tr>
<tr>
<td>P2Ox</td>
<td>−105</td>
<td>H167A</td>
<td>−150</td>
<td>[66]</td>
</tr>
<tr>
<td>BBE</td>
<td>+132</td>
<td>C166A</td>
<td>+53</td>
<td>[68]</td>
</tr>
<tr>
<td>ChitO</td>
<td>+131</td>
<td>C154A</td>
<td>+70</td>
<td>[70]</td>
</tr>
<tr>
<td>G0OX</td>
<td>+126</td>
<td>C130A</td>
<td>+61</td>
<td>[140]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H70A</td>
<td>~ +69$^a$</td>
<td>[140]</td>
</tr>
</tbody>
</table>

$^a$ The redox potential of this mutant protein could not be accurately measured.
enzyme could associate with the cytochrome c subunit, forming the heterocomplex, although it displayed lowered activity. For PCMH, the rationale for covalent flavinylation also appears to have its origin in an increased redox potential, and thereby the oxidative power of the enzyme. The redox potential of wild-type PCMH was +84–93 mV, whereas the noncovalent FAD in the PCMH [PchF(Y384F)] mutant had a redox potential of +34–48 mV [52,63]. This resulted in a decrease in $k_{\text{cat}}$ from 121 to 3.8 s$^{-1}$. Also, for PchF$^\text{C}$ (+62 mV) and PchF$^\text{NC}$ (−16 mV), $k_{\text{cat}}$ values were 2.2–4.4 s$^{-1}$ and 0.08 s$^{-1}$, respectively, again indicating that the same enzyme with covalently bound flavin is more active than the counterpart with noncovalently bound cofactor. It was suggested that the covalent bond facilitates effective electron transfer from FAD to the heme in the cytochrome c subunit; the electron would tunnel to PchC using a pathway that involves the 8α-carbon of FAD and the phenolic moiety of Tyr384 [38]. This rationale could also apply to the covalent FAD-containing and Fe–S cluster-containing reductase ThmD from Pseudonocardia, in which the flavin is involved in an electron transfer process [64]. Unfortunately, the exact mode of covalent flavin binding for this covalent flavoprotein is still unknown. A model structure of ThmD made using the crystal structure of benzoate dioxygenase reductase (Protein Data Bank: 1KRH) as a template suggests that the C8α of the flavin points towards the nearby Fe–S cluster (Fraaije, unpublished results). A C8α-FAD–protein linkage may be involved in covalently linking the cofactor, and could facilitate electron transfer from the reductase to the associated mono-oxygenase component. Intriguingly, the model indicates that there is no tyrosine, histidine or cysteine close to the C8α-methyl group of the flavin.

Cholesterol oxidase type II (CholO, 8α-N$^1$-histidyl–FAD) from Brevibacterium sterolicum catalyzes the oxidation of cholesterol and subsequent isomerization into cholest-4-en-3-one. Upon mutation of the respective His69 into an alanine, CholO could no longer covalently bind FAD, and this resulted in a drastic decrease in redox potential [29]. For wild-type CholO, a midpoint potential of −101 mV was determined, whereas the mutant enzyme displayed a midpoint potential of −204 mV [29]. A more recent study confirmed that the decrease in redox potential is responsible for a reduced rate of flavin reduction, which explains the 35-fold lowered catalytic activity [65]. The crystal structure of the CholO His69 mutant also revealed a distortion of the isoalloxazine ring moiety, which may contribute to the significant decrease in redox potential.

For pyranose 2-oxidase (P2Ox) from Trametes multi-color, removal of the histidine residue that covalently binds FAD decreases the $k_{\text{cat}}$ by a factor of 5, and lowers the reduction potential by 35 mV, as compared with wild-type P2Ox [66]. A comparable effect on redox potential and catalytic activity has been reported for MSOX [67].

Following the recent elucidation of the crystal structure of the bicovalent flavoprotein GOOX, several other bicovalent flavin-containing proteins were identified. This novel covalent binding mode raises the question of why a flavoprotein would require bicovalent attachment of a flavin to the polypeptide chain. A possible reason for bicovalent FAD binding in BBE was proposed. BBE from Eschscholzia californica, also referred to as reticuline oxidase, is involved in benzophenanthridine-type alkaloid biosynthesis in plants. In BBE, FAD is covalently linked to the protein via an 8α-histidyl and a 6-S-cysteinyl linkage [17]. The wild-type BBE and the C166A mutant, the latter containing FAD that is only covalently bound to His104, were compared with regard to their kinetic properties and redox potentials [68]. For wild-type BBE, a very high redox potential of +132 mV was found, whereas the C166A mutant exhibited a redox potential of +53 mV. The difference in potential was directly linked to the 360-fold decrease in the rate of flavin reduction by (S)-reticuline [68]. For BBE, it was concluded that the 6-S-cysteinyl–FAD linkage is also needed to increase the redox potential and thereby enhance the catalytic efficiency. For the histidine mutants of BBE, in which FAD is solely linked to Cys166 (H104A and H104T), and the double mutant H104T/C166A, no data could be obtained, owing to very low expression levels of the mutants [68]. The recently elucidated crystal structure of BBE has confirmed the bicovalent linkage of the flavin [69].

ChitO from Fusarium graminearum catalyzes the oxidation of chito-oligosaccharides at the C1 hydroxyl group to yield the corresponding lactones [21]. ChitO was also shown to contain a bicovalently linked FAD. In this fungal enzyme, the isoalloxazine moiety is tethered to His94 and Cys154 [70]. The H94A and C154A mutants were prepared, and their kinetic parameters and redox potentials were measured. In both mutant proteins, FAD was covalently attached to the remaining linking residue. This indicates that either covalent bond can be formed independently of the other, and removing either covalent bond has a major effect on activity. The observed reduction rates of FAD by N-acetyl-D-glucosamine decreased by a factor of approximately 700. For the C154A and wild-type ChitO, similar results with respect to redox properties...
were obtained as compared with the C166A and wild-type BBE. For the ChitO C154A mutant, a redox potential of +70 mV was measured, whereas for wild-type ChitO, a redox potential of +131 mV was determined. In this case, it was again shown that the covalent cysteinyl–FAD link is responsible for the change in redox potential and could also explain the lower rate of reduction. However, the C154A mutant also exhibited a marked increase in $K_m$ for the substrate $N$-acetyl-$\alpha$-glucosamine, suggesting that removal of this covalent bond also affects substrate binding. This suggests a role for the cysteinyl–FAD linkage in positioning FAD in a catalytically optimal conformation for substrate binding and flavin reduction. This idea is further supported by analysis of the H94A mutant of ChitO. For this mutant, similar effects on the $k_{\text{cat}}$, the $K_m$ and observed rate of reduction were found as for the C154A mutant. However, the redox potential for the reductive half-reaction was found to be even higher than that measured for the wild-type enzyme: +164 mV. This extremely high redox potential clearly does not correlate with the decreased $k_{\text{cat}}$ and lower rate of reduction, which suggests that both covalent bonds of FAD to the polypeptide chain of ChitO are required for correct positioning of the flavin to facilitate efficient catalysis. The double anchoring of FAD allows the protein to evolve a relatively open active site that can bind bulky substrates. In this context, it is striking to note that all recently reported bicovalent flavoprotein structures display remarkably open active sites, and these enzymes act on relatively bulky substrates (Fig. 6). In addition, ChitO may also benefit from the increased redox potential that is a result of both covalent bonds. The double mutant H94A/C154A ChitO could not be analyzed, owing to very low expression levels. For ChitO, the presence of one covalent bond could be necessary for the establishment of an increased redox potential, but the second covalent linkage is required for fixing FAD in the catalytically correct conformation, allowing the formation of a productive Michaelis complex [70].

In all of the cases described above, mainly histidyl–FAD-containing enzymes, it appears that the functional benefit of acquiring and retaining a covalent flavin–protein link is to increase the redox potential and thus also the oxidative power of the enzyme. From these data, it is tempting to assume that if a relatively high redox potential is beneficial for catalysis, flavoenzymes typically form a histidyl–FAD linkage. Similar observations were made with PCMH, which

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**Fig. 6.** Surface representations of several covalent flavoenzyme structures and the corresponding substrates, illustrating the open active sites of bicovalent flavoproteins. The protein structure images were generated with PYMOL [90].
contains tyrosyl–FAD. A thorough analysis of enzymes containing histidyl–FAD/FMN and cysteinyl–FAD/FMN and the respective noncovalent mutants is essential to understand further the specific role of the histidyl–flavin linkage.

Structural integrity

Another reason for covalent flavinylation could be to enhance protein stability. For several flavoenzymes, removing the covalent bond leads to the production of incorrectly folded apoenzyme. For aldiloxidase from Streptomyces coelicolor, it was shown that upon mutation of the respective histidine residue (His46), FAD could no longer bind to the protein, and approximately 50% of the expressed protein was insoluble [71].

For recombinant human monoamine oxidase A (MAO A), which contains covalent FAD (8ω-S-cysteinyl), a mutant was prepared that no longer covalently linked FAD (C406A), but the altered apo-MAO A was still incorporated into the outer mitochondrial membrane. The addition of FAD to C406A apo-MAO A resulted in the FAD being bound tightly, but noncovalently, and the activity was only 30% of that measured with wild-type MAO A [72]. However, after solubilization from the outer mitochondrial membrane, the mutant enzyme was found to be unstable, in contrast to the wild-type MAO A, which is stable under the same conditions. In this case, it appears that one role of covalent flavinylation is to stabilize the native conformation of the protein structure [72].

Results from a recent study on a sequence-related amine oxidase have hinted at another rationale for covalent flavinylation. The bacterial putrescine oxidase (PuO) was shown to contain equal amounts of tightly but noncovalently bound FAD and ADP [73]. On the basis of the high degree of sequence identity between mammalian MAO and PuO, only one dinucleotide cofactor is expected to bind to PuO. MS analysis revealed that PuO is isolated as a mixture of dimers containing either two molecules of FAD, two molecules of ADP, or one molecule of FAD and one molecule of ADP. This indicates that ADP is competing with FAD for binding. As ADP binding results in inactive enzyme, such a competitive event may be the driving force for creating a covalent FAD–protein linkage, as observed in MAO, as this would ensure full incorporation of FAD. To probe whether PuO could be converted to a covalent flavoprotein, an alanine residue corresponding to the linking cysteine in human MAO B was replaced by a cysteine. Intriguingly, the A394C PuO mutant was indeed able to form a covalent FAD–protein bond [73]. The ability to convert a noncovalent flavoprotein PuO into a covalent flavoprotein by a single amino acid replacement also confirms the self-catalytic nature of covalent flavinylation. A similar gene mutation event may have occurred during the evolution of MAOs or other covalent flavoproteins.

The effect of urea-induced unfolding was examined for wild-type and H69A CholO. It was clearly shown that unfolding of the mutant enzyme occurred at a lower urea concentration than was needed to unfold the wild-type enzyme. In addition, thermal denaturing experiments revealed that the mutant enzyme exhibited an approximately 10–15 °C lower melting temperature than wild-type CholO [74]. Thermal instability was also observed for apo-6-HDNO. In this case, the enzyme could be rescued upon incubation with FAD and subsequent covalent flavin linking [44].

For ChitO, substantial effects were observed on mutating the amino acids involved in covalent flavinylation. As mentioned before, removal of one of the covalent bonds affects the redox potential. However, on the basis of changes in the $K_m$ value, it also appears that loss of one covalent linkage prevents a stable, functional Michaelis complex from forming. The mutation also resulted in decreased structural stability, as, for the H94A mutant, protein aggregation was observed during redox potential measurements [70].

In the case of heterotetrameric sarcosine oxidase, it was shown that the $β$-subunit, which contains covalent histidyl–FMN, is catalytically inactive and forms labile heterotetrameric complexes when it cannot covalently bind FMN [75]. This indicates that the covalent link between FMN and the respective histidine is required for structural reasons, e.g. to form a stable heterotetrameric complex, and possibly to prevent cofactor loss. Also for MSOX, it has been found that disruption of the covalent FAD–protein bond prevents effective binding of the oxidized flavin [67].

In contrast, when His44 (to which FAD is normally bound covalently) of fumarate reductase from E. coli is changed to serine, cysteine or tyrosine, the complex heterotetrameric protein, which also contains three Fe–S clusters, assembles properly in the membrane of the bacterium, and FAD is tightly bound noncovalently [76]. All mutant forms of the enzyme show activity, albeit reduced, as compared with the wild-type enzyme. Also, FAD can be removed and reincorporated without loss of activity of the H44C, H44Y and H44R mutant forms of fumarate reductase. Hence, covalent binding of FAD to the polypeptide has little effect on structural integrity. In addition, Complex II of Saccharomyces cerevisiae assembles properly in the mitochondrial membrane when His90 (the residue...
covalently linked to FAD in the normal complex) is mutated. For normal Complex II, FAD becomes covalently bound in the mitochondrial matrix after the signal peptide is cleaved.

For PCMH from Pseudomonas putida, the flavoprotein subunit can be expressed in E. coli, and as its cytochrome subunit is not present, FAD is bound noncovalently to the isolated protein. The flavin is easily removed from this 'holo' enzyme, and the stable apo-protein can noncovalently rebind FAD. Exposure of this 'holo' subunit to its partner cytochrome subunit results in fully formed and fully active native flavocytochrome that has covalently bound FAD. A comparison of structure of the flavoprotein harboring covalently bound FAD [39] with the structures of the flavoprotein with noncovalently bound FAD or the apo-flavoprotein (F.S. Mathews & W.S. McIntire, unpublished) indicates that the flavin, whether covalently bound or not, or missing, does not affect the structural integrity of this protein.

A similar robustness of the apo form of a covalent flavoprotein has been observed for VAO. The crystal structures of H61T apo-VAO, ADP-complexed H61T VAO, and H61T holo-VAO and H422A holo-VAO, both containing noncovalently bound FAD, revealed that binding of FAD and formation of the covalent FAD–protein bond do not cause any significant structural changes [47,62]. Furthermore, it was recently shown that wild-type VAO can be produced and folded into a competent form to bind FAD in the absence of any FAD [48]. These results indicate that the apo forms of VAO and PCMH are able to fold into a stable protein structure that is preorganized to bind FAD and catalyze formation of the covalent FAD–protein linkage in a post-translational process.

**Flavin reactivity**

A third reason for covalent flavinylation has been suggested for TMADH, which oxidizes trimethylamine to form dimethylamine and formaldehyde [77]. TMADH contains FMN that is covalently linked to a cysteine via the C6 position of the flavin isalloxazine moiety. Removal of the covalent bond by mutating the Cys30 to an alanyl resulted in the formation of 6-hydroxy-FMN upon incubation with substrate [78]. The 6-hydroxy moiety that is formed after oxidation of the substrate-reduced mutant, results from the reaction of reduced FMN with molecular oxygen. It was suggested that the covalent 6-S-cysteinyl–FMN has evolved to prevent wild-type TMADH from forming the 6-hydroxy-FMN species, which renders the enzyme inactive [79].

**Enhanced lifetime of the holo-enzyme**

Although no germane studies are available, some enzymes may have evolved with covalently bound flavins to increase the in vivo lifetime of the protein. For an enzyme with noncovalently bound flavin, if flavin binding is not that tight or binding weakens as the protein ages, the flavin may dissociate. In general, apo-flavoproteins are less stable than the holo forms [80,81]. Furthermore, in cases where flavin reassociation is difficult or impossible, the enzyme may be rendered incompetent. This may be particularly important for membrane-bound and extracellular flavoenzymes, which, once formed and inserted into the lipid bilayer or excreted, would have limited access to free flavin [76].

The examples above illustrate that, besides an effect on redox potential, the covalent flavin–protein bond may also serve to, for example, increase the structural stability of the protein and/or ensure an optimal flavin conformation in the active site. This strongly suggests that the role of covalent flavinylation is enzyme-dependent.

**Artificial flavinylation**

In the previous sections, current views on the mechanism and function of covalent flavinylation have been discussed. The significance of covalent flavin binding can also be examined by studying the effects of covalent and noncovalent flavinylation with flavin analogs, which have shown to be powerful active site probes [82]. For several covalent and noncovalent flavoproteins, flavin analogs have been used to explore mechanisms and effects of flavin binding, and some examples are presented and discussed below.

A study of covalent flavinylation of the flavoprotein subunit (PchF) of PCMH was carried out using nine FAD analogs (FAD*) [40,83,84]. Analogs with an 8-methyl group bound tightly but noncovalently to apo-PchF [PchF(FAD*)NC] in the absence of the cytochrome subunit (PchC), but bound covalently when exposed to PchC; those analogs lacking 8-CH3 could not bind covalently. With PchC absent, the redox potential of a covalently bound FAD* congener was greater than it was when it was noncovalently bound to PchF, and the potential increased further on association of PchF(FAD*)C or PchF(FAD*)NC with PchC, while maintaining covalent or noncovalent FAD* binding. In other words, both covalent flavin attachment and a subunit association-induced conformational change [39] caused increases in the redox potential of bound FAD. As the potential increased for over 30 forms of PchF and PCMH, the catalytic
efficiency also increased. However, a better correlation was uncovered when the potentials of the substrate (Eₘ) and FAD⁺ (Eₚ) in the enzyme–substrate complex were taken into consideration; that is, \( \Delta n(k_{cat}) \) is a linear function of \( \Delta(E_m-E_p) \) [63].

For PCMH, it was found that the logarithm of rate constants for covalent flavinylation was a linear function of the redox potential of FAD analogs noncovalently bound to PchF [40]. The correlation would present itself if deprotonation of the 8-methyl group (step 1, Fig. 2) were rate-limiting; the pKₐ (or rate of deprotonation) of the 8-methyl proton should be a function of the electron affinity (i.e. redox potential) of the isoalloxazine ring. Alternatively, the attack by the nucleophilic amino acyl group on the vinyllic 8-carbon of the deprotonated flavo-iminoquinonoid intermediate (step 2, Fig. 2) may be the rate-limiting step. In this case, the rate constant for this step would be a function of the redox potential of this tautomer, which is assumed to be directly related to the potential of the normal form of the flavin.

The C406A mutant of MAO A, which lacks covalently bound FAD, was studied by reconstituting the apo mutant in vivo and in vitro with a large set of different FAD analogs. A clear effect was observed when high redox potential FAD analogs were used for reconstitution. To some extent, the flavin analogs could compensate for the decrease in redox potential due to the disrupted FAD–protein linkage. Moreover, lower redox potentials of FAD analogs as compared with normal FAD caused the catalytic activity to drop below the value that was determined for C406A holo-MAO A [72].

With the CholO type II H69A mutant, which no longer covalently binds FAD, an increase in redox potential from -204 to -160 mV (as compared with -101 mV for wild-type CholO) was observed upon reconstitution with 8-chloro-FAD. At the same time, this resulted in a 3.5-fold increase in activity, again showing that the flavin analog mimics the thermodynamic effects resulting from covalent FAD binding [29].

The examples above concern enzymes that naturally contain a covalent flavin and for which it has been shown that the covalent bond is necessary to raise the redox potential to a value that facilitates proper catalysis. On the other hand, there are also examples of proteins that normally do not contain a covalent flavin, but have been artificially covalently flavinylated. For example, the noncovalent flavoproteins lipoamide dehydrogenase, electron-transferring protein and lysine N6-hydroxylase [85–87] slowly covalently incorporated FAD when the respective apo-proteins are incubated with 8-Cl-FAD (FAD is linked via an 8-carbon rather than an 8α-carbon linkage). The covalent incorporation led to inactive enzymes, presumably because of a perturbed positioning of the flavin in the active site. In addition, in the noncovalent flavoprotein D-α-amino acid oxidase (DAO), the glycine at position 281 was mutated to a cysteine. Isolated G281C apo-DAO was incubated with the thiol-reactive 8-methylsulfonyl-FAD, which bound covalently to Cys281. This artificial covalent flavinylation (again, FAD is linked via an 8-carbon rather than 8α-carbon linkage) resulted in an increased \( k_{cat} \) value with D-alanine from 1.5 s⁻¹ for the mutant enzyme, containing noncovalently bound FAD, to 2.6 s⁻¹ for the FAD–S-mutant enzyme [88]. This rate is 26% of the respective value for wild-type DAO. The covalent binding of the flavin affected its mobility, which was also reflected in the 13-fold increased \( K_m \) value as compared with wild-type DAO [88]. Another example is the artificial covalent flavinylation of L-aspartate oxidase (LaspO) [89]. LaspO is involved in the biosynthesis of pyridine nucleotides in E. coli. FAD in LaspO is noncovalently and relatively weakly bound. To obtain an artificial covalent flavoprotein of LaspO, the apo-protein was incubated with the flavin analog \( N^\beta-(6-carboxyhexyl)-FAD \) suckinimidoester. The FAD analog was shown to be covalently linked to Lys38, and this resulted in a mutant protein that exhibited 2% of the activity that was found for the wild-type enzyme. Although some activity was measurable, it was clear that the microenvironment around the isoalloxazine moiety of the FAD analog cofactor was dramatically affected [89]. This shows that even though, in many cases, covalent flavinylation appears to be advantageous to the enzyme, not just any covalent bond between the flavin and the polypeptide chain will yield an improved enzyme.

**Concluding remarks**

From the elucidation of several crystal structures and many detailed analyses on covalent flavin binding, we are gradually obtaining more insights into the mechanism and function of covalent flavinylation. The proposed mechanisms for covalent flavinylation are similar and supported by structural, spectral and kinetic data. The recent finding of novel types of covalent flavin binding (FAD tethered via two amino acyl residues and threonyl–FMN) shows that many different covalent flavin–protein interactions have evolved, and that, some have probably not yet been identified.

For many enzymes, several reasons for covalent flavinylation have been put forward and supported by
Covalent flavinylation can increase protein stability, ensure cofactor binding, and/or induce a relatively high redox potential of the cofactor. Although the experimental data may show that a covalent bond is there to increase the redox potential, during the course of evolution this may have changed. It could be that, originally, an enzyme experienced evolutionary pressure to develop increased protein stability. The enzyme subsequently acquired a covalent protein–flavin bond for this purpose. Before that, the redox potential could have been optimized by some specific active site residues. However, the acquisition of the covalent flavin results in an inherent increase in redox potential, thereby relieving active site residues from the evolutionary pressure of maintaining the correct redox potential. During evolution, the respective active site residues may have mutated, resulting in a lower redox potential when the covalent bond is removed.

Probably, covalent flavinylation has been introduced in separate events in different flavoproteins during the course of evolution. This can be deduced from the existence of diverse flavin-binding folds in covalent flavoproteins, and also from the differences within one type of binding fold. With regard to the latter, in most VAO-type covalent flavoproteins, the flavin is covalently linked to an amino acid that is part of the FAD-binding domain, whereas in VAO, the corresponding histidine is part of the substrate-binding domain. In addition, for members of the VAO family, the flavin is most often linked via a histidyl linkage, and one, PCMH, has the tyrosyl–histidine, although a few have both a histidyl and a cysteinyl linkage, and one, PCMH, has the tyrosyl–FAD bond. Hence, over eons, this subclass of covalent flavoproteins has resorted to different strategies to link the flavin in order to gain an evolutionary advantage. Taking these considerations together, it can be concluded that, for the examined enzymes, removal of the covalent bond has dramatic effects on the flavin redox potential and enzyme activity. Additionally, it has been established that covalent flavinylation seems to enhance protein stability and, in some cases, can also affect Michaelis complex formation and protein oligomerization.

For a richer understanding of the role of covalent flavin binding to proteins, the scope of the research performed in this area should be broadened. Establishing the effects of (removing) the covalent bond on protein activity and other biochemical properties is fundamentally different from assigning the role of covalent flavinylation on the basis of these effects. Investigations aimed at assigning the importance of covalent flavin binding need to be linked to physiological and phylogenetic data. Information on the physiological fate of the enzyme, e.g. cellular localization and the reaction that is catalyzed, as well as in-depth knowledge of the evolution of this diverse class of cofactor-containing proteins, could yield new insights into the role of covalent flavinylation.

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