The Cytochrome Subunit Is Necessary for Covalent FAD Attachment to the Flavoprotein Subunit of p-Cresol Methylhydroxylase

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When p-cresol methylhydroxylase (PCMH) is expressed in its natural host Pseudomonas putida, or when the genes of the α and β subunits of the enzyme are expressed together in the heterologous host Escherichia coli, flavin-adenine dinucleotide (FAD) is covalently attached to Tyr384 of the α subunit and the correct αβ2 form of the enzyme is assembled. The apoflavoprotein has been expressed in E. coli in the absence of the β cytochrome c subunit and purified. While noncovalent FAD binding to apoflavoprotein in the absence of the cytochrome subunit could not be directly demonstrated, circumstantial evidence suggests that this indeed occurs. Covalent flavinylation requires one molecule each of FAD and cytochrome for each flavoprotein subunit. The flavinylation process leads to the 2-electron-reduced form of covalently bound FAD, and the resulting αβ2 enzyme is identical to wild-type PCMH. This work presents clear evidence that covalent flavinylation occurs by a self-catalytic mechanism; an external enzyme or chaperon is not required, nor is prior chemical activation of FAD or of the protein. This work is the first to define the basic chemistry of covalent flavinylation of an enzyme to produce the normal, active species, and confirms a long standing, postulated chemical mechanism of this process. It also demonstrates, for the first time, the absolute requirement for a partner subunit in the post-translational modification of a protein. It is proposed that the covalent FAD bond to Tyr384 and the phenolic portion of this Tyr are part of the essential electron transfer path from FAD to heme.

Since the discovery of enzymes containing covalently bound flavin-adenine dinucleotide (FAD) or flavin mononucleotide (FMN) over 40 years ago, five types of covalent bonds between flavin and proteins have been revealed (1, 2) (Fig. 1). In all this time, two major questions have remained unresolved: (a) what is the reason for covalent flavin binding in enzymes, and (b) what is the mechanism for covalent attachment of flavin?

No completely satisfying explanation has been provided for the first question. The attachment of an aminoacol group to the 8α- or 6-position of riboflavin does not confer any unusual properties on the flavin, either free in solution or in an enzyme, with the exception of the ultraviolet-visible spectrum of 6-S-cysteynilriboflavbin, which is quite different from that of other forms of free and bound aminoacol flavins (2, 3). While the oxidation-reduction potentials (E°') are about 50–60 mV more positive for aminoacol flavins than for unmodified forms (2), this increase in potential can also be achieved by noncovalent interactions with protein. Additionally, enzymes with covalently bound flavins do not catalyze a unique or specific set of reactions.

As to the second question, it is known that 2-electron reduction of protein-free 8α-O-tyrolylriboflavbin or 8α-S-cysteynilsulfonylriboflavbin cause expulsion of the aminoacol groups, thus producing unmodified, oxidized riboflavbin (2, 4). The principle of microscopic reversibility suggests that the reverse reaction could occur within an enzyme, with or without intervention of an external enzyme. A mechanism for covalent flavinylation, which has long been in the literature, is shown in Fig. 2 (5, 6). An analogous mechanism was proposed for nonenzymic base-catalyzed nuclophilic attack at the 8α-carbon of riboflavbin derivatives in organic solvents (7).

It has been suggested that covalent tethering might require prior activation of the flavin or proteins, e.g. a high energy phosphate bond (8). Of several enzymes studied to date, no specific enzyme has been implicated in the covalent modification process, which contrasts with examples of nonflavin-cofactor covalent attachment to apoenzymes (9–13).

The structural genes of several bacterial enzymes containing covalently bound flavin have been cloned into vectors for expression in new hosts: succinate dehydrogenase, fumarate reductase (14, 15), sarcosine oxidase (16), 6-hydroxy-α-nicotine oxidase (6-HDNO) from Arthrobacter aerogenes (15) (all containing 8α-N3 histidyl-FAD), trimethylamine dehydrogenase

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§§ V. K. compiled the GREENPATH program to run under the LINUX operating system on a 486 PC, and to run the program with the PCMH x-ray coordinates and generate the computer graphics. The molecular graphic image was produced using MidasPlus from the Computer Graphics Laboratory, University of California, San Francisco (supported by National Institutes of Health Grant RR-01081).

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§§ F. S. M.'s group at Washington University worked on the crystal structure of PCMH.

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The abbreviations used are: 6-HDNO, 6-hydroxy-D-nicotine oxidase; PAGE, polyacrylamide gel electrophoresis; PCMH, p-cresol methylhydroxylase; PchC, the α flavoprotein subunit of PCMH; pchC and pchF, the genes for PchC and PchF; pK-K-F, pK-K-C, or pK-K-CF, the pK expression vector harboring pchF, pchC, or both pchC and pchF, respectively; PES, phenazine ethosulfate; HPLC, high performance liquid chromatography.
derivatives. derivatives and represents ribityl-5'-diphosphoadenosine for FAD (PCMH) (8-p-cysteinyl-FMN) (17), and FAD to PCMH via Tyr384 of PchF. phenolate oxygen of Tyr384 attacks the 8-p-position during this step. B3H1 attacks the 8-p-position during this step. B3H1 extracts a proton from the 8-p-structure of FAD (structure 2). The stabilizing base at N-1/C-2 position during this step. B3H1 extracts a proton from the 8-p-structure of FAD (structure 2). The stabilizing base at N-1/C-2 atmosphere; Tyr172 is 4.5 Å from N-5. Reoxidation produces covalently bound reduced FAD (structure 1). In experiments with fumarate reductase, His44, which covalently binds to FAD, was replaced with Cys, Tyr, Arg, or Ser, amino acids capable of covalently binding to FAD at the 8-p-carbon. All site-specifically altered forms displayed lowered activity, relative to the normal enzyme, but in no case was FAD covalently bound (14). Obviously, precisely defined conditions are necessary for covalent flavinylation, in order to properly position the aminoacyl nucleophile, and the electrophilic center of the flavin.

The flavocytochrome PCMH oxidizes p-cresol (4-methylphenol) to 4-hydroxybenzyl alcohol and then oxidizes this alcohol to 4-hydroxybenzaldehyde in the periplasmic space of Pseudomonas putida and related bacteria (23). Electrons from these oxidations are funneled into the membrane electron-transport chain of the bacterium for ATP production. PCMH has an \( \alpha_2\beta_2 \) structure consisting of two \( \alpha \) flavoprotein subunits (PchF), each with covalently bound FAD, and two \( \beta \) c-type cytochrome subunits (PchC). PCMH and its related 4-ethylphenol monooxygenase are the only proteins known to contain FAD covalently bound via the 8-p-carbon of the flavin to the phenolic oxygen of a tyrosyl residue. The genes of both subunits (pchF and pchC) of form A of PCMH from P. putida NCIMB 9869 were cloned, sequenced, and expressed in E. coli (18). Whether PchC is expressed in the absence of or together with PchF, heme is covalently bound. When PchF and pchC are expressed together in E. coli, PchC is produced at about 20% the molar level of PchF. E. coli extracts were combined so that the final mixture contained equimolar amounts of PchC and PchF. PCMH purified from this mixture was identical to PCMH expressed by wild-type P. putida 9869. Gram quantities of pure heterologously expressed PCMH could be obtained in this way. EXPERIMENTAL PROCEDURES

Chemicals, Strains, and Culture Conditions—Chemicals and manufacturers: nitro blue tetrazolium, phenazine methosulfate, phenazine ethosulfate (PES), 3,5-xylene, Bi-Tris propane, sodium dodecyl sulfate (SDS), lysozyme (L-6876), nucleotide pyrophosphatase (P-7383), catalase (C-30), trypsin (T-8253), FAD, Sigma; alkaline phosphatase (713023), Boehringer Mannheim; glucose oxidase (31-617-2), Miles Laboratories; dithymotryptophan, Worthington Biochemical Corp.; sodium 2,6- dichlorophenol indophenol, General Biochemical, Inc., Chagrin Falls, OH; HPLC solvents, J.T. Baker; DEAE-52 (diethylaminoethyl cellulose), Whatman; Bio-Gel HTP hydroxyapatite, Bio-Rad; Sephadex G-200 Superfine, Sephadex G-100 Superfine, Pharmalyte ampholytes, Pharmacia Biotech, Inc. All other reagents were reagent grade. Polyclonal antibodies against PchF were raised in rabbit and purified by standard procedures (24). Anti-flavin antibodies (25) were a generous gift from Dr. Michael Barber, Department of Biochemistry, University of South Florida. We are very grateful to Dr. T. E. Meyers, Department of Biochemistry, University of Arizona, Tucson for generously supplying

FIG. 1. The structures of the five known aminoacyl flavin linkages in oxidoreductases. R represents ribityl-5'-phosphate for FMN derivatives and represents ribityl-5'-diphosphoadenosine for FAD derivatives.

FIG. 2. The proposed mechanism for covalent attachment of FAD to PCMH via Tyr384 of PchF. B1, B2, B3, and their protonated forms represent hypothetical bases on PchF that are predicted to be involved in the mechanism of covalent flavinylation. B1 extracts a proton from the 8-p-carbon of FAD (structure 1), yielding the iminonemethide form of FAD (structure 2). The stabilizing base at N-1/C-2 oxygen (B1H1) is probably Arg172 of PchF. This Arg is 3.3 and 2.5 Å from the N-1 and the C-2 oxygen positions, respectively (see Fig. 5). The phenolate oxygen of Tyr384 attacks the 8-p-methylene of the iminonemethide form to produce covalently bound reduced FAD (structure 3). One would expect a protonated base (B1H1) to transfer a proton to the N-5-position during this step. B1H1 could be hydronium ion that is stabilized by a side chain; Tyr172 is 4.5 Å from N-5. Reoxidation produces catalytically competent 8-p-O-tyrosyl-linked FAD (structure 4).

(6-S-cysteylnl-FMN) (17), and p-cresol methylhydroxylase (PCMH) (8-p-O-tyrosyl-FAD) (18) (Fig. 1). In some cases, normal enzymes with covalently bound flavin are produced. For the latter three, enzymes without bound flavin could be recovered. Further, only apo-6-HDNO and apo-PCMH could be subsequently converted to the holo forms in vitro. Analytical amounts of apo-6-HDNO were obtained from a pure Escherichia coli-expressed β-galactosidase-6-HDNO fusion protein (19). The in vitro flavinylation of 6-HDNO required Mg2+ and mercaptoethanol and was accelerated by millimolar amounts of three-carbon compounds such as glycerol 3-phosphate, glyceraldehyde 3-phosphate, phosphoenolpyruvate, or 45% glycerol (1, 15, 20). The phosphorylated compounds do not chemically activate FAD, but possibly act allosterically. It also has been reported that citric acid cycle intermediates accelerate covalent flavinylation of succinate dehydrogenase and fumarate reductase (14). These observations, together with results from GroE-dependent 6-HDNO refolding and flavinylation studies (21), and the characterization of a series of Cys to Ser replacements (22) in 6-HDNO, suggest that in vivo covalent incorporation of FAD occurs during the folding process after the entire protein chain has been synthesized. Contrary to expectation (21), a specific GroE/6-HDNO complex is not formed. It is not clear why such a myriad of factors should affect covalent flavinylation of 6-HDNO, and the relationship of these agents to in vivo A. aerogenes factor(s) is unknown. No experiments reported heretofore directly addressed the chemistry of covalent flavinylation.
FAD Attachment to p-Cresol Methylhydroxylase

Pseudomonas stutzerii cytochrome c₅₅₃, Pseudomonas aeruginosa cytochrome c₅₅₃, and halophilic Paracoccus (ATCC 12084) cytochrome c₅₅₃. Transformed E. coli DH5α strains were grown at 37 °C on 2 x YT medium with 100 mg/liter ampicillin. P. putida NCIMB 9869 was grown at 30 °C on Heiman’s medium (26) supplemented with Hunter’s “metals 44” solution and 0.03% (w/v) 3,5-xylenol (18). The bacteria were grown at 30°C on Hegeman’s medium (26) supplemented with Hunt’s medium (26) and 325 nmol of FAD in 2.1 ml of 10 mM potassium phosphate buffer, pH 7.0. The slurry was layered onto a glass plate (13 x 7.5 x 0.2 cm, length x width x depth) and dried to 75% of the original weight. The samples (1-50 mg of protein in 50-500 μl) were applied directly to the gel and focused at a constant power of 7 watts at 4°C. The separated subunits were eluted from the Sephadex matrix by repeated extractions with 16 mM potassium phosphate, pH 7.0, and then concentrated.

After the initial measurement, a known volume of a standardized riboflavin solution, and 2 mg/ml nitroblue tetrazolium and 0.2 mg/ml phenazine methosulfate, and incubated for 30 min in the dark, until a 100% gradient of FAD concentration in the cuvette. The resulting mixture was purified on the 15 μm Bis Tris propane buffer column of p-cresol methylhydroxylase in 2 h at room temperature. The resulting mixture was purified on the 15 μm Bis Tris propane buffer column of 81:19, H₂O:CH₃CN with 0.1% trifluoroacetic acid, and the flow rate was 1 ml/min. The reaction was quenched after 4.5 h by adding trifluoroacetic acid to 0.1% at 0°C. Two partially pure flavin-containing peptide fractions were obtained by this HPLC method with three injections of the peptide digest (retention times: 1 cm, 13.8 min; 2 cm, 14.1 min; broad tailing of flavin-containing material from 14 to 16 min). HPLC analysis of the nucleotide pyrophosphate-treated aliquots indicated that both peptides contained FAD. Sample 1 was further purified on a Pharmacia PepRPC HR 5/5 reverse phase column. The sample was injected onto the column equilibrated with 0.1% trifluoroacetic acid in H₂O. Peptides were eluted isocratically with 85:15, H₂O:CH₃CN + 0.1% trifluoroacetic acid with a 1 ml/min flow rate. Retention times were: FAD peptide, 13.8 min; peptide 2, 14.1 min. The FAD concentration in the HPLC fractions was determined by measuring the absorbance at 465 nm. For the kinetic assays, the reduction of the heme in PchC was followed by HPLC using a 15 μm Bis Tris propane buffer column of 81:19, H₂O:CH₃CN + 0.1% trifluoroacetic acid, 1 ml/min flow rate, and retention times for FMN peptides, 13.8 min (major peak, 12 nmol) and 9.2 min (minor peak). Treatment of the major flavin peptide with alkaline phosphatase, followed by HPLC analysis, confirmed that it contained FAD. Half the sample was lyophilized and not further treated. The remainder was lyophilized, dissolved in 200 μl of H₂O in a stopped tube, flushed with pure argon, and 20 μl of a 2 mg/ml sodium dithionite solution added. The yellow color bleached immediately upon addition of dithionite, indicating that the flavin was not bound to the protein. The solution was incubated under argon for 2 h, and the resulting FMN-free peptide (4) was purified on the Sephadex column in the same manner as the previous step: retention time of 24 min. All HPLC runs were monitored with a Hewlett-Packard (North Hollywood, CA) 1040A, Series II diode array detector. The peptide flavin peptides, with and without flavin attached, were sequenced in the laboratory of Professor Michael J. Barber, University of South Florida, Tampa, FL.

Steady-state Kinetic Assays—For these assays, reduction of PES by p-cresol-reduced PCMH was monitored at 600 nm due to the subsequent reduction of 2,6-dichlorophenol indophenol. The assays were done in 50 mM Tris HCl, pH 7.6, 6.3% at 25°C (32). In all assays, the sodium 2,6-dichlorophenol indophenol concentration was 92 μM. The concentration of PES was varied from 0.6 to 3.6 mM, and the concentration of p-cresol was varied from 5.25 μM to 0.6 mM. As in past studies with PCMH purified from P. putida (32), steady-state kinetic assays with the various forms of reconstituted enzyme produced parallel 1/ν versus [1/PES] plots for various p-cresol concentrations, and parallel 1/ν versus [1/p-cresol] plots for different PES concentrations. These patterns are indicative of ping-pong type kinetic behavior. The steady-state parameters reported in Table I are the true kcat and km values for each substrate.

Covalent Flavinylation Kinetics—These reactions were done in 16 mM potassium phosphate buffer, pH 7.0, at 30 °C. The reaction mixtures contained varying concentrations of apo-PchF₂, PchC, and FAD. Before mixing, there was a very slow linear increase in absorbance at 410 nm (1350-1000, 0.05 at 25°C) (32). In all assays, the sodium 2,6-dichlorophenol indophenol concentration was 92 μM. The affinity of apo-PchF₂ and PchC for the flavin were determined from reduction of PES as a function of [FAD] (33). The affinity of apo-PchF₂ for p-cresol was varied from 5.25 μM to 0.6 mM. As in past studies with PCMH purified from P. putida (32), steady-state kinetic assays with the various forms of reconstituted enzyme produced parallel 1/ν versus [1/PES] plots for various p-cresol concentrations, and parallel 1/ν versus [1/p-cresol] plots for different PES concentrations. These patterns are indicative of ping-pong type kinetic behavior. The steady-state parameters reported in Table I are the true kcat and km values for each substrate.
FAD Attachment to p-Cresol Methylhydroxylase

Apo-PchF Requires PchC for Its Covalent Flavinylation—In the course of E. coli DH5α expression experiments (18), anti-flavin (25), anti-PchF, or anti-PchC antibodies were used for Western blot analyses of whole cell extracts electrophoresed on denaturing SDS gels. It was determined that PchF was heterologously expressed as a noncovalently associated dimer (α2 or PchF2), while PchC was expressed as and exists as a monomer (β). For E. coli/pKK-CF, which produces both PchF2 and PchC, high levels of flavin were found associated with the PchF band on a denaturing gel. This indicated that PchF was covalently bound to this subunit. In earlier work, it was demonstrated that heme was covalently attached to E. coli-expressed PchF and that the protein displayed the properties of a typical c-type cytochrome (18). In analyses of extracts of E. coli expressing PchF2 but not PchC (E. coli/pKK-F), nearly undetectable amounts of flavin were found associated with PchF. Prolonged incubation of the extract with excess FAD did not alter the outcome. In contrast, after a 20-min incubation of an E. coli/pKK-F extract with an extract of E. coli expressing PchC but not PchF2 (E. coli/pKK-C), we detected a huge increase in the level of covalently bound flavin associated with the PchF band on an SDS gel. Addition of excess pure PchC to the E. coli/pKK-F extract yielded the same results. In either case, addition of excess FAD had no effect on the outcome. This indicated that E. coli extracts contain a relatively large amount of available FAD.

As progressively more pure PchC or an extract of E. coli/pKK-C was added to an extract of E. coli/pKK-F, progressively more FAD became covalently bound to PchF. Increasing the amount of E. coli/pKK-C extract or pure PchC added to the E. coli/pKK-CF extract also resulted in progressively more FAD becoming covalently bound. It was concluded that PchC was essential for covalent FAD attachment to PchF2. Subsequently, it was estimated that E. coli/pKK-CF manufactured PchF and PchC in a 5:1 ratio, which accounts for the incomplete flavinylation of PchF in E. coli/pKK-CF extracts.

A partially purified preparation of PchF/PchC from E. coli/pKK-CF was subjected to preparative isoelectric focusing using a pH gradient from 4 to 9. A red heme-containing PchC band focused at pl = 4.5, while two yellow bands focused at pl = 5.0 (Fα) and 5.4 (Fβ), respectively, and a broad colorless band focused at pl ~ 6.7. Fα was shown to be α2 flavoprotein (PchF2) with FAD covalently bound to both α subunits, and Fβ was found to be α3 flavoprotein with one α subunit containing covalently bound FAD and the other α subunit devoid of flavin (α-). The colorless protein was α2 flavoprotein devoid of FAD (apo-PchF2 or α2). Addition of FAD to pure apo-PchF2 did not result in covalent flavin attachment.

Using PAGE, addition of increasing amounts of PchC to α2, in the presence of excess FAD, indicated that a 1:1 ratio of α and β was required for complete flavinylation (Fig. 3). Incremental addition of FAD to α2α, in the presence of excess PchC, indicated that 1 mol of FAD bound covalently per 1 mol of flavoprotein subunit (data not shown). Covalent flavin binding did not occur when α2 was incubated with FMN, FMN and ATP, FMN and ADP, FMN and AMP, riboflavin, riboflavin and ATP, riboflavin and ADP, or riboflavin and AMP, in the presence of a large excess of PchC, even after 24-h incubations. PchC does not bind to α2 in the absence of FAD (see below). Horse heart cytochrome c, P. stutzeri cytochrome c550, P. aeruginosa cytochrome c551, or halophilic Paracoccus (ATCC 12084) cytochrome c550 did not substitute for PchC in promoting covalent binding of FAD to apo-PchF2. Soluble flavin was not released from trichloroacetic acid-precipitated samples containing holo-PchF, indicating that all FAD was covalently bound. Furthermore, the fluorescence quantum yield of FMN/riboflavin-containing, trypsin/chymotrypsin-digested PchF2 samples was much lower than expected for free FMN/riboflavin, but is in accord with the low quantum yield for 8α-O-tyrosyllavin (4).

Attempts to establish noncovalent binding of FAD to apo-PchF2 were inconclusive. A Kd value of 15 μM at 25 °C and pH 7.0 was determined by fluorescence quenching of bound FAD; however, the maximum fluorescence quenching was about 4 times greater than expected if specific binding of FAD to the flavin binding site apo-PchF2 had caused complete quenching of the FAD fluorescence. It was concluded that there was significant nonspecific quenching by apo-PchF2, possibly due to nonspecific FAD binding. The changes that occurred in UV-visible spectra during a FAD titration of apo-PchF2 were minor. Unless the Kd for specific FAD binding was extremely small (lapo-PchF2) = 19.7 μM), which apparently was not the case, the spectra of free FAD would always have swamped out the spectra of bound FAD; thus, these data were impossible to analyze. Additionally, there would be no way the separate spectral perturbations resulting from specific and nonspecific binding. Other experiments, such as equilibrium dialysis, sim-

### Table 1

| Recombined subunits | kαβ | kβα | kαβ | kβα |
|---------------------|-----|-----|-----|-----|
| PchF/PchCp         | 121 ± 7 | 15.1 ± 1.1 | 4.5 ± 0.40 | 4.5 ± 0.40 |
| αβ2                 | 147 ± 23 | 15.1 ± 2.1 | 6.42 ± 0.86 | 6.42 ± 0.86 |
| α2 + FAD + PchC     | 144 ± 24 | 19.2 ± 3.2 | 6.33 ± 1.08 | 6.33 ± 1.08 |
| PchF/PchCp         | 137 ± 8  | 15.7 ± 1.2 | 5.49 ± 0.48 | 5.49 ± 0.48 |
| PchF/PchCp         | 145 ± 12 | 13.3 ± 1.4 | 5.07 ± 0.62 | 5.07 ± 0.62 |
| PchF/PchCp         | 152 ± 18 | 13.7 ± 2.0 | 5.97 ± 0.98 | 5.97 ± 0.98 |

*The subscripts P and E refer to P. putida-expressed (wild-type) and E. coli-expressed subunits (recombinant), respectively. The lower activity for PchF/PchCp is due to the age of PCMH used (~3 years old). The enzyme loses about 5% activity/year under optimal storage conditions. The activity of PchF/PchCp is that expected for fresh, wild-type PCMH.*

**All activities were based on [FAD] or [PchC] ([FAD] = [PchC]), not [PchF], since the αβ2 species has 1/2 the FAD or PchC content per mole of protein compared to the other forms listed.
similarly would not discriminate specific and nonspecific binding. Band shifting was not observed on native gel electrophoresis gels of apo-PchF₂ in the absence of FAD, with and without PchC present, nor was a change in the retention time seen for molecular sieving chromatography of apo-PchF₂ in the absence and presence of excess PchC. Finally, PchC had no specific effect on the tryptophan fluorescence of apo-PchF₂. From these observations it was concluded that PchC does not bind to apo-PchF₂ when FAD is absent. This implies that FAD must bind to apo-PchF₂ before PchC can bind to catalyze the covalent flavinylation. Alternatively, there could be a fleeting complex between PchC and apo-PchF₂ to which FAD binds.

The αβα⁻ form of PCMH was made by adding excess PchC to Fₐ, in the absence of FAD. The αβ₂ form PCMH was prepared either by mixing α₂ with a 10-fold excess of FAD and a 2-fold excess of PchC, or by mixing PchC with Fₐ. Excess PchC and FAD were removed by gel filtration. Based on the heme or FAD content, within experimental error, the values for steady-state kinetic parameters were identical for these three forms of PCMH, and these parameters were the same as those for PCMH produced by wild-type P. putida 9869. It is interesting that the αβ portions of the αβα⁻ variant and αβ₂ enzyme display identical kinetic properties (Table I).

A peptic flavin-containing peptide was isolated from PCMH, prepared by mixing FAD, heterologously expressed apo-PchF₂ and PchC. The sequence of the peptide was Xaa-Trp-Asn-Arg-Gly-Gly-Gly-Ser-Met. The peptide was treated with sodium dithionite to induce reductive elimination of the flavin, as expected for 8α-O-tyrosylflavin. No other naturally occurring 8α-modified flavin undergoes this reductive-destruction reaction (4). The flavin-free peptide also was sequenced, and the N-terminal amino acid, Xaa, was identified as Tyr. This peptic flavin peptide is identical to the one previously isolated from wild-type PCMH purified from P. putida extracts (4, 18). These results prove that FAD attaches correctly to Tyr²⁸⁴ of PchF.

The Chemical Mechanism of Covalent FAD Attachment—In PCMH, 1- or 2-electron-reduced flavoprotein-bound FAD rapidly transfers an electron to the heme in PchC (k ≈ 200 s⁻¹) (32, 36). The long proposed mechanism for covalent FAD binding to the apo-PchF₂ subunit requires a 2-electron reduction of the i solafoxazine ring (Fig. 2) (5, 6). Thus, it would be expected that the reaction between FAD, apo-PchF₂, and PchC would result in reduction of the PchC-bound heme. Preliminary kinetic studies have shown that PchC is indeed reduced, and heme reduction provided a sensitive and convenient measure of the flavinylation reaction (ΔΔ₄₅₇(redox) = 91.4 mm⁻¹ cm⁻¹, and ΔΔ₅₅₅₂(redox) = 19 mm⁻¹ cm⁻¹ for the cytochrome subunit). Rate constants for reactions run at 30 °C, in 16 mM potassium phosphate buffer, pH 7.0, are presented in Table II. Fig. 4 presents some of the kinetic traces. Even though the concentrations of the various components were varied over a wide range ([apo-PchF₂], 0.21–0.70 μM; [PchC], 3.57–59.0 μM; [FAD], 1.16–116 μM), there was less than a 3-fold difference in the apparent first order rate constant for reduction of heme, i.e. covalent flavinylation (Table II). This suggests that chemical or conformational processes, not binding, have a disproportionate effect on the rate of covalent flavin binding at the conditions used for these studies. When 1.30 μmol of apo-PchF₂ were anaerobically exposed to 18.5 μmol of PchC and 92.3 μmol of FAD, at pH 7.0, 5.37 ± 0.05 μmol of heme were reduced. (This value was corrected for the linear heme reduction seen when FAD was absent; see Fig. 4.) In other words, for each molecule of apo-PchF₂, 4.13 ± 0.04

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**Table I**

| Reaction | Concentrations of components | Rate constant | Slope of linear phase² |
|----------|------------------------------|---------------|------------------------|
|          | Apo-PchF₂ | PchC | FAD | k x 10⁵ s⁻¹ | ΔA x 10⁸ s⁻¹ |
| Iᵇ | 0.70 | 11.6 | 23.1 | 6.78 ± 0.08 | 3.90 ± 0.13 |
| II | 0.70 | 11.6 | 116 | 8.54 ± 0.07 | 2.85 ± 0.07 |
| III | 0.70 | 59.0 | 23.1 | 7.20 ± 0.09 | 5.11 ± 0.11 |
| IV | 0.70 | 3.57 | 23.1 | 3.52 ± 0.04 | -2.51 ± 0.18 |
| V | 0.70 | 11.6 | 0 | NA | 4.10 ± 0.04 |
| VI | 0.21 | 11.6 | 1.16 | 2.93 ± 0.21 | 13.1 ± 0.06 |

ᵃ ΔA = A₂ – A₁.
ᵇ Reactions I–V were monitored at 552 nm. Reaction VI was monitored at 417 nm. Reactions I, IV, and V correspond to traces A, B, and C in Fig. 4, respectively.
ᶜ Not applicable.

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**Table II**

| Reaction | Concentrations of components | Rate constant | Slope of linear phase² |
|----------|------------------------------|---------------|------------------------|
|          | Apo-PchF₂ | PchC | FAD | k x 10⁵ s⁻¹ | ΔA x 10⁸ s⁻¹ |
| Iᵇ | 0.70 | 11.6 | 23.1 | 6.78 ± 0.08 | 3.90 ± 0.13 |
| II | 0.70 | 11.6 | 116 | 8.54 ± 0.07 | 2.85 ± 0.07 |
| III | 0.70 | 59.0 | 23.1 | 7.20 ± 0.09 | 5.11 ± 0.11 |
| IV | 0.70 | 3.57 | 23.1 | 3.52 ± 0.04 | -2.51 ± 0.18 |
| V | 0.70 | 11.6 | 0 | NA | 4.10 ± 0.04 |
| VI | 0.21 | 11.6 | 1.16 | 2.93 ± 0.21 | 13.1 ± 0.06 |

ᵃ ΔA = A₂ – A₁.
ᵇ Reactions I–V were monitored at 552 nm. Reaction VI was monitored at 417 nm. Reactions I, IV, and V correspond to traces A, B, and C in Fig. 4, respectively.
ᶜ Not applicable.
molecules of heme are reduced, or 2.07 ± 0.02 molecules of heme for each mole of FAD that becomes covalently bound. Apparently, each of two electrons from reduced, covalently bound FAD are transferred to different PchC molecules. PchC-bound PchC, which induced covalent binding, immediately becomes 1-electron reduced. This reduced PchC likely transfers an electron to another unassociated, oxidized PchC molecule. The reoxidized, associated PchC acquires the second electron from the remaining FAD radical.

Catalytic groups on the enzyme participating in flavinylation are unknown. We speculate that Arg477 stabilizes the negative charge that develops at N-1 nitrogen/C-2 oxygen of the flavin, when the iminoquinone methide form of FAD develops just prior to nucleophilic attack at the 8α-carbon by Tyr384 phenolate (Figs. 2 and 5). A guanidino nitrogen of Arg477 is 2.5 and 3.3 Å from the C-2 oxygen and N-1 nitrogen of FAD, respectively (Fig. 5).

The inability of PchC to bind to apo-PchF suggests that noncovalently bound FAD alters the structure of PchF such that PchC can bind. This structural change could be global, or local, only affecting the environment of PchF around the FAD site and the surface region where PchC binds. On the other hand, PchC binding to the apo-PchF/FAD complex must confer further structural changes in order to properly position catalytic groups, the 8α-carbon of FAD, and Tyr384 phenolate oxygen for efficient covalent tethering of flavin.

The mechanism in Fig. 2 presumes that a base is required to remove an 8α-hydrogen to produce the iminoquinone methide form of the dimethylbenzene portion of the isoaalloxazine ring of FAD. The closest group that could possibly act as a base is Asp440 in its anionic form (Fig. 5). One of its carboxylate oxygens is 5.5 Å from one of the 8α-hydrogens. Perhaps Asp440 moves subsequent to the formation of the FAD-Tyr384 bond, or there is an intervening H2O molecule that provides a bridge between Asp440 and the 8α-hydrogen. Alternatively, Tyr384 phenolate could remove the 8α-hydrogen and then lose the proton to another base such as Asp440 to regenerate the phenolate form for attack of the 8α-carbon.

Attack of Tyr384 phenolate at the 8α-carbon of FAD is a Michael-type addition that produces reduced covalently bound FAD (Fig. 2). The optimal stereochemistry for this addition would result in a dihedral angle of 90° between the plane of the flavin ring and the plane formed by the covalent bonds from the flavin to the phenolic oxygen (38). Inspection of the structure of covalently bound FAD on PCMH indicates a dihedral angle of 88°. This is also an optimal angle for elimination of Tyr384 from 2-electron-reduced PCMH-bound FAD. It is known that 2-electron reduction of enzyme-free, 8α-O-tyrosylflavin, results in facile elimination of tyrosine (4). However, 2-electron-reduced FAD, covalently bound in ω2β3 PCMH or ω2 flavoprotein, is not subject to elimination (18). The reason for stability of covalent linkage in the protein is speculative. Perhaps a base that is required to protonate Tyr384 phenolate as the 8α-electron bond is broken has moved as a result of a conformational change occurring when the covalent bond forms. Possibly newly formed protein-FAD interactions decrease the propensity for shifting electron density into the benzenoid portion of the isoaalloxazine ring system. Further refinement of the structure is under way. A more refined structure will provide valuable information regarding groups involved in the flavinylation reaction and may indicate a dihedral angle somewhat different from 90°.

Raison d’Être for Covalently Bound FAD in PCMH—As stated earlier, a major unanswered question concerns the purpose for covalent flavin binding in oxidoreductases. For PCMH, this linkage is likely involved in electron transfer from FAD to heme. On inspection of the 2.5-Å x-ray crystal structure of PCMH and of the 3.0-Å structure of the PCMH/p-cresol complex, it appeared that the most direct path for electron transfer from FAD in PchF to heme in PchC included the 8α-carbon of FAD and the phenolic moiety of Tyr384. The computer program GREENPATH, version 0.97, was used to calculate the optimal pathway for electron transfer from the N-5 position of FAD to the iron at the core of the heme group (35). By far the best calculated pathway is the one displayed in Fig. 5. An electron tunnels from the flavin C-8 Tyr384 phenolic ether bond through three other bonds of the phenol moiety, before “jumping” 2.96 Å to Ala49 of PchC. From Ala49, the electron travels to Met50, an axial ligand of heme-bound iron. Without the FAD-Tyr384 covalent bond, assuming minimal structural changes, and based on van der Waal’s radii, an electron would need to “jump” at least 2.4 Å through space from FAD to Tyr, on its journey to the heme group. This would decrease the rate of electron transfer (39).

While the covalent flavin bond may accelerate electron transfer in PCMH, this is not the case for the two covalently bound flavins of other flavoproteins with known structures. Diheme flavocytochrome c sulfide dehydrogenase from a purple phototrophic bacterium contains FAD covalently linked at its 8α-carbon to sulfurr of a Cys residue (40). Four potential electron-transfer pathways from FAD to the closest heme group were calculated. For two pathways, electrons jump to the polypeptide from the N-3 position of FAD; for another path, electrons jump to the peptide from the C-2 oxygen of FAD; for the fourth path, electrons exit via the N-5 position of FAD. The 8α-FAD bond to Cys52 is distal to the heme group and is not part of any “optimal” pathways (41).

For trimethylamine dehydrogenase from bacterium W3A1, the closest approach of covalently bound FMN and the Fe4S4 cluster is at the 8α-position of the flavin. The separation is about 6 Å (42). However, FMN is attached at its 6-position to...
the sulfur of Cys$^{30}$ (3, 42). It is extremely unlikely that electrons pass through the 6-position of FMN on the way to the Fe$_4$S$_4$ cluster.

We conclude that there is no dominant reason for covalent tethering of flavin in proteins. For PCMH, an obvious function revealed itself, but for other oxidoreductases, the reason remains obscure. At least in some cases, it is unlikely that covalent flavinylation is a fluke of nature. The linkage of FAD to the N-3 nitrogen of a specific, corresponding histidine is found in succinate dehydrogenase from all forms of life (14). If this linkage were not essential for stability, catalysis, electron transfer, etc., the linkage would not have survived eons of evolution.

The mechanism of post-translational covalent modification of PCMH is unique. We are not aware of any other example where a subunit seemingly stabilizes noncovalent binding of the modifying agent to its partner subunit, and then catalyzes formation of the covalent bond. In PCMH, this occurs without intermediating factors (e.g. external enzymes, chaperons, small molecule effectors), other than the subunits and FAD, nor is preactivation of apoprotein or flavin required.

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The Cytochrome Subunit Is Necessary for Covalent FAD Attachment to the Flavoprotein Subunit of \( p \)-Cresol Methylhydroxylase

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