8α-(*O*-Tyrosyl)flavin Adenine Dinucleotide, the Prosthetic Group of Bacterial *p*-Cresol Methylhydroxylase†

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ABSTRACT: 8α-(*O*-Tyrosyl)riboflavin has been synthesized by condensation of the copper complex of L-tyrosine with 8α-bromotetraacetylriboflavin. The structure of this synthetic product was proven by absorption and ^{1}H NMR spectroscopy and by chemical degradation, which yielded 1 mol of tyrosine per mol of flavin. The synthetic compound comigrated with the (aminoacyl)riboflavin isolated from the *p*-cresol methylhydroxylase of *Pseudomonas putida*, and both showed identical absorption and fluorescence spectral properties. 8α-(*O*-Tyrosyl)riboflavin as well as the flavin-containing decapeptide from *p*-cresol methylhydroxylase undergoes reductive cleavage to form riboflavin and FAD, respectively, on anaerobic treatment with dithionite. In contrast, the native enzyme, on reduction with dithionite, yields a reduced flavin via a red (anionic) flavosemiquinone intermediate, which remains covalently bound to the protein even under denaturing conditions. 8α-(*O*-Tyrosyl)riboflavin bound to apoflavodoxin is also not cleaved on reduction with dithionite, but, instead, a blue (neutral) semiquinone of tyrosylriboflavin is generated, which is resistant to further reduction with dithionite. Three *p*-cresol methylhydroxylases, isolated from different strains of *Pseudomonas putida*, differing in molecular weight and K_m values for substrates, contain the same peptide at the flavin site. These data provide definitive proof for the existence of 8α-(*O*-tyrosyl)riboflavin in nature.

p-Cresol methylhydroxylase is flavocytochrome isolated from *Pseudomonas putida* (N.C.I.B. 9866) when this organism is grown on 2,4-xylenol. The enzyme converts *p*-cresol to *p*-hydroxybenzyl alcohol and also catalyzes the further oxidation of the alcohol to *p*-hydroxybenzaldehyde. The enzyme is not a monooxygenase in that molecular oxygen is not required for the conversion of *p*-cresol to the alcohol. Hopper (1976) has proposed that the *p*-cresol is initially dehydrogenated and is then hydrated to the alcohol, followed by reoxidation of the enzyme by an external electron acceptor such as phenazine methosulfate (Hopper & Taylor, 1977). The further oxidation to *p*-hydroxybenzaldehyde is similarly an anaerobic reaction.

Two *p*-cresol methylhydroxylases have been isolated from *Ps. putida* (N.C.I.B. 9869) grown on 3,5-xylenol and *p*-cresol, respectively (Keat & Hopper, 1978). All three enzymes have been shown to contain two subunits of apparently equal molecular weights, and each contains one *c*-type cytochrome and one covalently bound flavin per dimer but differ in total molecular weight and substrate specificities (Hopper & Taylor, 1977; Keat & Hopper, 1978). Besides these enzymes, there are currently 18 proteins known to contain flavins which are covalently bound to an amino acid residue. These flavins fall into four categories, three of which comprise FAD moieties substituted in the 8α position to give variously 8α-(*S*-cysteinyl)-, 8α-(N^1-histidyl)-, and 8α-(N^3-histidyl)-FAD. The 6-(*S*-cysteinyl) derivative of FMN constitutes a fourth category (Kenney et al., 1979a,b; Weiner & Dickie, 1979; Kenney, 1980; Ohta-Fukuyama et al., 1980; Singer & Edmondson, 1980; Steenkamp, 1980).

A previous communication described the isolation and properties of a peptic flavin peptide from the *p*-cresol methylhydroxylase from *Ps. putida* (strain 9866) (McIntire et al., 1980). The visible absorption spectrum of this peptide suggested the presence of an 8α-substituted flavin. The presence of an 8α-histidyl linkage in the peptide was ruled out by the fact that the fluorescence remained constant in the pH range 3–7 (Singer & Edmondson, 1980), and an 8α-cysteinyl linkage could be discounted since acid hydrolysis of the flavin peptide with and without prior oxidation with performic acid failed to yield cysteine or cysteic acid, respectively (Kenney et al., 1977).

The following evidence suggested that the enzyme contains a novel form of covalently bound flavin, 8α-(*O*-tyrosyl)-FAD. Acid hydrolysis of the dansylated peptide yielded *N*-dansyltyrosine[1] but not *O*-dansyl- or *N*,*O*-didansyltyrosine. Hydrolysis of the flavin peptide with aminopeptidase M produced an (aminoacyl)flavin which gave approximately 1 mol of tyrosine per mol of flavin on acid cleavage. It was concluded that 8α-(*O*-tyrosyl)-FAD is at the N terminus of the peptide.

The present report describes the synthesis of 8α-(*O*-tyrosyl)riboflavin and its physical and chemical properties and demonstrates the identity of the synthetic product with the (aminoacyl)riboflavin isolated from the enzyme. In addition, this paper compares the flavin sites of the three bacterial proteins exhibiting *p*-cresol methylhydroxylase activity.

Experimental Procedures

Materials. Pepsin was obtained from Mann Research Laboratories, *Crotalus atrox* nucleotide pyrophosphatase from Sigma Chemical Co., bacterial alkaline phosphatase from Worthington Biochemicals, and aminopeptidase M from P-L Biochemicals. Silica gel 60 "high-performance" thin-layer chromatography plates were from E. Merck, Darmstadt, Germany, and microcrystalline cellulose sheets (F1440) and the micropolyamide sheets were from Schleicher & Schuell. Phosphocellulose was purchased from Gallard-Schlesinger, Florisil (60–100 mesh) from the Fisher Scientific Co., and Chelex-100 (100–200 mesh) as well as Bio-Gel P-2 (100–200

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[1] Abbreviation used: Dns, dansyl or 5-(dimethylamino)naphthalene-1-sulfonyl.

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mesh) from Bio-Rad Laboratories. $TiCl_3$ (20% solution in water) was obtained from Matheson Coleman and Bell. The source of [U-^{2}H]methanol, [U-^{2}H]dimethylformamide (both 99.5%), and tetramethylsilane was Merck Sharp and Dohme, Canada, Ltd., while that of 4 N methanesulfonic acid containing 0.2% (w/v) tryptamine and of 3 N 2-mercaptoethanesulfonic acid was Pierce Chemical Co. Apoflavodoxin from *Azotobacter vinelandii*, strain OP, was prepared according to a published procedure (Edmondson & Tollin, 1971a).

Methods. Peptic Peptides. *p*-Cresol methylhydroxylases were isolated from *Ps. putida* (N.C.I.B. 9866 and 9869) as previously described (Hopper & Taylor, 1977; Keat & Hopper, 1978). The former strain produces only one form of the enzyme and has been the source of the material for most of this study. The latter strain produces two forms of the hydroxylase, depending on the carbon source used (Keat & Hopper, 1978). FAD peptides from each of the three enzymes were prepared and purified as described (McIntire et al., 1980). Final purification involved additional chromatography on phosphocellulose columns [1 × 10 cm, 8% (v/v) formic acid as equilibrating and eluting medium, 50–100 nmol of flavin peptide applied].

(Aminoacyl)flavins. The pure FAD peptide (1–2 nmol) was dried in vacuo. To this were added 5 μL of a solution of nucleotide pyrophosphatase (1 mg/mL) in 50 mM *N*-ethylmorpholinium acetate and 15 mM $MgCl_2$, pH 7.4, and 5 μL of a 1 mg/mL solution of alkaline phosphatase in 50 mM *N*-ethylmorpholinium acetate, pH 8.0. Dephosphorylation was carried out for 20 min at 38 °C. After concentration to dryness, 5 μL of a 5 mg/mL solution of aminopeptidase M in 50 mM *N*-ethylmorpholinium acetate, pH 8.0, was added, and incubation at 38 °C was continued for 1 h. The resulting solution was applied directly to thin-layer plates for analysis.

*Bis(O-sodio-*L*-tyrosinato)copper(II).* The synthesis was based on the method of Yamashiro & Li (1973). Ten grams of L-tyrosine (55.5 mmol) in 54 mL of 2 N NaOH (54 mmol) was mixed with 6.8 g of $CuSO_4 \cdot 5H_2O$ (26 mmol) in 30 mL of water. The mixture was heated to 60 °C for 10 min to obtain a clear solution. The solvent was removed in vacuo at 60 °C. The solid was extracted with 100 mL of methanol and filtered to remove unreacted tyrosine and other salts. The product was precipitated on the addition of a minimum volume of ethanol or 2-propanol, filtered, redissolved in 100 mL of methanol, and dried at 60 °C in vacuo. Since the olive-green product is deliquescent, it was stored in vacuo over NaOH pellets. The yield was 15 g (35.6 mmol, 64%).

8α-Bromo-2′,3′,4′,5′-tetra-O-acetylriboflavin. This was prepared by the method of Walker et al. (1972).

Amino Acid and Sequence Analysis. The peptides (4–6 nmol) were hydrolyzed in 200 μL of 4 N methanesulfonic acid containing 0.2% (w/v) tryptamine for 16–18 h at 110–115 °C in vacuo (Simpson et al., 1976). The pH was raised to ~2 by adding 300 μL of 10% (w/v) NaOH, and the amino acid content was determined with a modified Hitachi Perkin-Elmer amino acid analyzer. Digestion with aminopeptidase M involved incubation of 4–6 nmol of the peptide with 25 μL of a 1 mg/mL solution of the peptidase in 50 mM *N*-ethylmorpholinium acetate, pH 7.5, for 16–18 h at 38 °C with toluene present to retard bacterial contamination. The pH was adjusted to ~2 and the solution applied to the amino acid analyzer, as above. Amino-terminal dansylation and sequencing were performed as outlined by Hartley (1970). Dns-amino acids were chromatographed on micropolyamide sheets (5 × 5 cm). When Dns-tryptophan was suspected to be present at the amino terminus, the peptide was hydrolyzed with 200 μL of 3 N mercaptoethanesulfonic acid for 16 h at 110 °C. After neutralization with 100 μL of concentrated NH_4OH, the solution was extracted twice with 0.4 mL of ethyl acetate (Giglio, 1977). The Dns-amino acid was chromatographed as before.

Solvent Systems. The following solvent systems were used in thin-layer chromatography: (1) $CHCl_3$–methanol–concentrated NH_4OH (2:2:1); (2) 1-butanol–acetic acid–water (12:3:5); (3) 1-propanol–water (3:1); and (4) 1-butanol–pyridine–acetic acid–water (15:3:10:12).

Other Methods. Absorption spectra were recorded with a Cary 219 or a Cary 14 instrument, interfaced with a Nova 2/4 computer. Corrected excitation fluorescence spectra were obtained with a Hitachi Perkin-Elmer MPF-3 instrument. Proton NMR spectra were recorded with a Varian 80-MHz instrument. The sample was dissolved in [U-^{2}H]methanol or [U-^{2}H]dimethylformamide with 0.2% (w/v) tetramethylsilane.

Anaerobic titrations with dithionite were performed at 25 °C and pH 7.0 as described by Edmondson & Singer (1973) and titrations to determine sulfite affinity by the method of Müller & Massey (1969). Titrations with $TiCl_3$ (0.2% (w/v)) were performed in 88% (v/v) formic acid.

Results and Discussion

Synthesis of 8α-(O-Tyrosyl)riboflavin. We have recently presented evidence that the FAD moiety of *p*-cresol methylhydroxylase from *Ps. putida* (strain 9866) is covalently bound, via the 8α carbon of the flavin, to the phenolic oxygen of a tyrosine residue (McIntire et al., 1980). Further proof of the structure assigned to this novel flavin and detailed characterization of its physical properties required larger quantities of the compound than could be obtained from natural sources. Thus, chemical synthesis of the (aminoacyl)flavin, in the dephosphorylated form, was undertaken.

Kenney & Walker (1972) described briefly a procedure for the synthesis of this compound, but the product (in our hands), using their procedure, was not 8α-(*O*-tyrosyl)riboflavin. A convenient alternate synthesis was worked out on the basis of the studies of Yamashiro & Li (1973) on the synthesis of tyrosine ethers from substituted benzyl bromides. Tyrosine forms a bidentate ligand with Cu^{2+}, with the carboxylate and amino functions of two tyrosine molecules forming the coordination sphere for a single cupric ion. The phenolate (sodium salt) in the resulting complex reacts under mild conditions with benzyl bromide to form a benzyl ether, without interference by the carboxyl or amino groups.

In the synthesis of 8α-(*O*-tyrosyl)riboflavin, 1 g of the copper–tyrosine complex (2.3 mmol) was dissolved in 50 mL of methanol and was added dropwise over a 2 h period to a solution of 8α-bromotetraacetylriboflavin (0.32 mmol) in 50 mL of methanol at room temperature. The mixture was heated to and maintained at 50 °C for 1 h. The greenish blue solution and brown precipitate obtained were diluted after being cooled, with 400 mL of water. Formic acid was added to 8% (v/v) concentration, and the solution was passed through a column of Chelex-100 resin (1.5 × 10 cm), equilibrated with 8% formic acid, to remove copper ions. The yellow-orange effluent was directly applied to a Florisil column (1.5 × 10 cm) previously equilibrated and washed with 5% (v/v) acetic acid. The column was washed first with 5% (v/v) acetic acid until the absorbance at 280 nm became negligible and then with water, and the flavin was eluted with a 20% (v/v) pyridine solution. The combined pyridine solutions were lyophilized and redissolved in 5% pyridine, and any Florisil carried over was removed by centrifugation. The solution was lyophilized

Table I: Absorption Maxima (nm) and Extinction Coefficients (mM^{-1} cm^{-1}) of 8α-Substituted Flavins[a]

compound	λ_1	λ_2	λ_3	λ_4
riboflavin[c]	448 (12.3)	372 (10.8)	268 (31.4)	223 (30.1)
8α-(*O*-tyrosyl)riboflavin[d]	446 (12.3)	359 (9.38)	268 (36.4)	224 (41.9)
8α-(*S*-cysteinyl)riboflavin[e]	448 (12.0)	367 (8.6)	270 (?)	ND[b]
8α-[*S*-(*N*-acetylcysteinyl)]riboflavin[f]	448 (12.0)	368 (8.7)	269 (33.0)	224 (40.0)
8α-(N^1-histidyl)riboflavin[g,h]	445 (12.0)	355 (9.3)	268 (35.3)	221 (37.4)
8α-(N^3-histidyl)riboflavin[h]	445 (12.0)	355 (8.7)	268 (35.3)	221 (37.4)

[a] All spectra taken in aqueous buffers at neutral pH. [b] ND, not determined. [c] Koziol (1971). [d] Extinction coefficients for this compound calculated assuming a value of 12.3 mM^{-1} cm^{-1} for the A_{446nm}. [e] Walker et al. (1971). [f] Falk et al. (1976). [g] Edmondson et al. (1976). [h] D. E. Edmondson, unpublished data.

and the residue dissolved in a minimum volume of 8% (v/v) formic acid. The product was chromatographed on a phosphocellulose column (6 × 20 cm) with 8% formic acid as solvent. Fractions were analyzed by high-performance thin-layer chromatography on silica gel plates in solvent system 3. Two fractions showed flavin-type color and fluorescence. The predominant one showed the same R_f and fluorescence as expected for 8α-(*O*-tyrosyl)riboflavin, suggesting that under the conditions of synthesis, deacetylation occurs on the ribityl side chain. The other product proved to be a partially deacetylated form of the compound since hydrolysis at 50 °C for 1 h in 1 N HCl converted it to a compound with the same R_f as the first fraction.

After deacetylation of the latter fraction, the two fractions were combined, applied to a column of Bio-Gel P-2 (2.5 × 95 cm), and eluted with water to give nearly pure 8α-(*O*-tyrosyl)riboflavin. Remaining trace impurities were removed by repeating the chromatography on phosphocellulose (1 × 30 cm) with 8% (v/v) formic acid, with the fractions analyzed as above. The product gave a single spot in thin-layer chromatography on silica gel 60 in solvent system 3. The yield of 8α-(*O*-tyrosyl)riboflavin was 4%, based on the flavin.

Proof of Structure of the (Aminoacyl)flavin. The absorption spectrum of the synthetic flavin (Figure 1) resembles those of other 8α-substituted riboflavins in showing a major hypsochromic shift of the near-ultraviolet absorption band. The maximum at 268 nm is 10–20% higher than in histidyl- or cysteinylflavins (Table I), in accord with the presence of tyrosine in the molecule, although not as high as we have reported earlier for the (aminoacyl)riboflavin isolated from the hydroxylase (McIntire et al., 1980), probably because of the presence of ultraviolet-absorbing impurities in the natural product.

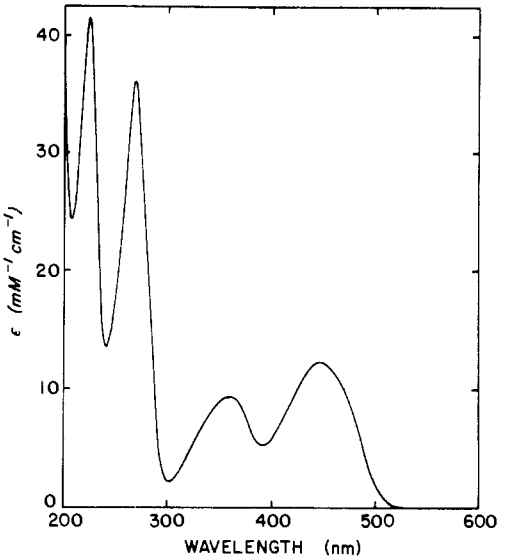


FIGURE 1: Absorption spectrum of synthetic 8α-(*O*-tyrosyl)riboflavin in 50 mM phosphate, pH 7.0.

Anaerobic acid hydrolysis [16 h at 110 °C in 4 N methanesulfonic acid containing 0.2% (v/v) tryptamine] of 5.8 nmol of the synthetic flavin produced 4.98 nmol of tyrosine, as determined by amino acid analysis. Dansylation of the synthetic compound, followed by acid hydrolysis under the same conditions, except in 6 N HCl, gave only *N*-dansyltyrosine, with no detectable *N*,*O*-didansyltyrosine, showing that tyrosine is linked to the flavin via the phenolic oxygen.

The NMR spectrum of the synthetic product in [U-^{2}H]-methanol (Figure 2) confirms the proposed structure of the flavin. Proof of 8α substitution is shown in that only one CH_3 is observed in the 2.0–2.5-ppm region of the spectrum, and a methylene peak is observed downfield at −5.26 ppm (Table II) in accord with published ^{1}H NMR data on other 8α-substituted flavins (Edmondson et al., 1976). The other protons attributable to the isoalloxazine ring system are assigned on the basis of chemical shifts reported for other flavins (Walker et al., 1972; Edmondson et al., 1976; Kenney & Singer, 1977). The doublet at 3.73 ppm may be attributed to either the β-methylene of the tyrosine or the C-1′ methylene of the ribityl chain. There is also a quartet centered around 7.11 ppm which appears to be due to an A_2B_2 splitting of the aromatic protons of tyrosine (Carrington & McLachlan, 1967). The large peaks at 3.27 and 4.75 ppm are produced by residual solvent protons. A group of smaller peaks between 2.9 and 5.1 ppm are assigned to the remaining ribityl and tyrosyl side-chain protons. A NMR spectrum in [U-^{2}H]dimethylformamide shows similar resonances (Table II), but in this case, solvent peaks at 2.7, 2.85, and 7.97 ppm and broad resonances between 3 and 5 ppm obscure much of the spectrum (not shown).

Table II: Proton NMR Chemical Shifts for the Synthetic Flavin[a]

solvent	flavin protons 6-H	7-CH_3	8-CH_2	9-H	ribityl C-1′ or tyrosyl β protons	aromatic[b] tyrosyl protons
[U-^{2}H]methanol	8.19 (0.83)	2.50 (2.98)	5.26 (1.79)	7.95 (1.07)	3.73 (1.79)	7.07 (2.38), 7.14 (2.26), J = 9.6 Hz
[U-^{2}H]dimethylformamide	8.43 (0.94)	2.54 (3.30)	5.39 (1.70)			7.18 (2.16), 7.29 (2.08), J = 8.8 Hz

[a] The chemical shifts are in ppm from Me_4Si. The numbers in parentheses are the peak intensities and are normalized to the average intensity of a single proton. [b] The chemical shifts for the A_2B_2 splitting of the 2,6 and 3,5 proton pairs for tyrosine are calculated according to Carrington & McLachlan (1976). J is the coupling constants for these protons.

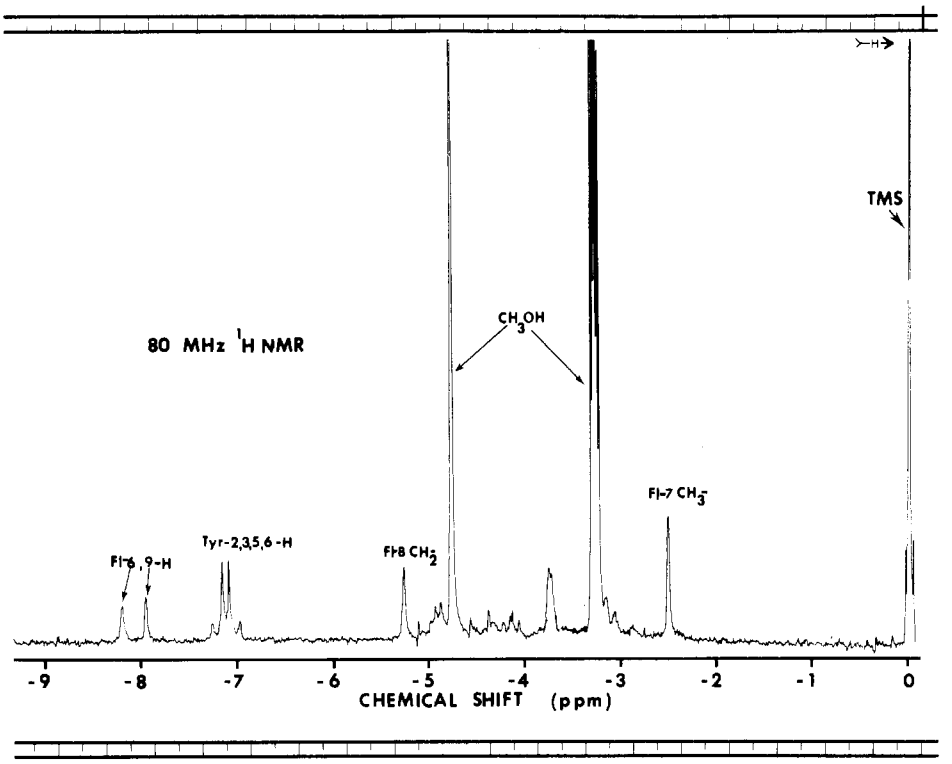


FIGURE 2: ^{1}H NMR spectrum of synthetic 8α-(*O*-tyrosyl)riboflavin, a saturated solution, in 99.5% [U-^{2}H]methanol at 80 MHz. Tetramethylsilane was used as an internal standard. The spectrum was obtained after Fourier transformation of 50 acquisitions (4-s acquisition time).

Table III: Fluorescence Properties of 8α-(*O*-Tyrosyl)riboflavin

solvent (emission maximum)	compound: riboflavin fluorescence[a]	compound: 8α-(*O*-tyrosyl)riboflavin fluorescence[a]	% fluorescence yield[b]
0.1 M sodium phosphate, pH 7.0 (525 nm)	77.6	1.13	1.5
0.1 M sodium citrate, pH 3.0 (525 nm)	74.6	1.14	1.5
methanol (515 nm)	100.7	6.69	6.6
dimethylformamide (515 nm)	95.7	11.74	12.3

[a] Fluorescence is given in arbitrary units. [b] Values relative to riboflavin fluorescence.

Fluorescence Properties. The fluorescence of 8α-(*O*-tyrosyl)riboflavin is only about 1.5% of that of riboflavin at both pH 3.0 and pH 7.0 (Table III). The fact that this slight fluorescence is due to the (aminoacyl)flavin and not to a trace of contaminating flavin may be concluded from the coincidence of the corrected fluorescence excitation spectrum (not shown) with the absorption spectrum (Figure 1). The fact that protonation of the α-NH_2 group of tyrosine does not restore any of the fluorescence of the flavin may be taken as additional evidence that the linkage of tyrosine to the flavin involves the phenol oxygen and not the amine function. In a series of lumiflavin derivatives substituted with various amines in the 8α position, protonation of the amine greatly enhanced the quenched fluorescence (Salach et al., 1972). A similar pH dependence of fluorescence yield was reported for 8α-(N^ϵ-lysyl)riboflavin (Kenney & Walker, 1972).

The fluorescence yield of the (aminoacyl)flavin was enhanced severalfold in organic solvents, while that of riboflavin increased only 20–30% (Table III). Similar observations were made by Falk & McCormick (1976) for 8α-(*S*-cysteinyl)-flavin. They pointed out that for these flavins fluorescence quenching is caused by the interaction of the nonbonding orbitals of the sulfur with the flavin π system. Such an effect can be decreased by the interaction of these orbitals with the solvent. Loss of quenching is also observed on oxidation of the thioether sulfur to the sulfone (Kenney & Singer, 1977) since in this oxidation state the sulfur no longer has nonbonding orbitals to interact with the flavin system. Similarly, the intense fluorescence quenching of 8α-N-substituted flavins at high pH and its reversal at lower pH values have been explained by the fact that the nonbonding electrons of the nitrogen are not available to interact with the flavin on protonation (Kenney & Walker, 1972; Salach et al., 1972; Singer & Edmondson, 1980). While other explanations cannot be ruled out, it seems reasonable that the same considerations may be applied to the intense fluorescence quenching of 8α-(*O*-tyrosyl)riboflavin; in this case, the nonbonding orbitals which interact with the molecular orbitals of the flavins are those of the phenolic oxygen.

Reductive Titrations. Since the oxidation–reduction properties of any new flavin isolated from biological material are of interest, it was decided to determine the redox potential of the tyrosylflavin by anaerobic titration with dithionite in the presence of indicator dyes. As a first experiment in this direction, the flavin was titrated anaerobically with dithionite in the absence of dyes with simultaneous monitoring of the absorption spectrum. The results are shown in Figure 3. The first molar equivalent (ca. two electrons) of dithionite caused rapid bleaching of the 446-nm band after each addition, followed by slow return of color, so that the net bleaching at this wavelength was small (Figure 3A). At 359 nm, the absorbance increased by a small extent with a pronounced shift of the maximum toward 372 nm. During the addition of the second molar equivalent of dithionite, the spectral changes corresponded to those associated with reduction of the flavin (Figure 3B). No clear indication of an anionic or neutral flavin

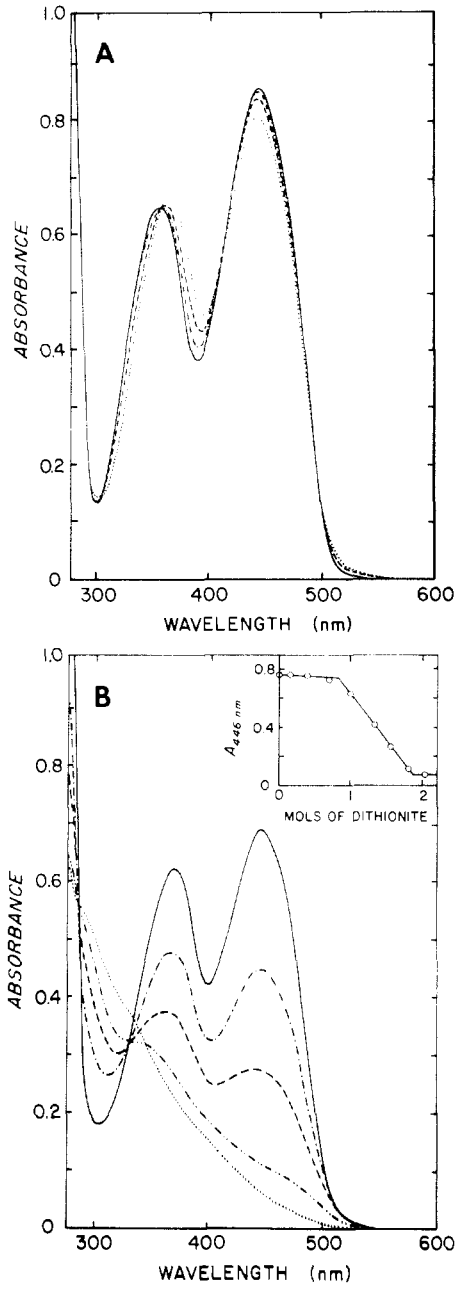
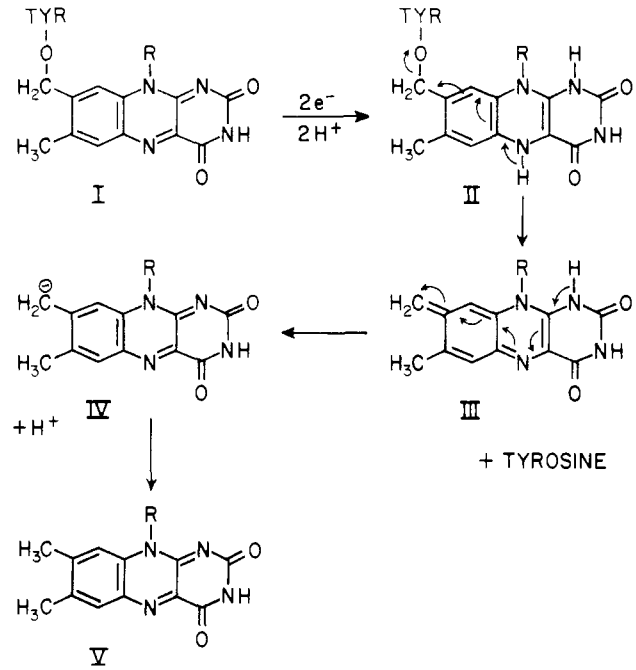


FIGURE 3: Effect of dithionite on the absorption spectrum of synthetic 8α-(*O*-tyrosyl)riboflavin. The titration was performed in 50 mM phosphate, pH 7.0, under anaerobic conditions. Spectra are uncorrected for volume changes on addition of dithionite (5.89 mM solution). (A) First phase of the titration: 150 nmol of fully oxidized flavin in 2.17 mL (—), 0.16 (–·––), 0.39 (– –), and 0.71 mol (···) of added dithionite per mol of flavin. (B) Second phase: 1.02 (—), 1.34 (–·––), 1.57 (– –), 1.80 (–··–··), and 2.04 mol (···) of total dithionite added per mol of flavin. The inset is a graph of the corrected absorbance at 446 nm vs. moles of dithionite added. Note spectra in the first phase were recorded 15 min after each addition of dithionite.

radical was noted during either phase. The inset for Figure 3B clearly shows that it is the second molar equivalent of dithionite which causes bleaching of the visible absorption. On admission of oxygen, an absorption spectrum typical of oxidized riboflavin was obtained (not shown). When compared with the starting material, this spectrum shows a bathochromic shift to 372 nm for the near-ultraviolet peak and intensification of the absorbance for this band.

The fluorescence of the final, reoxidized material equaled that of riboflavin. Thus, a greater than 60-fold increase in fluorescence accompanied the reduction–reoxidation cycle. All this strongly suggests that during the first phase of the reduction (Figure 3A), the tyrosylriboflavin is cleaved to its constituent moieties. Evidence for this interpretation was obtained by demonstrating that the flavin, produced after reduction and reoxidation of 8α-(*O*-tyrosyl)riboflavin, comigrated with riboflavin on a silica gel 60 plate in solvent system 3. In addition, amino acid analysis detected 0.91 mol of free tyrosine per mol of flavin.

Scheme I



Since reductive cleavage to riboflavin was virtually quantitative, comparison of the absorbance of the initial sample with the final, reoxidized one permitted calculation of the molar extinction coefficient of 8α-(*O*-tyrosyl)riboflavin. The value found was, within experimental error, the same as that of riboflavin (12.3×10^3 cm^{-1} M^{-1}).

The elimination of the 8α substituent on reduction of (aminoacyl)flavins has been previously observed. Edmondson & Singer (1973) demonstrated that the two-electron reduction of 8α-(*S*-cysteinyl sulfone)riboflavin with dithionite yielded cysteinylsulfinate and riboflavin. Kenney & Singer (1977) also reported the conversion of 8α-(*S*-cysteinyl)-8α-hydroxyriboflavin to 8α-(*S*-cysteinyl)riboflavin on treatment with dithionite. Scheme I suggests a mechanism to account for the experimental data observed for the reductive cleavage of tyrosylriboflavin. It is proposed that electrons rearrange in the reduced flavin nucleus, eliminating the substituent on the 8α-CH_3 group, with the production of a quinhydrone (III in Scheme I). Following tautomerization, a proton from the solvent is added either through the formation of intermediate IV or in a concerted reaction to produce oxidized riboflavin.

In all three of the examples quoted, reductive elimination occurred at neutral pH. It was of interest to see whether the cationic hydroquinone form of the flavin also undergoes reductive elimination. A solution of 8α-(*O*-tyrosyl)riboflavin in 88% (v/v) formic acid showed an absorbance maximum at 377 nm. Titration with $TiCl_3$ resulted in a similar biphasic absorbance change to that illustrated in the inset of Figure 3B. During the addition of the first 2 electron equiv, a gradual shift of the absorption maximum of the flavoquinone cation to 389 nm occurred, yielding a spectrum characteristic of the oxidized riboflavin cation. Further addition of $TiCl_3$ gave rise to the spectrum of the riboflavin cation radical, with maxima at 357 and 491 nm.

Table IV: Thin-Layer Chromatography of Natural and Synthetic Flavins[a]

flavin	solvent systems[b] 1	2	3	4
8α-(O-tyrosyl)riboflavin	0.64	0.28	0.31	0.14
(aminoacyl)flavin	0.59	0.24	0.33	0.14
both compounds (cochromatography)	0.58	0.22	0.31	0.14

[a] Numbers in the table are R_f values. [b] The solvent systems are as defined under Experimental Procedures. Thin-layer chromatography on silica gel 60 plates was used in association with solvent systems 1, 2, and 3, and micrycrystalline cellulose plates were used for chromatography in solvent system 4.

Since these observations precluded direct determination of the oxidation–reduction potential of 8α-(O-tyrosyl)riboflavin, we resorted to an indirect method of estimating the potential. A linear relationship exists between the midpoint potentials of flavins and the log of the dissociation constants of their sulfite complexes (Müller & Massey, 1969). This relationship also holds for 8α-substituted flavins (Edmondson & Singer, 1973). The K_D value for the sulfite complex of 8α-(O-tyrosyl)riboflavin was found to be 0.107 M at 25 °C, which corresponds to a calculated $E_{m,7} = -169$ mV, a value within the range observed for other 8α-substituted (aminoacyl)flavins.

Comparison of Synthetic 8α-(O-Tyrosyl)riboflavin with the Natural Product. A comparison of the data presented above for synthetic 8α-(O-tyrosyl)riboflavin with those summarized in our earlier paper for the (aminoacyl)riboflavin from *p*-cresol methylhydroxylase (McIntire et al., 1980) left little doubt about the identity of the two compounds. Further proof is offered in Table IV, which shows that, in thin-layer chromatography on silica gel 60 in three solvent systems and for thin-layer chromatography on microcrystalline cellulose in a fourth system, the synthetic and natural products comigrated as a single spot in mixtures of the two compounds.

Although the evidence indicates that the structure of the (aminoacyl)flavin from the hydroxylase is 8α-(O-tyrosyl)-FAD, as proposed, additional evidence for this assigment has come from the following experiment. When 2 nmol of the coenzyme peptide was reduced with dithionite under argon and reoxidized after 15 min with atmospheric oxygen, thin-layer chromatography in solvent system 3 indicated the formation of free FAD, confirming that the flavin is at the dinucleotide level. The flavin peptide therefore undergoes the reductive elimination described above. Dansylation of the material at the end of the dithionite treatment produced *N*,*O*-didansyltyrosine on acid hydrolysis, showing that the phenolic group was no longer blocked, whereas dansylation prior to dithionite reduction gave only *N*-dansyltyrosine. Dithionite treatment of the peptide followed by digestion with nucleotide pyrophosphatase also increased its fluorescence at pH 7.0 from ~2% of that of riboflavin to the level given by riboflavin and FMN, and the corrected fluorescence excitation spectrum now showed the maxima (375 and 445 nm) characteristic of free FMN. The large increase in fluorescence observed on reduction of the 8α-(O-tyrosyl)riboflavin peptide with dithionite and reoxidation by air may serve as the basis for the development of an analytical method for the determination of this type of flavin in biological materials since 8α-(O-tyrosyl)-riboflavin is the only naturally occurring flavin derivative so far known to show this effect.

Action of Dithionite on the Intact Enzyme. Since both the (aminoacyl)flavin and the flavin peptide are cleaved on reduction with dithionite, the interesting question arises whether the intact enzyme is also subject to reductive cleavage of the flavin and, if so, how this destruction of the catalytic site is prevented during reduction of the flavin by the substrate.

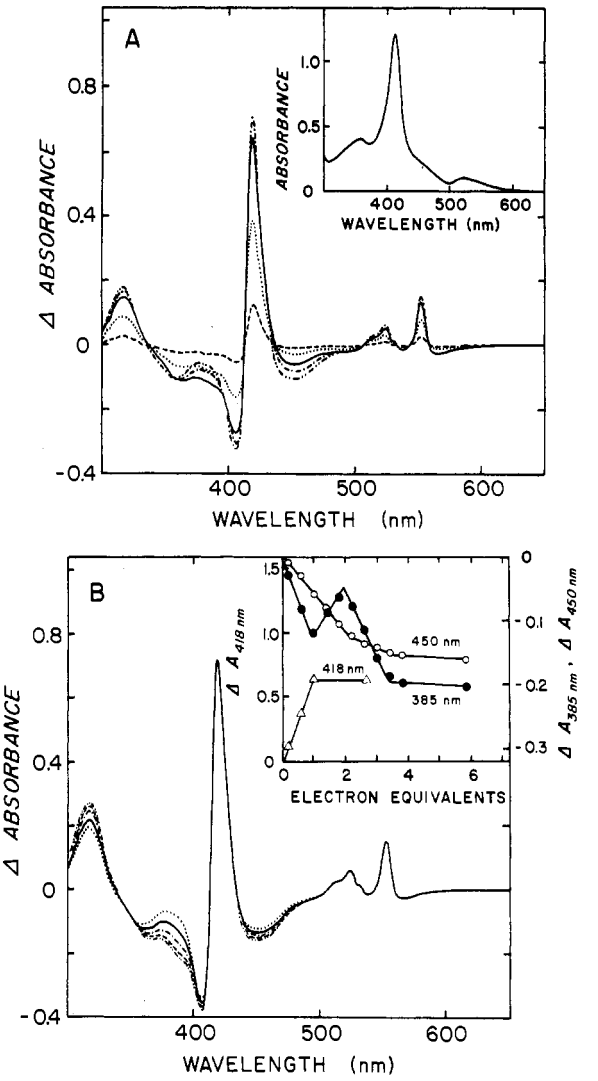


FIGURE 4: Spectral changes occurring on reduction of *p*-cresol methylhydroxylase. The enzyme (~0.86 mg in 0.732 mL of 50 mM phosphate buffer, pH 7.0) was titrated with 0.81 mM dithionite under anaerobic conditions. After each addition, the spectrum was recorded and stored in a computer, and corrections were made for volume changes. Each spectrum was then substracted from the spectrum of the oxidized enzyme. (A) Difference spectra after adding 0.21 (--), 0.62 (···), 1.08 (—), 1.44 (-·-·), and 1.84 (-··-··) electron equiv of dithionite per mol of enzyme. The inset shows the spectrum of oxidized enzyme for comparison. (B) Difference spectra after 2.25 (···), 2.66 (—), 3.06 (-·-·), 3.47 (--), and 3.88 (-···-···) electron equiv per mol of enzyme. The inset shows the relationship of the absorbance changes at 385, 415, and 450 nm to the amount of reducing agent added. Note that for the first two phases the spectra were recorded immediately after the addition of dithionite, while for the third phase the spectra were recorded 15–20 min after dithionite was added. The greater amount of dithionite consumed during the third phase of the titration than in the first two phases probably reflects the fact that the system was not completely at equilibrium. The occurrence of nonequilibrium conditions is supported by the gradual decrease in absorbance at 385 and 450 nm after the final phase of the titration, indicating that the enzyme has yet to reach its true fully reduced state. The spectrum for 5.84 electron equiv is not shown in (B).

In order to ascertain the effect of reduction on the native hydroxylase, the enzyme was titrated at pH 7.0 anaerobically with a dilute solution of sodium dithionite. The main panels of Figure 4 show the difference spectra after each addition of dithionite. The inset in Figure 4A gives the spectrum of the native oxidized enzyme and the inset in 4B the plots of absorbance changes at selected wavelengths vs. electron equivalents of reducing agent added.

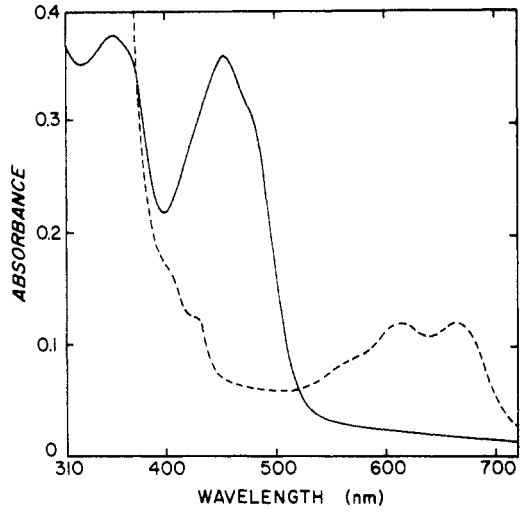


FIGURE 5: Dithionite reduction of 8α-(*O*-tyrosyl)riboflavin bound to apoflavodoxin. A 2-fold excess of apoflavodoxin was added to a solution of 113 nmol of flavin in 3.0 mL of 50 mM phosphate buffer, pH 7.0. The solid line represents the absorbance of the resulting oxidized flavoprotein. The dashed line is the spectrum recorded after addition of ~100-fold excess of dithionite under anaerobic conditions.

Inspection of Figure 4 reveals that the reduction consists of three phases and consumes just over 3 electron equiv of dithionite. The initial event is reduction of the heme which requires ~1 electron equiv of dithionite and is represented by increased absorbance at 418 nm (the Soret peak). This change is accompanied by a decrease in absorbance at 385 and 450 nm. During the addition of the second equivalent of dithionite, the flavin seems to be reduced to an anionic (red) flavosemiquinone radical which is seen by an increase in absorbance at 385 nm and a further decrease at 450 nm. The fact that bleaching of the absorbance at 450 nm is linear during the first two phases may reflect the similarity of the absorbance changes at this wavelength on reduction of the heme and the one-electron reduction of the oxidized flavin. During the third phase, reduction of the flavin radical to the hydroquinone occurs, as reflected by a sharp drop in absorbance at 385 nm (the absorption maximum of the anion radical) and a further decrease of the absorbance at 450 nm. The formation of an anionic flavin radical was not altogether unexpected since all native enzymes containing 8α-substituted covalently bound flavins, so far examined, generate this type of radical on one-electron reduction (Edmondson et al., 1980).

The reduced enzyme sample from the experiments in Figure 4 was precipitated anaerobically with 5% (w/v) trichloroacetic acid, exposed to oxygen, and centrifuged. Only a trace (~6%) of the flavin was found in the supernatant solution, and the rest was recovered as a decapeptide of 8α-(*O*-tyrosyl)-FAD after peptic digestion. Thus, despite the fact that dithionite reduces the flavin in the native enzyme to the dihydroflavin form as it does with the peptide and the (aminoacyl)flavin, in the former case reductive cleavage does not occur, as if, in some manner, noncovalent interactions with the protein prevented the electron migration (Scheme I) leading to cleavage.

It is of interest in this connection that a 100-fold molar excess of dithionite reduced the 8α-(*O*-tyrosyl)riboflavin bound to apoflavodoxin. This reduction does not result in cleavage of the tyrosine–flavin bond. Instead, despite the presence of a large excess of dithionite, reduction proceeds only to the radical stage (Figure 5). In this instance, however, the blue (neutral) radical is formed, with absorbance maxima at higher wavelengths (610 and 665 nm) than those found for typical neutral flavin radicals (580 and 615 nm) (Edmondson & Tollin, 1971b; Oestreicher et al., 1976; Massey & Hemmerich, 1980). Thus, reductive cleavage is prevented even when 8α-(*O*-tyrosyl)riboflavin is artificially combined with a protein other than that in which it occurs in nature. This experiment also illustrates the principle that the apoprotein determines whether a given (aminoacyl)flavin will yield an anionic or neutral flavosemiquinone radical on reduction.

Table V: Amino Acid Analysis of the Peptic Peptides[a]

amino acid	peptide from strain 9866	peptide from strain 9869	
		A	B
Asn[b]	1.10 (1)	0.89 (1)	0.91 (1)
Ser	1.21 (1)	0.85 (1)	0.81 (1)
Gly	4.60 (4–5)	4.07 (4)	4.15 (4)
Met	0.89 (1)	0.85 (1)	0.93 (1)
Arg	1.00 (1)	0.89 (1)	0.93 (1)
Tyr	0.90 (1)	1.09 (1)	1.06 (1)
Trp	0.67 (1)	0.45 (1)	0.67 (1)

[a] Numbers given are moles of amino acid per mole of flavin. The numbers in parentheses are the integer values. [b] The values given are for aspartic acid found in an acid hydrolysis. Aminopeptidase M digest indicates this residue is in fact asparagine.

Comparison of Flavin Peptides from Different p-Cresol Methylhydroxylases. Hopper & Taylor (1977) and Keat & Hopper (1978) have described the isolation of three proteins with *p*-cresol methylhydroxylase activity from *Ps. putida* (strain 9866 and 9869). All three are flavocytochromes and are dimeric in structure, but differ in molecular weight and K_m values for various substrates. It was of interest to determine whether the flavin sites of these three enzymes are identical. Peptic flavin peptides were isolated from the A and B hydroxylases from strain 9869 (Keat & Hopper, 1978) by the same procedure used in making the flavin peptide for the enzyme from strain 9866. The visible absorption spectra were vitrually identical for the three peptides, with maxima at 360–362 nm and 449–452 nm in each.

Sequential treatment with nucleotide pyrophosphatase, phosphatase, and aminopeptidase produced the same (aminoacyl)riboflavin for all three peptides, which was identified as 8α-(*O*-tyrosyl)riboflavin by thin-layer chromatography on silica gel 60 plates in solvent systems 1 and 3. Reduction of all three peptides with dithionite produced free FAD. Comparison of the amino acid analyses of acid hydrolysates of the three peptides is summarized in Table V. Except for the somewhat higher value for glycine in the peptide from strain 9866, the three peptides seem to have the same amino acid composition. It is by no means certain that strain 9866 peptide contains more than 4 mol of glycine since in other experiments a value close to 4 was observed. The occasional finding of "extra glycine" on analysis of flavin peptides has been frequently observed in this laboratory and results from breakdown of the isoalloxazine nucleus during acid hydrolysis. While the amino acid sequence has not been determined for the two peptides from strain 9869, the similarity of their amino acid compositions with that of the flavin peptide from strain 9866 and the presence of 8α-(*O*-tyrosyl)-FAD suggest that the flavin sites are identical in three proteins. The amino acid sequence of the peptide from strain 9866 has now been determined, except for the three C-terminal amino acids. The structure found is

FAD-Tyr-Asn-Trp-Arg-Gly-Gly-Gly-(Gly,Ser,Met)

The main reason for the remaining uncertainty at the C terminus of the peptide is due to the finding that, upon isolation, the methionine is always found at a higher oxidation level (probably sulfone), which precludes sequence analysis following

cyanogen bromide cleavage since this cleavage reaction occurs only with the thioether form.

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