Spectroscopic Studies of Pyridoxamine (Pyridoxine) 5′-Phosphate Oxidase. Equilibrium Dissociation Constants and Spectra for Riboflavin 5′-Phosphate and Analogues†

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ABSTRACT: Pyridoxamine (pyridoxine) 5′-phosphate oxidase (EC 1.4.3.5) has been shown to bind 1 mol of riboflavin 5′-phosphate (FMN) per mol of apoenzyme and is active with or inhibited by numerous FMN analogues [Kazarinoff, M. N., & McCormick, D. B. (1975) *J. Biol. Chem. 250*, 3436–3442]. The K_D values and spectra for selected apoenzyme–flavin complexes have been determined and used to elucidate some of the properties of the FMN-binding site of this flavoprotein. Alterations of the pyrimidinoid portion of the flavin ring decrease binding considerably. The absorption spectra for the protein complexes with 3-deaza-FMN and 8-hydroxy-FMN indicate the presence of a dipolar or positively charged protein group near N^1 and O^2. The substitution of methyl for hydrogen at N^3 apparently causes distortion of the interaction between the flavin ring and an active-site aromatic amino acid residue. Although binding is also decreased somewhat by substitutions at positions 8 and 8α, considerable bulk [e.g., 8-(diethylamino)-FMN and 8α-*S*-(*N*-acetylcysteinyl)-FMN] is accommodated. Hence, this portion of the flavin ring is probably oriented toward, possibly in contact with, solvent, as has been found for the flavodoxins. The importance of optimum interactions between the flavin and the apoprotein is further emphasized by large differences in the activity of flavin analogues that have similar midpoint potentials in solution.

Pyridoxamine (pyridoxine) 5′-phosphate[1] oxidase (EC 1.4.3.5) is an FMN-dependent enzyme involved in the conversion of vitamin B_6 to pyridoxal 5′-phosphate and may be important in regulating B_6 metabolism (Snell & Haskell, 1971; Merrill et al., 1978). It catalyzes oxidation of not only the 4-aminomethyl and 4-hydroxymethyl substituents of the phosphorylated vitamers (Wada & Snell, 1961) but also secondary amines at the 4 position (Kazarinoff & McCormick, 1973).

Previous studies have established that numerous FMN analogues are active or inhibitory with this enzyme (Tsibris et al., 1966; Kazarinoff & McCormick, 1974), revealing some information concerning the apoenzyme–FMN interactions. We describe herein a significant extension of these studies through examination of the dissociation constants and spectra for apooxidase with FMN and FMN analogues. In addition, ongoing investigations in this laboratory are using chemical modification techniques to elucidate the amino acid residues responsible for PMP (PNP) oxidase activity (McCormick et al., 1976; McCormick, 1977; Tsuge & McCormick, 1978; Horiike et al., 1979a,b). It is expected that these two approaches will prove complimentary and ultimately yield a detailed description of the active site of this enzyme.

It is additionally hoped that these findings may help promote a general understanding of how interactions between apoproteins and flavins explain differences among flavoproteins. Considerable progress has been made toward this goal by using standard methodologies [for example, Massey & Ganther (1965), Edmondson & Tollin (1971) and Ghisla et al. (1974)] and, more recently, X-ray crystallography (Ludwig et al., 1976; Watenpaugh et al., 1976; Schulz et al., 1978) and resonance Raman spectroscopy (Dutta et al., 1978; Nishina et al., 1978). For other flavoproteins, such as PMP (PNP) oxidase, that are difficult to obtain and/or crystallize, the method of choice continues to be the use of flavin analogues as spectroscopic and redox probes, especially considering the growing availability and variety of these compounds (Walsh et al., 1978).

Experimental Procedures

Materials. PMP (PNP) oxidase was purified and the apoenzyme prepared as described elsewhere [Kazarinoff & McCormick (1975) with the minor modifications of Merrill et al. (1979)]. The flavin analogues were obtained as follows. FMN 5-oxide (Yoneda et al., 1976), the 8-substituted riboflavins (Kasai et al., 1978), and the 8α-substituted riboflavins (Merrill & McCormick, 1978) were synthesized by published procedures; the 2-substituted FMN's, 3-methyl-FMN, 3-(carboxymethyl)-FMN (from W. Föry), 5-deazariboflavin (from G. Tollin), the 1- and 3-deazariboflavins, isoriboflavin, and 7,8-dichlororiboflavin (from Merck Sharp & Dohme Research Laboratories) were gifts. The flavin 5′-phosphates were synthesized, whenever possible, by using immobilized flavokinase (Merrill & McCormick, 1979). Otherwise, chemical phosphorylations were conducted by standard procedures (Flexner & Farkas, 1953; Scola-Nagelschneider & Hemmerich, 1976). All compounds were tested for purity by thin-layer chromatography and, when necessary, purified by thin-layer or paper chromatography prior to use. All other

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[1] Abbreviations used: PMP, pyridoxamine 5′-phosphate; PNP, pyridoxine 5′-phosphate; FMN, riboflavin 5′-phosphate; CD, circular dichroic; UV, ultraviolet; EtMe-FMN, 8-(ethylmethylamino)-FMN; roseo-FMN, 8-(dimethylamino)-FMN.

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chemicals were of the highest available commercial grade.

Determination of Dissociation Constants. The dissociation constants (K_D) were determined by fluorescence quenching experiments in 0.1 M Tris-HCl buffer, pH 8 (except where otherwise noted), at 25 °C. A sulfhydryl-protecting agent (0.05 mM β-mercaptoethanol) was included to retard denaturation of the apoenzyme; no other effect of this reagent on FMN binding or fluorescence was observed. Microliter aliquots from stock flavin solutions were added to the apoenzyme (usually 60 to 300 nM), and the fluorescence of either the flavin or the protein was measured. The K_D values were obtained graphically by using the equations with linear regression analysis of the data. For the protein quenching data, the following equation was used (derived from Heyn & Weischet, 1975):

$$1/(1 - \gamma) = K_D^{-1}[F]_t/\gamma - [E]_t/K_D$$

where $[E]_t$ and $[F]_t$ are the total enzyme and flavin concentrations and γ is the fraction of total enzyme bound by flavin. Values for γ are calculated from $\gamma = (Fl_i - Fl_0)/(Fl_\infty - Fl_0)$, where the Fl terms are protein tryptophanyl fluorescence intensities in the absence (Fl_0), at an intermediate level (Fl_i), and with saturation (Fl_∞) of apoenzyme by flavin. Inner-filter corrections, where necessary, were made as described previously (Wu et al., 1970). For the flavin quenching data the following equation was used:

$$(1 - \alpha)/\alpha = -K_D^{-1}(1 - \alpha)[F]_t + [E]_t/K_D$$

where α is the fraction of total flavin that is not protein bound. Values for α are calculated from Fl_{obsd}/Fl_{ref}, where the Fl terms are flavin fluorescence in the presence (Fl_{obsd}) and absence (Fl_{ref}) of apoenzyme, because protein binding completely quenches flavin fluorescence. The use of this equation is only practical when the flavin 5'-phosphates are absolutely pure, because residual fluorescence distorts the curve considerably.

These equations assume that 1 mol of flavin is bound per mol of apoenzyme, which has been shown previously for this flavoprotein (Kazarinoff & McCormick, 1975).

Spectroscopic Measurements. Samples were protected from light, except during analysis, to minimize photodecomposition. Absorption spectra were obtained with an Amico DW-2 spectrophotometer. Circular dichroic spectra were recorded by using a Cary Model 60 spectropolarimeter. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer for analyses at fixed wavelengths and with a Hitachi Perkin-Elmer MPF-3 fluorescence spectrophotometer for fluorescence spectra.

For analogues with low K_D values, holoenzyme was prepared by mixing apoenzyme and a slight molar excess of flavin, and unbound flavin was removed by Sephadex G-25 chromatography. For analogues with higher K_D values (e.g., 3-methyl-FMN), the concentration ratio for apoenzyme to flavin was adjusted so that greater than 90% of the flavin was protein bound.

Reduced holoenzyme was prepared photochemically by using a catalytic amount of 5-deazariboflavin (Massey & Hemmerich, 1977) in cuvettes fitted with a rubber septum and solutions flushed with vanadous sulfate washed (oxygen-free) nitrogen. Upon reintroduction of air to the reduced enzyme, the initial spectrum of the oxidized enzyme was obtained.

Activity Measurements with Flavin Analogues. The V_{max} values were determined from Lineweaver–Burk plots of the enzymatic activity of apooxidase with varying concentrations of flavin 5'-phosphate using otherwise standard assay conditions (Merrill et al., 1979). Values for the solution midpoint potentials were from published sources as follows: for the 8-substituted flavins, Kasai et al. (1978); for the 8α-substituted flavins, Falk et al. (1976) and Singer & Edmondson (1974); for the deazaflavins, Walsh et al. (1978); for iso-, 7,8-dichloro-, and 2-thioflavins, Müller & Massey (1969); for 3-methyl- and 3-(carboxymethyl)flavins, the midpoint potentials were assumed to be similar to that of FMN. The values were normalized by subtracting from the E_m for each analogue the E_m for riboflavin reported by the same workers.

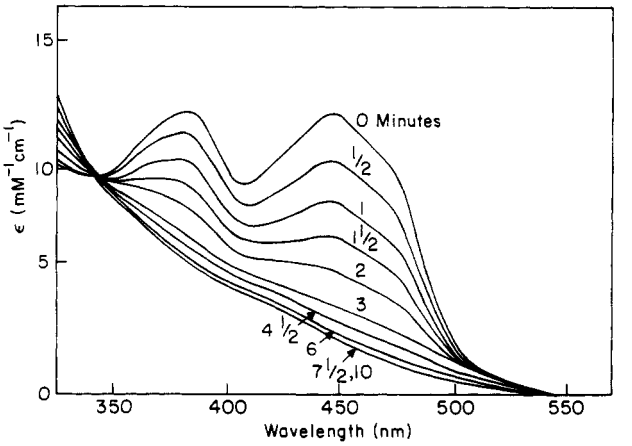


FIGURE 1: Absorption spectra for oxidized and partially to fully reduced PMP (PNP) oxidase. The photoreductions were carried out under anaerobic conditions in the presence of 0.25 μM 5-deazariboflavin and 1 mM EDTA with illumination by white light for the indicated times. The spectra were obtained at 25 °C with 10 μM enzyme in 0.1 M potassium phosphate (pH 8); the reference cuvette contained all components except holoenzyme.

Results

Equilibrium Dissociation Constants for Apo-PMP (-PNP) Oxidase and Flavin 5'-Phosphates. These, plus K_m (or K_i), are summarized in Table I. A number of previously determined values have been included for comparison with those determined by this study. In general, the K_D and K_m (or K_i) are in reasonable agreement, which would be expected if coenzyme binding is rapid compared to turnover of this enzyme.

Upon protein binding, all FMN analogues quenched protein fluorescence to the same extent as did FMN. Similarly, fluorescent flavins were completely quenched upon binding, with the exception of 3-methyl-FMN.

Spectra of Apoenzyme with and without FMN. The absorption spectra of apo- and holoenzyme were found to be similar to those reported previously (Kazarinoff & McCormick, 1975) with a slightly less pure preparation. Molar extinction coefficients at λ_{max} for the holoenzyme are 11 800 (450 nm), 10 700 (380 nm), and 68 000 (276 nm). Despite some broadening of the 450-nm absorption band (Figure 1), the holoenzyme spectrum would be classified as "unresolved" (Palmer & Massey, 1968); i.e., there is little resolution of the long-wavelength vibronic bands. The 372-nm band of free FMN is shifted to 380 nm upon protein binding, as has been occasionally observed for other flavoproteins (Palmer & Massey, 1968). The spectra of partially and completely reduced holoenzyme are also shown in Figure 1. The spectra are isosbestic at 342 nm, and those for partial reduction appear to reflect a mixture of fully reduced and fully oxidized enzyme. Further evidence for the absence of a protein-stabilized flavin semiquinone is the lack of absorbances at wavelengths above those shown (up to 800 nm). Neither the oxidized nor the reduced spectra are detectibly different from those shown in Figure 1 throughout the pH range of 4.5–10.5, beyond which PMP (PNP) oxidase is unstable.

Table I: Equilibrium Dissociation Constants, K_m, and/or K_i for Flavin 5'-Phosphates and Apopyridoxamine-5'-Phosphate Oxidase at 25 °C

flavin	pH	K_D[a] (nM)	K_m or K_i[b] (nM)	$\Delta F°$[c] (kcal/mol)
riboflavin 5'-phosphate	5	30		−10.3
riboflavin 5'-phosphate	6	16		−10.6
riboflavin 5'-phosphate	7	11		−10.9
riboflavin 5'-phosphate	8	13	20, 40[d]	−10.8
riboflavin 5'-phosphate	9	21		−10.5
1-deaza-FMN	8	190		−9.2
2-thio-FMN	7, 8	520[e]	250[d]	−8.6
2-(methylthio)-FMN	7, 8	900[e]		−8.3
2-(hydroxyethylimino)-FMN	8		6000[d]	(−7.1)
3-methyl-FMN	8	260	220[d]	−9.0
3-deaza-FMN	8	180		−9.2
3-(carboxymethyl)-FMN	8	21	40[d]	−10.5
5-deaza-FMN	8	20	20[d]	−10.5
FMN 5-oxide	8	260[f]		−9.0
iso-FMN	8		60[d]	(−9.9)
7,8-dichloro-FMN	8		41[d]	(−10.1)
8-hydroxy-FMN	5	62		−9.8
8-hydroxy-FMN	8	29		−10.3
8-(dimethylamino)-FMN	8	76		−9.7
8-(diethylamino)-FMN	8	120		−9.5
8α-*S*-(*N*-acetylcysteinyl)-FMN	8		70	(−9.8)
8α-*S*-(mercaptopropionic acid)-FMN	8		90	(−9.6)
8α-N^3-(*N*-acetylhistidyl)-FMN	8		1000	(−8.2)
8α-*N*-(1,6-diaminohexyl)-FMN	8		800	(−8.3)

[a] Determined by fluorescence titration as described in the text. [b] Determined by Lineweaver-Burk plots. [c] The standard molar free energy of binding was calculated from $\Delta F° = -RT \ln K_a$; $K_a = K_D^{-1}$. Unitary free energies can be obtained by subtracting 2.4 kcal/mol for the cratic entropy value of 8 eu (Kauzmann, 1959). Estimates for $\Delta F°$ based on K_m or K_i are indicated by parentheses. [d] Values from Kazarinoff & McCormick (1975). [e] Measured at pH 7 to minimize hydrolysis to FMN. [f] An approximate value; the analogue was contaminated with lumichrome.

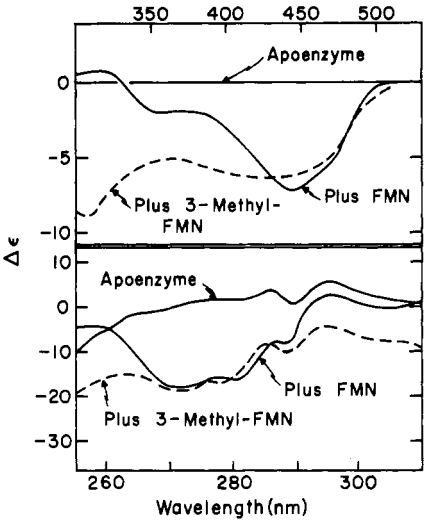


FIGURE 2: CD spectra of PMP (PNP) oxidase with FMN and 3-methyl-FMN at 10 °C. The spectra were measured for equimolar apoenzyme and flavin in 20 mM potassium phosphate (pH 7) by using a cylindrical cell of 2.5-cm light path. The intensity of the signals was calibrated by using a methanolic solution of *d*-camphor (Cassim & Yang, 1969).

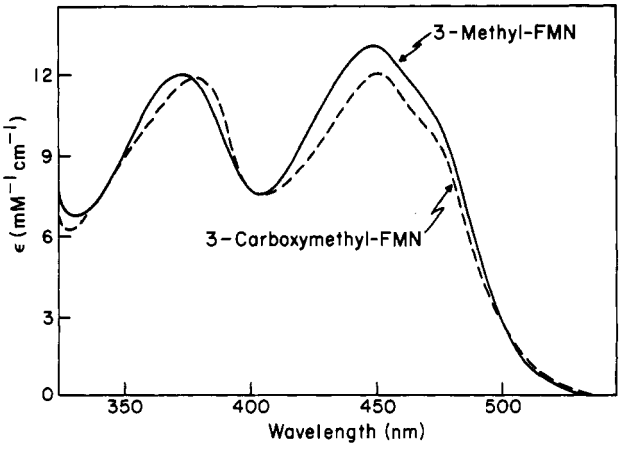


FIGURE 3: Absorption spectra for PMP (PNP) oxidase with 3-methyl-FMN and 3-(carboxymethyl)-FMN at pH 8. Spectra were obtained at 25 °C by using 5 μM 3-methyl-FMN plus 10 μM apoenzyme and equimolar 3-(carboxymethyl)-FMN and apoenzyme (5 μM).

The fluorescence excitation and emission spectra for apo- and holoenzyme (spectra not shown) both have excitation and emission maxima for protein fluorescence of 285 and 330 nm, respectively, although FMN binding greatly (83%) quenches the fluorescence. The fluorescence emission maximum for tryptophanyl residues alone (obtained by excitation at 300 nm) is 335 nm for apoenzyme and is bathochromically shifted by ~3 nm upon flavin binding. Since the emission maximum for tryptophan in aqueous solutions is ~360 nm (Teale, 1960), the six tryptophanyl residues of PMP (PNP) oxidase (Tsuge & McCormick, 1978) are, on the average, in a relatively nonpolar environment. The shift in fluorescence emission maximum for holoenzyme may be due to relocation of some tryptophanyl residues into more polar environments, but it more likely means that the residual fluorescence is due to tryptophans already in such environments.

The CD spectrum (Figure 2) for holoenzyme contains strong negative ellipticities, especially in the region of the 450-nm flavin absorption band. The lower UV trough at 290 nm likely reflects reorientation changes affecting tryptophanyl residues (Strickland et al., 1969; Edmondson & Tollin, 1971).

A resonance Raman spectrum with laser irradiation at 488.0 nm was attempted; however, unlike for riboflavin-binding protein (Nishina et al., 1978), residual fluorescence masked the Raman signal.

Spectra of Apoenzyme with 3-Methyl-FMN or 3-(Carboxymethyl)-FMN. The absorption (Figure 3) and CD (Figure 2) spectra of apoenzyme plus 3-methyl-FMN are considerably different from those for the FMN-reconstituted enzyme. While the 450-nm absorption band resembles that for holo-

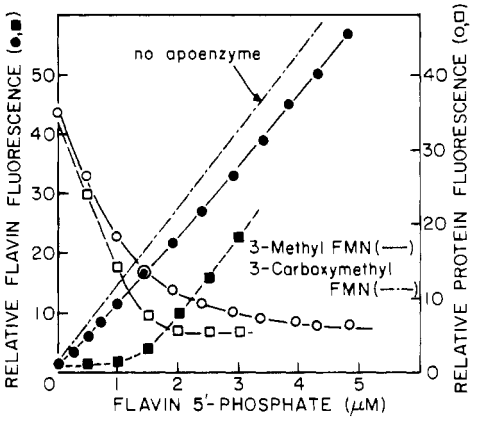


FIGURE 4: Fluorescence titration of apo-PMP (-PNP) oxidase with 3-methyl-FMN or 3-(carboxymethyl)-FMN. Microliter aliquots of the flavins were added to 1.38 μM apoenzyme in 2.5 mL of 0.1 M Tris buffer (pH 8) containing 10 μM β-mercaptoethanol at 25 °C. Flavin fluorescence was measured with excitation at 445 nm and emission at 530 nm; the fluorescence intensity for the flavins in the absence of apoenzyme is also shown. Protein fluorescence was monitored with excitation at 295 nm and emission at 335 nm.

enzyme, the 372-nm band of free 3-methyl-FMN is only shifted to 374 nm, compared to an 8-nm bathochromic shift for the FMN–holoenzyme. Furthermore, the CD spectrum differs in shape and magnitude, having strongest ellipticity at 308 nm.

Titration of apoenzyme with 3-methyl-FMN is shown in Figure 4. Protein fluorescence is nearly completely quenched, the same as occurs with FMN, but only partial quenching of bound 3-methyl-FMN fluorescence results. The fluorescence of bound 3-methyl-FMN, calculated from Figure 4 and the K_D, is diminished by 30% upon binding, and the flavin emission maximum is hypsochromically shifted by 3–5 nm. Protein fluorescence, conversely, is bathochromically shifted by 5–7 nm.

The absorption and fluorescence spectra for 3-(carboxymethyl)-FMN are essentially identical with those for FMN (cf. Figures 3 and 4). Flavin fluorescence is nearly completely quenched upon protein binding, and the 372-nm absorption band of the free flavin is shifted to 379 nm.

Spectra of Apoenzyme with 3-Deaza-FMN at pH 4.7 and 8. These spectra (Figure 5A) both show primarily the spectral features of the flavinyl anion, which, in solution, has a pK_a of 5.8 (Walsh et al., 1978). Hence, protein binding decreases the pK_a by more than 1 unit. A precise determination of the bound pK_a was prevented by precipitation of the protein at lower pH, as became apparent at pH 4.7 by the light scattering at lower wavelengths. Also shown (Figure 5B) is the effect of decreasing solvent polarity on the absorption spectra of neutral and anionic 3-deazariboflavin. Thus, the splitting of the absorption band that is observed upon protein binding apparently reflects the lower general polarity of the flavin environment.

Spectra of Apoenzyme with 8-Hydroxy-FMN at pH 5.0 and 7.0. These spectra (Figure 6) indicate that the protein-bound 8-hydroxy-FMN anion (pK_a = 4.8) is only partially shielded from changes in the pH of the bulk solution. The absorption maximum is bathochromically shifted by ~6 nm with the appearance of a shoulder at 515 nm, and there is a dramatic hypochromic effect (ϵ_{472nm} for the anion in solution is ~42000).

General Properties of the Spectra of Other Apoenzyme–Flavin Complexes. Protein binding consistently resulted in bathochromic shifts in the long-wavelength absorption bands

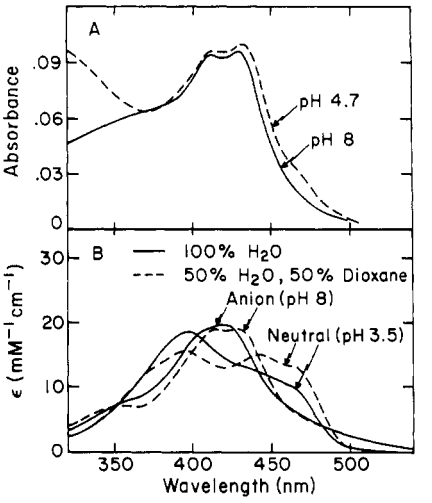


FIGURE 5: Absorption spectra for 3-deaza-FMN with PMP (PNP) oxidase (A) or in solution (B). Approximately equimolar apoenzyme and 3-deaza-FMN were mixed at pH 8 (0.05 M potassium phosphate plus 0.05 M potassium acetate), and most free flavin was removed by using a small column of Sephadex G-10. The spectrum was recorded, the pH was carefully lowered to 4.7 by addition of HCl, and the spectrum was rerecorded (panel A). The spectra for neutral and anionic 3-deaza-FMN were measured at the approximate pH values and in the solvents shown in panel B.

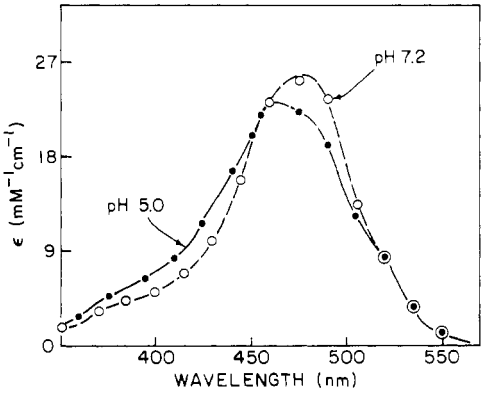


FIGURE 6: Spectra of 8-hydroxy-FMN with PMP (PNP) oxidase at pH 5 and 7.2. Equimolar flavin and apoenzyme (3.5 μM) were mixed in 0.1 M potassium acetate (pH 5) or 0.1 M potassium phosphate (pH 7.2).

for the flavin analogues examined. This effect was greatest with roseo-FMN and 2-thio-FMN, which had changes in λ_{max} of 15 and 20 nm, respectively.

Relationship between PMP (PNP) Oxidase Activity and E_m for the Flavin. The relationship between the midpoint potential for a number of these compounds and V_{max} when assayed with apoenzyme is shown in Figure 7. The midpoint potentials are expressed relative to FMN to normalize differences among the determinations. While the expected correlation exists for the few analogues with E_m more negative than FMN (i.e., activity increases as the flavin becomes a more powerful oxidant), the opposite is observed for higher midpoint potentials.[2] Such behavior, indicating a change in the rate-limiting step, might be expected for a flavoprotein that has a relatively slow rate of reoxidation, which preliminary evidence suggests may be the case for PMP (PNP) oxidase (Horiike et al., 1979a). More relevant to this study, however,

[2] Clearly, no definite assignment for the relationship $\Delta E_m/\Delta \log k$ can be made from such scattered data, but a reasonable range of values might be 60 (solid line) to 120 mV (dashed line).

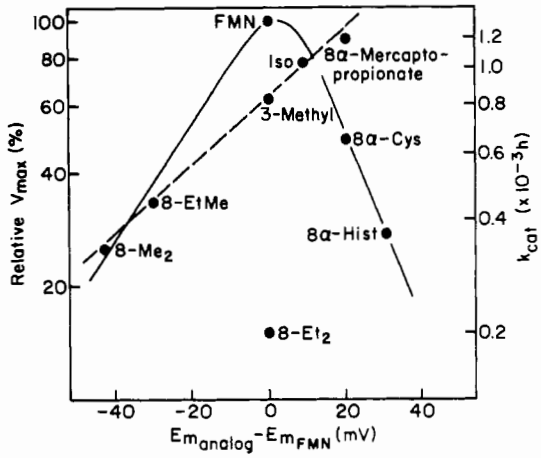


FIGURE 7: Relationship between the solution midpoint potential (pH 7) for FMN analogues and the activity with PMP (PNP) oxidase. The V_{max} and k_{cat} were determined from Lineweaver–Burk plots for apooxidase with varying concentrations of flavin 5′-phosphate. Midpoint potentials are expressed relative to that of FMN; values for the midpoint potentials were from the references cited in the text. Also determined but not shown because they were inactive were 8-hydroxy- (−123 mV), 1-deaza- (−72 mV), 3-deaza- (−32 mV), 5-deaza- (−103 mV), and 7,8-dichloro-FMN (+81 mV). Also not shown was 25% activity with 2-thio-FMN (+127 mV) because this may reflect contamination of the sample with small amounts of FMN.

are results for flavin analogues that significantly deviate from this trend. That additional structural considerations are crucial is best shown by the decreased activity with 3-methyl-FMN (62%), 3-(carboxymethyl)-FMN (<1%), and 8-EtMe-FMN (10%) which have E_m values nearly identical with FMN.

Discussion

Earlier studies (Kazarinoff & McCormick, 1974) established that PMP (PNP) oxidase has nearly absolute specificity for a D-ribityl side chain with a 5′-phosphate group. Preferred binding of the phosphate dianion was indicated, and this has been confirmed by the present study (cf. Table I). Numerous FMN analogues which contain substitutions at the 7,8-dimethylisoalloxazine ring were found active or inhibitory with apoenzyme. Determination of the actual K_D values for many of these compounds (hence avoiding the limitations inherent in inferring binding information from K_m or K_i) and for others has yielded a more complete description of the FMN-binding site of this enzyme. These results are summarized diagrammatically in Figure 8.

This oxidase joins the growing list of flavoproteins that apparently bind the coenzyme with either or both methyl groups of the dimethylbenzene ring relatively unhindered and in contact with the bulk solution (Abramovitz & Massey, 1976; Ludwig et al., 1976; Watenpaugh et al., 1976). Hence, while some decrease in binding is observed for the substitution of fairly bulky groups at position 8 (e.g., 8-(dimethylamino)- and 8-(diethylamino)flavins), even larger groups are accommodated at the 8α position (e.g., *N*-acetylcysteine or mercaptopropionic acid). Interestingly, there is a marked decrease in binding when an amino group is present at the 8α-methylene; this is not due to the amine being protonated, since the pK_a values for both compounds are considerably less than the pH of these experiments.

An additionally informative analogue, as has been shown by others (Ghisla & Massey, 1976; Walsh et al., 1978), is 8-hydroxy-FMN. This compound has a pK_a (4.8) lower than that of simple phenol due to considerable delocalization of the negative charge. Model studies (Ghisla & Mayhew, 1976; Ghisla & Massey, 1976) have been interpreted to mean that

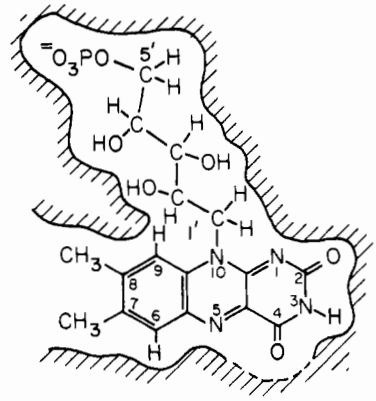
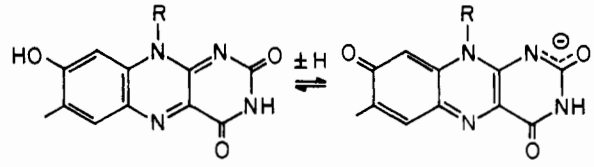


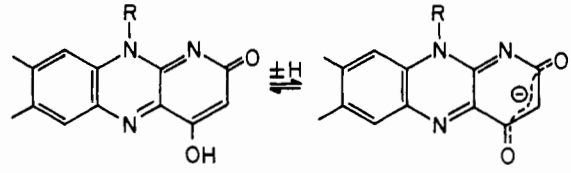
FIGURE 8: Diagrammatic representation of the structural specificity of PMP (PNP) oxidase for the flavin coenzyme.

the negative charge density for the anion is located at N^1 and O^2, but the phenol tantomer predominates for the neutral flavin:



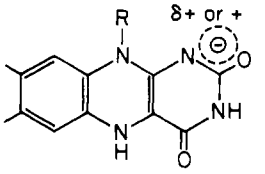
Apo-PMP (-PNP) oxidase binds 8-hydroxy-FMN very well, and the absorption spectrum substantiates that the anionic species is bound at pH 8. This spectrum is nearly identical with that for D-amino acid oxidase (Ghisla & Massey, 1976), for which the binding of 8-hydroxy-FAD has been suggested to indicate the presence of a positive charge near N^1. The K_D and spectrum at pH 5 show that there is little change in the pK_a of PMP (PNP) oxidase bound 8-hydroxy-FMN. This analogue does not yield enzymatic activity with PMP (PNP) oxidase, which further substantiates that the paraquinoid form is present (Ghisla & Mayhew, 1976; Walsh et al., 1978).

Even small modifications of the pyrimidinoid ring, on the other hand, result in large decreases in binding. Substitution of CH for N at position 1, S for O at position 2, or CH_3 for H at position 3 each increases K_D by greater than 10-fold. The nature of some of the interactions occurring at this portion of the ring can be elucidated by examination of 3-deaza-FMN. This analogue has been shown to exist as the enol tantomers shown below (Walsh et al., 1978) and upon dissociation of a proton (pK_a = 5.8) yields a resonance stabilized anion:



The absorption spectra for this analogue with apooxidase show that the anion is clearly bound preferentially, most likely because the protein proffers a cationic group or strong dipole that stabilizes the negative charge. While this protein group may be positioned at many sites along the pyrimidinoid edge of the flavin ring, there are reasons to believe that the primary association may occur proximal to N^1 and O^2, such as the already discussed behavior with 8-hydroxy-FMN and the strong sensitivity of K_D to alterations at N^1 and O^2. Somewhat more speculative support for this assignment is the lack of appearance of a stabilized semiquinone upon partial reduction. Model studies have indicated (Hemmerich, 1976) and the X-ray crystallographic studies of the flavodoxins have con-

firmed that the semiquinone is stabilized by interactions at N^5 but destabilized by those at N^1. The fact that PMP (PNP) oxidase shows no semiquinone would lead one to predict an ionic interaction at N^1.[3] Lastly, the presence of a strong dipolar or electrostatic interaction[4] at N^1 may serve to delocalize the dihydroflavinyl anion, resulting in the observed, apparently pH-independent reduced spectra which most closely resemble those for a neutral dihydroflavin.



Because the pK_a for 3-deaza-FMN is shifted more than that for 8-hydroxy-FMN, this protein group may be more closely associated with O^2 in the oxidized flavoprotein; this orientation may change upon reduction of the flavin.

This analogue is different from FMN in a second respect; i.e., the 3 position has been converted from an H donor to an H acceptor. That this may account for the higher K_D for 3-deaza-FMN is revealed by the K_D for 3-methyl-FMN. The spectral behavior with 3-methyl-FMN further raises the intriguing possibility that this position influences the orientation of the flavin and aromatic amino acid residue(s). The precedent for this possibility is seen in the X-ray crystallographic structure of the flavodoxin from *Desulfovibrio vulgaris* (Watenpaugh et al., 1976) for which hydrogen 3 appears to be H bound to the portion of the peptide backbone (at amino acid 100) which also contains the tryptophan (position 98) that stacks with the flavin ring. A tryptophan residue has been shown to be present in the flavin-binding site of PMP (PNP) oxidase (McCormick, 1977), and the further involvement of a tyrosine is indicated by preliminary data (Horiike and McCormick, unpublished experiments). These interactions are also suggested by the changes in the CD spectrum of PMP (PNP) oxidase upon flavin binding. Such active-site aromatic amino acid residues are common for flavoporteins and are primarily responsible for static quenching of flavin fluorescence.

Hence, that 3-methyl-FMN is bound without complete quenching of the flavin fluorescence (30% quenching of 3-methyl-FMN vs. 99+% for FMN) is consistent with alteration of the flavin–aromatic amino acid complex reponsible for quenching. Further support for this proposal is the loss of an 8-nm bathochromic shift in the 372-nm flavin absorption band upon protein binding of 3-methyl-FMN compared to FMN. Model studies of flavin–aromatic amino acid complexes have established that one spectroscopic result of complexing is a bathochromic shift in this band (McCormick, 1977). The spectra for 3-(carboxymethyl)-FMN rule out the possibility that these effects are due to deprotonation of the N^3-hydrogen by a protein base. Apparently, 3-(carboxymethyl)-FMN compensates for loss of the 3-hydrogen by alternate interactions involving the carboxyl group, since the K_D is near that for FMN. Most importantly, while the enzyme is completely inactive with this analogue, both the quenching of flavin fluorescence and the bathochromic shift in the 372-nm band are similar to those of FMN.

[3] Similarly, these arguments suggest that the interaction at N^5 resulting in the higher K_D with FMN 5-oxide is nonpolar (because an *N*-oxide is a dipolar ion) or sterically restrictive.

[4] Chemical modification studies (Tsuge & McCormick, 1978) indicate that this may be a sulfhydryl group.

Undoubtedly, additional critical interactions occur between the flavin and the apoenzyme, and others may be induced by substrate binding and reduction of the flavin. Some of these are probably reflected in the lack of a simple relationship between the midpoint potential for the flavin and the enzymatic activity, especially for analogues that have not been modified at redox-active portions of the ring (for example, 3-methyl-FMN and 3-(carboxymethyl)-FMN, as opposed to 5-deaza-FMN). In addition, large changes in the spectra of PMP (PNP) oxidase are observed upon changing the properties of the bulk solution (Horiike et al., 1979a) or adding pyridoxyl 5′-phosphates (Merrill & Tsuge, 1978).

These conclusions at least superficially explain the somewhat enigmatic spectroscopic changes observed upon protein binding of all flavin 5′-phosphates with apooxidase. Although the longest wavelength absorption band is consistently shifted to longer wavelength, as is seen when flavins are placed in nonpolar solvent (Harbury et al., 1959; Koziol, 1966), other features of the spectra, such as resolution of vibronic bands or shifts in the next longest wavelength band, are not as analogous with simple solvent systems, even when hydrogen bonding is considered (Kotaki et al., 1970; Nishimoto et al., 1978). Apparently, the combination of specific ring stacking plus strong polar interactions at the pyrimidinoid ring, as described above, accounts for the observed spectral properties of PMP (PNP) oxidase. That these are common among flavoproteins can be seen in the evidence for similar types of interactions among diverse flavoproteins, for example, D-amino acid oxidase (Massey & Ganther, 1965; Ghisla & Massey, 1976), glucose oxidase (Dutta et al., 1978), and the flavodoxins (Edmondson & Tollin, 1971; Ludwig et al., 1976; Watenpaugh et al., 1976).

Altogether, these results can be interpreted with reasonable satisfaction by considering the flavin environment of PMP (PNP) oxidase as being basically similar to those seen in the X-ray crystallographic structures for the flavodoxins. While the details of the interactions are certainly different, as they are even among the flavodoxins, the basic properties are that the ribityl 5′-phosphate side chain is intimately associated with apoprotein groups, the faces of the 7,8-dimethylisoalloxazine ring are in contact with nonpolar groups (at least one aromatic amino acid residue), and the perimeter of the ring is in contact with polar protein groups (for the pyrimidinoid edge), solvent (for the dimethylbenzene edge), and unspecified protein groups near positions 5 and 6. Furthermore, the major distinction between these two types of flavoproteins, the strength of the interaction at N^1 and O^2, may be an important clue to understanding their different redox behavior.

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References

Abramovitz, A. S., & Massey, V. (1976) *J. Biol. Chem. 251*, 5327–5336.

Cassim, J. Y., & Yang, J. T. (1969) *Biochemistry 8*, 1947–1951.

Dutta, P. K., Nestor, J., & Spiro, T. G. (1978) *Biochem. Biophys. Res. Commun. 83*, 209–216.

Edmondson, D. E., & Tollin, G. (1971) *Biochemistry 10*, 124–132.
Falk, M. C., Johnson, P. J., & McCormick, D. B. (1976) *Biochemistry 15*, 639–645.
Flexner, L. A., & Farkas, W. G. (1953) U.S. Patent 2610177; (1957) *Chem. Abstr. 47*, 8781*a*.
Ghisla, S., & Massey, V. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 334–340, Elsevier, Amsterdam.
Ghisla, S., & Mayhew, S. G. (1976) *Eur. J. Biochem. 63*, 373–390.
Ghisla, S., Massey, V., Lhoste, J.-M., & Mayhew, S. G. (1974) *Biochemistry 13*, 589–597.
Harbury, H. A., LaNove, K. F., Loach, P. A., & Amick, R. M. (1959) *Proc. Natl. Acad. Sci. U.S.A. 45*, 1708–1717.
Hemmerich, P. (1976) *Fortschr. Chem. Org. Naturst. 33*, 451–527.
Heyn, M. P., & Weischet, W. O. (1975) *Biochemistry 14*, 2962–2968.
Horiike, K., Merrill, A. H., & McCormick, D. B. (1979a) *Arch. Biochem. Biophys.* (in press).
Horiike, K., Tsuge, H., & McCormick, D. B. (1979b) *J. Biol. Chem.* (in press).
Kasai, S., Kubo, Y., Yamanaka, S., Hirota, T., Sato, H., Tsuzukida, Y., & Matsui, K. (1978) *J. Nutr. Sci. Vitaminol. 24*, 339–350.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.
Kazarinoff, M. N., & McCormick, D. B. (1973) *Biochem. Biophys. Res. Commun. 52*, 440–446.
Kazarinoff, M. N., & McCormick, D. B. (1974) *Biochim. Biophys. Acta 359*, 282–287.
Kazarinoff, M. N., & McCormick, D. B. (1975) *J. Biol. Chem. 250*, 3436–3442.
Kotaki, A., Naoi, M., & Yagi, K. (1970) *J. Biochem. (Tokyo) 68*, 287–292.
Koziol, J. (1966) *Photochem. Photobiol. 5*, 41–54.
Ludwig, M. L., Burnett, R. M., Darling, G. D., Jordan, S. R., Kendall, D. S., & Smith, W. W. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 393–404, Elsevier, Amsterdam.
Massey, V., & Ganther, H. (1965) *Biochemistry 4*, 1161–1173.
Massey, V., & Hemmerich, P. (1977) *J. Biol. Chem. 252*, 5612–5614.
McCormick, D. B. (1977) *Photochem. Photobiol. 26*, 169–182.
McCormick, D. B., Kazarinoff, M. N., & Tsuge, H. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 708–719, Elsevier, Amsterdam.
Merrill, A. H., & McCormick, D. B. (1978) *Anal. Biochem. 89*, 87–102.
Merrill, A. H., & Tsuge, H. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, Abstract 431.
Merrill, A. H., & McCormick, D. B. (1979) *Biotechnol. Bioeng. 21*, 243–252.
Merrill, A. H., Horiike, K., & McCormick, D. B. (1978) *Biochem. Biophys. Res. Commun. 83*, 984–990.
Merrill, A. H., Kazarinoff, M. N., Tsuge, H., Horiike, K., & McCormick, D. B. (1979) *Methods Enzymol. 62*, 568–574.
Müller, F., & Massey, V. (1969) *J. Biol. Chem. 244*, 4007–4016.
Nishimoto, K., Watanabe, Y., & Yagi, K. (1978) *Biochim. Biophys. Acta 526*, 34–41.
Nishina, Y., Kitagawa, T., Shiga, K., Horiike, K., Matsumura, Y., Watari, H., & Yamano, T. (1978) *J. Biochem. (Tokyo) 84*, 925–932.
Palmer, G., & Massey, V. (1968) in *Biological Oxidations* (Singer, T. P., Ed.) pp 263–300, Interscience, New York.
Schulz, G. E., Shirmer, R. H., Sachsenheimer, W., & Pai, E. F. (1978) *Nature (London) 273*, 120–124.
Scola-Nagelschneider, G., & Hemmerich, P. (1976) *Eur. J. Biochem. 66*, 567–577.
Singer, T. P., & Edmondson, D. E. (1974) *FEBS Lett. 42*, 1–14.
Snell, E. E., & Haskell, B. M. (1971) *Comp. Biochem. 21*, 47–71.
Strickland, E. H., Horwitz, J., & Billups, C. (1969) *Biochemistry 8*, 3205–3213.
Teale, F. W. J. (1960) *Biochem. J. 76*, 381–388.
Tsibris, J. C. M., McCormick, D. B., & Wright, L. D. (1966) *J. Biol. Chem. 241*, 1138–1143.
Tsuge, H., & McCormick, D. B. (1978) in *Flavins and Flavoproteins* (Yagi, K., & Yamano, T., Eds.) (in press) Japan Scientific Societies Press, Tokyo.
Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089–2095.
Walsh, C., Fisher, J., Spencer, R., Graham, D. W., Ashton, W. T., Brown, J. E., Brown, R. D., & Rogers, E. F. (1978) *Biochemistry 17*, 1942–1951.
Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 405–410, Elsevier, Amsterdam.
Wu, F. Y.-H., Tu, S.-C., Wu, C.-W., & McCormick, D. B. (1970) *Biochem. Biophys. Res. Commun. 41*, 381–385.
Yoneda, F., Sakuma, Y., Ichiba, M., & Shinomura, K. (1976) *J. Am. Chem. Soc. 98*, 830–835.