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Synthesis of a Phosphorothioate Analogue of Flavin Mononucleotide: Reconstitution of the FMN-Free Form of NADPH–Cytochrome P-450 Reductase†

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Received December 17, 1986; Revised Manuscript Received April 8, 1987

ABSTRACT: The chemical synthesis of riboflavin 5′-phosphorothioate (5′-FMNS) is described. 5′-FMNS is obtained from the alkaline hydrolysis of riboflavin 4′,5′-cyclic phosphorothioate, which is produced upon reaction of riboflavin (RB) with thiophosphoryl chloride in trimethyl phosphate. 5′-FMNS has been tested for enzymatic reconstitution of NADPH–cytochrome P-450 reductase (EC 1.6.2.4) depleted of its FMN prosthetic group, but containing its full complement (1 equiv) of FAD. The synthesis, purification, and characterization of 5′-FMNS are reported, and documentation of its efficacy in reconstituting the reductase by fluorometric and absorbance spectrophotometric measurements, as well as enzymatic activity, is presented. Data indicate that 5′-FMNS is totally competent in reconstituting NADPH–cytochrome *c* reductase activity, which requires the presence of both FAD and a flavin mononucleotide, and its fluorescence is completely quenched upon addition to FMN-free NADPH–cytochrome P-450 reductase.

The use of structurally modified substrates and coenzyme analogues in the investigation of enzyme mechanisms, structure, and function has increased in recent years. Much attention has been focused on the use of modified nucleotides in mechanistic studies. One particular class of nucleotide analogues that is very useful in mechanistic studies is that of nucleoside phosphorothioates, first introduced by Eckstein (1979, 1985). These are compounds in which a nonbridging oxygen atom of a phosphate group has been replaced by a sulfur atom. Large numbers of phosphorothioate derivatives have been synthesized and applied to the study of various enzyme systems (Eckstein, 1985).

In the study of flavoproteins, modified flavin analogues have been very useful in unraveling the role of the isoalloxazine ring of the flavin in the binding of the flavin to the flavoprotein and in its participation in the catalytic mechanism of the flavoprotein in question (Ghisla & Massey, 1986). A few examples are the use of 6-substituted flavins (Ghisla et al., 1986), 1-carba-1-deazariboflavin (1-deazariboflavin; Spencer et al., 1977), 5-deazaflavins (Edmondson et al., 1972; Fisher et al., 1976), and ^{13}C and ^{15}N isotopically substituted flavins (Vervoort et al., 1985; Moonen et al., 1984a,b; Franken et al., 1984; Van Schagen & Müller, 1981). In their review of "artificial" flavins as active-site probes, Ghisla and Massey classified the various analogues into four categories on the basis of the type of information obtained: spectral probes, chemically active probes, mechanistic probes, and photoaffinity labels.

Since the work in McCormick's laboratory in the 1960s (McCormick et al., 1964; Tsibris et al., 1965, 1966; Roth et al., 1966), very little emphasis has been placed on substitutions aside from those directly involving the isoalloxazine ring with the exception of the 8-azido- (adenine) FAD, which has been used as a photoaffinity label for D-amino acid oxidase (Koberstein, 1976). During our investigation of NADPH–cytochrome P-450 reductase, the only mammalian flavoprotein known to contain 1 mol each of FAD and FMN, we became interested in studying the effects of a phosphorothioate moiety in the ribityl side chain of FMN on the catalytic, spectral, and fluorescent properties of the enzyme. In this paper, the chemical synthesis and characterization of riboflavin 5′-monophosphorothioate (5′-FMNS) and preliminary studies of its interaction with FMN-depleted NADPH–cytochrome P-450 reductase are reported. This is the first report of a flavin phosphorothioate analogue utilized in reconstituting a flavoprotein.

Materials and Methods

General. FAD, FMN, Trizma base, and cytochrome *c* (type VI) were purchased from Sigma Chemical Co. The flavins were purified by DEAE chromatography and shown to be >95% pure by the reverse-phase HPLC procedure of Light et al. (1980) as detected by absorbance at 444 nm. FAD was shown to be homogeneous, and FMN preparations were shown to contain 86% 5′-FMN and 14% 4′-FMN by ^{31}P NMR. NADPH was obtained from Pharmacia P-L Biochemicals.

† Supported by a grant from the National Institutes of Health (HL 30050) to B.S.S.M.
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0006-2960/87/0426-5344$01.50/0

Riboflavin (RB), $PSCl_3$, charcoal, and trimethyl phosphate [$(MeO)_3PO$] were purchased from Aldrich Chemical Co. DEAE-Sephadex A-25 was obtained from Pharmacia. KBr was purchased from Mallinckrodt. All other chemicals were of reagent grade or of the highest purity obtainable.

The DEAE-Sephadex A-25 was converted to the HCO_3^- form by the following procedure: the resin was suspended in 0.5 M HCl for 1 h, washed with water until neutral, resuspended in 0.1 M NaOH for 1 h, washed with 0.1 M NaOH until chloride free, and then washed with water until neutral. The resin was then resuspended in 1 M $(NH_4)_2CO_3$ for several hours, washed with water until neutral, resuspended in 0.5 M triethylammonium bicarbonate ($Et_3NH^+HCO_3^-$) for 2 h, washed until neutral, and finally stored in 0.05 M Et_3NH^+-HCO_3^- until used.

HPLC Conditions. All analyses were performed with a Varian 5000 liquid chromatograph interfaced to a Varian UV-SD variable-wavelength detector set at 446 nm. A Bio-Rad Hi-Pore RP-318 column (4.6 × 250 mm) was used for analytical measurements at a flow rate of 1.2 mL/min with a solvent system of 40% MeOH and 60% 20 mM tetrabutylammonium phosphate [$(Bu_4N)_3PO_4$] in an isocratic elution procedure. For preparative work a Bio-Rad Hi-Pore RP-318 column (10 × 250 mm) at a flow rate of 4 mL/min was used. The solvent system consisted of 30% MeOH and 70% 60 mM tetrabutylammonium acetate (Bu_4NOAc) in an isocratic elution procedure.

Preparation of Riboflavin 4′,5′-Cyclic Phosphorothioate. Riboflavin (3.0 g, 8.0 mmol) was suspended in 20 mL of trimethyl phosphate [$(MeO)_3PO$] at 100 °C. The suspension was quickly cooled to 0 °C, and then thiophosphoryl chloride (2.4 mL, 24.0 mmol) was added. The mixture was allowed to stir in the dark at room temperature for 120 h in order to maximize product formation. The mixture was added to 80 mL of 10% aqueous barium acetate, and the resulting suspension was stirred for 45 min at 10–15 °C. The suspension was brought to pH 9 by the addition of 20 mL of triethylamine, and then 240 mL of 95% ethanol was added to complete the precipitation. The solid was isolated by suction filtration, washed with 3 × 80 mL of 70% ethanol, and extracted with 6 × 150 mL of water. The combined aqueous extracts were loaded onto a DEAE-Sephadex A-25 (HCO_3^- form) column (25 × 210 mm), and the column was washed with 1500 mL of water to complete the removal of unreacted riboflavin. The product was eluted first with a linear gradient [1 L, 0.0–0.5 M triethylammonium bicarbonate (Et_3NH^+-HCO_3^-)] followed by 500 mL of 0.5 M $Et_3NH^+HCO_3^-$. The product began eluting at about 0.4 M $Et_3NH^+HCO_3^-$. The peak fractions (monitored by A_{446} and HPLC) were combined and evaporated at 35 °C (20 mm) from H_2O and subsequently from MeOH to yield 1.4 g (3.1 mmol, 40% yield) of riboflavin 4′,5′-cyclic phosphorothioate (cFMNS) as characterized by ^{31}P and ^{13}C NMR (Figures 1 and 2). The diastereomers exhibit one peak by reverse-phase HPLC.

Preparation of 5′-FMNS. Riboflavin 4′,5′-cyclic phosphorothioate (50.0 mg, 0.110 mmol) was dissolved in 2.0 mL of 0.50 M KOH and heated at 40 °C for 1 h in the dark. Disappearance of the two cFMNS diastereomers and concomitant appearance of several positional isomers of FMNS were monitored by ^{31}P NMR. After completion of the hydrolysis, the solution was adjusted to approximately pH 7 with concentrated HCl. Preparative reverse-phase HPLC of the hydrolysis product mixture gave two major peaks eluting at 15.1 and 17.6 min. The peak at 15.1 min (10 mg, 0.021 mmol of mixture) was shown to contain a 1:1 mixture of 5′-FMNS

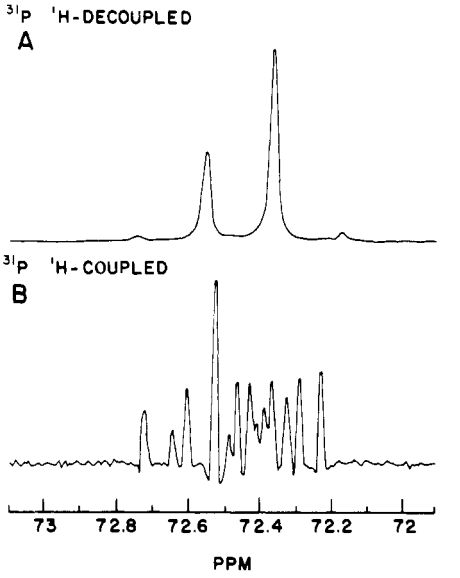


FIGURE 1: ^{31}P NMR spectra of riboflavin 4′,5′-cyclic phosphorothioate (cFMNS). Spectra of 60 mM riboflavin 4′,5′-cyclic phosphorothioate at pH 7 were obtained under (A) ^1H-decoupled and (B) ^1H-coupled conditions. Both spectra were resolution enhanced by Gaussian filtration.

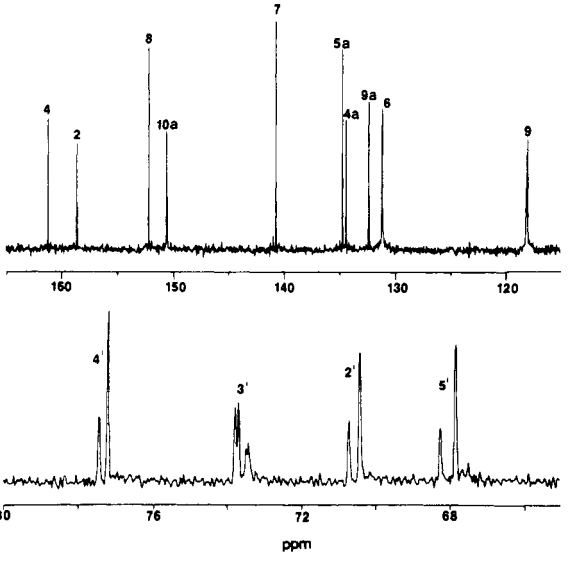


FIGURE 2: Aromatic (top) and ribitol (bottom) regions of ^1H-decoupled ^{13}C NMR spectrum of 60 mM cFMNS at pH 7. Resonance assignments were based on those of FMN (Moonen et al., 1984; Breitmaier & Voelter, 1972) and supported by off-resonance decoupling experiments.

and a positional isomer (2′, 3′, or 4′) by ^{31}P NMR (Figure 3). After addition of EDTA to a concentration of approximately 1 mM, the isomer mixture was incubated at 37 °C for several days. During this time, ^{31}P NMR indicates that both isomers slowly decompose, though the 5′-FMNS does so substantially more slowly than the other isomer. From the new signals that appear in the ^{31}P spectrum at 1.7 ppm (corresponding to inorganic phosphate) and 18.0 ppm (corresponding to FMN cyclic phosphate), it is concluded that desulfurization by an as yet unknown mechanism is occurring during the decomposition. Following the complete disappearance of the resonance from the undesired FMNS isomer after 3–5 days, the remaining 5′-FMNS (40–50% of that present initially) was purified by preparative HPLC and characterized by ^{31}P and ^{13}C NMR. The purified 5′-FMNS was stored at 4 °C.

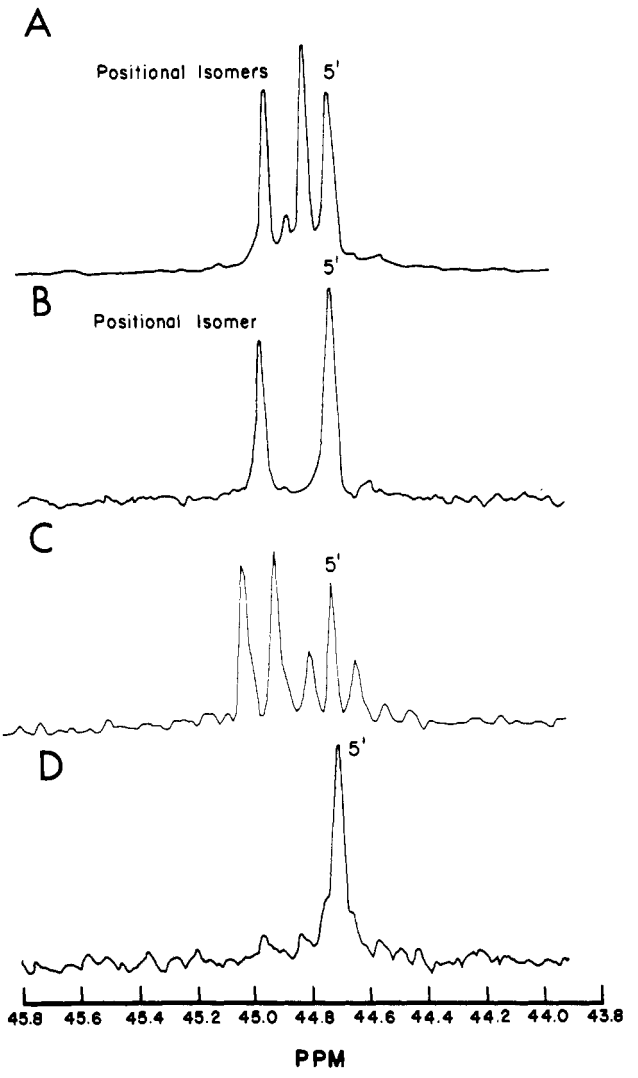


FIGURE 3: ^{31}P NMR spectra of hydrolysis products of riboflavin 4',5'-cyclic phosphorothioate (cFMNS). ^{1}H-decoupled spectra of (A) the product mixture from the alkaline hydrolysis of cFMNS following neutralization to pH 7.3, (B) the mixture of 5'-FMNS and the positional isomer that coelute during preparative HPLC, (C) the ^{1}H-coupled spectrum of the mixture in (B), and (D) 5'-FMNS isolated after thermal decomposition of the positional isomer and a second purification by preparative HPLC.

NMR Methods. ^{31}P and ^{13}C NMR spectra were measured at 101.2 and 62.9 MHz, respectively, on a Bruker WM-250 spectrometer. All spectra were recorded at 10 ± 2 °C with or without broad-band proton decoupling on samples containing 10% D_2O as a field frequency lock. ^{31}P chemical shifts are referenced to external 85% phosphoric acid and ^{13}C shifts to external Me_4Si.

Spectrophotometric and Fluorometric Determinations. All absorbance spectra were measured on a Hewlett-Packard 8450A diode array spectrophotometer with an HP 89100A electronic temperature control accessory. Fluorometric measurements of the FAD and FMN content of the NADPH–cytochrome P-450 reductase holoenzyme and FMN-free reductase were performed on an Aminco SPF-500 ratio spectrofluorometer equipped with an Aminco SPF-500 recorder. The flavin content of NADPH–cytochrome P-450 reductase was determined as described previously (Masters et al., 1975) by the method of Faeder and Siegel (1973).

Preparation of FMN-Free NADPH–Cytochrome P-450 Reductase. NADPH–cytochrome P-450 reductase was purified and characterized as described previously (Otvos et al., 1986). FMN-free reductase was prepared by the method of Alexander (1981) with some modifications. Samples for depletion were diluted to 2.5 μM protein in 50 mM Tris-HCl buffer, pH 7.65, containing 10% glycerol, 0.1 mM EDTA, and 0.02% NaN_3. Samples were then dialyzed against a similar buffer containing 3 M KBr and 0.5% charcoal for 72–96 h at 4 °C. Aliquots were removed at various times during dialysis and tested for completeness of FMN depletion, estimated by absorbance measurements at 452 and 383 nm. The samples were then dialyzed for 48 h against 50 mM Tris-HCl buffer, pH 7.65, containing 10% glycerol, 0.1 mM EDTA, and 0.02% NaN_3. Finally, the FMN-free reductase preparations were filtered through a fine sintered-glass filter and concentrated on an Amicon PM-10 ultrafiltration membrane. Recoveries typically ranged from 37 to 45% of the starting protein. FMN-free reductase preparations were shown to be from 90 to 97% FMN free, based on the method of Faeder and Siegel (1973). FMN-free reductase preparations had specific activities of approximately 2.7 μmol of cytochrome *c* reductase activity min^{-1} (mg of protein)$^{-1}$. After incubation for 10 min at 37 °C with a 10-fold excess of FMN, these same samples had specific activities of approximately 27.6 μmol min^{-1} mg^{-1}. These activities compared favorably with those obtained with enzyme containing both flavin constituents prepared by conventional methods (19–28 μmol min^{-1} mg^{-1}; Otvos et al., 1986). Preparations of FMN-free reductase consistently reveal fewer proteolytic degradation products than the holoenzyme preparations as evidenced by 10% SDS–PAGE (Laemmli, 1970).

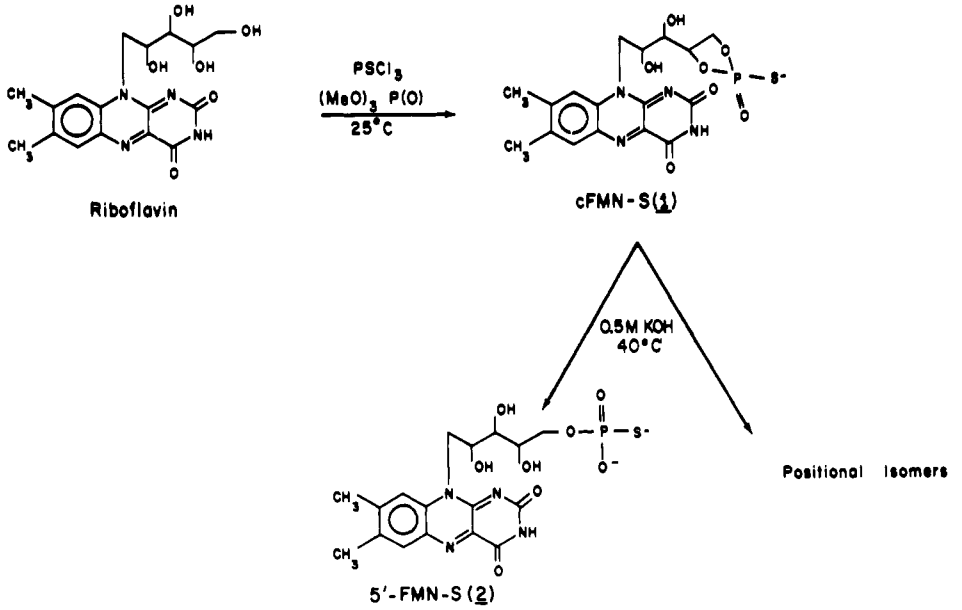
Reconstitution Titration and Semiquinone Experiments with Purified 5'-FMNS. The titration of FMN-free reductase with various quantities of FMN or purified 5'-FMNS was performed as indicated specifically in the legend to Figure 7. The enzyme used for reconstitution with 5'-FMNS in the semiquinone experiment (Figure 6) was incubated with 10-fold excess flavin for 2 h in the dark. Excess flavin was removed by washing on an Amicon PM-10 membrane.

RESULTS

Synthesis of 5'-FMNS. The method, first developed by Murray and Atkinson (1968), using $PSCl_3$ to thiophosphorylate nucleosides has become a standard chemical procedure to obtain nucleoside phosphorothioates and was used here to thiophosphorylate riboflavin. There is one report of a successful synthesis with the sugar ribose (Murray et al., 1969), albeit in very low yield (8.6%).

The anticipated product of the reaction of $PSCl_3$ with RB on the basis of literature precedent is 5'-FMNS. However, the only isolable product, and by far the major product determined by HPLC (11.2 min), is identified by ^{31}P and ^{13}C NMR as riboflavin 4',5'-cyclic phosphorothioate (cFMNS; structure **1**, Scheme I; Figures 1 and 2). The ^{1}H-decoupled ^{31}P NMR spectrum shows two peaks at 72.37 and 72.56 ppm arising from the two diastereomers of cFMNS (Figure 1A). The chemical shifts of these resonances identify them as arising from 5-membered, rather than 6-membered, cyclic phosphorothioates (Eckstein et al., 1972, 1974). The ^{1}H-coupled spectrum (Figure 1B) shows a complex splitting pattern that clearly indicates phosphorus coupling to three rather than two protons, as expected only for the 4',5'-cyclic ester. The ^{13}C NMR spectrum (Figure 2) confirms this assignment. The two diastereomers of the cyclic ester give rise to the duplicate resonances observed for the ribitol carbons 2', 3', 4', and 5' as well as for carbons 2 and 10a of the isoalloxazine ring. The only resonance that exhibits ^{13}C–^{31}P spin–spin splitting is that of carbon 3', consistent with previous observations with FMN

Scheme I



that only the 3-bond, but not 2-bond, coupling is large enough to be resolved (Breitmaier & Voelter, 1972).

Formation of a cyclic phosphate during the phosphorylation reaction of RB has been reported previously. Forrest and Todd (1950) found that the reaction of RB with $POCl_3$ in wet pyridine yields the riboflavin 4′,5′-cyclic phosphate as the only isolable product. Scola-Nagelschneider and Hemmerich (1976) also found that riboflavin 4′,5′-cyclic phosphate is a common intermediate in many riboflavin phosphorylations.

The reaction conditions were varied over a wide range to accomplish two objectives: (1) maximization of the yield of cFMNS; (2) diversion of the course of the reaction from the production of cFMNS to that of 5′-FMNS. The ratio of RB to $PSCl_3$ was varied over a range of 1:1 to 1:40, the temperature was varied from 0 to 50 °C, and the reaction time was extended from 1 to 120 h. Under all of these conditions, only unreacted starting material and cFMNS were observed by HPLC with a maximum isolated yield of 40% for cFMNs.

Yoshikawa et al. (1967) and Flexser and Farkas (1953) reported that in the reactions of unprotected nucleosides with $POCl_3$, in which the production of diphosphates was a problem, the amount of diphosphates produced could be reduced with a concomitant increase in the 5′-nucleotide yield by first treating the $POCl_3$ with 1 equiv of water in trialkyl phosphate to make the dichloridate, $P(O)(OH)Cl_2$, and then adding the nucleoside. This approach was tried with a ratio of RB to $P(O)(OH)Cl_2$ varying from 1:1 to 1:40. Under these conditions and with reaction times as long as 120 h, only a trace of product was seen, and this was, again, cFMNS.

Scola-Nagelschneider and Hemmerich (1976) demonstrated that, for the 5′-monophosphorylation of riboflavins, the preferred intermediate was the carefully prepared homogeneous monochloridate, $P(O)(OH)_2Cl$. When this procedure was tried using the thiophosphate analogue, $P(S)(OH)_2Cl$, and RB, there was no detectable formation (by HPLC) of any monothiophosphate esters even after prolonged reaction times (6 days).

The hydrolysis of cFMNS in 0.50 M KOH at 40 °C gave rise to three major products identified by their ^{31}P NMR chemical shifts of 44.71, 44.80, and 44.95 ppm (Figure 3A) as phosphorothioate monoesters (Eckstein, 1979). The most upfield signal in the ^{1}H-coupled spectrum (not shown) is a triplet, consistent with its assignment as 5′-FMNS (**2**). The other two peaks are doublets, consistent with any of the other positional isomers (4′, 3′, or 2′). The hydrolysis mixture was separable into two major peaks by semipreparative reverse-phase HPLC eluting at 15.1 and 17.6 min. By ^{31}P NMR analysis, the peak at 15.1 min contains a mixture of the 5′-FMNS (triplet) and another positional isomer (doublet) of the monophosphorothioate (Figure 3B,C). The isolated yield of this approximately 1:1 mixture of 5′-FMNS and other isomer after semipreparative HPLC is approximately 20%. All attempts to separate the two isomers chromatographically were unsuccessful. However, the positional isomer was found to be fortuitously more thermally labile at pH 7 and 37 °C than 5′-FMNS, thereby providing a means of obtaining pure 5′-FMNS following removal of the decomposition products by preparative HPLC (Figure 3D). As yet, we do not know the mechanism(s) of the reaction(s) responsible for this preferential decomposition of the unwanted isomer of FMNS. Observation of a ^{31}P NMR signal from a decomposition product at the chemical shift (18.0 ppm) expected for FMN cyclic phosphate suggests, however, that cyclization and desulfurization processes are involved.

Fluorescence Quenching by FMN-Free NADPH–Cytochrome P-450 Reductase of FMN and FMNS. The data presented in Figure 4 show that the fluorescence of FMN or FMNS is partially quenched by the addition of a substoichiometric amount of FMN-free NADPH–cytochrome P-450 reductase, which exhibits no fluorescence of its *bound* prosthetic groups. The fully complemented flavoprotein contains 1 equiv each of FAD and FMN/mol of reductase (Iyanagi & Mason, 1973), but it was possible to obtain an FMN-free preparation by a modification of the procedures of Vermilion and Coon (1978), Alexander et al. (1980), and Alexander (1981). Addition of 4.4 nmol of FMN-free reductase to 2.0 mL of a 3 μM solution of FMN results in the tracing shown in Figure 4A. Similarly, addition of 4.4 nmol of FMN-free reductase to 2.0 mL of a 3 μM solution of FMNS produces the time-dependent quenching shown in Figure 4B. It is to be noted that the absolute fluorescence of FMNS is approximately one-fourth that of FMN, indicating

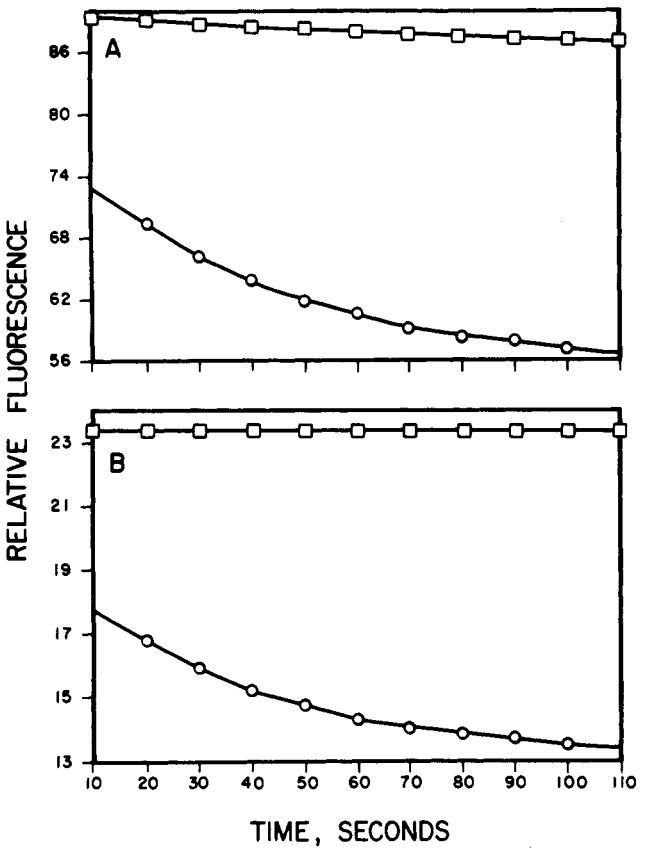


FIGURE 4: Fluorescence quenching of FMN or FMNS by FMN-free reductase. A 200-μL portion of a 22 μM stock of FMN-free reductase or buffer was added to a cuvette containing 2 mL of a 3 μM solution of FMN (A) or FMNS (B), and fluorescence (excitation 448 nm, emission 535 nm) quenching was monitored as a function of time. The fluorescence of the flavins was measured without added FMN-free reductase (open squares) and with a substoichiometric addition of FMN-free reductase (open circles).

Table I: Comparison of FMN- vs. FMNS-Reconstituted Reductase

	FMN-free reductase	FMN reconsti-tuted	FMNS reconsti-tuted
flavin turnover no. [mol of cyt *c* reduced min^{-1} (mol of flavin)$^{-1}$]	223	1282[a]	1195[a]
A_{452}/A_{383} (oxidized protein)[b]	1.06	1.12	1.16
A_{452}/A_{383} (1e reduced)[b]		0.91	0.93

[a] Turnover numbers, based on starting flavin concentrations of FMN-free reductase, were divided by 2. These turnover numbers were obtained with a 10-fold excess of FMN or FMNS added. [b] The absorbance data were obtained with dialyzed enzyme preparations free of excess flavin.

that sulfur atom substitution is affecting the isoalloxazine ring electron distribution.

Catalytic Competence of FMN- and FMNS-Reconstituted NADPH–Cytochrome P-450 Reductase. Table I shows the catalytic activities of NADPH–cytochrome P-450 reductase from which FMN has been removed and to which either FMN or FMNS has been added in 10-fold excess. It can be seen that the reductase is reactivated to a turnover number of 1282 min^{-1} with FMN and to 1195 min^{-1} with FMNS in this experiment, demonstrating the efficacy of the phosphorothioate analogue of FMN in reconstituting enzyme activity.

Spectral Characterization of the FMN- and FMNS-Reconstituted Preparations of NADPH–Cytochrome P-450 Reductase. Figure 5 shows the spectra of FMN- and FMNS-reconstituted preparations of NADPH–cytochrome P-450 reductase for qualitative comparison. It is not possible

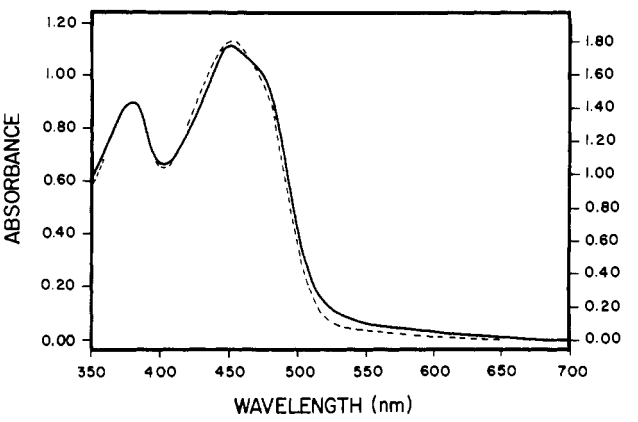


FIGURE 5: Absorbance spectra of FMN- and FMNS-reconstituted reductase. Spectra of FMN-free reductase (83.6 μM) incubated for 60 min at 4 °C with a 4-fold excess of FMN (dashed line, right *y* axis) and FMN-free reductase (52.1 μM) incubated with a 4-fold excess of FMNS (solid line, left *y* axis) for 60 min at 4 °C. Spectra were obtained after exhaustive dialysis to remove free flavin for both incubations. The lower than expected absorption for both samples resulted from handling losses incurred during NMR experiments carried out prior to the measurement of the absorbance spectra.

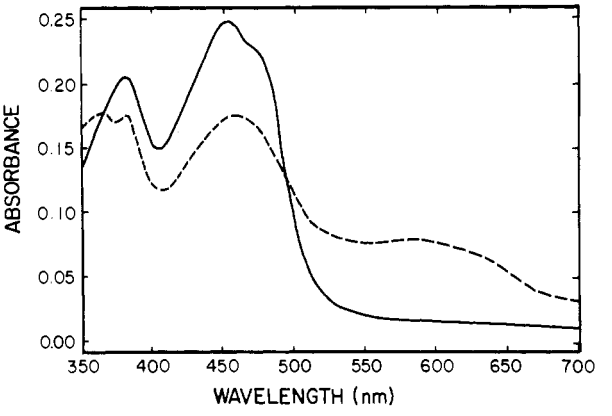


FIGURE 6: Absorbance spectra of oxidized and 1e-reduced FMNS-reconstituted FMN-free reductase. The 1e-reduced form of the reductase was generated by adding approximately 1 equiv of anaerobic NADPH to a sample of FMNS-reconstituted reductase that had been previously bubbled with purified Ar to remove O_2. The sample was then exposed to air prior to acquiring the absorbance spectrum (dashed line). The spectrum of oxidized protein (solid line) was taken before the addition of NADPH to the sample.

to compare the absolute concentrations of these two spectra due to differential handling losses upon dialysis and subsequent ultrafiltration. It was observed in the fluorescence quenching experiments that FMNS exhibited approximately 25% of the fluorescence of FMN. For comparison, FAD exhibits approximately 10% of the fluorescence of FMN (Tsibris et al., 1965). The structural explanation for the partial quenching in FMNS is not known.

Formation of the One-Electron-Reduced Semiquinone of NADPH–Cytochrome P-450 Reductase from FMNS-Reconstituted Flavoprotein. As documented in Table I, NADPH–cytochrome *c* reductase activity of the FMN-free enzyme can be reconstituted fully by either FMN or FMNS. The early experiments of Masters et al. (1965a,b) and later documentation that electron cycling must involve the 1e-reduced semiquinone form of the reductase (Iyanagi et al., 1974; Oprian & Coon, 1982) place the requirement for maintenance of a stable semiquinone upon the flavin or flavin analogue that occupies the FMN site. Figure 6 shows the spectral characteristics of the air-stable 1e-reduced form of NADPH–cytochrome P-450 reductase reconstituted with FMNS. The spectrum is typical of a neutral blue semiquinone and is very

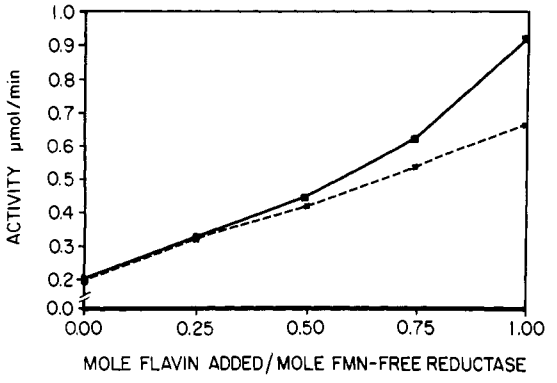


FIGURE 7: Reconstitution of NADPH–cytochrome *c* reductase activity from FMN-free reductase using FMN or FMNS. Aliquots of FMN (solid line) or FMNS (dashed line) were added to individual samples of FMN-free reductase (0.5 nmol each) and incubated for 30 min at 25 °C, followed by exhaustive dialysis to remove free flavin. NADPH–cytochrome *c* reductase activity was measured after dialysis and plotted vs. mole of flavin added per mole of FMN-free reductase in the starting mixtures.

similar to that obtained with the native reductase or the FMN-reconstituted enzyme (Masters et al., 1965a,b; Vermilion et al., 1981).

Titration of Enzymatic Activity with FMN or FMNS Addition to FMN-Free NADPH–Cytochrome P-450 Reductase. In the experiment shown in Figure 7, the FMN-free reductase was titrated with increasing quantities of either FMN or FMNS and tested for reconstitution of NADPH–cytochrome *c* reductase activity. Vermilion and Coon (1978) showed that FMN-depleted reductase was catalytically incompetent with cytochrome *c* as an electron acceptor. The data in Figure 7 indicate that both FMN and FMNS are capable of reconstituting this enzyme activity, with FMNS giving 65% of the activity of an equivalent amount of FMN (1 mol of flavin added/mol of FMN-free reductase). It should be noted that at 2 mol/mol excess flavin added the activity is still rising and is 1.19 μmol/min for FMN addition and 0.92 μmol/min for FMNS addition (data not shown). These data are interesting in light of the results in Table I that show FMNS and FMN to be equipotent in reconstituting cytochrome *c* reductase activity at a 10-fold molar excess.

Discussion

The use of flavin analogues in probing the active center of flavoenzymes originated in McCormick's laboratory (McCormick et al., 1964; Tsibris et al., 1965, 1966; Roth et al., 1966). In one of these studies (Tsibris et al., 1966), modifications of the constitution of the N(10) side chain were made to elucidate the mode of binding of flavin in flavoproteins. Most studies since then, however, have been restricted to modifications of the isoalloxazine ring system or the adenine ring of FAD. The use of analogues has served as a complementary approach to crystallographic studies to probe the environment of the flavoproteins being examined [see the review by Ghisla and Massey (1986)].

In the case of NADPH–cytochrome P-450 reductase, several types of flavin analogues have been utilized to examine the redox, spectral, and catalytic properties of FMN analogue substituted protein. The pioneering studies with FMN-free or FMN analogue substituted NADPH–cytochrome P-450 reductase were performed in Coon's laboratory (Vermilion & Coon, 1978; Vermilion et al., 1981) and have been supplemented by the reports of Alexander et al. (1980) and Alexander (1981). The first experiments with the FMN-free NADPH–cytochrome P-450 reductase were performed by Vermilion and Coon (1978) to determine the flavin requirements for reduction by NADPH and reaction with electron acceptors. In this study (Vermilion & Coon, 1978) and in collaboration with Ballou and Massey (Vermilion et al., 1981), they established the low-potential (FAD) and high-potential (FMN) flavins of the reductase. These pioneering studies utilized several substituted flavin analogues as probes of the catalytic mechanism of the reductase, demonstrating the spectral shifts unique for these analogues and the necessity of semiquinone formation for catalytic function.

The experiments undertaken in this work have been directed toward the synthesis of a unique type of spectral probe suggested by the elegant studies of Eckstein and his co-workers (Eckstein, 1985; Miziorko & Eckstein, 1984) in which the utility of nucleoside phosphorothioates as substrate analogues were demonstrated. The recent report from this laboratory using ^{31}P NMR as a means of examining the domains of the two prosthetic flavins of NADPH–cytochrome P-450 reductase (Otvos et al., 1986) has demonstrated the location of the free radical of the air-stable semiquinone on FMN and the differential perturbation of the two flavins by Mn(II). These experimental results led us to pursue the synthesis of a phosphorothioate analogue of FMN in order to test its efficacy in reconstituting FMN-free preparations of the reductase and as a possible spectral probe.

This synthesis is useful, the yields are reasonable (40%), the starting materials are not prohibitively expensive, and the amount of cFMNS obtained from 3.0 g of riboflavin is sufficient for a large number of experiments. The intermediate products obtained after DEAE-Sephadex A-25 chromatography, alkaline hydrolysis, reverse-phase HPLC, and heat treatment have been well characterized by ^{13}C and ^{31}P NMR spectroscopy (Figures 1–3). One of the unique properties of the phosphorothioate monoester analogues of FMN is a marked low-field ^{31}P (~45 ppm) chemical shift. Its resonance would, therefore, always be easily resolved from those of the enzyme-bound prosthetic flavins, FAD or FMN, and nucleotide cofactors, NADPH or $NADP^+$, if the FMN-free reductase could be reconstituted with 5′-FMNS.

As shown in Figures 1–3, the initial product of the reaction of $PSCl_3$ with riboflavin in trimethyl phosphate was a cyclic product, 4′,5′-cFMNS, which could be hydrolyzed subsequently in basic solution in the dark at 40 °C. The mixture of 5′-FMNS and positional isomer isolated by semipreparative HPLC from the cFMNS hydrolysis mixture was capable of binding to the reductase, and in a fluorescence quenching experiment, the fluorescence of either the FMNS mixture or FMN was shown to be quenched when it was added to the FMN-free reductase. Table I shows the ability of a 10-fold excess of purified 5′-FMNS to reconstitute cytochrome *c* reductase activity of FMN-free reductase to a turnover number based on flavin of 1195 min^{-1} compared to 1282 min^{-1} for FMN-reconstituted flavoprotein and 720–900 min^{-1} for conventionally prepared flavoprotein (Otvos et al., 1986).

A critical experiment, supporting the notion that an analogue capable of restoring the catalytic activity of NADPH–cytochrome P-450 reductase must be able to form the 1e-reduced semiquinone, was performed as shown in Figure 6. The reductase is apparently stabilized at this oxidation–reduction state after anaerobic reduction with NADPH and reoxidation in air of the 2e-reduced enzyme (either $FMNH_2$ or $FMNSH_2$). The spectrum is typical of a neutral blue semiquinone and does not reveal any remarkable spectral features uniquely different from those of the FMN-reconstituted counterpart or the holoreductase. Furthermore, titration of catalytic activity with

less than stoichiometric amounts of FMN or FMNS (Figure 7) resulted in a recovery of NADPH–cytochrome *c* reductase activity less than that obtained with excess flavin addition (Table I). The calculations were based on flavin added, and no attempt was made in this experiment to determine the actual FMN (or FMNS) content by fluorometry after each addition. When this is done with FMN (Alexander, 1981), the reductase is fully reconstituted based on the actual amount of flavin bound.

Recent preliminary experiments utilizing ^{31}P NMR spectroscopy have shown that FMNS-reconstituted NADPH–cytochrome P-450 reductase exhibits, as expected, an NMR spectrum distinct from that of the native oxidized reductase (unpublished experiments; J. D. Otvos, J. P. Calhoun, H. M. Miziorko, D. P. Krum, and B. S. S. Masters, manuscript in preparation), with the resonance from bound FMNS appearing at approximately 43 ppm. Further studies utilizing the FMNS analogue are in progress. The utility of this phosphorothioate derivative of FMN in probing its domain in NADPH–cytochrome P-450 reductase suggests that it may be useful for other FMN-binding proteins as well.

ACKNOWLEDGMENTS

We are indebted to Dr. Vincent Massey for his suggestion of the use of charcoal during KBr dialysis to remove FMN quantitatively for these studies.

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