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Chemical and Physical Characterization of the Shethna Flavoprotein and Apoprotein and Kinetics and Thermodynamics of Flavin Analog Binding to the Apoprotein*

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ABSTRACT: A molecular weight of 23,000 g mole^{-1} has been determined for the Shethna flavoprotein from sedimentation equilibrium data, sedimentation and diffusion coefficients, and amino acid analysis. The amino acid composition of the Shethna flavoprotein is similar to those reported for *Peptostreptococcus elsdenii* and *Clostridium pasteurianum* flavodoxins. Similarities are also noted in the folding of the protein chain as indicated by the shape of the far-ultraviolet circular dichroism spectra of the three flavoenzymes. An improved method for resolving the FMN group from the Shethna flavoprotein is described. Ultraviolet circular dichroism spectra and other properties indicate a reversible binding of the coenzyme to the apoprotein. The kinetics and thermodynamics of the binding of flavin analogs to the Shethna apoprotein have been studied. The 5′-phosphate group contributes 2–3 kcal/mole to the binding energy and is important in determining the redox properties of the bound flavin. Modification of the isoalloxazine ring decreases the binding energy, whereas removal of the side-chain hydroxyl groups has a negligible effect. The steric properties of the ribityl side chain are shown to be important by the facts that acetylation of the hydroxyl groups completely prevents binding and that approximately three-fourths of the binding energy is contributed by the isoalloxazine ring. The intramolecular ring-side-chain hydroxyl and the flavin–protein interactions are involved in the quenching of flavin fluorescence upon binding to the protein.

An increase in binding rate upon removal of the terminal phosphate or the side chain hydroxyl groups indicates that the initial, and rate-determining, step in the binding process involves a flavin side-chain–protein interaction which occurs within the protein structure.

The recent isolation of relatively simple, low molecular weight flavoenzymes has provided systems which are particularly amenable to the study of protein–flavin interactions. One of these, the Shethna flavoprotein (Shethna *et al.*, 1965; Hinkson and Bulen, 1967), upon reduction forms a semiquinone species which is quite resistant to air oxidation (Hinkson and Bulen, 1967), and can be resolved into an apoprotein which is stable to denaturation (Hinkson, 1968).

A more complete characterization of the chemical and physical properties of the Shethna flavoprotein is essential to an understanding of the protein–flavin interaction. Furthermore, a demonstration of the reversibility of resolution and recombination of the FMN molecule with the protein is required if flavin binding is to be used to obtain this type of information.

As shown in paper I of this series, circular dichroism spectroscopy provides evidence for similarities in the flavin environments of the flavodoxins isolated from *Clostridium* and *Peptostreptococcus elsdenii* and the Shethna flavoprotein. Inasmuch as the latter species does not have any of the biological activities of the flavodoxins (Hinkson and Bulen, 1967), a comparison of the chemical compositions and overall protein structure (as revealed by circular dichroism spectroscopy in the far-ultraviolet spectral region) is of interest.

We have also studied the effect of flavin structural modifications on the kinetics and thermodynamics of binding to the Shethna apoprotein. Previous investigations of flavin analog binding to a variety of apoflavoenzymes (Tsibris *et al.*, 1966; Arsenis and McCormick, 1964; Chassey and McCormick, 1965) have explored the positions on the isoalloxazine ring and ribityl side chain which are important in the restoration of catalytic activity. In this work, the physicochemical aspects of the flavin–protein interaction are emphasized.

Hinkson (1968) has measured the association constants for FMN, riboflavin, and FAD binding to the Shethna apoprotein and has determined a second-order rate constant for FMN binding. The present investigation represents an extension of this work to a larger number of flavin analogs.

Experimental Section

Materials

Isoriboflavin was obtained from Calbiochem and used without further purification. The FMN used in the binding studies was isolated from the Shethna flavoprotein and puri-

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fied on DEAE-cellulose. 3-Methylriboflavin[1] was a gift from Dr. D. McCormick, Division of Biological Sciences, Cornell University, Ithaca. Lumiflavin was synthesized using the method of Guzzo and Tollin (1963). *N*-10-ω-Carboxybutylisoalloxazine was a gift from Dr. D. McCormick, Cornell University, Ithaca, The 2-thioFMN analog was a gift from Dr. P. Hemmerich, University of Konstanz, Germany. All flavin derivatives were homogeneous upon thin-layer chromatography on cellulose plates, using either 5% K_2HPO_4 or butanol–acetic acid–water (4:1:5, v/v) as the developing solvent. Most flavin concentrations were determined spectrophotometrically at 445 nm using a molar extinction of 12,200 l. $mole^{-1}$ cm^{-1}. IsoFMN and isoriboflavin concentrations were determined spectrophotometrically at 448 nm using a molar extinction of 6700 l. $mole^{-1}$ cm^{-1} (Berezovskii and Radinova, 1958). A molar extinction of 11,300 at 450 nm was used in determining FAD concentrations (Penzer and Radda, 1967).

Protein concentrations were estimated from the flavin absorbance in the 450-nm region using published extinction coefficients. The Shethna apoprotein concentration was estimated by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a standard. All other chemicals were obtained as described in paper I of this series or were of reagent grade from commercially available sources. Deionized water, prepared by passing distilled water through a Barnstead mixed-bed ion-exchange resin, was used in the preparation of all solutions.

Instruments. Circular dichroism spectra were measured as described in paper I. Cylindrical quartz cells (1 mm) were used in the 250–190-nm spectral region. To avoid absorption artifacts, the dynode voltage across the photomultiplier tube was never allowed to exceed 0.45 kV.

Absorption spectra were recorded with a Cary 14R spectrophotometer. A Gilford Model 240 spectrophotometer was used for single-wavelength absorbance measurements.

Florescence quenching was measured using a fluorimeter built in our laboratory. This instrument utilized band pass primary and secondary interference filters. The excitation beam was mechanically chopped and the emission signal was detected utilizing a lock-in amplifier. Pyrex rectangular cells (10 mm) were used in all experiments. Signals were recorded on a Sanborn Model 151 recorder.

Analytical ultracentrifugation experiments were performed with a Spinco Model E ultracentrifuge utilizing schlieren optics. Photographic plates were analyzed on a Nikon microcomparator.

Methods

Amino acid analysis on dried, salt-free samples of the flavoprotein and of the apoprotein were performed by the AAA Laboratory, Seattle, Wash. Cysteine was estimated as cysteic acid after performic acid oxidation (Hirs *et al.*, 1956) and as carboxymethylcysteine with and without prior reduction by mercaptoethanol. Tryptophan was determined after $Ba(OH)_2$ hydrolysis (Dreize and Reith, 1956). Tryptophan was also determined in our laboratory spectrophotometrically (Goodwin and Morton, 1946) and by *N*-bromosuccinimide oxidation (Peters, 1959). Both of these determinations were made on the apoprotein as the flavin chromophore interferes with the analysis.

Preparation of the Apoprotein. The procedure used for resolving the flavin from the protein is a modification of that described by Hinkson (1968). Dithiothreitol was added to an aqueous solution of the flavoprotein (≃1 mg/ml) to a final concentration of 1×10^{-3} M. At 0° and under dark conditions, a 30% w/v aqueous solution of trichloroacetic acid was added to the protein solution to a final concentration of 3%. The precipitated protein was collected by centrifugation at 12,000*g* for 15 min. After decantation, the pellet was washed with 3% trichloroacetic acid and recentrifuged. Normally after one washing the protein pellet was colorless. If any yellow color still remained, the precipitate was washed again. The precipitated apoprotein was dissolved in 1–2 ml of 0.1 M Tris buffer (pH 8.5).

Any precipitated protein not solubilized was removed by centrifugation. At pH 8.5, the apoprotein was stable for several weeks. The apoprotein was stable for only several days at pH 7.0. A protein concentration of 1 mg/ml, as determined by the Lowry *et al.* (1951) method had an absorbance at 280 nm of 1.270. If the precautions of adding dithiothreitol and light exclusion were not observed, a large molecular weight protein species was observed upon column chromatography on Sephadex G-100. Similarly, two electrophoretic bands were observed upon cellulose acetate electrophoresis. This high molecular weight material had different ultraviolet absorption and circular dichroism spectra than the apoprotein and would not rebind FMN. No systematic investigation was made into the structure of this material.

The technique of dialyzing the holoprotein in 2 M KBr, as was used in resolving the FMN group from *P. elsdenii* flavodoxin (Mayhew and Massey, 1969), was not successful in dissociating the FMN–protein complex of the Shethna flavoprotein.

Analytical Ultracentrifugation Experiments. The sedimentation coefficient of the Shethna flavoprotein was measured on four samples simultaneously using two double-sector cells (G. Adams and P. Adams, unpublished observation, 1970). To separate the sedimenting refractive index gradients on the photographic plate, a positive and a negative wedge window were used on the two cells. A rotor speed of 59,767 rpm and a temperature of 20° were used in the experiment.

The diffusion coefficient was measured utilizing a double-sector synthetic boundary cell. The rotor speed in these experiments was 10,000 rpm and the temperature was 20°.

The molecular weight of the flavoprotein was calculated from the sedimentation and diffusion coefficients at infinite dilution using the procedures as outlined by Svedberg and Pedersen (1940). The partial specific volume of the protein was calculated from the amino acid composition by the method of Cohn and Edsall (1943).

The molecular weight of the Shethna flavoprotein was also determined by the short-column sedimentation equilibrium technique of Yphantis (1964). The rotor speed was 20,000 rpm and the temperature was 20°. Equilibrium was established after 20 hr.

Determination of Binding Constants. The association constants of flavin analogs with the apoprotein were determined by fluorescence quenching experiments in 0.025 M phosphate, pH 7.0 at room temperature. A microliter quantity of apoprotein was added to 2 ml of flavin solution and the residual flavin fluorescence at 530 nm was measured at equilibrium. The micropipet was calibrated spectrophotometrically by pipetting the protein solution into 2 ml of buffer and measuring the absorbance at 280 nm. Flavin concentrations were measured spectrophotometrically at 445 nm. For most of the

[1] Because of the unexpected results obtained with this derivative (see below), its identity was confirmed using thin-layer chromatography and infrared spectroscopy.

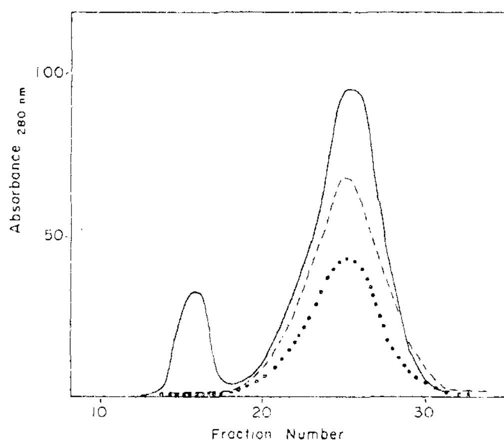

FIGURE 1: Sephadex G-100 column chromatography of the Shethna flavoprotein and apoprotein. (-----) Flavoprotein, (· · · ·) apoprotein prepared in the dark and in the presence of 1×10^{-3} M dithiothreitol, and (—) apoprotein prepared in the light without dithiothreitol. The buffer used was 0.025 M phosphate (pH 7.0) and the column dimensions were 1.8 × 50 cm.

flavins, the fluorescence was assumed to result from only unbound flavin. In the case of deoxyriboflavin, N-10-ω-carboxybutylisoalloxazine, and FAD, the residual fluorescence upon adding a 5-fold molar excess of apoprotein was assumed to result from the protein-flavin complex. The quenching data were corrected for this residual fluorescence.

To calculate the association constant, a simple 1:1 equilibrium was assumed: P + F ⇄ PF. This results in the following expression

$$\frac{\alpha}{[F]} = K_a(1 - \alpha)$$

where α is the ratio of PF to total protein. A plot of α/[F] *vs.* $1-\alpha$ will give a linear plot with a slope of K_a. Most association constants were determined in this manner.

Since lumiflavin fluorescence is only slightly quenched upon binding to the protein, the association constant was determined by difference spectroscopy using the 0–0.1 absorbance unit slide-wire in the Cary 14R spectrophotometer. A tandem cell arrangement was used to correct for any possible light scattering from the added protein. Absorbance changes at 432 nm were monitored to calculate the amount of bound lumiflavin. The difference in extinction between the bound and unbound lumiflavin was determined by adding a 5-fold molar excess of protein.

Results

Molecular Weight. Hinkson and Bulen (1967) have reported a minimal molecular weight for the Shethna flavoprotein of 31,200 g mole^{-1} based on flavin content and 31,500 g mole^{-1} based on sulfhydryl content. More recently, Hinkson (1968) measured the flavin binding weight as 27,000 g mole^{-1}. To provide a more accurate value, the molecular weight was determined by sedimentation equilibrium, from experimentally measured values of the sedimentation coefficient and of the diffusion coefficient, and from the amino acid composition.

The sedimentation coefficient of the Shethna flavoprotein was determined at five different protein concentrations ranging from 4 to 8 mg per ml. The protein samples gave only a single, symmetric refractive index gradient. A plot of ln x *vs.* time was linear, where x is the distance in centimeters from the center of the boundary to the axis of rotation. Little or no concentration dependence of the value of the sedimentation coefficient, $s_{20,w}$, was observed. The average of five determinations gave a value for $s_{20,w}$ of 2.33×10^{-13} sec.

TABLE I: Amino Acid Composition of the Shethna Flavoprotein.

Amino Acid	Apoprotein: Micromole of Amino Acid	Flavoprotein: Micromole of Amino Acid	Residues/ Mole of Protein
Lysine	0.173	0.173	13
Histidine	0.001	0.003	0
Arginine	0.078	0.075	6
Aspartic acid Asparagine	0.263	0.270	21
Threonine	0.107	0.104	8
Serine	0.190	0.189	15
Glutamic acid Glutamine	0.293	0.294	23
Proline	0.072	0.069	5
Glycine	0.270	0.270	21
Alanine	0.193	0.196	15
Valine	0.127	0.125	10
Methionine	0.015	0.013	1
Isoleucine	0.077	0.074	6
Leucine	0.272	0.258	20
Tyrosine	0.070	0.069	5
Phenylalanine	0.177	0.168	13
½-Cystine	0.013	0.013	
Cysteic acid	0.014	0.016	1
Carboxymethylcysteine	0.010	0.013	
Tryptophan [Ba(OH)$_2$ hydrolysis]	0.053	0.058	4
Tryptophan	Spectrophotometric titration		3.8
Tryptophan	N-Bromosuccinimide oxidation		3.7

The diffusion coefficient was determined at three different protein concentrations (from 2.0 to 7.0 mg per ml). A plot of A^2/H^2 *vs.* time was linear, where A is the area under the schlieren peak and H is maximum height of the peak at time t. The diffusion coefficient did vary with concentration and the value, corrected to infinite dilution, is 9.03×10^{-7} cm^2 sec^{-1}.

The molecular weight of the Shethna flavoprotein was calculated using the above values of the diffusion coefficient and the sedimentation coefficient and a value for the partial specific volume (0.723) which was calculated from the amino acid composition (Cohn and Edsall, 1943). Using the Svedberg equation, the molecular weight was calculated to be 22,600 g mole^{-1}.

The molecular weight was also determined by sedimentation equilibrium using the short-column technique of Yphantis (1964). A plot of ln y/r *vs.* r^2 was linear, where r is the distance in centimeters from the refractive index increment to the axis of rotation and y is the height of the refractive index gradient

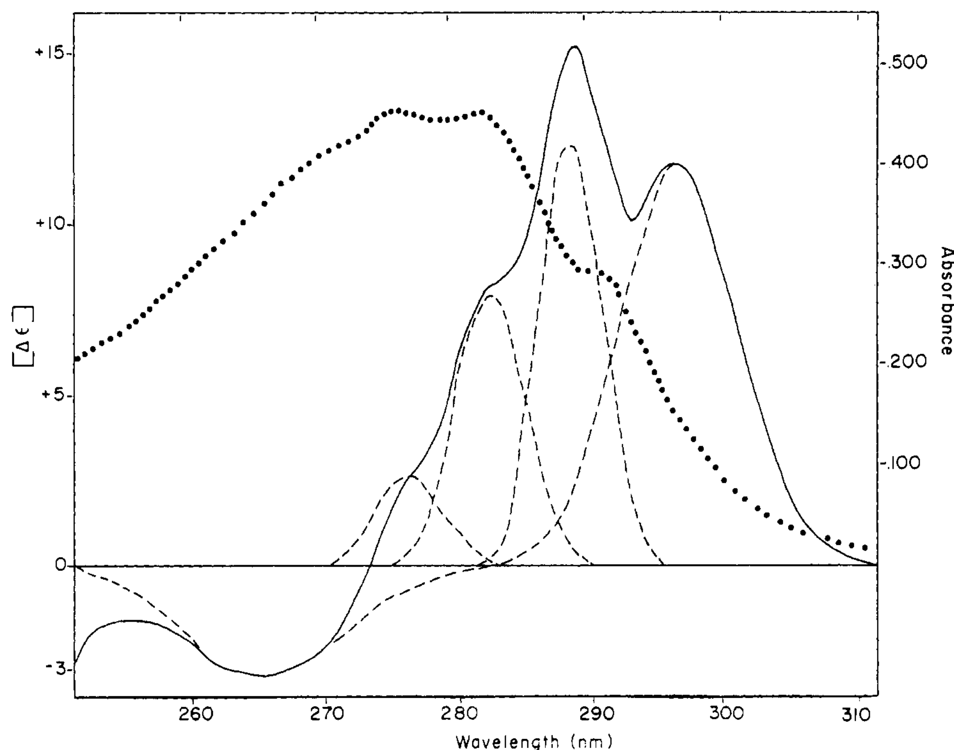

FIGURE 2: Near-ultraviolet circular dichroism and absorption spectra of the Shethna apoprotein. The spectra were measured on protein solutions in 0.025 M phosphate buffer (pH 7.0). (····) Absorption spectrum, (——) circular dichroism spectrum, and (– – – –) curve resolution.

at distance r. The molecular weight values determined at protein concentrations of 2.3, 3.5, and 7 mg per ml were 26,500, 24,300, and 23,600 g $mole^{-1}$, respectively. An average of the three determinations gave a molecular weight for the Shethna flavoprotein of 24,800 g $mole^{-1}$.

The amino acid composition (see below) gives a value of 21,300 g $mole^{-1}$. This is calculated from the combined weight of the substituent amino acids minus the water contribution. The weight of amide ammonia was not included inasmuch as the amide content has not been determined. The average of the three methods gives a molecular weight of 23,000 g $mole^{-1}$ for the Shethna flavoprotein.

Amino Acid Composition. The amino acid analyses of the Shethna flavoprotein and the apoprotein were identical (as indicated by Table I). Assuming 1 mole of cysteine/mole of protein, the number of residues of amino acid per mole was calculated. Since the calculated molecular weight is similar to the molecular weight determined by hydrodynamic measurements (see above) the assumption of 1 mole of cysteine/mole of protein seems justified. Independent methods of tryptophan determination (Table I) also gave the same value as the one based on the total amino acid analysis.

The amino acid composition indicates no histidine, one cysteine, four tryptophans, five tyrosines, and a high percentage of glutamic acid, aspartic acid, and glycine. The composition determined for the Shethna protein is similar to those obtained for *P. elsdenii* flavodoxin (Mayhew and Massey, 1969) and *C. pasteurianum* flavodoxin (Knight and Hardy, 1967). The two flavodoxins also have no histidine or cystine and contain four tryptophans. *C. pasteurianum* flavodoxin has one cysteine residue while *P. elsdenii* flavodoxin has two cysteines. The similarities in the compositions of the three flavoproteins are quite striking considering that the molecular weights of the two flavodoxins are 15,000 g $mole^{-1}$, as compared to 23,000 g $mole^{-1}$ for the Shethna flavoprotein. All three flavoproteins contain one FMN per mole of protein (Knight and Hardy, 1967; Mayhew and Massey, 1969; Hinkson and Bulen, 1967). The Shethna flavoprotein, in spite of its similar chemical composition, does not have any of the biological activities which are known for the flavodoxins (Hinkson and Bulen, 1967).

Resolution of the Shethna Flavoprotein. The FMN chromophore is readily dissociated from the protein moiety by treatment with 3% trichloroacetic acid (Hinkson, 1968). The precipitated protein easily dissolves in basic buffer and will rebind FMN. This technique gives rise to a variable amount of a high molecular weight protein component. This was shown by using cellulose acetate electrophoresis or gel filtration column chromatography on Sephadex G-100 (Figure 1). This high molecular weight material was not formed when the trichloroacetic acid treatment was done in the dark in the presence of 10^{-3} M dithiothreitol (Figure 1). Although no confirming experiments were carried out, it is likely that this substance is a dimeric form of the apoprotein with an intermolecular disulfide bond linking the two protein molecules. Since flavin is a good oxidizing agent in acid solution and is also a good photooxidant, the precautions taken also protect against possible oxidation of susceptible amino acids such as tryptophan, methionine, and cysteine.

The ultraviolet absorption spectrum (Figure 2), as well as the visible spectrum of the apoprotein, indicates no residual flavin and is similar to the general ultraviolet absorption spectra of many proteins containing aromatic amino acids. The circular dichroism spectrum (Figure 2) shows optical activity due to transitions of the aromatic amino acids tryptophan and tyrosine. Resolution of the circular dichroism spectrum into a minimum number of Gaussian components gives five bands with four positive transitions occurring at

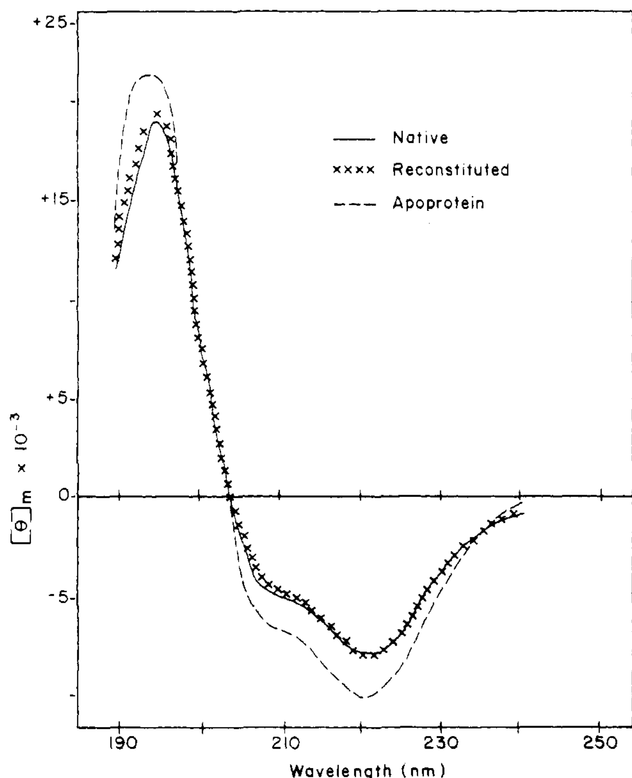

FIGURE 3: Far-ultraviolet circular dichroism spectra of the Shethna flavoprotein, apoprotein, and reconstituted flavoprotein. The spectra were measured on protein solutions in 0.025 M phosphate buffer (pH 7.0); 1-mm path-length quartz cells were used in all measurements. A mean residue weight of 130 was used to calculate the mean residue ellipticity.

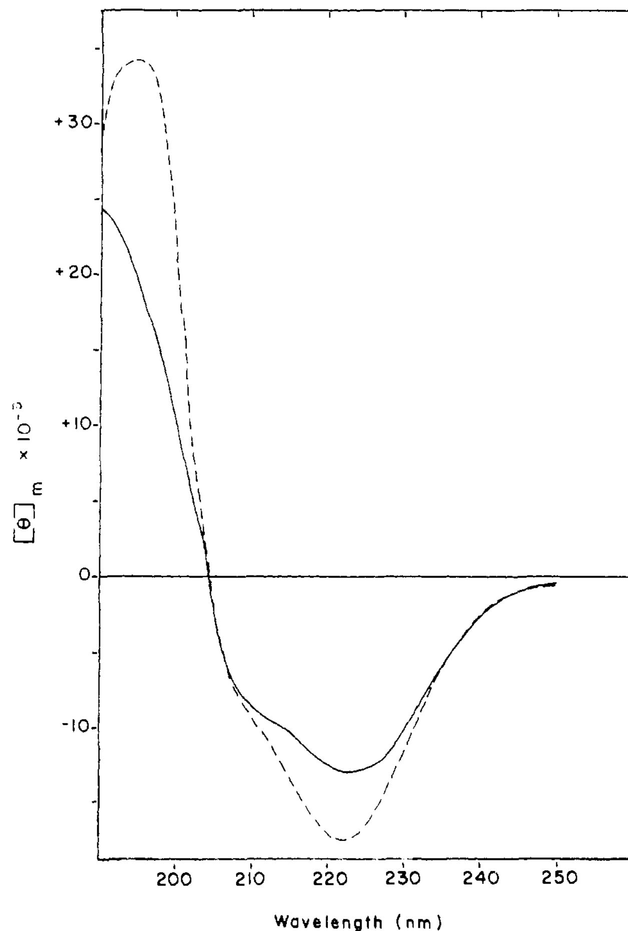

FIGURE 4: Far-ultraviolet circular dichroism spectra of flavodoxins. (—) Clostridial flavodoxin and (- - -) *P. elsdenii* flavodoxin. The buffer was 0.025 M phosphate (pH 7.0). A mean residue weight of 100 was used in calculating the Clostridial enzyme curve and a value of 117 was used in the case of *P. elsdenii* flavodoxin.

297, 288, 282, and 276 nm and a negative band at 265 nm. Based on the results of Strickland *et al.* (1969), the circular dichroism seems to be due primarily to indole electronic transitions, although tyrosine excitations probably overlap below 285 nm. The negative indole circular dichroism transition at 303 nm seen in the spectrum of chymotrypsinogen (Strickland *et al.*, 1969) is not present in the Shethna apoprotein (Figure 2), even though the other dichroic bands are quite similar. Both ^1L_A and ^1L_B indole transitions are apparent which, according to the Strickland analysis, suggests that some of the tryptophyl residues may be exposed and some "buried" with respect to the solvent. The fluorescence emission spectrum of the tryptophan residues is intermediate between the emission maxima expected for tryptophan in nonpolar solvents and in polar solvents (J. D'Anna and G. Tollin, unpublished data, 1970).

A comparison of the far-ultraviolet circular dichroism spectrum of the apoprotein with that of the native flavoprotein, indicates no extensive denaturation upon flavin removal (Figure 3). The differences seen in the circular dichroism spectra of these two species indicate a slightly different conformation for the apoprotein. Rebinding of FMN gives a complex whose far-ultraviolet circular dichroism spectrum is indistinguishable from that native flavoprotein (Figure 3), thereby providing evidence for a reversible flavin-protein recombination. Likewise, the circular dichroism and absorption spectra of the reconstituted flavoprotein in the near-ultraviolet and visible region are indistinguishable from those of the native flavoprotein.

A comparison of the amide chromophore Cotton effects of the Shethna flavoprotein and apoprotein with those of model polypeptides (Beychok, 1968) suggests the presence of both α helix and β structure in the secondary conformation of the polypeptide chain. The negative circular dichroism bands at 222 nm and at 208 nm are characteristic of an α helix. Using the values of the mean residue ellipticities at 222 nm (Figure 3) (-7800 deg cm^2 dmole^{-1} for the holoprotein and $-10{,}000$ deg cm^2 dmole^{-1} for the apoprotein) helix contents of 25 and 33% are estimated, respectively. A 30% helix content is estimated from the magnitude of the 233-nm trough in the optical rotatory disperion spectrum of the apoprotein.

α-Helical polypeptides generally have a positive circular dichroism band at 190–192 nm, whereas polypeptides with a large amount of β structure show a positive dichroism at 195 nm (Beychok, 1968). Thus, the occurrence of a positive circular dichroism band at 195 nm in the spectra of the apoprotein and flavoprotein suggests a substantial amount of this type of structure. The presence of β structure would also be expected to increase the magnitude of the 222-nm negative band, inasmuch as a peptide chain having that conformation gives a negative circular dichroism band at 217–218 nm (Beychok, 1968). That this is the case is shown in Figure 3, where it can be seen that the 208-nm band is approximately 60% as intense as the 222-nm band in both the flavoprotein and the apoprotein. In a true α-helical polypeptide, the magnitude of both of these bands would be approximately the

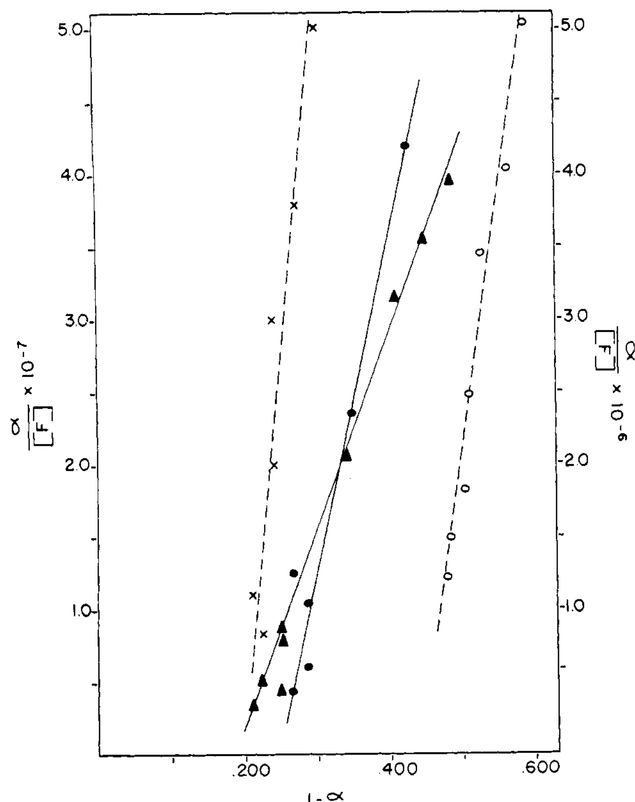

FIGURE 5: Equilibrium binding of FMN analogs to the Shethna apoprotein. The solid-line plots correspond with the values on the left-hand ordinate while the dashed-line plots correspond with the values on the right-hand ordinate. (●) FMN, (▲) dFMN, (×) isoFMN, and (○) 3-MeFMN. All measurements were made in 0.025 M phosphate buffer (pH 7.0) at 25 ± 2°.

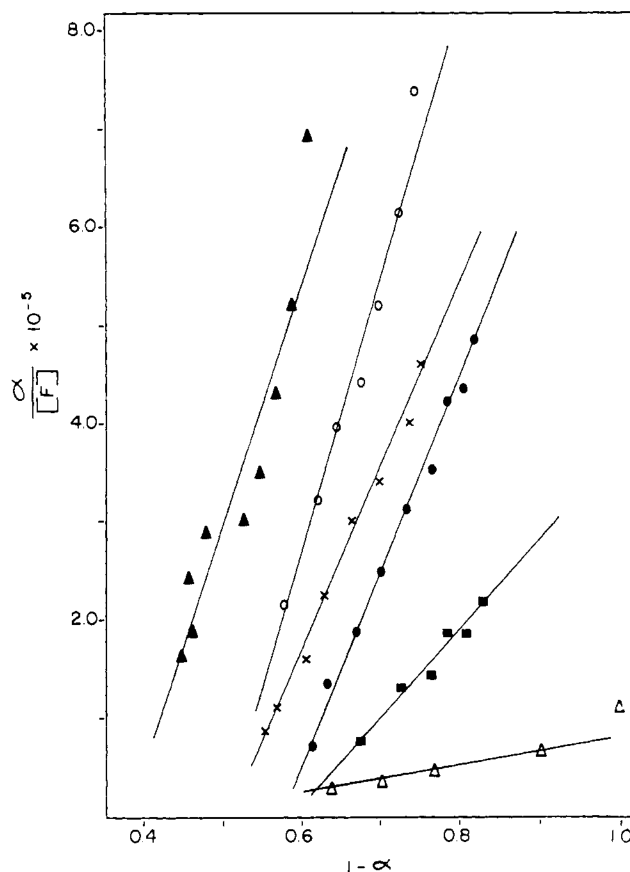

FIGURE 6: Equilibrium binding of riboflavin analogs and FAD to the Shethna apoprotein. (▲) Deoxyriboflavin, (○) N-10-ω-carboxybutylisoalloxazine, (×) isoriboflavin, (●) riboflavin, (■) FAD, and (△) lumiflavin. All measurements were made in 0.025 M phosphate buffer (pH 7.0) at 25 ± 2°.

same (Beychok, 1968). This constitutes further evidence for the presence of substantial amounts of β structure in the Shethna protein and also indicates that the estimated percentage of α helix is probably twice as large as is actually present. Therefore, a helical content of approximately 15% represents a more realistic value.

The far-ultraviolet circular dichroism spectrum of *P. elsdenii* flavodoxin is quite similar in shape to that of the Shethna flavoprotein, with a major negative band at 222 nm, a negative shoulder at 208 nm, and a positive 195-nm band (Figure 4). The intensities of the bands are much larger, however, thus suggesting a more compact polypeptide chain folding. The shape of the circular dichroism spectra also indicates the presence of a substantial amount of β structure, as discussed above.

The far-ultraviolet circular dichroism spectrum of Clostridial flavodoxin differs from those of the other two proteins in that the positive dichroic band is blue shifted to below 190 nm (Figure 4). However, the shape of the circular dichroism spectrum above 200 nm is quite similar to that of the Shethna flavoprotein and of *P. elsdenii* flavodoxin, and has a mean residue ellipticity intermediate between the values for those flavoproteins. The circular dichroism spectral data suggest that Clostridial flavodoxin has a larger ratio of α-helical to β structure than does the *P. elsdenii* flavodoxin or the Shethna flavoprotein.

Equilibrium Binding of Flavin Analogs to the Apoprotein. The equilibrium binding of flavin analogs to the Shethna apoprotein was measured by the quenching of flavin fluorescence for all analogs except lumiflavin, for which optical difference spectra were used. The binding of all analogs to the protein was in a 1:1 molar ratio, as indicated by the linearity of the binding plots (Figures 5 and 6). The data are presented in tabular form in Table II.

Circular Dichroism Spectra of Protein-Bound Riboflavin Analogs. In order to be certain that the riboflavin analogs and FAD are bound in a specific manner, the circular dichroism spectra of these protein–flavin complexes were measured. The circular dichroism of the protein complexes of riboflavin, FAD, and isoriboflavin are quite similar to that of the FMN–protein complex (see paper I) both with respect to shape and intensity. Similarly, the circular dichroism spectra of the protein complexes of lumiflavin, deoxyriboflavin, and N-10-ω-carboxybutylisoalloxazine resemble that of the dFMN–protein complex (see paper I). The low rotational strength of the long-wavelength positive dichroic band is evident in the complexes of all three of the deoxy analogs, as was found for the dFMN–protein complex (see paper I). This is again evidence that the optical activity in the long-wavelength region reflects ring–side-chain interaction. The circular dichroism of the 2-thioFMN–protein complex is much different in shape and has a greater intensity than the spectrum of the unbound 2-thioFMN (which is quite similar to the circular dichroism spectrum of free FMN although red shifted).

The similar circular dichroism spectra of the bound riboflavin analogs and the protein-bound FMN analogs indicate

TABLE II: Equilibrium Binding Constants of Flavin Analogs to the Shethna Apoprotein.[a]

Flavin	K_a	Correlation Coefficient	$\Delta F°$ (kcal/Mole)	ΔF_u (kcal/Mole)
FMN	2.2×10^8	0.960	−11.3	−13.7
dFMN	1.3×10^8	0.986	−11.0	−13.4
3-MeFMN	3.7×10^7	0.983	−10.3	−12.7
IsoFMN	4.8×10^7	0.905	−10.4	−12.8
Riboflavin	1.8×10^6	0.991	−8.5	−10.9
Deoxyriboflavin	2.4×10^6	0.931	−8.7	−11.1
3-Methylriboflavin	Not bound			
Isoriboflavin	1.7×10^6	0.912	−8.5	−10.9
FAD	1.3×10^6	0.915	−8.3	−10.7
Lumiflavin	2.2×10^5	0.959	−7.2	−9.6
N-10-ω-Carboxybutylisoalloxazine	3.1×10^6	0.972	−8.8	−11.2
Tetra-O-acetylriboflavin	Not bound			
2-ThioFMN	Not determined			

[a] The association constant (K_a) was estimated from a least-squares analysis of the binding plots (Figures 5 and 6). The correlation coefficient is given for each plot. The standard molar free energy of binding ($\Delta F°$) was calculated from the expression: $\Delta F° = -RT \ln K_a$. The unitary free energy of binding (ΔF_u) was calculated using the standard Molar free energy less the eratic entropy value of 8 eu (Kauzmann, 1959).

TABLE III: Second-Order Rate Constants for Flavin Binding to the Shethna Apoprotein.[a]

Flavin	k (M^{-1} sec^{-1})
FMN	2.0×10^5
IsoFMN	2.9×10^5
3-MeFMN	1.7×10^5
dFMN	5.7×10^5
Riboflavin	$t_{1/2} = 0.5$ sec
Isoriboflavin	$t_{1/2} = 0.5$ sec
Deoxyriboflavin	Too fast to measure

[a] All kinetic rate constants were determined at a concentration of 2×10^{-6} M flavin and 2×10^{-6} M protein in 0.025 M phosphate buffer (pH 7.0) at room temperature ($25 \pm 2°$).

no major differences in interaction (although minor differences certainly exist), and thus suggest that the same protein binding site is involved. The apoprotein had very little effect on the circular dichroism spectra of 3-methylriboflavin and tetra-O-acetylriboflavin showing that any nonspecific interactions which may occur have no effect on flavin circular dichroism properties.

Kinetics of Flavin Binding to the Apoprotein. Further evidence for differences in the interactions of the various flavin analogs with the apoprotein can be seen in the binding kinetics. FMN binding follows second order kinetics with a rate constant of 2.0×10^5 M^{-1} sec^{-1} (Figure 6 and Table III). As indicated in Figure 7, equimolar quantities of FMN and protein gave linear second-order plots at three different concentrations. The 3-MeFMN and isoFMN analogs gave results which were quite similar to FMN whereas dFMN is bound considerably more rapidly (rate constant about three times that of FMN (Table III). Riboflavin and isoriboflavin were also bound more rapidly, with half-times of about 0.5 sec, as compared to 2.5 sec for FMN. The binding rate was too rapid to determine whether the process was first or second order.

Discussion

The results obtained here, in conjunction with those of Hinkson (1968) on FMN combining weights, indicate that the Shethna flavoprotein is a low molecular weight species consisting of a single polypeptide chain with one FMN molecule per mole of protein. The flavin can be reversibly resolved and reconstituted as judged by the criteria of far-ultraviolet, near-ultraviolet, and visible circular dichroism spectroscopy, which monitor the overall protein conformation and the protein–flavin and flavin–side-chain interactions (Edmondson and Tollin, 1971a). Further evidence for reversibility is given by a comparison of the semiquinone redox properties for the native and reconstituted flavoproteins (Edmondson and Tollin, 1971b).

The similarities in amino acid composition and overall protein conformation which exist among the three flavoenzymes considered here is indeed striking in view of the differences in molecular weight and the fact that the Shethna flavoprotein does not function as a flavodoxin in either the phosphoroclastic reaction or in the light-induced chloroplast reduction of TPN (Hinkson and Bulen, 1967). However, the Shethna flavoprotein can be reduced to the semiquinone form by illuminated chloroplasts at rates which are substantially faster (Benemann *et al.*, 1969) than photoreduction using EDTA as an electron donor (Massey and Palmer, 1966; Edmondson and Tollin, 1971b). The apparent reason for this inertness toward TPN reduction must await further experimentation. Of particular significance will be redox potential measurements. Any correlation of the similarities between amino acid composition and protein structure among the flavoproteins considered here and biological function must await the elucidation of the physiological role of the Shethna flavoprotein in *Azotobacter*.

As is shown in Table II, FMN is quite strongly bound to the protein. This K_a value is in good agreement with that published by Hinkson (1968). That the side-chain hydroxyl groups do not appreciably contribute to the binding energy is apparent from the similar values for FMN and dFMN and for riboflavin and deoxyriboflavin (Table II). The binding energy contributed by the terminal phosphate group is 2.8 kcal mole^{-1} for FMN, 2.3 kcal mole^{-1} for dFMN, and 1.9 kcal mole^{-1} for isoFMN (by comparison of the phosphate analogs with the nonphosphorylated flavins). A dramatic difference is observed in the binding properties of 3-MeFMN and 3-methylriboflavin. 3-MeFMN is bound quite strongly while 3-methylriboflavin is not bound at all. No fluorescence quenching, absorption spectral perturbations, or changes in circular dichroism spectrum were observed upon adding apoprotein to a solution of 3-methylriboflavin.

Modification of the isoalloxazine ring results in a decrease in binding energy of $\simeq$1 kcal mole^{-1} (by comparison of the binding energies of isoFMN and 3-MeFMN with that of FMN). The association constant for the 2-thioFMN–protein complex was not determined inasmuch as 2-thioFMN is nonfluorescent (Mitchell and Hastings, 1969). However, this flavin analog is bound quite strongly since it cannot be dialyzed away from the protein. A difference spectrum titration of 2-thioFMN with the apoprotein also indicated a large binding constant. The high concentration required ($\simeq 4 \times 10^{-5}$ M) made the determination of an equilibrium constant impossible.

The steric and electrostatic importance of the ribityl phosphate side chain is evident in the binding properties of FAD, *N*-10-ω-carboxybutylisoalloxazine, lumiflavin, and tetra-*O*-acetylriboflavin (Table II). FAD is bound with about 3 kcal mole^{-1} less energy than FMN, even though a negatively charged pyrophosphate group is present. *N*-10-ω-Carboxybutylisoalloxazine is bound with about 2 kcal mole^{-1} less energy than dFMN, although the former compound has a negatively charged carboxyl group. Lumiflavin is bound considerably less strongly than any of the other analogs. The bulky acetyl side-chain groups on tetra-*O*-acetylriboflavin prevent binding altogether, indicating a close fit between protein and flavin side chain.

The lumiflavin binding constant shows that the majority of the free energy (9–10 kcal mole^{-1}) in flavin–protein binding is derived from the isoalloxazine–protein interactions (the difference in binding energy between lumiflavin and deoxyriboflavin is only 1.5 kcal mole^{-1}). This finding, in conjunction with the observed lack of binding of tetra-*O*-acetylriboflavin, suggests that the ribityl side chain and the isoalloxazine ring are buried within the protein structure in close proximity to one another. The latter point is substantiated by circular dichroism and optical spectra (Edmondson and Tollin, 1971a).

Inasmuch as the complexing energy of flavin with phenols or indoles is about 3 kcal mole^{-1} (Tollin, 1968), it seems likely that several protein interactions occur with the isoalloxazine ring. The differences in circular dichroism spectra observed upon the substitution of the 3 position of the FMN molecule (Edmondson and Tollin, 1971a) indicates that this is probably one site of interaction. This is further substantiated by the shift in pK of the N-3 proton from $\sim$10 in unbound FMN (Ehrenberg and Hemmerich, 1968) to above 11 for the Shethna flavoprotein (as determined by measuring the optical difference spectrum in the visible region as a function of pH). A true pK value is technically impossible to obtain due to major changes in protein structure which occur above pH 12.0 (as evidenced by circular dichroism spectra). The positive free-energy contribution to binding from the 3-methyl group in 3-methylriboflavin must be quite large since it overcomes the negative free energy from the flavin ring and side-chain interactions with the protein. Such a large free-energy term could not be expected to be counteracted by the 2.5–3 kcal mole^{-1} estimated for the phosphate interaction with the protein. Thus, to explain the lack of binding of 3-methylriboflavin and the strong binding of 3-MeFMN it is necessary to postulate that the protein–flavin interactions are different with the FMN analogs than with the riboflavin analogs. The possible cause of such a different interaction could be a phosphate-induced change in the flavin binding site in the protein (see below for further discussion).

Fluorescence Quenching of the Flavin Analogs upon Binding. The flavin fluorescence is completely quenched in all of the FMN analog–protein complexes. With the riboflavin analogs, the flavin fluorescence is completely quenched only for riboflavin and isoriboflavin. For those analogs without side-chain hydroxyl groups (deoxyriboflavin, *N*-10-ω-carboxybutylisoalloxazine, and lumiflavin), the ratio of fluorescence of the protein–flavin complex to that of the unbound flavin is 0.49, 0.48, and 0.88, respectively. These data suggest that the hydroxyl groups play a role in the nonradiative dissipation of singlet energy in the riboflavin analogs (see paper I for evidence that ring–side-chain interaction is increased upon binding FMN to apoprotein) and that there is more than one mechanism for the total quenching of protein-bound flavin fluorescence (since dFMN fluorescence is totally quenched). These differences in fluorescence quenching are not due to binding energy differences, since, for example, deoxyriboflavin is bound as strongly as riboflavin (Table II). The total quenching of the fluorescence of dFMN demonstrates that quenching by protein groups is a much more efficient process with this analog than is the case with deoxyriboflavin. This is a further indication of the difference in protein–flavin interactions in the FMN analogs and the riboflavin analogs. The ratio of flavin fluorescence of the FAD–protein complex to that of free FAD is 0.34. This is difficult to compare directly with the other quenching data since FAD has only one-fifth the fluorescence of the other flavin analogs (Penzer and Radda, 1967). It is clear, however, that the AMP group does interfere with the quenching processes.

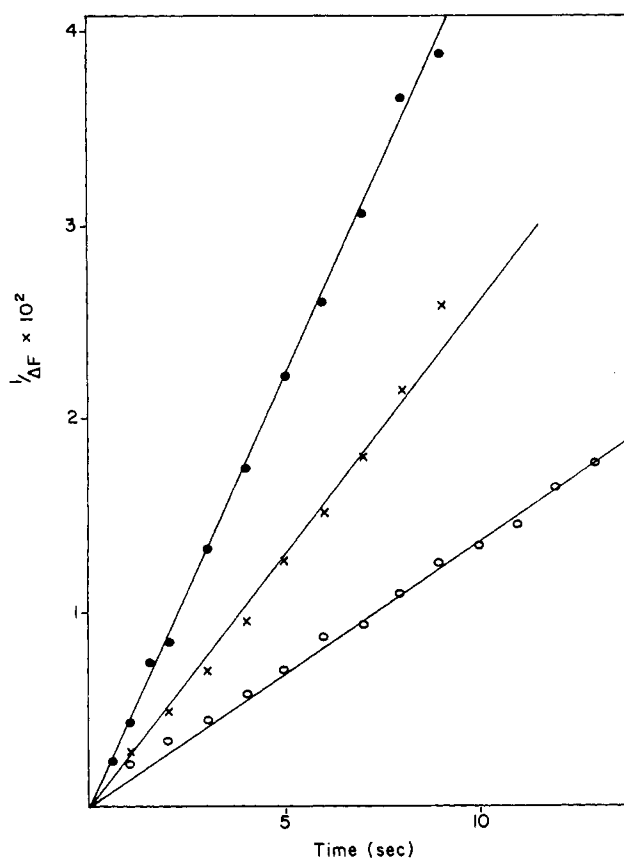

FIGURE 7: Second-order rate plot of FMN binding to the Shethna apoprotein. The rate of binding was determined fluorometrically in 0.025 M phosphate buffer (pH 7.0) at 25 ± 2°. The individual plots are for the following equimolar concentrations of FMN and apoprotein: (●) 2×10^{-6} M, $k = 2.0 \times 10^5$ M^{-1} sec^{-1}; (×) 1×10^{-6} M, $k = 1.8 \times 10^5$ M^{-1} sec^{-1}; and (○) 4×10^{-7} M, $k = 2.0 \times 10^5$ M^{-1} sec^{-1}.

The kinetic data in Table III clearly demonstrate the importance of the side chain in the binding process. The large increase in binding rate upon removal of the terminal phosphate group indicates that the phosphate–protein interaction occurs within the protein structure and not on the surface, in agreement with our previous conclusion. Tryptophan fluorescence quenching is also seen upon binding of the flavin analogs (D'Anna and Tollin, 1971). The second-order rate constants for tryptophan quenching are approximately the same as for flavin quenching for each of the analogs.

The higher binding rate of dFMN is also qualitatively seen in comparing deoxyriboflavin with riboflavin (Table III). The rate of deoxyriboflavin binding was too fast to be measured under the conditions employed. Thus, the side-chain hydroxyl groups also play a role in the binding process, although as we have seen, they do not significantly contribute to the binding energy (Table II). Since flavin ring modifications do not appreciably affect the binding rate, the side-chain interactions with the protein probably represent the initial, and rate-determining, step in the binding process.

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