Flavin–Protein Interactions and the Redox Properties of the Shethna Flavoprotein*

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ABSTRACT: The redox properties of several flavin analog complexes of the Shethna apoprotein and of some flavodoxins have been studied. Modification of the flavin ring and side chain affect the photoreduction, dithionite reduction, and oxygen oxidation behavior of the analog proteins. Evidence is presented which indicates a role of the side-chain hydroxyls and phosphate in stabilizing the flavoprotein semiquinone. Dithionite reduction of the Shethna flavoprotein to the hydroquinone form is autocatalytic between pH 7.0 and 8.0. The semiquinone form is shown to be an intermediate in this process. The pH dependence suggests that the ionization state of the protein-bound flavohydroquinone determines whether or not full reduction can occur. The pH dependence of oxygen oxidation of the Shethna flavoprotein semiquinone is interpreted in terms of the conversion of this species from the neutral into the anionic radical form. A correlation is found to exist between the ultraviolet spectra and oxygen reactivity of the various flavoprotein semiquinones which implies that the protein environment is important in determining redox behavior. The tyrosyl titration properties of the Shethna protein show that flavin–tyrosine interactions occur upon flavin binding. Circular dichroism spectra indicate that no major protein conformational changes occur upon flavin reduction to either the semiquinone or hydroquinone forms.

Massey and Palmer (1966) have proposed the identification of the red and blue flavoprotein semiquinones with the anionic and neutral flavin radical species, based on the pH dependence of the spectra of the glucose oxidase semiquinone. Verification of this has come from spectral and electron spin resonance work (Ehrenberg *et al.*, 1967) and pulse radiolysis studies (Land and Swallow, 1969). The pK for the ionization of the unbound neutral semiquinone is 8.3–8.4, as shown by potentiometric titration (Draper and Ingraham, 1968), pulse radiolysis (Land and Swallow, 1969), electron spin resonance studies (Ehrenberg *et al.*, 1967), and flash photolysis (S. P. Vaish and G. Tollin, 1970, manuscript in preparation).

The Shethna flavoprotein forms a blue (neutral) semiquinone which displays unusual redox behavior toward oxygen and dithionite (Hinkson and Bulen, 1967). This presents an interesting contrast to the semiquinones of a number of other flavoenzymes (Massey and Palmer, 1966). Because these properties are modified by urea treatment, Hinkson and Bulen (1967) have postulated that they are caused by protein–flavin interactions.

The reversible resolution and recombination of the Shethna flavoprotein (see paper II of this series) provides an opportunity to identify the portions of the flavin molecule important in the interactions which determine the redox properties of the protein-bound semiquinone. As a comparison, the redox properties of several other flavoenzymes have been examined, as well as the effect of flavin redox form on protein properties such as ultraviolet circular dichroism spectra and tyrosyl ionization behavior.

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FMN was obtained from Calbiochem, Los Angeles, Calif., and was purified by DEAE-cellulose column chromatography. FAD was from Sigma Chemical Co., St. Louis, Mo. All other materials were as described in papers I and II of this series.

Absorption and difference spectra were measured on a Cary Model 14R spectrophotometer. A Gilford Model 240 or a Coleman Model 124 spectrophotometer were used for other spectral measurements. pH measurements were made using a Leeds and Northrup pH meter and the Leeds and Northrup miniature pH electrode assembly. All other methods were as described in papers I and II.

Tyrosine Spectrophotometric Titration. The tyrosyl ionization behavior of the apoprotein and of the holoprotein were followed spectrophotometrically at 245 nm. Absorbance measurements were made directly after adding microliter amounts of 5 N NaOH to the protein solution in 0.1 M glycine buffer. The pH of the solution was determined in the spectrophotometer cell after the absorbance measurement.

The tyrosyl ionization curve of the semiquinone form of the protein was measured anaerobically on individual samples. A calculated amount of 5 N NaOH was added to the side arm of a quartz Thunberg cuvet. After thoroughly flushing out any oxygen by alternate evacuation with an oil pump and purging with oxygen-free nitrogen, the base was tipped into the protein solution and the change in absorbance at 245 nm immediately determined. The change at 580 nm was quite small during this procedure indicating negligible loss of semiquinone. The pH of the solution was determined after opening the cuvet.

To calculate the extent of tyrosyl ionization, the change in molar extinction at 245 nm was assumed to be 11,500 M^{-1} cm^{-1} (Wetlaufer, 1962). The ionization of the semiquinone form had to be followed anaerobically and quickly due to the increased rate of oxidation by O_2 and to disproportionation of the radical at high pH. For this latter reason the measurements represent initial values for all three forms. No determinations were made of the total number of tyrosines titrated upon long standing at a given pH. Reverse titrations of the apoprotein and holoprotein indicated reversibility of ionization. A reverse titration could not be performed on the semi-

TABLE I: Photochemical Semiquinone Production and Chemical Reduction of Flavoenzymes and FMN Analog–Protein Complexes at pH 7.0.

Flavoenzyme	Rate of Photoreduction, $t_{1/2}$ (hr)	Dithionite Stability of FH·	Rate of Dithionite Reduction, $t_{1/2}$
Shethna flavoprotein	70	Yes	16 min
FMN–apoprotein	63	Yes	
IsoFMN–apoprotein	125	No	Fast[a]
dFMN–apoprotein	5.5	Yes	5 min
3-MeFMN–apoprotein	4.8	No	Fast
2-ThioFMN–apoprotein	3.7	No	Fast
R. rubrum flavodoxin	28.8	Yes	*b*
P. elsdenii flavodoxin	1.5	No	*b*
Clostridial flavodoxin	0.4	No	*b*
Shethna flavoprotein in 3 M $(NH_4)_2SO_4$	0.3	No	Fast
Shethna flavoprotein + 5 mole % FMN	5.0	Yes	*b*

[a] This was beyond the time resolution of our procedure (≃1 sec). [b] Not measured.

quinone form because of the previously mentioned technical difficulties.

Preparation of Semiquinone Form of Flavoenzymes. The Shethna flavoprotein can be isolated from *Azotobacter* in its semiquinone form under anaerobic conditions. Preliminary experiments indicated no change in the spectral properties of the semiquinone prepared by other methods compared to those of the naturally reduced material.

Flavoprotein free radicals were ordinarily prepared by illumination of the samples in the presence of EDTA, according to the method of Massey and Palmer (1966). In all photoreduction experiments, the EDTA concentration was 0.05 M. The enzyme solutions were made anaerobic in a Thunberg cuvet by alternate evacuation and purging with oxygen-free nitrogen. After approximately six cycles of evacuation and flushing, the enzyme solution was left under a slight positive pressure of nitrogen.

Illumination was carried out at 4° in a cold room with a 150-W tungsten lamp approximately 20 cm from the sample. The sample was kept cold by circulating water through a small glass dewar flask from a reservoir in the cold room. This apparatus was used to measure all rates of photoreduction. In some instances where a more rapid photoreduction was desired, the sample was placed in a beaker of ice water and illuminated with a 625-W Sylvania sun gun lamp at a distance of approximately 30 cm. A heat filter and two focusing lenses were used in this apparatus.

Since the photoreduction of the Shethna flavoprotein was quite slow, a method was devised for a more rapid production of semiquinone. When the flavoprotein was irradiated in the presence of 2 M $(NH_4)_2SO_4$ and 0.05 M EDTA at pH 7.0, semiquinone formation was complete in about 12 hr. After reduction, the sample was desalted by passing through a Sephadex G-10 column (2 × 25 cm) made anaerobic by equilibration and elution with nitrogen-purged buffer. No differences were noted in the properties of the semiquinone prepared in this way or as described above.

Results

Photochemical Reduction. A striking difference between riboflavin and FMN analog complexes of the Shethna apoprotein is that stable semiquinones are formed only with the FMN complexes. Upon photoreduction, only fully reduced forms are generated from the protein complexes of riboflavin analogs or of FAD. When oxygen is added, they are very rapidly oxidized all the way to the fully oxidized flavoproteins.

The rate of photoreduction of the Shethna flavoprotein is quite slow (Table I), in agreement with earlier studies (Massey and Palmer, 1966; McCormick *et al.*, 1967). The reconstituted FMN–protein complex is photoreduced at approximately the same rate as the native protein, indicating no irreversible changes upon resolution and reconstitution (*cf.* paper II of this series). The protein complexes of 3-MeFMN, dFMN, and 2-thioFMN are reduced more rapidly than the FMN protein, whereas the isoFMN derivative is reduced somewhat more slowly (Table I). The photoreducibility of bound FMN analogs is thus quite sensitive to the presence of side-chain hydroxyl groups and to modifications in the pyrimidine ring, but less sensitive to modification in the benzenoid portion of the flavin ring system.

A plot of A_{450} *vs.* A_{580} should be linear for a one-electron reduction of the flavoproteins, but will become nonlinear if further reduction of the flavin to the hydroquinone occurs. Figure 1 indicates that very little hydroquinone is produced in the photoreduction of the protein complexes of FMN and isoFMN, while some complete reduction occurs with the dFMN, 3-MeFMN, and 2-thioFMN analogs. This is consistent with the rate studies. Substantial hydroquinone formation, however, occurs when the photoreduction of the Shethna flavoprotein is carried out in 3 M $(NH_4)_2SO_4$ (Figure 1). Some flavin hydroquinone formation was also found to occur with *Peptostreptococcus elsdenii* and Clostridial flavodoxins, but not with the *Rhodospirillum rubrum* flavodoxin.

R. rubrum flavodoxin is photoreduced at an intermediate rate, while the *P. elsdenii* and Clostridial flavodoxins are photoreduced much more rapidly than any of the other species (Table I). The rate of photoreduction of the Shethna flavoprotein is increased quite dramatically by high salt concentration (3 M $(NH_4)_2SO_4$) or by the addition of free flavin (Table I). The enhanced rate and increased formation of flavohydroquinone in the presence of $(NH_4)_2SO_4$ is probably due partly to free flavin dissociated from the protein and also to changes in protein structure. The latter is suggested by the lack of semiquinone stability to dithionite reduction under these conditions (see below). The circular dichroism spectra of the oxidized and semiquinone forms of the Shethna flavoprotein in 3 M $(NH_4)_2SO_4$ were found to be identical in the visible and the near-ultraviolet spectral regions to those of the flavoprotein in low ionic strength buffer (0.025 M phosphate–0.05 M EDTA). Thus, any protein structural changes were not reflected in the circular dichroism data and consequently are probably fairly localized.

An enhanced rate of photoreduction of D-amino acid oxidase upon adding free FMN or FAD has also been observed (McCormick *et al.*, 1967). Such effects are probably due to the rapid reduction of free flavin by illumination, followed by reduction of the protein-bound flavin by free flavin hydroquinone.

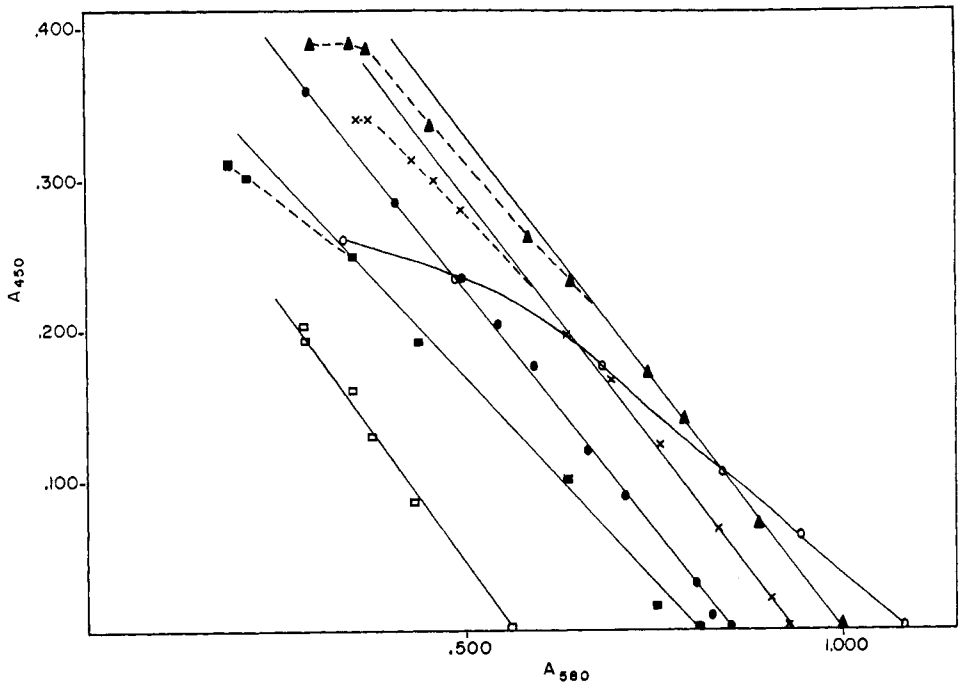

FIGURE 1: Relation between decrease of oxidized flavoprotein absorbance and increase in semiquinone absorbance during photoreduction. All photoreductions were carried out 4° in 0.025 M phosphate–0.05 M EDTA buffer (pH 7.0). Further details are given in the Experimental Section. (●) Shethna flavoprotein (native and reconstituted). (○) Shethna flavoprotein plus 3 M $(NH_4)_2SO_4$. The FMN analog–Shethna apoprotein complexes are: (×) dFMN, (▲) 3-MeFMN, (□) isoFMN, and (■) 2-thioFMN (for this analog the ordinate is the absorbance at 490 nm and the abscissa the absorbance at 670 nm).

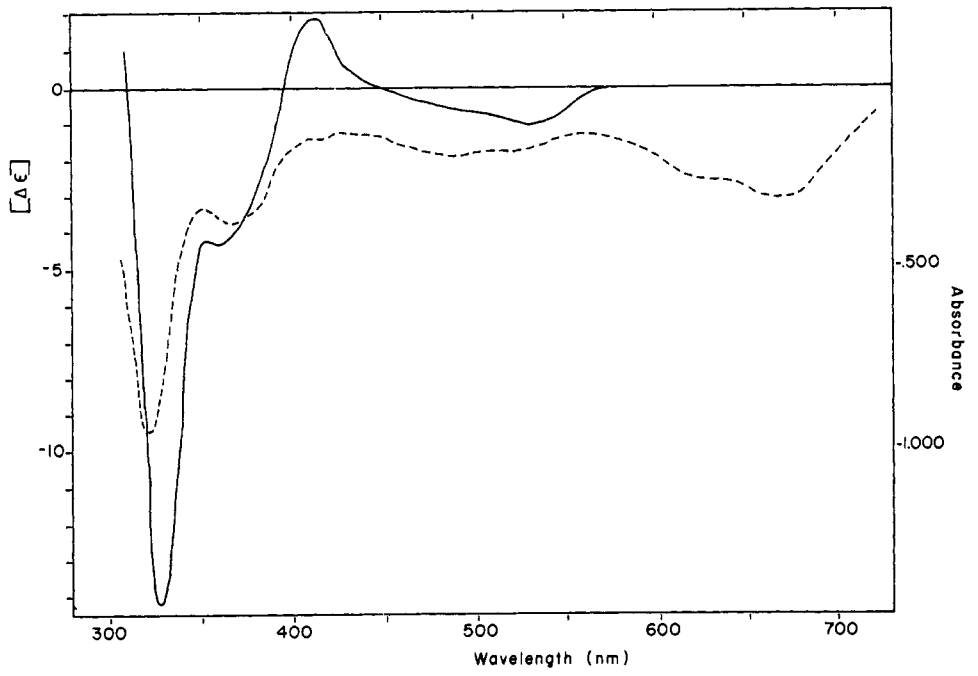

FIGURE 2: Circular dichroism and absorption spectra of the semiquinone form of the 2-thioFMN–Shethna apoprotein complex in the visible region. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA buffer (pH 7.0). Note that the absorption spectrum (dashed line) is inverted to facilitate comparison.

The absorption and circular dichroism spectra of the 2-thioFMN–Shethna protein semiquinone are presented in Figure 2. The long-wavelength absorption band (670 nm) indicates that this semiquinone is also in its protonated form. The photoreduced material was paramagnetic, as were the semiquinones of all the other flavin analogs (determined by electron spin resonance spectrometry). The circular dichroism spectrum (Figure 2) shows no optical activity in the long-wavelength (600-nm) region, suggesting weak side-chain–ring interaction see paper I of this series). The other bands in the absorption spectum have their counterparts in the circular dichroism spectrum. The circular dichroism and absorption spectra of

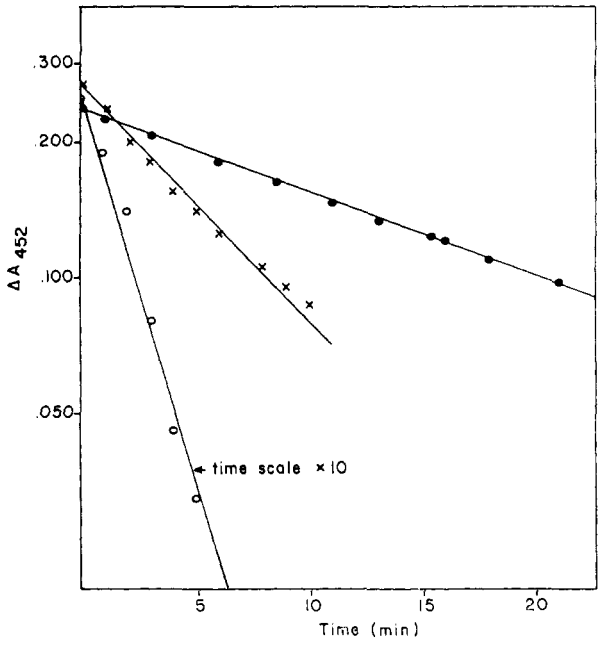

FIGURE 3: First-order rate plot of flavoprotein reduction by dithionite. All reactions were carried out in 0.025 M phosphate buffer (pH 7.0) at room temperature. (●) Shethna flavoprotein, (○) Shethna flavoprotein plus a catalytic amount of methyl viologen (1 mole %), and (×) dFMN–Shethna apoprotein complex. The reductions were carried out under anaerobic conditions with a large excess (≃1 mg) of dithionite.

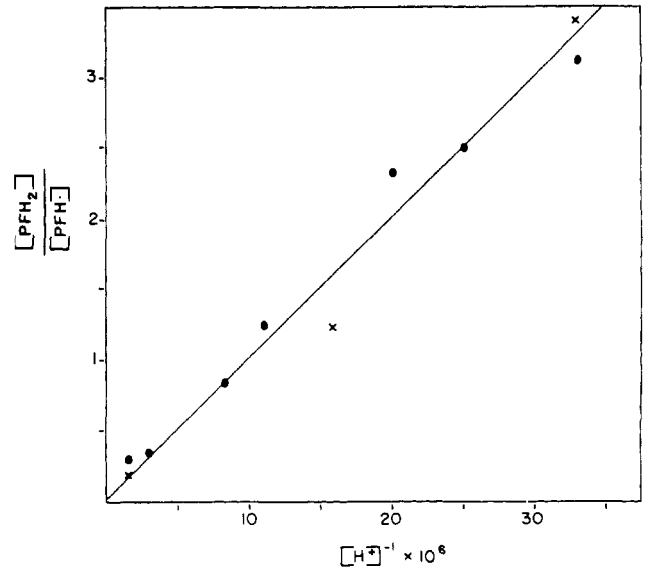

FIGURE 5: Ratio of Shethna flavoprotein hydroquinone to semiquinone as a function of the reciprocal hydrogen ion concentration. The semiquinone (PFH·) concentration was estimated from the absorbance at 580 nm at equilibrium. The hydroquinone (PFH_2) was assumed to account for the rest of the total flavin concentration. The buffers used were 0.1 M Tris–0.33 M phosphate above pH 7.0 and 0.1 M phosphate below pH 7.0. No differences were observed with the different buffers at the same pH. The closed circle denotes the presence of a catalytic amount of methyl viologen and the ×'s are samples with no methyl viologen. The temperature was 2.5 ± 2°. A large excess of dithionite (≃1 mg) was added to the flavoprotein under anaerobic conditions. The pH was measured before and after the measurement.

the reoxidized material were indistinguishable from those of the nonirradiated 2-thioFMN–protein.

Chemical Reduction by Dithionite. At neutral pH and low ionic strength, the semiquinone form of the Shethna flavoprotein cannot be further reduced, even with a large excess of dithionite (Hinkson and Bulen, 1967). The reconstituted FMN–protein complex also retains this property (Hinkson, 1968). Our results (Table I) verify these observations. Also of interest is that ring-modified FMN analog–protein complexes are reduced to the hydroquinone form, whereas removal of the hydroxyl groups (dFMN) has no effect on semiquinone stability to dithionite. In the latter case, the rate of reduction of the oxidized form is increased to about 2.5 times that of the FMN–protein complex (Table I and Figure 3). In both cases, the reduction rate follows pseudo-first-order kinetics (Figure 3). All of the riboflavin analog–protein complexes were rapidly reduced to the hydroquinone form, again indicating the importance of the terminal phosphate group in determining protein-bound flavin properties.

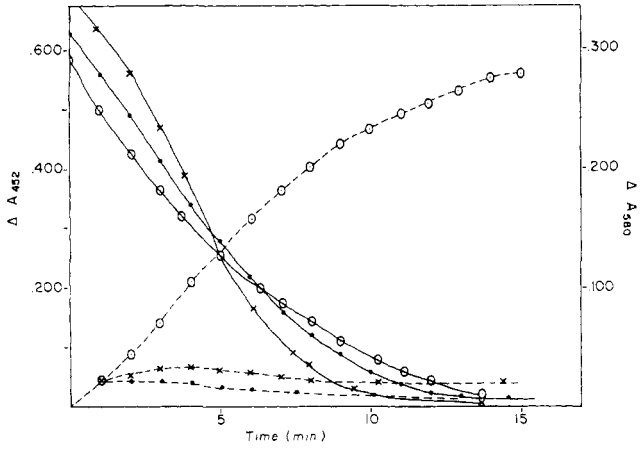

FIGURE 4: Rate of Shethna flavoprotein reduction by dithionite as a function of pH. The reactions were carried out in 0.1 M Tris buffer at the appropriate pH under anaerobic conditions with an excess (≃1 mg) of dithionite at 25 ± 2°. The solid lines refer to the change in absorbance at 452 nm while the dashed lines designate the change in absorbance at 580 nm. The absorption changes at both wavelengths were monitored during the same reaction. (○) pH 7.0, (×) pH 7.7, and (●) pH 10.4.

R. rubrum flavodoxin is also resistant to reduction past the semiquinone form by dithionite at pH 7.0 (M. Cusanovich, 1970, unpublished data), whereas *P. elsdenii* and Clostridial flavodoxins are reduced to the hydroquinone form (Mayhew *et al.*, 1969; Knight and Hardy, 1967). Circular dichroism spectral properties which correlate with this chemical behavior have been discussed previously (see paper I of this series).

High salt concentration (3 M $(NH_4)_2SO_4$) removes the semiquinone stability of the Shethna flavoprotein to dithionite reduction, while added free flavin has no effect (Table I). The addition of a catalytic amount of methyl viologen (1 mole %) increases the rate of reduction by a factor of 80 (Figure 3) but has no effect on semiquinone stability to further reduction. The reaction rate again follows pseudo-first-order kinetics (Figure 3).

At pH 8.0, the Shethna flavoprotein semiquinone is no longer resistant to further reduction. The semiquinone form is, however, still an intermediate in the conversion of the oxidized protein to the hydroquinone form, as is shown in Figure 4. Thus, one observes a steady state in semiquinone con-

TABLE II: Oxygen Oxidation of the Neutral Semiquinone Forms of Flavoenzymes and FMN Analog–Shethna Apoprotein Complexes.[a]

Flavoenzyme	$t_{1/2}$ (min)	k (min^{-1})
Shethna flavoprotein	2000	3.5×10^{-4}
Shethna flavoprotein, no EDTA	3000	2.3×10^{-4}
Shethna flavoprotein in 3 M $(NH_4)_2SO_4$	41	1.7×10^{-2}
FMN–apoprotein	2000	3.5×10^{-4}
dFMN–apoprotein	78	9.0×10^{-3}
2-ThioFMN–apoprotein	41	1.7×10^{-2}
3-MeFMN–apoprotein	108	6.4×10^{-3}
IsoFMN–apoprotein	44	1.6×10^{-2}
Clostridial flavodoxin	115	6.0×10^{-3}
P. elsdenii flavodoxin	80	8.7×10^{-3}
R. rubrum flavodoxin	260	2.6×10^{-3}
Glucose oxidase	1.2	0.58

[a] The rates of oxidation were determined at room temperature in 0.025 M phosphate–0.05 M EDTA (pH 7.0) except where otherwise indicated. The rate of oxidation of the glucose oxidase semiquinone was determined in 0.1 M acetate–0.05 M EDTA (pH 6.0). Saturating conditions of oxygen ($\approx 10^{-3}$ M) were maintained by bubbling air through the solutions at intervals. Semiquinone concentrations were 5–8 $\times$ 10^{-5} M in all cases.

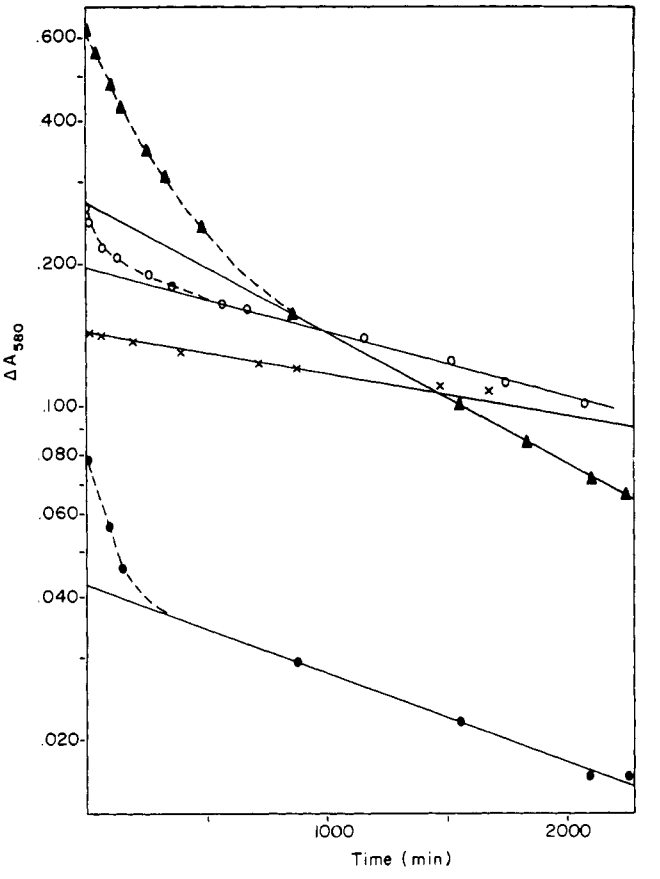

FIGURE 6: First-order rate plot of Shethna flavoprotein semiquinone oxidation by oxygen. Photoreduced PFH· in 0.025 M phosphate–0.5 M EDTA (pH 7.0). (▲) 1.14×10^{-4} M; (●) 1.46×10^{-5} M; (○) dithionite-reduced PFH· (5×10^{-5} M) in 0.025 M; phosphate–0.5 M EDTA buffer (pH 7.0); (×) PFH·photoreduced in 3 M $(NH_4)_2SO_4$, 0.025 M phosphate–0.05 M EDTA (pH 7.0). The semiquinone was freed from salts by anaerobic Sephadex G-10 column chromatography. The buffer present during the kinetic run was 0.025 M phosphate (pH 7.0).

centration with a time course consistent with its being an intermediate species.

At pH 7.0, the absorption changes at 452 nm and at 580 nm upon addition of excess dithionite follow a monophasic (exponential) curve, while at pH's between 7.0 and 8.0, the decrease in absorbance at 452 nm is initially slow and then changes more rapidly at later times (Figure 4). Above pH 8.0, monophasic kinetics are again followed. This type of behavior is indicative of an autocatalytic reduction of the oxidized flavoprotein to the hydroquinone form *via* the semiquinone. The latter can be generated by a disproportionation reaction and, in order to account for these results, would have to react more rapidly with dithionite than the oxidized form

$$PF \xrightarrow[\text{slow}]{S_2O_4^{2-}} PFH\cdot \xrightarrow[\text{fast}]{S_2O_4^{2-}} PFH_2 \quad (1)$$

$$PF + PFH_2 \xrightarrow{\text{fast}} 2PFH\cdot \quad (2)$$

The addition of flavoprotein in the hydroquinone form to oxidized material results in a very rapid production of semiquinone (less than 5 sec), thereby establishing that reaction 2 could be operative. This reaction seems to be essentially irreversible, inasmuch as no disproportionation of semiquinone can be observed in this pH region. In contrast with the riboflavin derivatives, upon adding oxygen to the hydroquinone form, a rapid oxidation to the semiquinone results at all pH's. Thus, no change in semiquinone stability to oxygen occurs in going from pH 7.0 to 8.0 (see below). These results are in agreement with the pathway of oxygen oxidation of the dehydrogenase hydroquinones proposed by Massey *et al.* (1969).

The pH dependence of hydroquinone formation by an excess of dithionite was investigated. The reaction was allowed to proceed for a long enough time to assure that equilibrium had been achieved. The fact that this is an equilibrium process can only be explained if the species which is ionizing is PFH_2. If either PF or PFH· were ionizing, the excess dithionite would serve to drive the reaction to completion. If a one-proton process is occurring, a plot of the ratio of PFH_2 to PFH· *vs.* $1/[H^+]$ should be linear. As is shown in Figure 5, this is the case. Note that the plot goes through the origin. This is consistent with an ionization of the hydroquinone species rather than the semiquinone or oxidized forms. No differences were caused by the addition of a catalytic amount of methyl viologen, although the equilibrium was established much faster when the viologen was present. The slope of the plot gives an apparent pK for the ionization of 7.0.

Oxidation by Molecular Oxygen. The semiquinone form of the Shethna flavoprotein is unique because of its low reactivity toward oxygen. As indicated in Figure 6 and Table II, the half-time for the oxidation is 2000–3000 min. Semiquinone forms prepared either by photoreduction or by dithionite reduction are oxidized at the same rate. However, the presence of EDTA was found to cause a fast initial phase in the oxidation which is independent of semiquinone concentration (Fig-

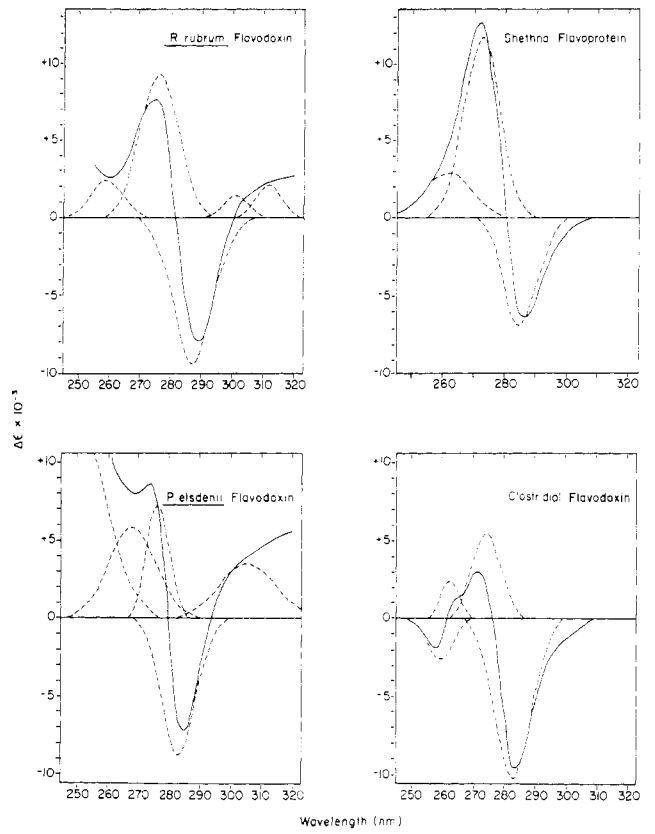

FIGURE 7: Resolved ultraviolet difference spectra of flavoenzyme semiquinone minus oxidized forms. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA buffer (pH 7.0).

ure 6). When the semiquinone was prepared by photoreduction in the presence of 2 M $(NH_4)_2SO_4$ and 0.05 M EDTA and then freed from the salts by anaerobic Sephadex G-10 column chromatography, no initial fast phase was observed. Subsequent addition of EDTA to this material greatly increased its rate of oxidation. The dithionite-prepared semiquinone also showed an initial fast phase in the rate of oxidation when EDTA was present (Figure 6). These results indicate that the faster oxidation rate is caused by EDTA and not by mechanistic complexities in the pseudo-first-order oxidation of the semiquinone by oxygen or by irreversible protein changes upon photoreduction. It is possible that EDTA–trace metal complexes could act as catalysts in the initial oxidation and become ineffective at a later stage due to poisoning.

A faster initial rate of oxidation in the presence of EDTA was also observed with the semiquinones of the 3-MeFMN–apoprotein complex, the Shethna flavoprotein semiquinone in 3 M $(NH_4)_2SO_4$, glucose oxidase, and the dFMN–apoprotein complex. None of the other semiquinone species showed this behavior. In Table II, the rates of oxidation given are for the slow component of the rate curve. All rates followed pseudo-first-order kinetics beyond the initial stage.

The semiquinone form of the reconstituted Shethna flavoprotein was oxidized at the same rate as the native form (Table II), thereby providing further evidence that no irreversible changes occurred upon resolution.

The reactivity of the protein-bound FMN semiquinone with oxygen is quite sensitive to modification of the isoalloxazine ring, as well to the absence of side-chain hydroxyl groups (Table II). The 2-thioFMN and isoFMN semiquinones were oxidized at a rate 50 times that of FMN, while the 3-MeFMN analog was oxidized 20 times faster. The absence of ribityl hydroxyl groups (dFMN) increased the rate of oxidation by a factor of 25. The presence of high salt concentration also increases the oxidation rate of the Shethna flavoprotein (a factor of 50) thereby establishing that the interactions which stabilize the semiquinones to oxygen oxidation are sensitive to ionic strength.

The semiquinone forms of *P. elsdenii* and Clostridial flavodoxin are oxidized about 20–25 times faster than the Shethna flavoprotein (Table II). The pseudo-first-order rate constant for *P. elsdenii* flavodoxin found here is in good agreement with a value determined by Mayhew *et al.* (1969) of 8×10^{-3} min^{-1} at pH 5.2.

The neutral semiquinone of glucose oxidase is oxidized at a rate which is 10–1000 times faster than that of the other flavoprotein semiquinones (Table II). This large increase in rate could be due to the presence of a small amount of the anionic radical in equilibrium with the neutral radical. Flash photolysis work has established that the unbound anionic flavin radical reacts with oxygen at least 10^4 times faster than the neutral radical, with a second-order rate constant of 3×10^8 M^{-1} sec^{-1} (S. P. Vaish and G. Tollin, 1970, manuscript in preparation). Since the p*K* of the glucose oxidase semiquinone is 7.5 (Massey and Palmer, 1966), at pH 6.0 some of the anionic radical would be present. The possibility that this faster rate of oxidation is due to the anionic radical precludes a direct comparison of glucose oxidase semiquinone reactivity toward oxygen with that of the other flavoenzyme semiquinones. No spectral evidence for the presence of anionic semiquinone has been obtained for *P. elsdenii* flavodoxin (Mayhew and Massey, 1969) or for the Shethna flavoprotein in this study, even at pH 11. This indicates that for these two dehydrogenases, the p*K* of the flavin semiquinone is shifted from 8.4 to values greater than 11. The differences in oxygen reactivities which we have observed for these proteins at pH 7.0 therefore are probably not due to small concentrations of anion radical in equilibrium with the neutral form.

It is of considerable interest that the redox properties of the semiquinone of *R. rubrum* flavodoxin are in many respects intermediate between those of the Shethna protein and the other flavodoxins, in view of the similar relationship which exists in the circular dichroism spectra (paper I of this series). This provides a further indication that circular dichroism properties are a sensitive indicator of flavin environment and hence redox behavior.

Near-Ultraviolet Absorption Spectra of Oxidized and Semiquinone Flavoproteins. In an attempt to correlate the oxygen reactivity of the flavoprotein semiquinones with structural parameters, the ultraviolet absorption and circular dichroism spectra of the oxidized and semiquinone forms were determined. The ultraviolet difference spectrum (semiquinone form minus oxidized form) of the Shethna flavoprotein shows a negative band at 287 nm and a positive band at 272 nm (Figure 7). The extinction differences are much too large to ascribe to an aromatic amino acid perturbation.

Oxidized free FMN has an absorption band at 267 nm with a molar extinction of about 32,000 M^{-1} cm^{-1} (Figure 8). The ultraviolet spectrum of protein-bound FMN (Figure 8) shows a hypochromic effect at this wavelength of about 20%, with little or no wavelength shift. A hyperchromic effect is observed at longer wavelengths (280–300 nm). From the absorption spectrum of unbound, neutral flavin semiquinone (Land and Swallow, 1969) one would expect an ultraviolet absorption band in this species to be below 260 nm. On the basis of these

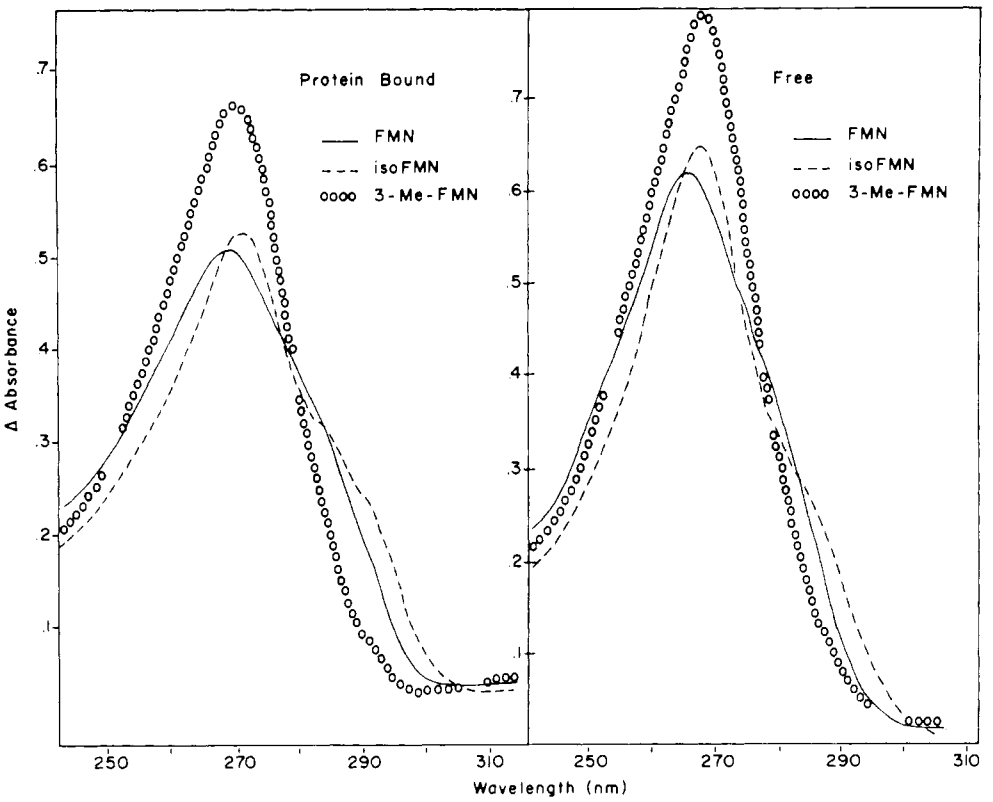

FIGURE 8: Ultraviolet spectra of protein-bound and free FMN analogs. The spectra of the protein-bound flavins were measured as difference spectra of protein–flavin complex minus apoprotein in 0.025 M phosphate buffer (pH 7.0). In both cases, flavin concentration was 2.0×10^{-5} M.

observations, it is possible to ascribe the negative band in the 280-nm region of the difference spectrum (Figure 7) to the decrease in absorbance at this wavelength of the oxidized flavin in going to the semiquinone form. The increase in extinction at 272 nm in the difference spectrum could be due to a red shift of the ultraviolet absorption band of the semiquinone (by at least 10–15 nm) in going from water to the environment provided by the protein. If this is the correct explanation, one can infer that either the protein environment of the semiquinone form is quite different from that of the oxidized flavin for which no major spectral shifts are evident (Figure 8), or that the semiquinone spectrum is more sensitive to environmental changes than is the spectrum of the oxidized form. Another alternative is to ascribe this increased absorption to a charge-transfer type of interaction between the semiquinone and an aromatic amino acid residue. The actual assignment of the cause of this increased extinction must await further experimentation.

The same difference spectral pattern is also present in all of the flavoenzyme and FMN analog–Shethna apoprotein complexes studied. In the case of the 3-MeFMN analog, the increased extinction of the oxidized flavin (Figure 8) cancels out the positive extinction of the semiquinone form at 275 nm. The 2-thioFMN analog exhibits the same general behavior, although the spectral changes are red shifted to above 300 nm.

In order to obtain a more accurate estimation of the positive absorbance of the various semiquinones, the difference spectra were resolved into Gaussian components. This procedure minimizes cancelling effects by the negative absorption difference, as well as additive effects caused by adjacent absorption bands, as is evidently occurring with the *P. elsdenii* flavodoxin (Figure 7). More error is involved in fitting the 3-MeFMN spectrum than in those cases in which the difference spectra have a shape similar to that of the FMN protein. An error of approximately 10% is assumed, as judged by the range of successful alternatives observed in fitting the spectra. The analyses obtained are shown by the dashed curves in Figure 7.

As is indicated in Figure 9, a relatively linear relationship is found between the extinction of the positive band in the difference spectrum and the negative logarithm of the rate of oxygen oxidation of the semiquinone form of the flavoenzyme or FMN analog–Shethna apoprotein complex. This suggests that the interactions which cause this increase in extinction also increase the activation energy for the reaction with molecular oxygen.

Near-Ultraviolet Circular Dichroism Spectra of Oxidized and Semiquinone Flavoproteins. The circular dichroism properties of the Shethna flavoprotein in its oxidized and semiquinone forms also reflect the patterns seen in the optical difference spectrum (Figure 10). The negative Cotton effect at 270 nm in the oxidized form is increased by about 40% upon semiquinone formation. Also, an increase in positive dichroism is apparent at 285 nm. The semiquinone form of isoFMN shows only a slightly increased negative 270-nm circular dichroism band, while this band is decreased in rotational strength in the case of the 3-MeFMN semiquinone form (Figure 10). The dFMN–protein complex parallels the FMN–protein complex quite closely in circular dichroism spectral properties.

The rotational strengths of the circular dichroism bands in the FMN analog complexes between 280 and 300 nm are also significantly increased when compared to these bands in the apoprotein (Figure 10). That this is probably due mostly

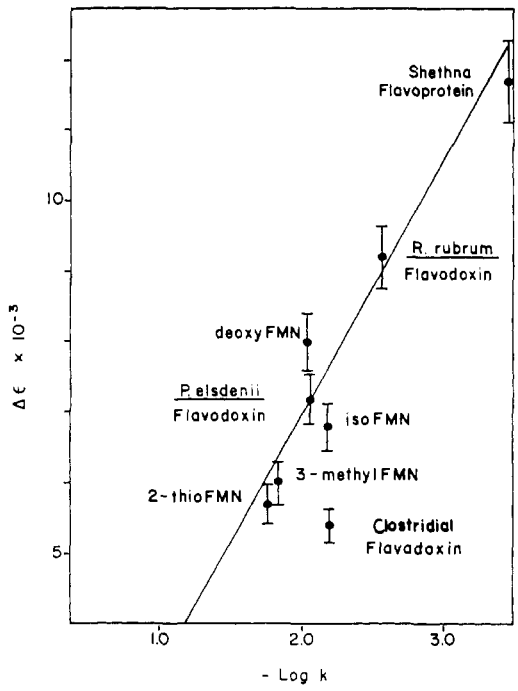

FIGURE 9: Relation between semiquinone absorptivity in the ultraviolet and oxygen reactivity. $\Delta\epsilon$ is the positive increase in semiquinone extinction of the most prominent resolved band in the difference spectra (Figure 7). The abscissa is the negative logarithm of the pseudo-first-order rate constant for flavoenzyme semiquinone oxidation by oxygen (Table II).

to flavin transitions rather than to perturbation of the tryptophan bands is indicated by the circular dichroism spectrum of the oxidized and semiquinone forms of the 2-thioFMN–Shethna apoprotein complex (Figure 11). The sulfur group shifts the flavin transition to the red so that the tryptophyl circular dichroism bands can be observed without overlay. No appreciable differences[1] in rotational strength are observed in the tryptophan circular dichroism bands of the 2-thioFMN–Shethna apoprotein complex in either the oxidized or semiquinone forms when compared to the circular dichroism properties of the apoprotein. Thus, there is no evidence from circular dichroism data for a tryptophan–flavin interaction, although the possibility does exist that tryptophan residues which do not contribute to the circular dichroism spectra might interact with the flavin.

The near-ultraviolet circular dichroism of the *P. elsdenii* and Clostridial flavodoxins are similar to the Shethna flavoprotein, although in these cases very little change in rotational strength is observed at 270 nm upon semiquinone formation. The circular dichroism spectral properties of *R. rubrum* flavodoxin are again intermediate between those of the other flavodoxins and the Shethna flavoprotein. The positive circular dichroism band in this protein at 290 nm lacks the resolution seen in the Shethna flavoprotein. This could mean that there is little optical activity arising from tryptophyl residues or that these residues are in a more polar environment than in the other flavoproteins.

Although the circular dichroism spectra of the flavoenzymes and FMN analog–Shethna apoprotein complexes in their oxidized and semiquinone forms reflect to some extent the absorption spectral properties, no correlation could be made with the oxygen reactivity of the semiquinone form. This is probably due to the complex overlapping of optically active transitions both from the protein and from the flavin.

Effect of pH on Semiquinone Oxidation by Oxygen. Although the semiquinone form of the Shethna flavoprotein is air oxidized very slowly at neutral pH, this stability is considerably decreased upon increasing the pH. Above pH 10, the flavin radical is very rapidly oxidized by oxygen (Figure 12). A plot of the reciprocal of the initial velocity *vs.* $[H^+]$ is linear (inset, Figure 12). This suggests a one proton ionization event which results in an increase in radical reactivity towards oxygen. This ionization could involve a group in the protein responsible for semiquinone stability or the ionization of the neutral radical to the anionic form, which is known to react much more rapidly with oxygen (see above).

The possibility of semiquinone disproportionation to the fully reduced form (which would react quickly with oxygen) is eliminated since at pH 11.0 the half-time of disproportionation is 4.7 min (second-order rate constant of 5×10^3 M^{-1} min^{-1}) while the rate of oxidation has a half-time of 0.57 min (pseudo-first-order rate constant of 1.2 min^{-1}) (Figure 13). This lack of agreement between the rate of disproportionation and the rate of oxygen oxidation is observed at other pH values and has also been observed with the semiquinone form of *P. elsdenii* flavodoxin (Mayhew *et al.*, 1969).

If the ionization of a protein group resulted in the increase in oxidation rate, a tyrosyl residue would be suspect, inasmuch as the pK for the ionization of a phenolic proton usually occurs in this pH region. The tyrosyl ionization properties of the Shethna flavoprotein, however, indicate little difference between the oxidized and semiquinone forms (Figure 14). This suggests that tyrosyl ionization is probably not involved in increasing the rate of semiquinone oxidation by oxygen. The possibility of the involvement of another protein group, such as a guanidino group of arginine whose pK is shifted, cannot be entirely eliminated. However, it would seem that the more plausible explanation of this effect is the ionization of the neutral semiquinone to the anionic form. The semiquinone form of *P. elsdenii* flavodoxin also reacts more rapidly with oxygen as the pH is increased (Mayhew *et al.*, 1969). With this protein, the rate increase occurs about one pH unit lower than in the case of the Shethna flavoprotein. This would imply that the pK for the semiquinone ionization is also about one pH unit lower for *P. elsdenii* flavodoxin than for the Shethna flavoprotein. This is not unreasonable in view of the fact that the reactivity of the flavodoxin semiquinone is considerably greater than that of the Shethna protein semiquinone (see above), suggesting less effective stabilization of the neutral radical.

From the values of the slope and intercept of the plot in the inset of Figure 12, it is in principle possible to evaluate the ionization and rate constants for the processes involved in radical oxidation. However, because of the high pK and the difficulty of obtaining rate measurements at high pH's, it is impossible to get an accurate value for the intercept. If we assume a pK of approximately 11.5 (see Figure 12), the rate constant (evaluated from the slope of the line in the inset of Figure 12) for the oxidation is 2.4×10^8 M^{-1} sec^{-1}. This can be compared to a value of 3×10^8 M^{-1} sec^{-1} obtained for the reaction of the lumiflavin anion radical with O_2 by flash photolysis (S. P. Vaish and G. Tollin, 1970, manuscript in preparation). Thus, if the reason for the increase in oxida-

[1] The changes which are observed in the 285–310-nm region are considerably smaller than those which are seen with the other FMN analogs. The differences which appear between 270 and 280 nm might be due to tyrosine perturbations.

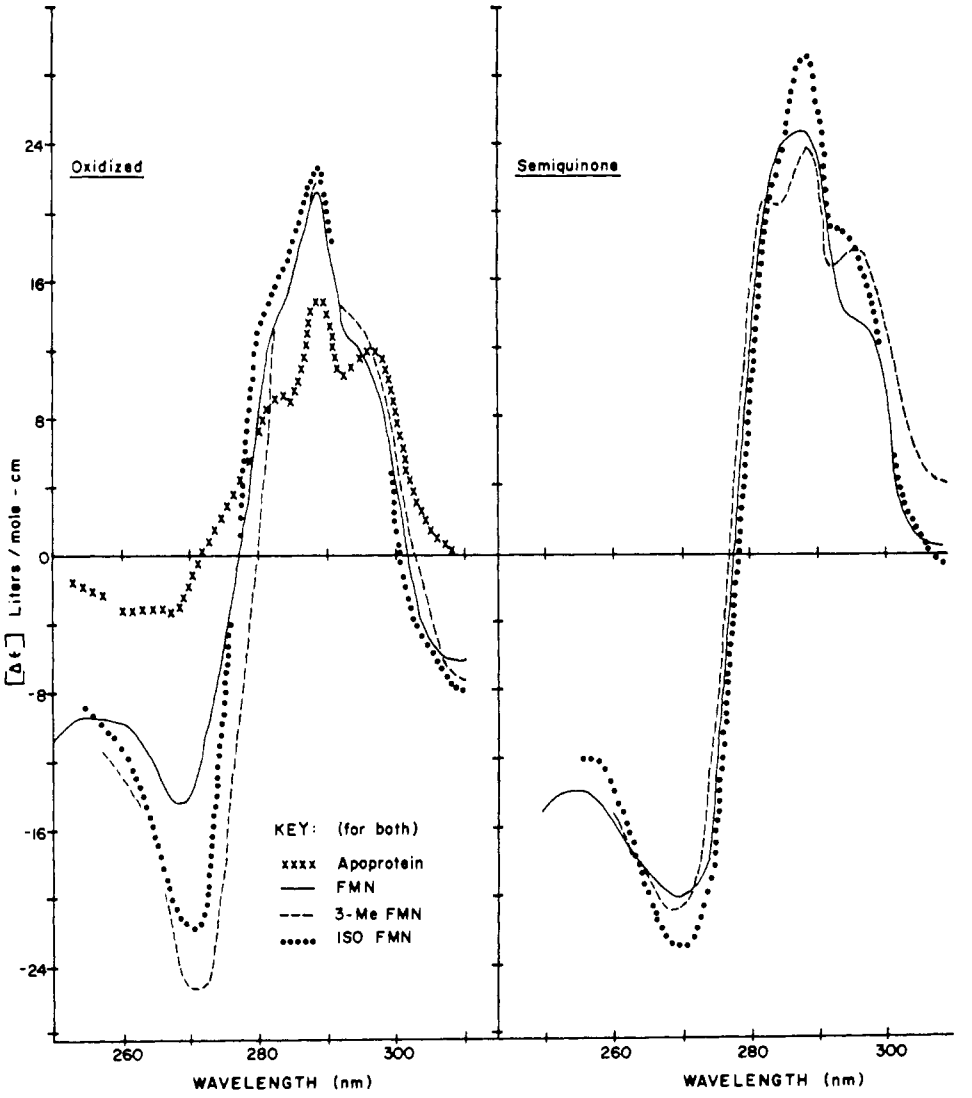

FIGURE 10: Ultraviolet circular dichroism spectra of the oxidized and semiquinone forms of FMN–, 3-MeFMN–, and isoFMN–Shethna apoprotein complexes. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA buffer (pH 7.0).

tion rate of the Shethna protein semiquinone as the pH is increased is that the neutral radical becomes converted into the anion radical, then it is possible to conclude that the protein-bound species is approximately 10^5 times less reactive toward O_2 than is the free anion radical. Using the apparent rate constant for glucose oxidase semiquinone oxidation by oxygen (Table II) and a pK value of 7.5 for radical ionization (Massey and Palmer, 1966), a rate constant of 3.3×10^2 M^{-1} sec^{-1} is calculated. This value is not unreasonable when compared with the constant calculated for the Shethna flavoprotein, in view of the different pH's employed for the rate measurements.

All five tyrosyl residues are reversibly titrated in the Shethna apoprotein (Figure 14). The difference in tyrosyl ionization between the apoprotein and the holoprotein reflects a difference in tyrosine environment upon FMN binding. This change in environment could be due to a protein conformational change upon FMN binding. This is suggested by the far-ultraviolet circular dichroism spectra (see paper II of this series).

Relation between Flavin Redox Form and Protein Conformation. Far-ultraviolet circular dichroism spectra are sensitive to alterations in protein conformation which are reflected in the environment of the amide bond. No change in rotational strength or shape is observed in the circular dichroism spectrum from 240 to 190 nm between the semiquinone form and the oxidized form of the Shethna protein at pH 7.0 (see paper II for the spectrum of the oxidized flavoprotein). At pH 8.0, the spectra are the same as at pH 7.0. However, as seen above, the addition of dithionite at pH 8.0 reduces the flavin to the hydroquinone form. The circular dichroism intensity at 222 nm of this form of the protein is also identical with that obtained with the oxidized and semiquinone forms. Due to the absorption of dithionite and its degradation products, the spectrum in this case could not be extended below 210 nm. The results of these measurements indicate no major protein conformational changes upon flavin reduction to either the semiquinone[2] or hydroquinone forms.

[2] 220-MHz proton magnetic resonance spectra of the oxidized and semiquinone forms of the Shethna flavoprotein also indicate no major protein conformational changes. The authors are grateful to Drs. W. D. Phillips and J. Glikson of the DuPont Co. for their collaboration on these measurements.

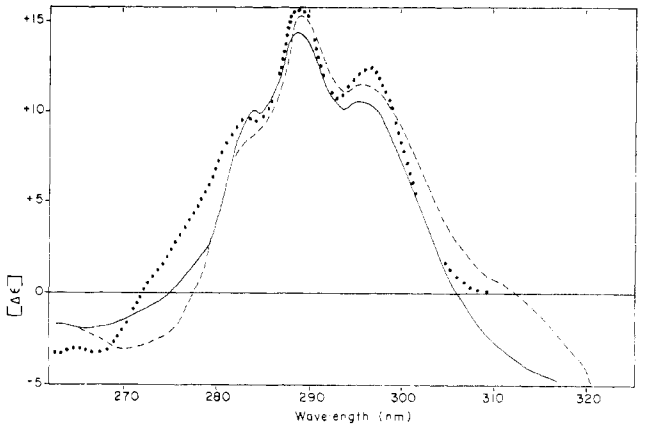

FIGURE 11: Ultraviolet circular dichroism spectra of the oxidized and semiquinone forms of the 2-thioFMN–Shethna apoprotein complex. (– – –) Semiquinone form, (——) oxidized form, and (· · · ·) apoprotein. The buffer for all three spectra was 0.025 M phosphate–0.05 M EDTA (pH 7.0).

Discussion

Dithionite Reduction of the Shethna Flavoprotein Semiquinone. The pH dependence of semiquinone reduction by dithionite (Figure 5) indicates that the removal of a proton from either a protein group or the flavin molecule is responsible for the loss of stability of the flavin semiquinone to dithionite reduction. The identical behavior with and without methyl viologen establishes that any changes in dithionite reducing potential at different pH values are not important. Also, any redox potential changes which do occur (Munemori, 1958) would not effect the reduction since a large excess (≃1 mg) of dithionite was used. The only protein group which normally ionizes in this pH region (7–8) is the imidazole ring. However, inasmuch as the Shethna flavoprotein does not contain histidine (see paper II of this series), a carboxyl or amino group whose pK's were shifted would have to be considered. The large pK changes which would have to occur for either of these groups to ionize at pH 7.0 makes this possibility unlikely, although still feasible.

A more likely candidate is the ionization of the FMN-terminal phosphate group. The second ionization of phosphoric acid is at pH 7.2. Removal of a proton could disrupt the phosphate–protein bond thereby changing the environment of the bound flavin so as to make the semiquinone form susceptible to further reduction. In agreement with this, protein-bound riboflavin analogs are completely reduced by dithionite (see above).

A third possibility which can account for these data is the ionization of the neutral flavin hydroquinone to the anion form. Free flavin hydroquinone ionizes with a pK = 6.2 (Ehrenberg and Hemmerich, 1968). Since flavin hydroquinone exists in a bent "butterfly" configuration (Ehrenberg and Hemmerich, 1968), steric constraints in the flavin binding site may allow only the planar oxidized and semiquinone flavin rings to exist in the Shethna flavoprotein.[3] Ionization of the flavin hydroquinone may bring about changes in the flavin binding site (induced, for example, by changes in solvation at N-1) which could permit the bent configuration to exist. The effect of high salt (*e.g.*, 3 M $(NH_4)_2SO_4$) would be expected to shift the apparent pK of 7.0 toward the true pK of 6.2. This could explain the effect of high salt concentrations in eliminating the stability of the semiquinone toward dithionite reduction. The modifications in the flavin ring (2-thio-, 3-Me-, and isoFMN analogs) might also be expected to influence the apparent pK of the hydroquinone form either directly (possible only with the 2-thio- and isoFMN analogs) or by modifying the protein–flavin interactions. Such changes in flavin structure would not be expected to affect the phosphate ionization. For this reason, of the three possibilities, the last-mentioned seems the most attractive. The effect of the phosphate group could be ascribed to changes in the flavin binding site which relax the steric constraints on hydroquinone formation. That such changes probably occur is shown in paper II of this series.

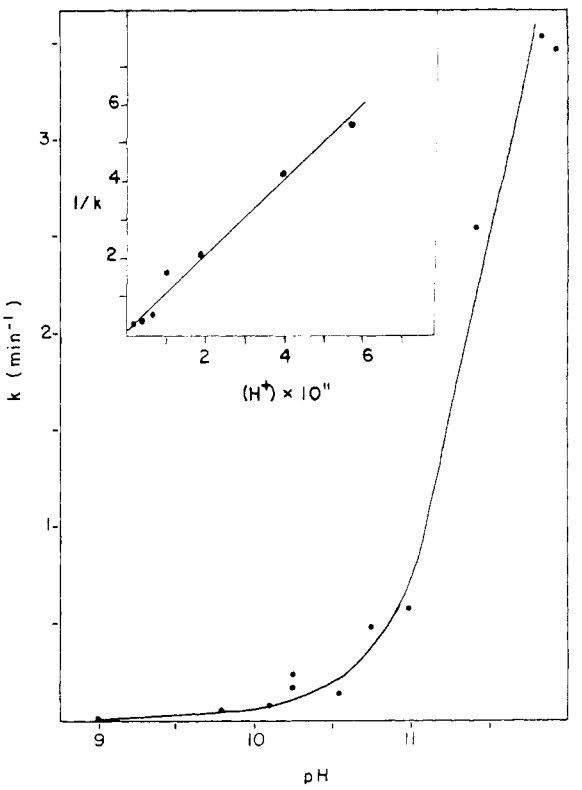

FIGURE 12: The influence of pH on the pseudo-first-order rate of oxidation of the Shethna flavoprotein semiquinone by oxygen. The buffer was 0.1 M glycine. Microliter quantities of flavoprotein semiquinone were added to air-saturated buffers at the appropriate pH. pH values were determined after the rate measurement. Inset: the relation between the reciprocal of the first-order rate constant for semiquinone oxidation by oxygen and hydrogen ion concentration. $1/k$ is expressed in minutes.

The shift of the pK of FH_2 from 6.2 in the free species to 7.0 in the Shethna protein could be due either to a direct protein–flavin interaction at N(1) or to inductive effects from hydrogen bonding of the 2 and 4 position carbonyl oxygens to the protein, or both. A steric constraint on the positioning of the flavin ring in the binding site is suggested by the importance of an unhindered N(3)–protein interaction, as shown by the fact that the 3-MeFMN–apoprotein complex is completely reduced at pH 7.0. It has been demonstrated that the presence of an alkyl group at the N(3) position does not affect the pK for N(1) ionization of free flavin hydroquinone (Ehrenberg and Hemmerich, 1968). Although no direct experimental evidence is available, the hydroquinone forms of the 2-thio- and isoFMN analogs, because of electronic effects mediated through the flavin–π system, might be expected to have a dif-

[3] This is very likely the case whatever the ionizing group is.

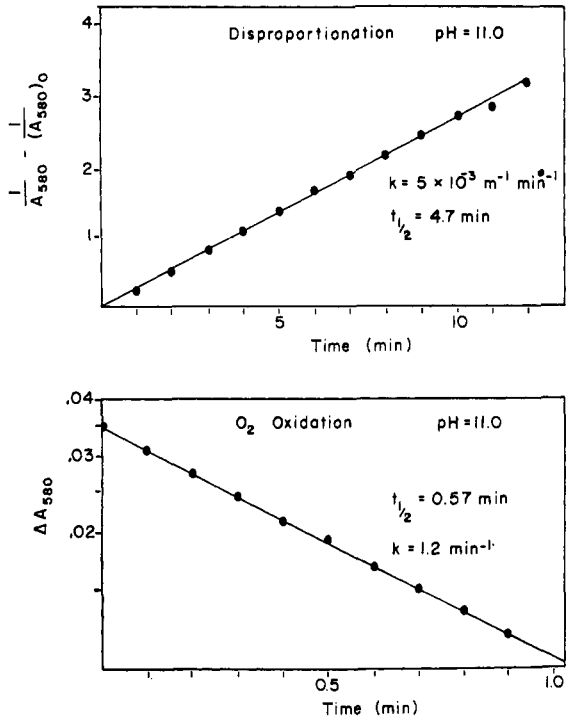

FIGURE 13: A comparison of the rate of Shethna flavoprotein semiquinone disproportionation and oxygen oxidation at pH 11.0.

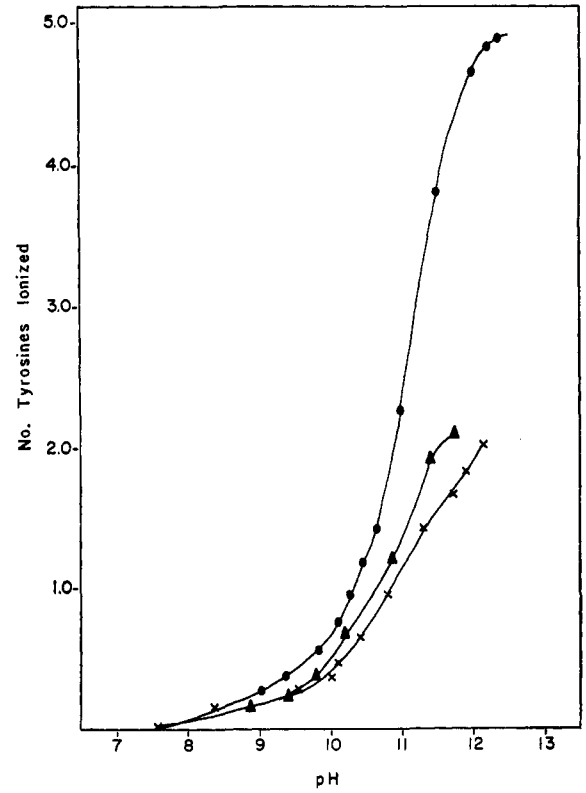

FIGURE 14: Tyrosyl ionization in the oxidized and semiquinone forms of the Shethna flavoprotein and in the Shethna apoprotein. The buffer used was 0.1 M glycine. (-●-●-) Apoprotein, (-▲-▲-) semiquinone form, and (-×-×-) oxidized form.

ferent ionization constant than that of $FMNH_2$. Some indication of this is given by the large differences in absorption spectra between these analogs and FMN. There is also the possibility of pK shifts through altered protein–flavin interactions.

The flavin hydroquinone has been suggested to be in a bent configuration in *both* its neutral and anionic forms (Ehrenberg and Hemmerich, 1968). It is thus necessary to explain how the Shethna protein can impose steric limitations preventing this nonplanarity for the neutral form but not for the anionic form. This can easily be accomplished by postulating an alteration of protein structure through an electrostatic interaction between the flavin hydroquinone anion and a positively charged protein group (*e.g.*, a protonated amino group) which would change the binding site geometry sufficiently to allow the bent flavin ring to fit. This proposed structural alteration of the protein would have to be localized, inasmuch as no major changes in peptide backbone structure were observed in the comparison of the far-ultraviolet circular dichroism spectrum of the flavoprotein hydroquinone with those of the semiquinone and oxidized forms. The negatively charged flavin hydroquinone, formed in a predominantly nonpolar milieu, would also increase its solvation which could in turn cause a localized swelling of the dimensions of the binding site, thus providing more room for the bent configuration. Proton magnetic resonance studies might be useful in providing support for these ideas.

Oxidation of the Shethna Flavoprotein Semiquinone by Oxygen. The neutral semiquinone form of the native Shethna flavoprotein reacts slowly with molecular oxygen ($k = 3 \times 10^{-4}$ min^{-1}) at pH 7.0 to form oxidized flavoprotein and presumably the superoxide ion ($O_2 \cdot^-$) as the reaction products. The rate of oxygen oxidation of the unbound flavin neutral semiquinone is not known, but it is at least 10^5 times slower than its rate of disproportionation (S. P. Vaish and G. Tollin, 1970, manuscript in preparation). On the other hand, the flavin anion radical reacts very rapidly with oxygen (S. P. Vaish and G. Tollin, 1970, manuscript in preparation) with a rate close to diffusion controlled. One can account for this large difference in reactivity by assuming that oxygen reacts by removing an electron (the pK of the $O_2 \cdot^-$ ion is 4.3) from the N(5) position of the flavin ring. Thus, it should be relatively easy to accomplish this with the electron-rich ring system of the anion radical, compared to the more difficult task of removing an electron from the positively charged N(5) position of the neutral semiquinone.

The differences in oxygen reactivity among the flavoenzyme neutral semiquinones could be due to the degree of protein stabilization of the protonated N(5) nitrogen and the negatively charged N(1) position. This stabilization seems to be reflected in the correlation we have observed between the increase in ultraviolet extinction of the flavoenzyme neutral semiquinones and oxygen reactivity (Figure 9). The amount of stabilization is also quite sensitive to modifications of flavin structure, as evidenced by the fact that the FMN analog–Shethna apoprotein complexes are oxidized more rapidly and have a lower ultraviolet extinction than the FMN–protein complex. Modification of the flavin aromatic ring system (as with 2-thio- and isoFMN) would be expected both to have a direct influence on the basicity of the N(5) position of the flavin semiquinone and to affect the protein-induced stabilization. This is supported by the observation of a more rapid oxidation of the semiquinone forms of 2-thioFMN and isoFMN compared to 3-MeFMN and dFMN (Table II). The increase in the rate of semiquinone oxidation as the pH is increased (Figure 12) can be explained simply in terms of

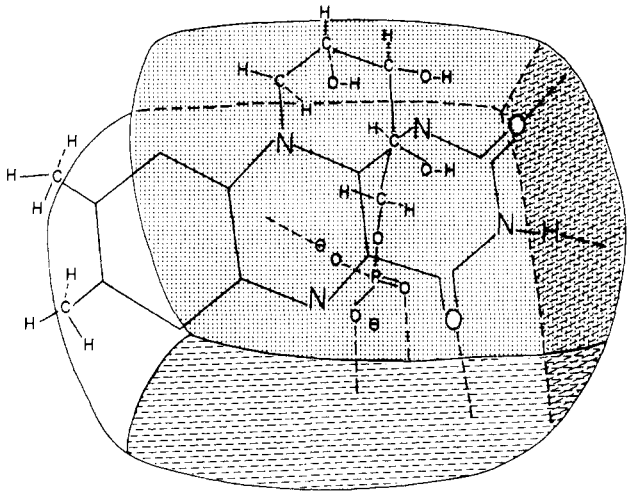

FIGURE 15: Schematic depiction of flavin conformation in Shethna protein binding site. The dashed lines refer to interactions with protein groups. The enclosure suggests the overall shape of the protein binding site.

the postulated relation to the state of protonation of N(5) if one supposes that the oxidation of the neutral semiquinone proceeds *via* the anion radical at high pH.

Other types of supporting evidence for the key role of the N(5) position in determining protein semiquinone reactivity toward oxygen are electron spin resonance experiments on unbound flavin radicals (Guzzo and Tollin, 1964) which show a high unpaired spin density at this position and the sulfite addition studies of Massey *et al.* (1969). Müller and Massey (1969) have determined, from model systems, that sulfite adds to the N(5) position of the isoalloxazine ring. Only those flavoenzymes which form anion radicals can add sulfite (*i.e.*, the oxidases). Those flavoproteins (the dehydrogenases) which form neutral semiquinones do not form sulfite adducts. These results suggest that the N(5) position is unreactive in the oxidized form as well as in the semiquinone form in the dehydrogenases. The proposal that the nature of a charged protein group in the vicinity of the flavin determines the type of semiquinone species and reactivity with sulfite (Massey *et al.*, 1969) cannot be proven or disproven by the present study.

The phosphate group also may be *specifically* involved in radical stabilization toward oxygen. This is suggested by the fact that fully reduced riboflavin analog proteins do *not* generate radical upon air oxidation, whereas the fully reduced FMN protein does, even at pH's greater than 8 where presumably the binding site is loosened. This could be accomplished by placing the negative charges of the phosphate group in proximity to the positive charge at N(5). There is evidence that this occurs in the free coenzyme. Thus, the pK of the FMN semiquinone is a few tenths of a pH unit higher than that of riboflavin (Draper and Ingraham, 1968; S. P. Vaish and G. Tollin, 1970, manuscript in preparation).

Little difference in protein conformation is observed for the oxidized, semiquinone, and fully reduced forms of the Shethna flavoprotein. The principle evidence for this is the comparison of the far-ultraviolet circular dichroism spectra, the proton magnetic resonance spectra, and the tyrosyl ionization properties. Further support is provided by the fact that it is possible to photoreduce oxidized flavoprotein crystals to the neutral semiquinone form without disruption of the crystals. Although the protein crystals used in this experiment were irregular and not characterized by X-ray methods, the change of redox form within the same crystals indicates no major protein conformational changes. The electron spin resonance spectrum of a semiquinone protein crystal suspension was also identical to that obtained with the solution. These results are of interest in connection with the suggestion of a protein conformational change upon semiquinone formation based on crystallographic studies of Clostridial flavodoxin (Ludwig *et al.*, 1969). Although the crystals of the oxidized and semiquinone forms of this flavoprotein are isomorphous as far as cell dimensions are concerned, the diffracted intensities are changed. The authors interpret this to be due to changes in the positions of the atoms in the unit cell upon flavin reduction. However, for this protein, the semiquinone is red in the crystalline form and blue in solution, thereby directly indicating some changes in flavin environment upon crystallization.

The change in tyrosyl ionization behavior upon flavin binding indicates the possibility of tyrosyl residues at or near the flavin binding site. A recent study (Hinkson, 1968) has shown that bound flavin protects two of the five tyrosines in the Shethna flavoprotein from nitration. Nitration of four of the five tyrosines in the apoprotein practically eliminates all flavin binding capacity.

Circular dichroism studies on the apoprotein and on the 2-thioFMN complex in the oxidized and semiquinone forms provide no clear evidence for a tryptophan–flavin interaction (Figure 11), although the presence of a tyrosyl perturbation is suggested. Tryptophan fluorescence is quenched upon flavin binding but the lack of any wavelength shift in the fluorescence emission spectra of the apoprotein and oxidized and semiquinone flavoproteins suggests an indirect effect (D'Anna and Tollin, 1971), perhaps mediated *via* energy transfer from tyrosyl groups.

It is of interest to attempt to summarize the results of the present series of studies in the form of a model which relates the structural features of the flavin molecule to its binding site on the Shethna protein. This would be of interest in its own right and can also serve as a guide to further investigation. Such a model is shown in Figure 15. The flavin molecule is presumed to be almost completely buried in a cavity or cleft in the protein. This is based upon several lines of evidence: the large free energy of binding which implies multiple sites of flavin–protein interaction, the nonpolar environment of the flavin chromophore, the fact that changes in any part of the flavin molecule cause modifications of binding and redox properties, the thermodynamic barrier to PFH_2 formation below pH 8.0 and the slow rate of reduction of the Shethna flavoprotein even with as strong a reductant as dithionite.

Specific protein–flavin interactions are shown to occur at the 2-carbonyl and the N(3) positions, based upon the redox differences observed between the apoprotein complexes of 2-thioFMN and 3-MeFMN and the native protein. The lower binding constant of 3-MeFMN, the difference in binding between riboflavin and its 3-methyl analog, and the shift in pK of the N(3) proton in the FMN–protein complex provide further evidence for this interaction.

One side of the binding site cavity is shown to be open with the benzene ring partly buried. The methyl groups on this portion of the flavin ring are probably important in sterically maintaining the position of the flavin ring (suggested by the lower binding constant of isoFMN as well as the different redox properties of this semiquinone form relative to FMN. The rearrangement of the 8-position methyl group could change this interaction and generate sterically unfavorable

interactions at the 6 position, thus shifting the position of the ring system. The partly open nature of this region is suggested by the ability of oxidants such as ferricyanide and cytochrome *c* to quickly oxidize PFH· (Hinkson and Bulen, 1967; C. Sigwart and G. Tollin, unpublished observations, 1970). This could occur by electron withdrawal *via* the benzene ring π system, which has a high unpaired spin density in the free flavosemiquinone (Guzzo and Tollin, 1964; Ehrenberg *et al.*, 1967).

The side-chain hydroxyl groups are shown in close proximity to the flavin ring system within the cavity. This is suggested for both the oxidized and semiquinone forms. Such a concept is supported by the red shift of the circular dichroism and difference spectra of protein-bound complexes of dFMN relative to FMN, by the low rotational strength of the long wavelength band in the circular dichroism of those protein-bound analogs without side-chain hydroxyl groups, by the increase in long-wavelength rotational strength upon binding of flavin, by the role of hydroxyl groups in fluorescence quenching and by the lack of binding of tetra-*O*-acetylriboflavin. The low rotational strength of the long-wavelength bands in the circular dichroism spectrum of the semiquinone form of dFMN compared to the FMN protein, suggests that this is true in the semiquinone also.

The low rotational strengths of the semiquinone forms of the 2-thioFMN– and isoFMN–protein complexes in the long-wavelength bands suggests that those modifications position the flavin ring away from the side chain, although the circular dichroism indicates strong interactions in the oxidized form. The reduced stability of the dFMN protein semiquinone provides evidence that the side-chain hydroxyl groups play a role in stabilizing the radical. This could be accomplished by interactions at N(1) which stabilize the negative charge at this position in the semiquinone (for both the neutral and anion forms).

The crucial importance of the phosphate group is most evident in the observation that it is essential for stable semiquinone formation. That this phenomenon is not due only to the extra binding energy is indicated by the comparison of the association constants for binding to the protein of 3-MeFMN and 3-methylriboflavin with FMN and riboflavin (paper II). Thus, the phosphate group must act to help maintain the binding-site geometry. The relative flavin fluorescence yields of the protein complexes of deoxyriboflavin and dFMN compared to FMN and riboflavin provides further evidence for different interactions in the protein complexes of the phosphorylated flavins than those which occur with the riboflavin analogs. These differences are probably to be associated with the protein moiety, inasmuch as the circular dichroism spectra of the protein-bound riboflavin analogs are quite similar to those of the FMN analogs. Still further support for this concept is to be found in the fact that bound riboflavin derivatives will form PFH_2 very readily upon reduction. It is interesting that a carboxyl group will not substitute for the phosphate; thus the interaction is highly specific.

The slower binding rate of the FMN analogs relative to the riboflavin analogs indicates that the phosphate–protein interaction is within the protein structure and not on the surface. This difference in rate has also been observed by Hinkson (1968). The rate of FMN binding increases upon lowering the pH (with an apparent $pK = 7.0$) (Hinkson, 1968). The ionizing group here is probably the phosphate, which, when rendered uncharged by protonation, leads to an increase in binding rate. This is in accord with the difference in rate observed between riboflavin and FMN. The rather slow rate observed with FMN ($k = 2 \times 10^5$ M^{-1} sec^{-1}) may be a reflection of the difficulty of burying a highly charged group within the protein structure. The ring-modified FMN analogs show essentially no differences in binding rate, while removal of the hydroxyl groups increases the rate of binding. This suggests that the side chain is bound first (presumably through a phosphate–protein electrostatic interaction) and is then followed by the ring-binding process(es).

The phosphate group also may be *specifically* involved in radical stabilization toward oxygen. This is suggested by the fact that fully reduced riboflavin analog proteins do *not* generate radical upon air oxidation, whereas the fully reduced FMN protein does, even at pH values greater than 8 where presumably the cavity is loosened. This could be accomplished by having the negative charges of the phosphate group in proximity to the positive charge which exists in the semiquinone at N(5). Finally, the model depicts the top of the cleft as relatively open to the solvent. This is necessary to explain FAD binding, since it will allow the adenosine moiety to be oriented away from the protein, as is indicated by the circular dichroism of the FAD protein and the similarity between the binding constants of FAD and riboflavin. Furthermore, it is consistent with the pH dependence of the dithionite reduction (if N(1) of FH_2 is involved) and the fact that the protein can be reduced at all.

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