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Circular Dichroism Studies of the Flavin Chromophore and of the Relation between Redox Properties and Flavin Environment in Oxidases and Dehydrogenases*

Dale E. Edmondson† and Gordon Tollin‡

ABSTRACT: Flavin electronic transitions in the 300–500-nm region have been investigated using absorption and circular dichroism spectroscopy. Data from model flavin compounds in polar and nonpolar solvents, as well as from flavin bound in dehydrogenase-type flavoproteins, indicate the occurrence of a minimum of six vibronic bands corresponding to three $\pi \rightarrow \pi^*$ transitions. No evidence was obtained for $n \rightarrow \pi^*$ transitions. The intensities of the flavin circular dichroism bands are increased by acetylating the side-chain hydroxyl groups and by the environment provided by the binding site of the flavoprotein. The magnitude and sign of the rotational strength of the long-wavelength vibronic bands is particularly sensitive to the interaction of the ribityl hydroxyl groups with the isoalloxazine ring. This interaction is greatly increased when flavin is bound to protein. An analysis of the band positions and rotational strengths of the vibronic transitions in the visible circular dichroism spectra of the oxidized, semiquinone, and hydroquinone forms of several flavoprotein dehydrogenases indicates that the flavin environments in these proteins are quite similar. Such a correlation is also found to exist for some flavoprotein oxidases, although the nature of the flavin–protein contacts in these enzymes are clearly different from those in the dehydrogenases. Thus, circular dichroism spectroscopy provides a useful probe of the relations between flavoenzyme redox properties and the interactions which occur within the coenzyme binding site.

The spectral properties of the flavin chromophore (isoalloxazine) have been the subject of several theoretical and experimental studies (Fox *et al.*, 1967; Weber, 1966; Tollin, 1968; Kurtin and Song, 1968; Song, 1969). The information gained from this work is relevant to an understanding of protein–flavin interactions and to the relation between flavin environment and enzymic properties in flavoenzymes.

In most cases, the binding of flavin to a protein moiety results in wavelength shifts in the 300–500-nm region of the absorption spectrum. A partial resolution of the 450-nm band into three bands is also observed in many flavoproteins (Penzer and Radda, 1967). Similar spectral shifts and increases in resolution are found upon dissolving unbound flavins in nonpolar solvents (Harbury *et al.*, 1959; Kotaki *et al.*, 1967).

The shoulder which appears on the long-wavelength side of the 450-nm band has been attributed to an $n \rightarrow \pi^*$ transition (Kotaki *et al.*, 1966). However, since the polarization of the flavin fluorescence is constant across this absorption region, the resolved bands have alternatively been ascribed to vibronic structure (Weber, 1966). Fluorescence polarization is also constant across the 365-nm band (Weber, 1966), suggesting that only a single transition occurs in this spectral region. On the other hand, molecular orbital calculations (Fox *et al.*, 1967) and circular dichroism and magnetic circular dichroism spectra (Tollin, 1968) indicate the presence of a third $\pi \rightarrow \pi^*$ transition at approximately 340 nm. Recent fluorescence polarization measurements (Kurtin and Song, 1968) and circular dichroism studies (Miles and Urry, 1968) have provided evidence for the presence of still another transition (possibly $n \rightarrow \pi^*$) at 300 nm.

The present work represents an extension of the analysis of the circular dichroism and absorption spectra of free flavin (Tollin, 1968) and of a number of flavoenzymes. Inasmuch as the isoalloxazine ring is optically inactive, any circular dichroism bands observed are due to the asymmetric environment provided by the optically active ribityl side chain and, in the case of flavoenzymes, also to the asymmetric environment of the protein moiety. Due to a high degree of conformational mobility of the side chain, the circular dichroism spectra observed with unbound FMN and riboflavin reflect an average of the various interactions which can occur between the side chain and the isoalloxazine ring. This permits the simultaneous occurrence of both positive and negative dichroic bands for the same transition, and is probably responsible for the weak circular dichroism bands which are observed (Tollin, 1968). The low rotational strength (particularly in the 450-nm region) makes definitive interpretation quite difficult. An increase in rotational strength (5–20-fold) is observed upon binding the flavin to its site on a protein[1] or by increasing

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[1] Similar effects are observed in the circular dichroism spectra of tryptophan analogs and the tryptophyl residues of chymotrypsinogen (Strickland *et al.*, 1969).

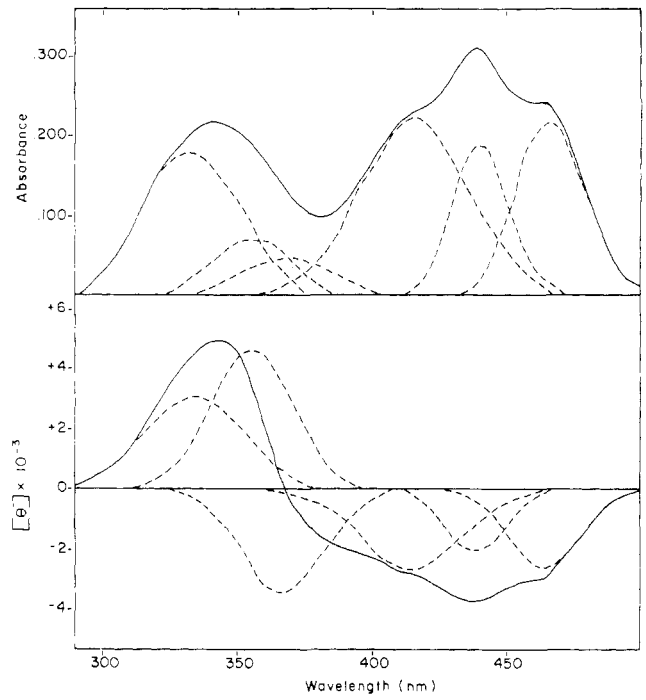

FIGURE 1: Resolved absorption and circular dichroism spectra of tetra-*O*-acetylriboflavin in 1,2-dichloroethane. Solutions in this figure and Figure 2 were saturated in flavin.

the size of the ribityl side chain through acetylation of the hydroxyl groups. This effect, plus the solubility of acetylated flavin in organic solvents, has permitted further insight into the electronic structure of the flavin chromophore to be obtained.

A classification of flavoenzymes as dehydrogenases and oxidases has been proposed by Massey *et al.* (1969a) on the basis of several properties. The dehydrogenases generate neutral semiquinones upon either photoreduction in the presence of EDTA or dithionite reduction. They do not form sulfite adducts and their hydroquinones react with oxygen to produce the semiquinone and superoxide ion (Massey *et al.*, 1969b).

The oxidases generate anionic semiquinones upon photoreduction and readily form sulfite adducts (Massey *et al.*, 1969a). Oxygen oxidation of their hydroquinones does not produce measurable quantities of superoxide ion, thereby suggesting that the radical form is not an intermediate in the oxidation (Massey *et al.*, 1969b).

The presence of a charged group on the protein (either positive or negative) in the vicinity of the bound flavin has been proposed to account for these differences in redox properties (Massey *et al.*, 1969a). Alternatively, one could suppose the existence of a more general similarity in the bound flavin conformation and the protein environment about the flavin coenzyme within each class, which would lead to similar reactivities. For those flavoenzymes in which the overall environment of the flavin chromophore is similar one would expect to see similarities in the circular dichroism spectra in the wavelength region in which flavin electronic transitions occur. As will be demonstrated in this paper, this expectation is borne out for a group of flavoprotein dehydrogenases and oxidases.

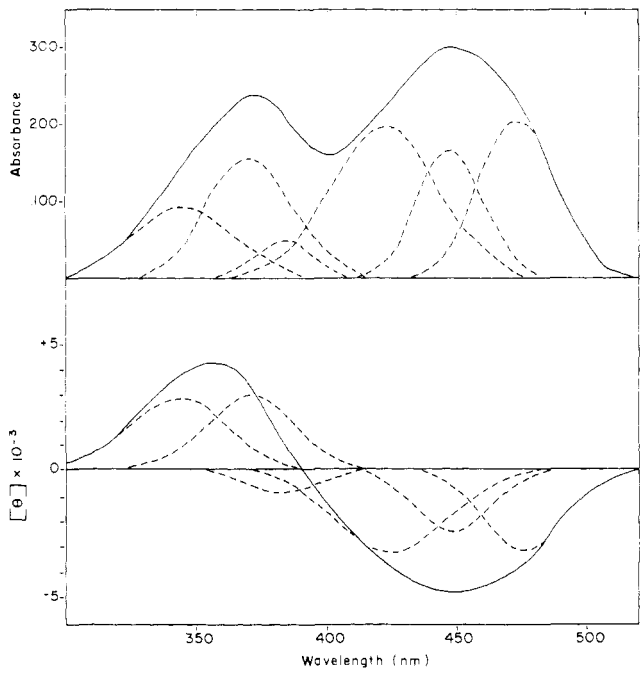

FIGURE 2: Resolved absorption and circular dichroism spectra of tetra-*O*-acetylriboflavin in 0.025 M phosphate buffer (pH 7.0).

Experimental Section

Methods

Circular dichroism spectra were measured using a Cary Model 60 spectropolarimeter equipped with the Model 6001 circular dichroism attachment. The intensity of the signals was calibrated using an aqueous solution of *d*-10-camphorsulfonic acid. Sample absorbance never exceeded 2.0 in the spectral region scanned. A fixed slit width of 0.5 mm was used in the 600–700-nm region. Cylindrical quartz cells (10 mm) were normally used; quartz Thunberg cells were used for spectral measurements on anaerobic solutions of flavoprotein semiquinones. Due to low solubility, 25- and 50-mm cells were used to measure the spectrum of tetra-*O*-acetylriboflavin in aqueous solutions. Absorption spectra were recorded on the Cary Model 14R spectrophotometer. Curve resolution was carried out on a DuPont 310 curve resolver.

Flavoprotein free radicals were 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.

Materials

Riboflavin, FAD, and FMN were obtained from Calbiochem and used without further purification. Tetra-*O*-acetylriboflavin was obtained from Dr. V. Massey. The 3-MeFMN and isoFMN analogs were gifts from Dr. P. Hemmerich, University of Konstanz, Germany. *N*-10-ω-Hydroxypentylisoalloxazine was a gift from Dr. D. McCormick. This compound was phosphorylated according to Flexser and Farkas (1952) to form dFMN (*i.e.*, FMN with the 2′-, 3′-, and 4′-hydroxyls replaced by hydrogen). Dichloroethane was dried and distilled over P_2O_5. All other chemicals were of reagent grade from commercially available sources.

The Shethna flavoprotein from *Azotobacter vinelandii* was purified according to Hinkson and Bulen (1967). The flavin-

TABLE I: Analysis of the Optical and Circular Dichroism Spectra of Free and Protein-Bound Flavins.

Compound	Band I	II	III	IV	V	VI
Tetra-*O*-acetylriboflavin in dichloroethane	4650 Å 0–0[a]	4400 0 + 1200 cm^{-1}	4130 0 + 1200 + 1500	3680 0–0	3550 0 + 1000	3320 0–0
Tetra-*O*-acetylriboflavin in 0.025 M phosphate, pH 7.0	4750 0–0	4490 0 + 1200	4240 0 + 1200 + 1500	3830 0–0	3700 0 + 900	3450 0–0
FMN in 0.1 M phosphate, pH 7.0	4730 0–0	4480 0 + 1200	4180 0 + 1200 + 1600	3820 0–0	3600 0 + 1600	3350 0–0
Riboflavin in 0.1 M phosphate, pH 7.0	4700 0–0	4490 0 + 1000	4200 0 + 1000 + 1500	3810 0–0	3590 0 + 1600	3340 0–0
Shethna flavoprotein	4750 0–0	4460 0 + 1400	4230 0 + 1400 + 1200	3780 0–0	3610 0 + 1200	3410 0–0
dFMN–Shethna apoprotein complex	4690 0–0	4420 0 + 1300	4210 0 + 1300 + 1100	3720 0–0	3610 0 + 800	3400 0–0
Clostridial flavodoxin	4720 0–0	4430 0 + 1400	4250 0 + 1400 + 1000	3800 0–0	3610 0 + 1400	3400 0–0
P. elsdenii flavodoxin	4710 0–0	4420 0 + 1400	4230 0 + 1400 + 1000	3780 0–0	3590 0 + 1400	3320 0–0
R. rubrum flavodoxin	4870 0–0	4570 0 + 1300	4300 0 + 1300 + 1400	3920 0–0	3780 0 + 900	3500 0–0
Ferredoxin–TPNH reductase	4900 0–0	4610 0 + 1300	4420 0 + 1300 + 900	3850 0–0	3610 0 + 1700	3350 0–0
3-MeFMN–Shethna apoprotein complex	4800 0–0	4520 0 + 1300	4320 0 + 1300 + 1000	3820 0–0	3680 0 + 1000	3480 0–0
Glucose oxidase	4810 0–0	4510 0 + 1400	4240 0 + 1400 + 1400	3870 0–0	3610 0 + 800	3400 0–0
L-Amino acid oxidase	4970 0–0	4650 0 + 1400	4380 0 + 1400 + 1300	4050 0–0	3910 0 + 900	3620 0–0

[a] 0–0 refers to the electronic transition from the 0 vibrational level in the ground state to the 0 vibrational level in the excited state. Similarly, 0 + 1200 cm^{-1} indicates a transition from the 0 vibrational level in the ground state to a 1200-cm^{-1} vibrational level in the excited state. 0 + 1200 + 1500 cm^{-1} denotes a transition from the 0 vibrational level in the ground state to a (1200 + 1500)-cm^{-1} vibrational level in the excited state (Suzuki, 1967).

free Shethna apoprotein was prepared by a modification[2] of the method used by Hinkson (1968). Clostridial and *Peptostreptococcus elsdenii* flavodoxins were obtained from Dr. Steven Mayhew, University of Michigan. L-Amino acid oxidase was isolated from the venom of *Crotalus adamanteus* by the method of Wellner and Meister (1960). The dried venom was obtained from Sigma Chemical Co., St. Louis, Mo. *Rhodospirillum rubrum* flavodoxin was a gift from Dr. M. Cusanovich of this Department. Glucose oxidase, from *Aspergillus niger*, was purchased from Worthington Biochemical Corporation, Freehold, N. J. It was further purified by column chromatography and ammonium sulfate precipitation, following the final stages of the purification procedure of Swoboda and Massey (1964). Ferredoxin–TPNH reductase was isolated from fresh spinach leaves, obtained at a local market, by the method of Shin *et al.* (1963). The concentrations of flavoenzymes were determined spectrophotometrically using published extinction coefficients.

Results

Circular Dichroism and Absorption Spectra of Unbound Flavins. The low solubility of most flavin derivatives in nonpolar solvents makes it difficult to use solvent shifts as an aid in spectral interpretation. However, acetylation of the side-chain hydroxyl groups eliminates this handicap. In Figures 1 and 2 are shown the circular dichroism and absorption spectra of tetra-*O*-acetylriboflavin in dichloroethane and in aqueous buffer. Note that the resolution of the 450-nm band is quite apparent in the circular dichroism spectrum as well as in the absorption spectrum when dichloroethane is the solvent. This resolution is lost and the bands shift to the red in phosphate buffer.

The circular dichroism spectra of riboflavin and of FMN are less intense but otherwise quite similar to that of tetra-*O*-acetylriboflavin in aqueous solution (Tollin, 1968). The most notable difference is that the apparent positive 335-nm dichroic band observed in FMN and in riboflavin is shifted to 360 nm in the tetra-*O*-acetyl derivative. This must reflect a change in the nature of the interaction between the side chain and the isoalloxazine ring upon substitution of the side-chain hydroxyl groups. Small differences are also observed between the circular dichroism spectra of riboflavin and FMN. This has been noted by Miles and Urry (1968) and can be attributed to an effect of the terminal phosphate on the side-chain–ring interactions.

To provide a better comparison of the spectral differences, the absorption and the circular dichroism spectra of the dif-

[2] This will be described in paper II of this series.

TABLE II: Oscillator and Rotational Strengths of the Resolved Spectral Bands of Free and Protein-Bound Flavin.

	Band											
	I		II		III		IV		V		VI	
	f_i[a]	$R_i \times 10^{40}$[b]	f_i	$R_i \times 10^{40}$	f_i	$R_i \times 10^{40}$	f_i	$R_i \times 10^{40}$	f_i	$R_i \times 10^{40}$	f_i	$R_i \times 10^{40}$
Tetra-*O*-acetylriboflavin in dichloroethane	0.046	−1.8	0.039	−1.4	0.099	−3.7	0.025	−4.5	0.033	+5.4	0.123	+4.9
Tetra-*O*-acetylriboflavin in 0.025 M phosphate (pH 7.0)	0.055	−3.1	0.044	−2.2	0.089	−5.1	0.015	−0.8	0.078	+4.3	0.054	+5.6
FMN in 0.1 M phosphate (pH 7.0)	0.049	−0.8	0.060	−0.7	0.064	−1.1	0.050	−0.5	0.110	+0.8	0.008	+1.6
Riboflavin 0.1 M phosphate (pH 7.0)	0.068	−1.4	0.053	−0.7	0.080	−1.2	0.073	0	0.071	+1.2	0.003	+3.0
Shethna flavoprotein	0.067	+10.5	0.029	+2.7	0.080	−5.3	0.061	−25.4	0.017	−6.0	0.109	−35.5
dFMN–Shethna apoprotein complex	0.083	+5.0	0.017	+1.3	0.091	−14.3	0.048	−22.0	0.029	−3.8	0.114	−42.2
Clostridial flavodoxin	0.041	+7.7	0.026	+5.0	0.101	+4.9	0.027	−10.1	0.057	−27.5	0.060	−4.8
P. elsdenii flavodoxin	0.053	+10.4	0.023	+4.7	0.097	+7.2	0.049	−18.2	0.052	−18.9	0.038	−3.8
R. rubrum flavodoxin	0.070	+25.9	0.026	+7.7	0.093	−3.6	0.007	−13.4	0.053	−10.7	0.171	−59.1
Ferredoxin–TPNH reductase	0.025	−6.1	0.023	−6.1	0.127	+6.1	0.044	+25.2	0.061	+26.8	0.032	+23.3
3-MeFMN–Shethna apoprotein complex	0.031	+6.1	0.013	+3.3	0.092	+4.3	0.017	−6.3	0.026	−19.8	0.085	−34.2
Glucose oxidase	0.064	0	0.079	+0.1	0.090	+4.6	0.027	+2.4	0.076	+17.9	0.149	0
L-Amino acid oxidase	0.044	+11.2	0.065	+16.8	0.074	−17.2	0.008	−12.3	0.092	+46.9	0.108	0

[a] The oscillator strength (f_i) is calculated from the expression: $f_i = 4.32 \times 10^{-9}\, \epsilon_i \Delta\nu_i$, where ϵ_i is the molar extinction at the curve maximum and $\Delta\nu_i$ is the band width at half-maximal intensity (Suzuki, 1967). [b] The rotational strength (R) is calculated from the expression: $R_i = 1.23 \times 10^{-42} (\theta_i \Delta_i / \lambda_i)$, where θ_i is the molar ellipticity at the curve maximum, Δ_i is the bandwidth in nanometers at half-maximal intensity, and λ_i is the wavelength in nanometers at maximum intensity (Miles and Urry, 1968).

ferent analogs were resolved into a minimum number of Gaussian functions. By simultaneously fitting both the absorption and circular dichroism curves, varying only the sign and intensity of the individual functions, it was possible to fit all of the spectra in the 300–500-nm region with six functions. This must be considered as representing the minimum number of transitions which occur in this region. Some of the limitations of this type of analysis are discussed by Miles and Urry (1968). Additional error is introduced into the fit by the relative broadness of the observed spectra. The confidence in the analysis is increased by the fact that it is possible to fit the more complex circular dichroism spectra of the protein-bound flavins using a similar set of functions (see below). Since the absorption and circular dichroism spectra can be matched with the same set of Gaussian functions, solvation effects which could shift the circular dichroism bands relative to the absorption bands (Moscowitz *et al.*, 1963) do not seem to be operative. The resolutions obtained by the analysis are shown by the dashed curves in Figures 1 and 2. A detailed listing of the energy spacings, oscillator strengths, and rotational strengths are given in Tables I and II.

A flavin transition occurring at about 300 nm has been detected by fluorescence polarization measurements (Kurtin and Song, 1968) and by circular dichroism measurements (Miles and Urry, 1968). Because of overlapping bands, it is difficult to decide whether or not this transition appears in the circular dichroism spectra of the flavoproteins. Miles and Urry (1968) have suggested that this is an $n \rightarrow \pi^*$ transition because of its low oscillator strength. If this assignment is correct, one should observe an appreciable red shift in going from polar to nonpolar solvents. In Figure 3 is shown the circular dichroism spectra in the 280–320-nm region of concentrated solutions of tetra-*O*-acetylriboflavin, FMN and FAD in water and in dichloroethane, along with the results of a curve resolution. The solvent polarity is seen to have only a small effect on the 300-nm transition (a small blue shift in going from water to dichloroethane). The loss in resolution which occurs in dichloroethane is almost entirely due to the large blue shift of the 383-nm band (see Table I). The transition is seen to be quite side-chain dependent, however, both in sign and in position. It is possible that effects from dimerization could be operative at the concentrations employed, although no changes were seen upon a 5-fold dilution. Thus, there is presently no experimental evidence to assign this band as being due to an $n \rightarrow \pi^*$ transition.

Circular Diochroic and Absorption Spectra of Protein-Bound Oxidized Flavin. The changes in the absorption spectra of flavins when they are bound to protein are small compared to the large changes which are observed in the circular dichroism spectra (Figures 4 and 5). The flavoproteins which we are considering here all have one FMN per protein molecule, are metal free, and have only a single polypeptide chain. This

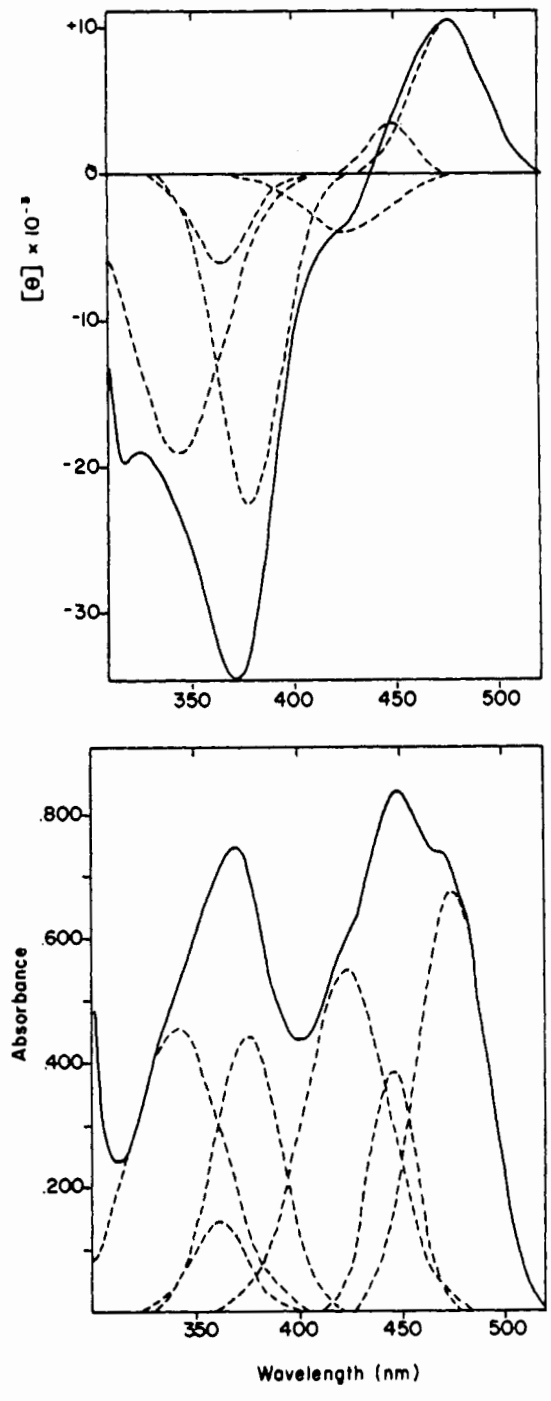

FIGURE 3: Resolved circular dichroism and absorption spectra of the Shethna flavoprotein. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA (pH 7.0) buffer.

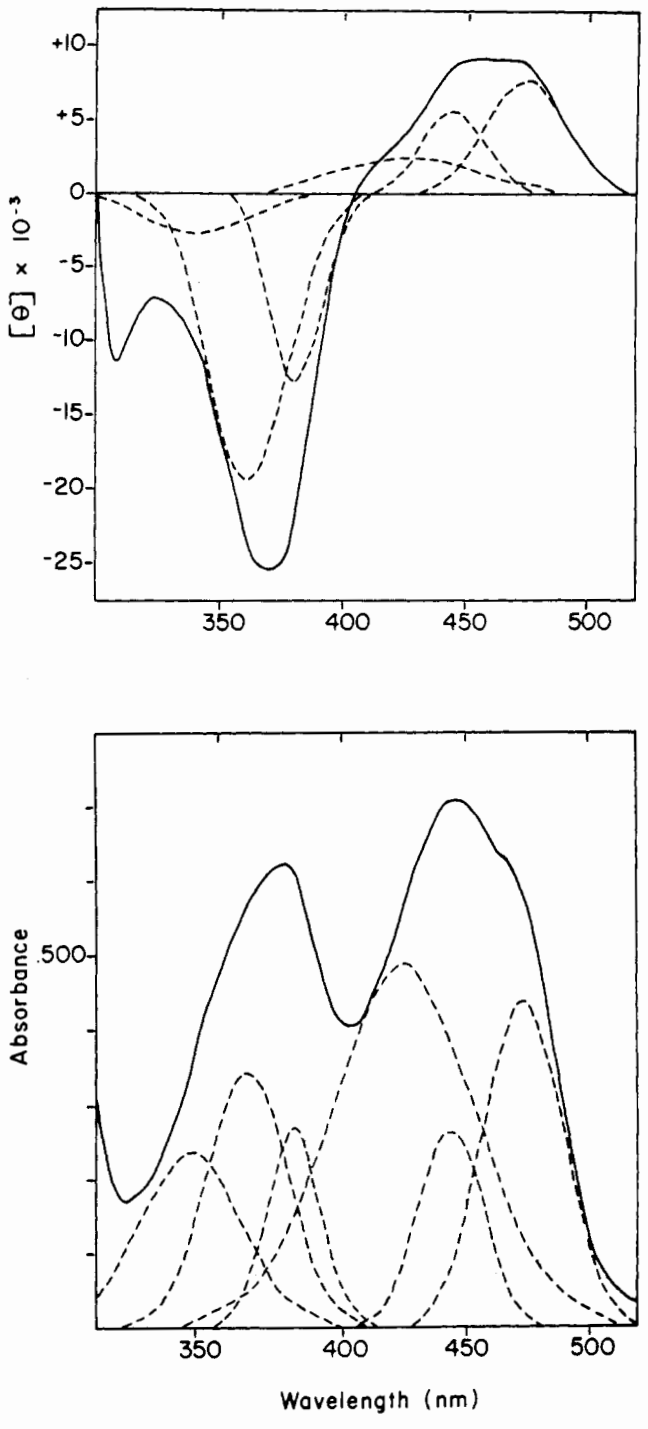

FIGURE 4: Resolved absorption and circular dichroism spectra of Clostridial flavodoxin. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA (pH 7.0) buffer.

assures that there are no interfering effects from metal chromophores, from flavin–flavin interactions, or from protein subunit interactions.

As mentioned previously, resolution of the circular dichroism and absorption spectra of these flavoenzymes was achieved using six Gaussian functions, as was the case with the unbound flavins. The negative band observed at 310 nm (Figures 4 and 5) was not included in the analysis due to overlap by the strong positive dichroic bands of the aromatic amino acids occurring below 300 nm. This dichroic band could originate either from the flavin chromophore (see below) or from a tryptophyl residue. A negative dichroic band at 303 nm has been observed for chymotrypsinogen (Strickland *et al.*, 1969) and ascribed to an indole transition. The energy spacings between the resolved bands follow the same pattern as was observed with the unbound flavins (Table I).

A comparison of the rotational strengths of the circular dichroism bands of free FMN with those for FMN bound to the Shethna flavoprotein indicates that the 0–0 transitions are increased from 5- to 20-fold (Table II). In contrast, the rotational strengths of the vibronic bands are increased to a lesser extent. The FMN bands in Clostridial flavodoxin show a

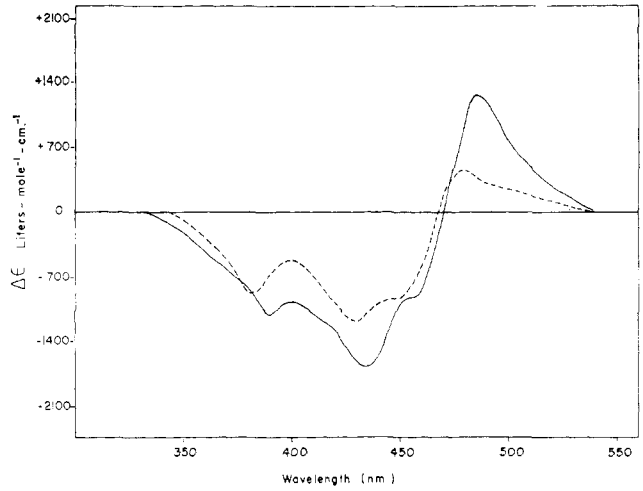

FIGURE 5: Difference spectra of shethna apoprotein-bound flavin minus unbound flavin. (—) FMN–protein complex minus FMN in 0.025 M phosphate (pH 7.0). (- - -) dFMN–protein complex minus dFMN in 0.025 M phosphate (pH 7.0).

somewhat different pattern of change in the rotational strengths. In addition, the sign of band III is opposite to that of the Shethna flavoprotein. The reasons for these differences are unclear. However, they must reflect differences in the two environments of the FMN molecule in the two flavoproteins, even though the overall shape of their circular dichroic spectra are quite similar (Figures 4 and 5).

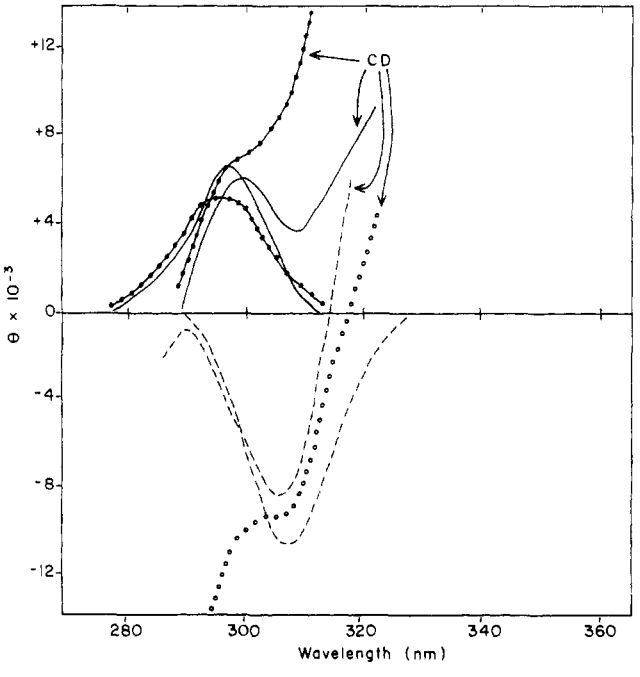

FIGURE 6: Circular dichroism spectra of flavin solutions in the near-ultraviolet region. (○○○) FAD (4.4×10^{-4} M) in H_2O. (- - -) FMN (9.4×10^{-4} M) in H_2O. (●●●) Tetra-*O*-acetylriboflavin (11.3×10^{-4} M) in 1,2-dichloroethane. (—) Tetra-*O*-acetylriboflavin (4.15×10^{-5} M) in H_2O. The ellipticity values are multiplied by three. Short path-length cells (10 mm) were used for all measurements except for tetra-*O*-acetylriboflavin in water in which a 50-mm path-length cell was used. The arrows point to the measured spectra. The other curves are obtained from curve resolution into Gaussian functions for the appropriate spectrum. The FAD spectrum was not resolved.

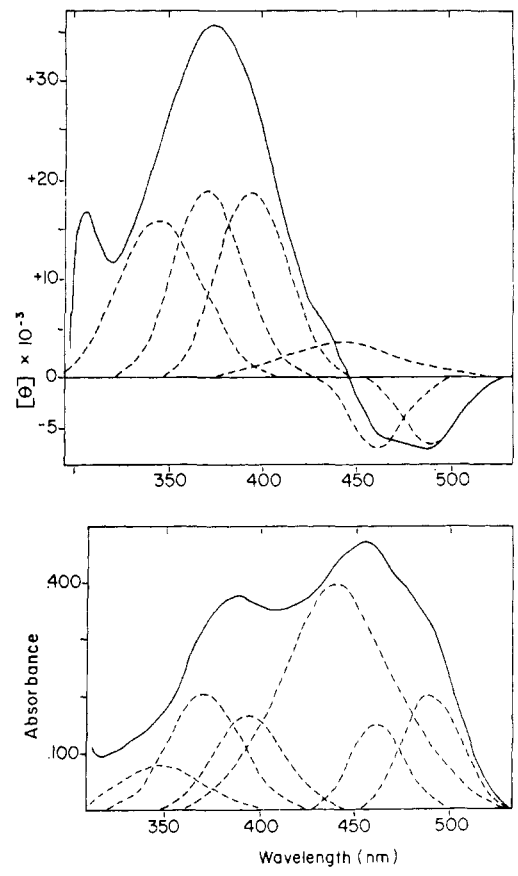

FIGURE 7: Resolved absorption and circular dichroism spectra of spinach ferredoxin–TPNH reductase in the visible region. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA (pH 7.0) buffer. The dashed curves are the resolved vibronic bands.

The circular dichroism spectrum of the dFMN–Shethna apoprotein complex[3] (Tables I and II) is a result of only protein-induced asymmetry, inasmuch as the flavin side chain has no asymmetric carbons. The overall shape of the spectrum is quite similar to that for the FMN protein. However, some differences are observed. One such difference is a red shift of the absorption bands of the FMN–protein complex relative to those of the dFMN–protein complex (Table I). This indicates that the hydroxyl groups provide a polar environment for the isoalloxazine ring when it is bound to the protein. This spectral shift can be seen more clearly in the optical difference spectra of the bound minus unbound flavins (Figure 6). Note also that a hypochromic effect and a "tailing" of the long-wavelength band to the red are observed for both derivatives. Also significant is the weaker positive dichroism at 470 nm for the bound dFMN as compared to the bound FMN. These effects are also seen with the protein-bound riboflavin and deoxyriboflavin analogs (see paper II of this series).

The circular dichroism spectrum of *P. elsdenii* flavodoxin is nearly identical with that of Clostridial flavodoxin while that of *R. rubrum* flavodoxin differs somewhat from the *P. elsdenii* and Clostridial enzymes, although again the overall shape is

[3] The binding of flavin analogs to the Shethna apoprotein is discussed in paper II of this series. The circular dichroism spectrum of the FMN–Shethna apoprotein complex is identical with that of the native flavoprotein.

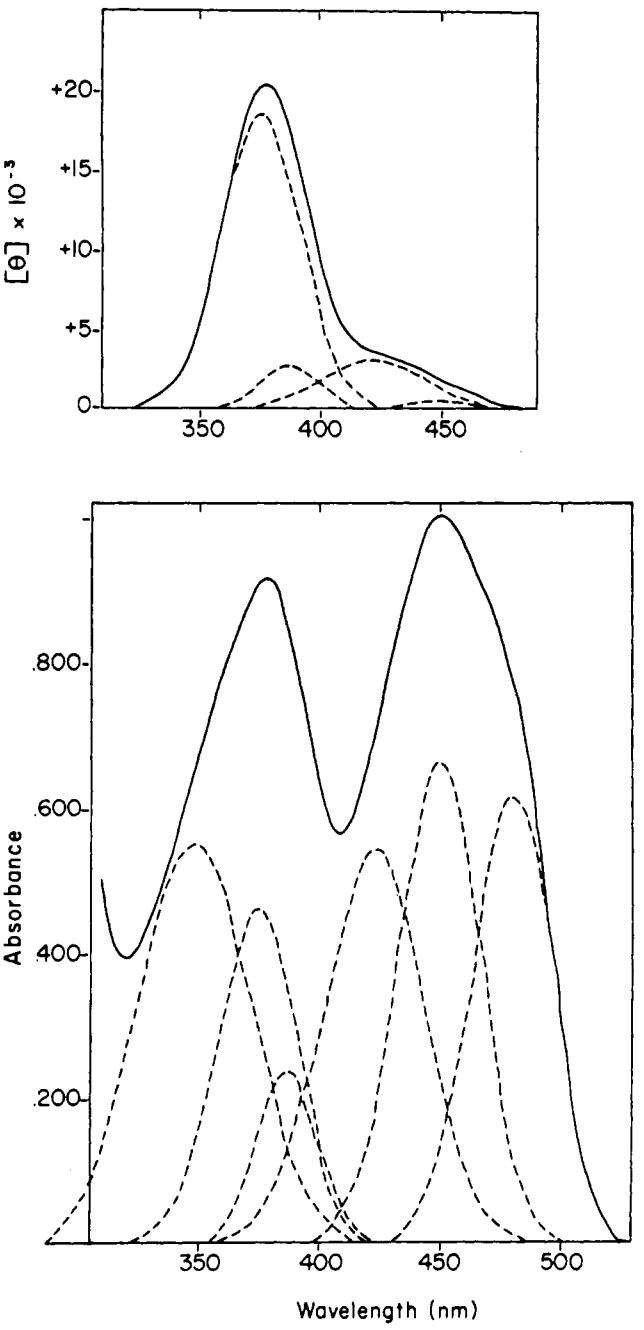

FIGURE 8: Resolved circular dichroism and absorption spectra of glucose oxidase in the visible region. The spectra were measured on protein solutions in 0.1 M acetate–0.05 M EDTA (pH 6.0) buffer. The dashed curves are the resolved vibronic bands.

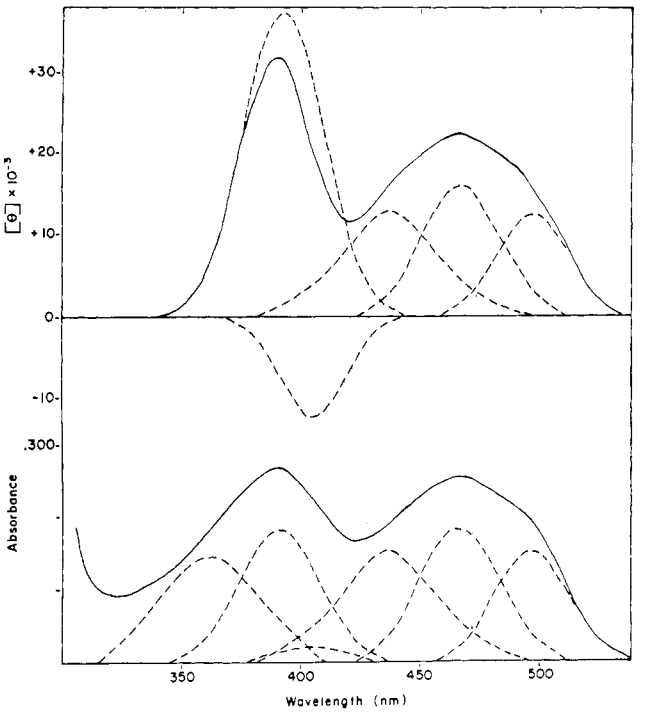

FIGURE 9: Resolved circular dichroism and absorption spectra of snake venom L-amino acid oxidase (*C. adamanteus*) in the visible region. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA (pH 7.0) buffer. The dashed curves are the resolved vibronic bands.

similar (see Tables I and II). It is interesting that the flavin dichroic bands of *R. rubrum* flavodoxin more closely resemble those of the Shethna flavoprotein than they do the other flavodoxins. This is also reflected in redox properties (see paper III of this series).

Since all of the above-mentioned flavoprotein dehydrogenases contain FMN, it was of interest to measure the circular dichroism spectrum of an FAD-containing dehydrogenase. In Figure 7 is shown the circular dichroism spectrum of spinach ferredoxin–TPNH reductase. It can be seen that this spectrum resembles those of the other dehydrogenases, except that all of the bands are reversed in sign. This indicates that the flavin environment is like that which is found in the other proteins, but that the perturbations induce opposite polarizations in the flavin transitions. The spectrum also suggests that the adenine–isoalloxazine interaction is broken upon binding of the FAD. It is of interest to note that this flavoenzyme transfers electrons to, and forms complexes with, ferredoxins and flavodoxins (Foust *et al.*, 1969).

The circular dichroism spectra of the oxidase flavoenzymes are quite different in shape from those of the dehydrogenases (Figures 8 and 9). The most prominent dichroic band in glucose oxidase and in L-amino acid oxidase is at 380 nm. Positive bands are observed at longer wavelengths in the case of L-amino acid oxidase, but are very weak in glucose oxidase. The circular dichroism spectrum of D-amino acid oxidase (Kotaki *et al.*, 1968) is similar to that of glucose oxidase both in band positions and in intensity.

Curve resolution of these oxidase and dehydrogenase circular dichroism and absorption spectra indicates the presence of six vibronic bands, as was found to be the case with the free flavins (paper I). Inasmuch as the band spacings are similar to those occurring in the unbound flavins (Table I), the bands can be assigned in the same manner.

Inspection of the rotational strengths and signs of the resolved circular dichroism bands of the dehydrogenases indicates two general patterns (Table II). The second vibronic band (band III) is opposite in sign to the 0–0 band (band I) in the circular dichroism spectra of the Shethna flavoprotein, the dFMN–Shethna apoprotein complex, *R. rubrum* flavodoxin, and ferredoxin–TPNH reductase. In contrast, band III has the same sign as the 0–0 band (band I) in the circular dichroic spectra of Clostridial flavodoxin and *P. elsdenii* flavodoxin. The circular dichroism spectrum of the 3-MeFMN–Shethna apoprotein complex (Tables I and II) indicates a pattern quite similar to that of the Clostridial and *P. elsdenii* flavo-

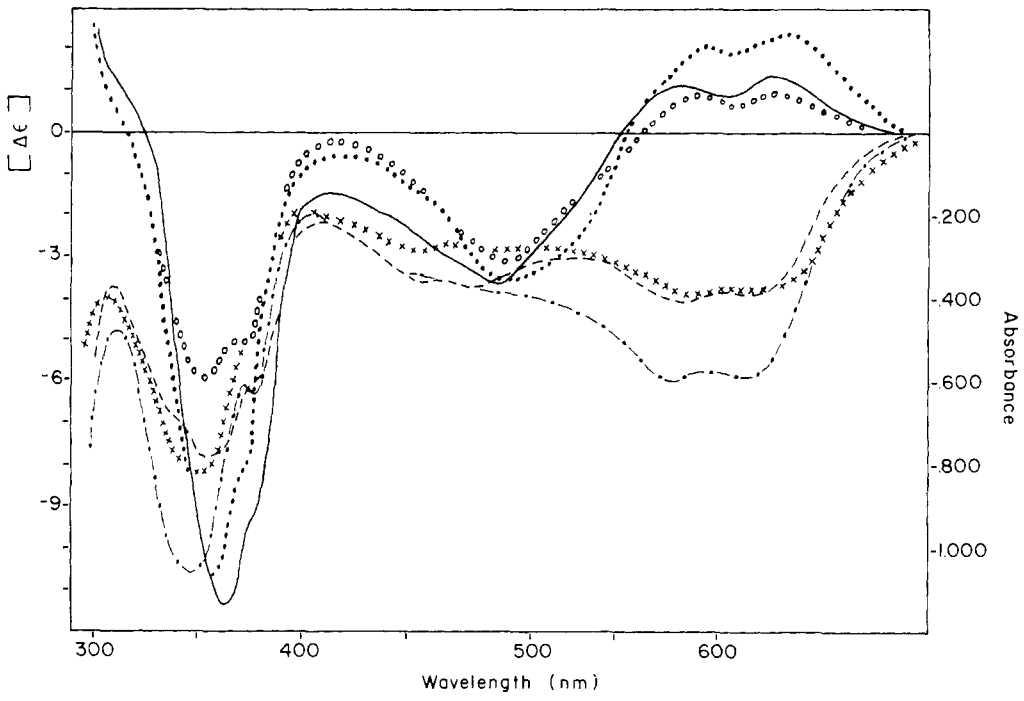

FIGURE 10: Absorption and circular dichroism spectra of the semiquinone forms of some dehydrogenase flavoproteins in the visible region. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA buffer (pH 7.0). (OOOO) Shethna flavoprotein–circular dichroism. (–·–·–·) Shethna flavoprotein absorption. (····) 3-MeFMN–Shethna apoprotein complex—circular dichrosim. (××××) 3-MeFMN–Shethna apoprotein complex—absorption. (—) *R. rubrum* flavodoxin—circular dichroism. (– – –) *R. rubrum* flavodoxin–absorption. Note that the absorption spectra are inverted to facilitate comparison.

doxins, in that the sign of band III is the same as that for band I. As will be shown in paper III of this series, some of the redox properties of this derivative also resemble those of the flavodoxins. It is of interest to note that the semiquinone forms of those flavoproteins in which band III is of opposite sign from band I in the circular dichroism spectra (with the exception of ferredoxin–TPNH reductase) are resistant to further reduction by dithionite at neutral pH, while those flavoenzymes in which the sign of band III is the same as that of band I are reduced to the flavin hydroquinone form. The structural significance of this apparent relationship is unclear.[4]

A second pattern which is observed is that the third 0–0 transition (band VI) is of comparatively low rotational strength in the Clostridial and *P. elsdenii* flavodoxins, but has a large rotational strength in *R. rubrum* flavodoxin, ferredoxin–TPNH reductase, the 3-MeFMN– and the dFMN–Shethna apoprotein complexes and in the Shethna flavoprotein. The 3-MeFMN protein derivative therefore does not have all of the circular dichroic properties of the Clostridial and *P. elsdenii* flavodoxins.[5]

The circular dichroism spectrum of the isoFMN–Shethna apoprotein complex (Tables I and II) is similar in shape and intensity to that of the native Shethna flavoprotein. A slight red shift of the 478-nm positive circular dichroism band to 488 nm is also apparent in the absorption spectrum. Since the flavin transitions are modified by the methyl shift from the 8 to the 6 position, a direct comparison of the individual bands cannot be made. However, the loss of the methyl group at the 8 position and the methyl addition to the 6 position does not drastically alter the environment of the bound flavin.

The most notable feature of the oxidase circular dichroism spectra is the lack of any measurable rotational strength in band VI. The low rotational strength in the 450-nm region in the case of glucose oxidase (Figure 8) could indicate the absence of side-chain–isoalloxazine ring interaction.

On the other hand, the circular dichroism spectrum of L-amino acid oxidase (Figure 9) indicates that such an interaction does occur in this protein. Both oxidase flavoenzymes contain FAD as the flavin group and have two FAD molecules per molecule of protein (Swoboda and Massey, 1964; Wellner and Meister, 1960).

Circular Dichroism and Absorption Spectra of the Semiquinone and Hydroquinone Forms of Flavoenzymes. In order to further compare the flavin environments in the dehydrogenases and oxidases, the circular dichroism and absorption spectra of the flavin semiquinone forms were measured. Figure 10 shows these spectra for the semiquinone form of the Shethna flavoprotein. The circular dichroism spectrum reflects the absorption spectrum except for the large negative dichroic band at 500 nm, which has a low extinction in the absorption spectrum, and a lack of optical activity in the 400–450-nm region. The spectra of the semiquinone forms of the *R. rubrum* flavodoxin (Figure 10) and the 3-MeFMN–Shethna apoprotein complex (Figure 10) resemble those of the Shethna flavoprotein. The semiquinone form of the dFMN–Shethna apoprotein complex also has the same type of circular dichroism spectrum, except for the low rotational strength of the two bands in the 600-nm region (Table II). Thus, the longest wavelength region of the circular dichroism spectrum again reflects side-chain–ring interaction. A similar decrease of rotational strength is found in the circular di-

[4] The sign of band III does not change in the Shethna flavoprotein upon raising the pH to above 8.0, where the semiquinone is reduced by dithionite (see paper III).

[5] The 3-MeFMN–Shethna apoprotein complex also does not function as a flavodoxin in the chloroplast-mediated photoreduction of TPN (D. E. Edmondson and G. Tollin, unpublished observations).

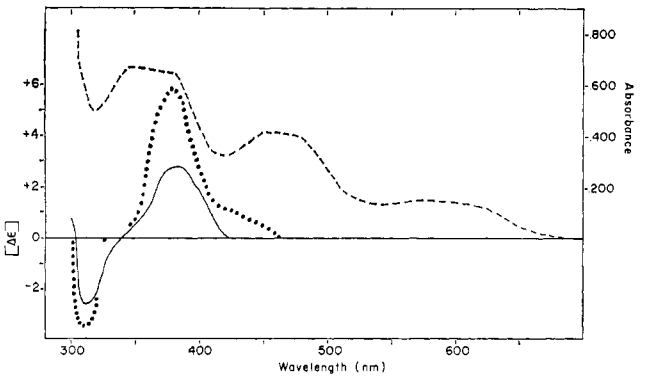

FIGURE 11: Circular dichroism and absorption spectra of the neutral semiquinone form of glucose oxidase in the visible region. (– – –) Absorption spectrum, (——) circular dichroism spectrum. (· · · ·) Circular dichroism spectrum of reoxidized material. All spectra were measured on protein solutions in 0.1 M acetate–0.05 M EDTA buffer (pH 6.0).

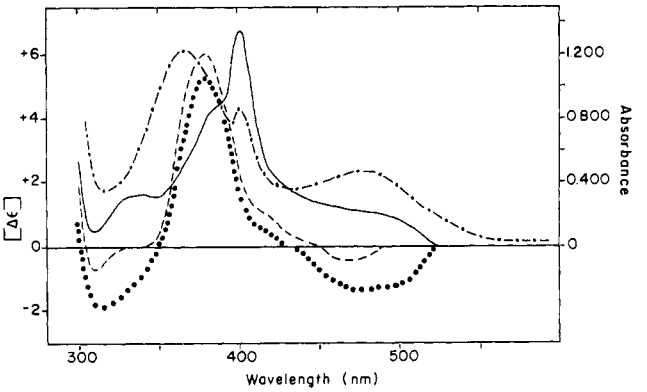

FIGURE 12: Circular dichroism and absorption spectra of the anionic semiquinone form of glucose oxidase in the visible region. (· – · – ·) Absorption spectrum, (——) circular dichroism spectrum. (– – –) Circular dichroism spectrum of nonirradiated material. (· · · ·) Circular dichroism spectrum of reoxidized material. All spectra were measured on protein solutions in 0.1 M glycine–0.05 M EDTA buffer (pH 9.5).

chroism spectrum of the isoFMN–Shethna apoprotein complex. The absorption spectrum of this derivative is appreciably different from those of the other flavoenzyme semiquinones.

The absorption spectral characteristics of the Clostridial and the *P. elsdenii* semiquinones are more similar to those of the isoFMN semiquinone, even though both of these flavoenzymes have FMN as their chromophore. The circular dichroism spectra of these two flavoprotein semiquinones are almost identical, and have the same general characteristics as the spectra of the semiquinone forms of the other dehydrogenases. The small differences which are observed are a furhter verification of the minor variations in flavin environment which are suggested by the observations on the oxidized proteins.

No curve analysis was done on the semiquinone spectra since it is technically impossible to determine the circular dichroism spectrum of unbound flavin radical due to its high rate of disproportionation. Also, to date, there are no molecular orbital calculations on flavin semiquinones. The spectral properties of the ferredoxin–TPNH reductase semiquinone were not determined due to the difficulty in preparing substantial amounts of this species.

Since both the neutral (blue) and anionic (red) semiquinone forms of glucose oxidase can be prepared (Massey and Palmer, 1966), this flavoenzyme provides a "bridge" for comparing the semiquinone environments of the dehydrogenases and oxidases. The absorption spectrum of the neutral semiquinone resembles that of this form of the dehydrogenase flavoenzymes (Figure 11). The circular dichroism spectrum, however, is quite different, principally in the lack of optical activity at wavelengths longer than 400 nm. The positive band at 375 nm is about one-third the intensity of the negative 355-nm band in the Shethna flavoprotein. The negative band seen at 310 nm appears to be an artifact resulting from irradiation, since it does not disappear upon reoxidation. The circular dichroism spectra of all of the other flavoproteins were identical upon reoxidation to those of the nonirradiated proteins. The nature of this irreversible effect is not known. The absorption spectrum of the reoxidized material is identical with the nonirradiated flavoenzyme and no enzymic activity is lost (Massey and Palmer, 1966).

The absorption spectrum of the anionic glucose oxidase semiquinone (Figure 12) is close to that found for this species of L-amino acid oxidase (Figure 13), although the very sharp band seen at 400 nm in glucose oxidase does not appear in the L-amino acid oxidase spectrum. This latter band is the major circular dichroism absorption in glucose oxidase (Figure 12), while the 370-nm maximum observed in the amino acid oxidase spectrum can be identified as a shoulder on the 400-nm band. The circular dichroism spectrum of the L-amino acid oxidase anionic semiquinone has a major band at 400 nm which seems to be a component of the asymmetric 395-nm absorption maximum. The broad "tail" in the circular dichroism spectra of both oxidase semiquinones in the 450–500-nm region reflects the 490-nm band apparent in both absorption spectra. As was observed at pH 6.0, an irreversible change occurs in the photoreduction of glucose oxidase at pH 9.5. This can be seen by comparing the circular dichroism spectrum of the reoxidized material with the nonphotoreduced material (Figure 11).

Further evidence for differences in flavin environment between the dehydrogenases and oxidases can be obtained from a comparison of the circular dichroism spectra of the hydroquinone forms of the dehydrogenases (Figure 14) and the

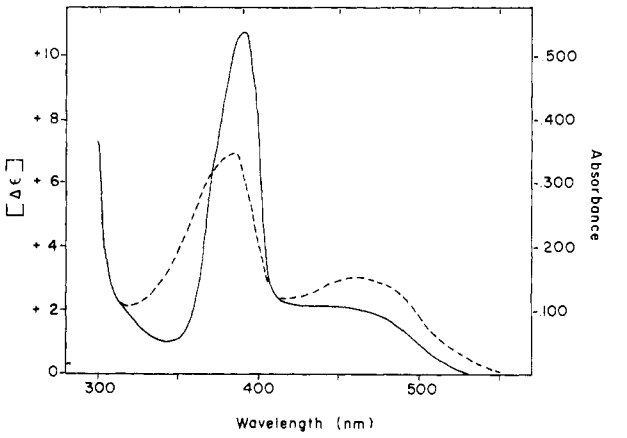

FIGURE 13: Circular dichroism and absorption spectra of the anionic semiquinone form of L-amino acid oxidase in the visible region. (– – –) Absorption spectrum. (——) Circular dichroism spectrum. The spectra were measured on protein solutions in 0.025 M phosphate–0.05 M EDTA buffer (pH 7.0).

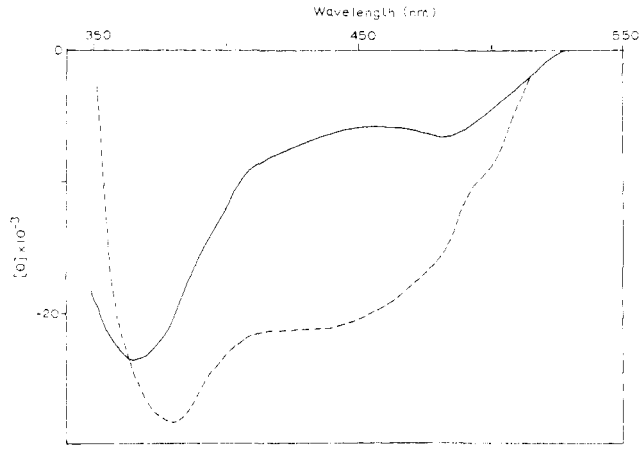

FIGURE 14: Circular dichroism spectra of dehydrogenase hydroquinone forms. (——) *P. elsdenii* flavodoxin. (– – –) Shethna flavoprotein. The flavoproteins were reduced with a small excess of dithionite in 0.1 M Tris buffer (pH 8.0).

published spectrum of the D-amino acid oxidase hydroquinone (Yagi *et al.*, 1969). The latter species has a positive circular dichroism band at 350 nm and a negative band at 420 nm (Yagi *et al.*, 1969), whereas the circular dichroism spectra of the two dehydrogenase hydroquinones are characterized by a broad negative band occurring between 400 and 500 nm and a second negative band in the 370-nm region (Figure 14).

Discussion

The resolved spectra of tetra-*O*-acetylriboflavin in dichloroethane and in phosphate buffer show that all of the vibronic bands are shifted 10–15 nm to the blue in the nonpolar solvent (Table I). No evidence for an n → π^* transition in this spectral range is obtained; the spectral shifts can all be ascribed to solvation effects on the $\pi \rightarrow \pi^*$ transitions. An analysis[6] of the band separations suggests that bands I, IV, and VI are 0–0 transitions (Table I). Such an assignment is in agreement with theoretical calculations which predict three $\pi \rightarrow \pi^*$ transitions in this spectral range (Fox *et al.*, 1967). It should be pointed out that the accuracy of the band positions presented is estimated to be approximately ± 200 cm^{-1}.

Analysis of the separations of bands II, III, and V indicate a vibronic origin (Table I). Band II is assigned as a vibronic transition associated with the lowest energy 0–0 band. Band III is also assigned as a vibronic overtone of the lowest energy 0–0 transition. That this is probably not a progression of the first vibronic band (band II) is indicated by the fact that it changes sign relative to the lowest energy 0–0 band in the circular dichroism spectrum of the Shethna flavoprotein (see Figure 3). This would suggest that it belongs to a vibrational mode of different symmetry than band II. The assignment of band III as vibronic and not as a separate electronic transition is based on the following considerations. (1) Theoretical calculations (Fox *et al.*, 1967; Song, 1969) do not predict another $\pi \rightarrow \pi^*$ transition in this region of the spectrum. (2) The spectra of tetra-*O*-acetylriboflavin in polar and in nonpolar solvents (Figures 1 and 2) do not provide evidence for an n → π^* transition. (3) Recent theoretical work (Caldwell, 1969) predicts the possibility that a vibronic band can have a different sign from the 0–0 band in the circular dichroism spectrum. This phenomenon has also been observed in low-temperature circular dichroism studies of phenylalanine and its derivatives (Horwitz *et al.*, 1969).

The energy spacing (Table I) is consistent with an assignment of band V as a vibronic transition associated with the second 0–0 band (band IV). On the basis of the same arguments presented for band III (see above), it seems unlikely that this is a separate electronic transition. It is noteworthy that band V has the same sign as the 0–0 band in the circular dichroism spectra of the flavoproteins (see below) and of FMN but is of opposite sign in the circular dichroism spectra of tetra-*O*-acetylriboflavin (Figures 1 and 2). This band is so weak in the circular dichroism spectrum of riboflavin that its sign cannot be unambiguously determined. The large range in energy spacing relative to the 0–0 band (Table I) which is observed for band V could be due to the inherent error in resolving the broad featureless 370-nm spectral region. Another possibility is that the vibration excited in this transition involves the side-chain–isoalloxazine ring interaction, since a consistently smaller spacing is observed in tetra-*O*-acetylriboflavin and the dFMN–protein derivative (Table I).

The band occurring in the spectra at 330–340 nm (band VI) is assigned as the third 0–0 transition. This is based on the molecular orbital calculations (Fox *et al.*, 1967) and on circular dichroism and magnetic circular dichroism studies (Tollin, 1968). The energy spacing (2000 cm^{-1}) seems too large for it to be a vibronic overtone of the 375-nm transition. On the basis of the solvent studies presented, this band must be considered as resulting from a $\pi \rightarrow \pi^*$ transition rather than from an n → π^* transition as suggested by Miles and Urry (1968). The fact that a transition in this spectral region is not observed in fluorescence polarization measurements could indicate that the angle between the transition moment and the emission oscillator is similar to that for the 375-nm transition.

The optical difference spectra provide insights into the environment of the flavin chromophore on the protein. As is shown in Figures 1 and 2, the absorption spectrum of flavin in a nonpolar solvent is partially resolved in the long-wavelength region. Little or no hypochromism is observed and no appreciable tailing of the 450-nm band occurs (Kotaki *et al.*, 1967; Palmer and Massey, 1968). These latter effects, however, do occur when flavins are complexed with other aromatic molecules, *e.g.*, with phenols (Fleischman and Tollin, 1965) or with adenine in FAD. In the flavoproteins considered here, increased resolution, hypochromism and long-wavelength tailing are observed, although no blue shifts are apparent. Thus, it may be that the binding of FMN to the Shethna apoprotein, as well as that of Clostridial flavodoxin, results in placing the isoalloxazine ring in a less polar environment than in water and close to one or more aromatic amino acid residues of the protein. It is conceivable that this combination of effects accounts for the lack of a blue shift of the vibronic bands.

The circular dichroism data also show that the rotational strength of the 0–0 band in the 470-nm region is particularly sensitive to side-chain hydroxyl group interaction with the isoalloxazine ring and that this interaction is greatly increased in magnitude when FMN is bound to the proteins considered here (the optical difference spectra are also consistent with this). Such a correlation could explain the occurrence of strong optical activity in the 450-nm region of certain FAD-containing flavoenzymes, for example, *p*-hydroxybenzoate

[6] Because of the similarities in numbers of vibronic bands and in energy spacings, it is suggested that the analysis to be presented is valid for all of the flavin species investigated here.

hydroxylase (Hesp *et al.*, 1969). Free FAD has little or no optical activity in this spectral region (Miles and Urry, 1968; Hesp *et al.*, 1969). This may be because the ribityl side chain cannot interact strongly with the isoalloxazine ring as a result of steric limitations caused by the complexation with the adenine ring. Specific protein–FAD interactions could break up this complex and could position the ribityl side chain and the isoalloxazine ring in such a manner as to produce the observed circular dichroism spectrum. This is evident in the circular dichroism spectrum of the FAD–Shethna apoprotein complex (see paper V of this series). In the case of lipoyl dehydrogenase, another FAD flavoprotein, the circular dichroism data show no optical activity in the long-wavelength region (Brady and Beychok, 1969). This would suggest that the side-chain–ring interactions are weak, even though the flavin–adenine complex is no longer intact, as evidenced by the increase in fluorescence of the bound FAD (Palmer and Massey, 1968).

More experiments are needed to verify this hypothesis. However, the interpretation is reinforced by the observation that FAD analogs containing no ribityl hydroxyl groups have essentially indentical absorption and fluorescence properties as does FAD (Chassey and McCormick, 1965). A comparison of circular dichroism properties of FAD and its deoxy analog would be of interest. In addition, the suggestion from circular dichroism data that there is little or no isoalloxazine ring–ribityl side-chain interaction in free FAD could perhaps be more unequivocally indicated by nmr studies, since the possibility of a cancelling effect by overlapping positive and negative circular dichroism bands could be operative.

Massey *et al.* (1969a) have proposed the classification of flavoenzymes as dehydrogenases and oxidases on the basis of oxygen reactivity, sulfite addition, and semiquinone ionization form. In the present work, we have shown that circular dichroism spectroscopy provides another basis for such a classification. Specifically, circular dichroism spectra represent a direct structural probe by means of which correlations between flavin environment and reactivity may be made. Thus, the results presented here, in conjunction with other studies (Nishikimi and Yagi, 1969; Kotaki *et al.*, 1968), clearly establish the existence of similar flavin environments among the dehydrogenases and among the oxidases, for the oxidized, semiquinone, and hydroquinone forms.

The circular dichroism spectra of several other flavoenzymes in their oxidized forms (egg-white flavoprotein (Nishikimi and Yagi, 1969), *p*-hydroxybenzoate hydroxylase (Hesp *et al.*, 1969) and lipoyl dehydrogenase (Brady and Beychok, 1969)) all are fairly similar in shape to the dehydrogenases discussed here. The egg-white flavoprotein has no known redox function but forms a blue semiquinone on dithionite titration (Nishikimi and Yagi, 1969). Lipoyl dehydrogenase forms a nonparamagnetic long-wavelength-absorbing species (Palmer and Massey, 1968). A redox-active disulfide bond is implicated in the catalytic mechanism of this enzyme, so that a direct comparison with the dehydrogenases studied here would not be proper. No semiquinone was detected in the dithionite titration of *p*-hydroxybenzoate hydroxylase (Hesp *et al.*, 1969).

The data presented in this paper and in paper III of this series demonstrate that within the dehydrogenases, correlations may be made between circular dichroism spectra and redox properties (*e.g.*, Clostridial and *P. elsdenii* flavodoxins *vs. R. rubrum* flavodoxin *vs.* Shethna flavoprotein). These correlations seem to be based upon differences in environment which affect the ionization behavior of the flavin in its various redox forms and thus control its redox reactivity (paper III). Thus, as one might expect *a priori*, the flavin–protein interaction is a key determinant in redox behavior. Circular dichroism spectroscopy, because of its ability to probe this interaction, provides the enzymologist with a powerful tool for investigating certain aspects of flavin conformation in flavoproteins.

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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*

Dale E. Edmondson† and Gordon Tollin‡

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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