which is activated by another endogenous ligand, AMet. There is suggestive evidence that AMet may activate the enzyme in a hysteretic manner (Figure 3).

There are two main factors which may contribute to the time-dependent change of reaction velocity in the synthesis of AMet. (a) Time-dependent formation of endogenous AMet which is required for activation of tripolyphosphatase activity. The significance of this effect on the overall enzymatic reaction has been shown by adding small amounts of exogenous AMet to the reaction system (Figure 2). (b) Time-dependent activation of tripolyphosphatase by AMet. The finding that externally added AMet did not eliminate the lag period completely (Figure 3) is in favor of this possibility.

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Studies of Flavin–Protein Interaction in Flavoproteins Using Protein Fluorescence and Circular Dichroism†

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ABSTRACT: Preparation of the apoproteins of *Desulfovibrio vulgaris* and *Rhodospirillum rubrum* flavodoxins and the equilibrium constants for riboflavin binding to these proteins are reported. Far-ultraviolet circular dichroism (CD) spectra of the apo- and holoproteins of *Clostridium pasteurianum*, *Peptostreptococcus elsdenii*, *D. vulgaris*, and *R. rubrum* flavodoxins indicate that significant changes in protein secondary structure accompany FMN binding. Protein fluorescence maxima and excitation spectra have also been measured for these materials and found to correlate with riboflavin binding ability and visible CD spectra of the holoproteins. The kinetics of FMN and protein fluorescence quenching upon coenzyme binding to apoprotein have been found to be second order (first order in flavin and first order in protein) and rate constants have been determined. The visible CD spectra of *C. pasteurianum* and *D. vulgaris* flavodoxins indicate that these proteins may be classified into two previously distinguished subclasses of dehydrogenases (Edmondson, D. E., and Tollin, G. (1971a,c) *Biochemistry 10*, 113, 133). These subclasses are shown to differ in their ability to bind riboflavin. Trends among these various types of measurements are tabulated and are shown to be consistent with the classification based upon CD spectroscopy and to correlate with enzymic activity. Fluorescence quenching studies of glucose oxidase apoprotein during binding of FAD have shown that protein fluorescence quenching is a faster process than is flavin fluorescence quenching. Protein fluorescence is not affected by ADP which binds to the apoprotein and which competes for FAD binding sites. Possible quenching mechanisms are discussed.

Flavin absorption and fluorescence properties have been widely utilized in studying coenzyme environment in flavoproteins (Swoboda, 1969a,b; Edmondson and Tollin, 1971a–c; Mayhew, 1971). Protein fluorescence (Massey and Curti, 1966; D'Anna and Tollin, 1971) and circular dichroism (CD) spectroscopy (Edmondson and Tollin, 1971a,b) have been less frequently employed. The use of these latter

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techniques, particularly far-uv CD, protein fluorescence emission, and excitation spectra and protein fluorescence quenching kinetics, in conjunction with absorption and CD spectra in the visible region, may reveal protein secondary and/or tertiary structural changes which accompany flavin binding. Such information, coupled with the binding thermodynamics of specifically modified flavin derivatives, can be helpful in assessing the importance of protein rearrangement to flavin binding.

Among the simpler flavoproteins are the various flavodoxins (Knight and Hardy, 1966; Mayhew and Massey, 1969; Dubourdieu and LeGall, 1970; Cusanovich and Edmondson, 1971) and the Shethna flavoprotein from *Azotobacter vinelandii* (Shethna *et al.*, 1965). These flavoproteins have low molecular weights (15,000–25,000), possess one FMN prosthetic group per molecule and have no subunit structure. Previous CD studies of some of the flavodoxins and of the Shethna protein (Edmondson and Tollin, 1971a) have indicated that the FMN binding sites are similar in these materials. On the other hand, the visible absorption spectra, while all showing more vibronic structure than free FMN, differ considerably in their band maxima, extinction coefficients, and band ratios (see below). Additionally, the flavodoxins and the Shethna protein show variations in susceptibility to different methods of apoprotein resolution and in flavin fluorescence quenching kinetics upon coenzyme binding to the apoprotein. Thus, although FMN is similarly bound in these flavoproteins, environmental differences do exist. A study of protein physical properties in conjunction with those of the flavin coenzyme may help to elucidate the nature of these differences.

A more complex flavoprotein which has received considerable attention is the glycoprotein, glucose oxidase (Swoboda and Massey, 1964). Swoboda (1969a,b) has recently reported the preparation and several of the properties of the apoprotein of this oxidase. It was shown that flavin fluorescence quenching upon binding to the apoprotein could be resolved into two first-order processes. Fluorescence polarization measurements indicated that flavin fluorescence quenching followed flavin–protein interaction. It was also shown that the apoprotein and holoprotein differ in sedimentation coefficient and intrinsic viscosity. ADP (among other adenosine derivatives) was demonstrated to bind to the apoprotein. The ADP–apoprotein complex would not bind FAD and was shown to have the same sedimentation coefficient as the holoprotein.

Several questions arise from these experiments. (1) Do the CD spectra of the apoprotein, holoprotein and ADP–apoprotein complex reflect the changes observed in the ultracentrifuge experiments? (2) Is protein fluorescence quenched upon FAD and ADP binding? (3) How do protein fluorescence quenching kinetics compare with flavin quenching kinetics?

In this paper we report the far-uv CD spectra of the apo- and holoproteins of several flavodoxins and of glucose oxidase. We have also measured the fluorescence emission and excitation spectra of the apoproteins and protein fluorescence quenching and flavin fluorescence quenching kinetics. Visible CD spectra for two flavodoxins are also reported. The occurrence of protein rearrangement upon flavin binding, differences in the visible CD, absorption, and fluorescence spectra and the importance of the ribityl phosphate group to flavin binding in the flavodoxins are discussed. The results of the above-mentioned experiments with glucose oxidase are considered in terms of possible fluorescence quenching mechanisms.

Experimental Section

Materials

Rhodospirillum rubrum flavodoxin was isolated according to the method of Cusanovich and Edmondson (1971). Final purification was achieved by ammonium sulfate fractional precipitation. The flavodoxin from *Clostridium pasteurianum* (Knight and Hardy, 1966) was a gift from Dr. J. L. Fox of the University of Texas at Austin; the enzyme from *Desulfovibrio gigas* was from Drs. M. Dubourdieu and J. LeGall, C. N. R. S., Marseilles (France). *Peptostreptococcus elsdenii* flavodoxin was obtained from Dr. Stephen Mayhew, University of Michigan, Ann Arbor. Glucose oxidase was purchased from Worthington Biochemical Corp. Further purification of this enzyme using column chromatography and fractional precipitation (Swoboda and Massey, 1964) did not affect the spectral properties nor the yields of apoprotein obtainable. FMN and FAD, from Calbiochem, were purified by the method of Massey and Swoboda (1968). Riboflavin, also obtained from Calbiochem, was used without further purification.

Methods

The apoproteins of *C. pasteurianum* and *P. elsdenii* flavodoxins were prepared by the KBr dialysis method of Mayhew (1971). His reported extinction coefficients of 25,000 l. mole^{-1} cm^{-1} (278 nm) and 25,200 l. mole^{-1} cm^{-1} (282 nm) were used for the respective apoproteins.

It was possible to resolve the apoproteins of *D. vulgaris* and *R. rubrum* using either the KBr method or 3% trichloroacetic acid containing 10^{-3} M dithiothreitol (Edmondson and Tollin, 1971b). As with the other flavodoxins, yields of active apoprotein varied from preparation to preparation. However, the best yields of *D. vulgaris* apoprotein were obtained using the KBr method, whereas better yields were obtained from *R. rubrum* by acid treatment. For approximately equal concentrations of holoprotein (10^{-4} M), complete diffusion of flavin from solutions dialyzed against KBr required 24 hr. for *R. rubrum* flavodoxin, about 20 hr for *P. elsdenii* flavodoxin and about 12 hr for the other flavodoxins.

An extinction coefficient of 35,000 l. mole^{-1} cm^{-1} at 276 nm was obtained for the *R. rubrum* apoprotein using the Lowry method (Lowry *et al.*, 1951) with holoprotein as the standard. Such a value for the extinction coefficient is consistent with the presence of four tryptophan and nine tyrosine residues (Cusanovich and Edmondson, 1971). For *D. vulgaris* apoprotein, an extinction coefficient of 20,000 l. mole^{-1} cm^{-1} (278 nm) was obtained by titrating the apoprotein with FMN while monitoring flavin and protein fluorescence quenching. All experiments with the flavodoxins were performed in 0.025 M phosphate buffer (pH 7.0).

Glucose oxidase apoprotein was prepared according to the acid ammonium sulfate method of Swoboda (1969a). An extinction coefficient of 19.2×10^4 l. mole^{-1} cm^{-1} at 278 nm was obtained by the biuret method (Gornall *et al.*, 1949) using bovine serum albumin as standard. Experiments with glucose oxidase were performed in 0.10 M phosphate buffer (pH 6.1). The enzymatic activity of apoprotein plus excess FAD paralleled the ability of the apoprotein to quench flavin fluorescence. The activity was determined by monitoring oxygen uptake during glucose oxidation by use of a YSI oxygen electrode.

Other instrumentation and techniques have been previously described (D'Anna and Tollin, 1971; Edmondson and Tollin, 1971a,b).

Results and Discussion

Visible CD Spectra. The visible CD spectra of *D. vulgaris* and *C. pasteurianum* flavodoxins are shown in Figure 1. These spectra are very similar in shape and ellipticities to those of other flavodoxins and of the Shethna flavoprotein (Edmondson and Tollin, 1971a). The positions of the maxima of the positive CD bands reflect the red shift of the first electronic absorption transitions of *D. vulgaris* flavodoxin relative to that of *C. pasteurianum*. It should be noted, however, that the region of positive ellipticity in the *D. vulgaris* spectrum is narrower in breadth compared to the corresponding portion of the *C. pasteurianum* spectrum. Also, the ellipticity changes sign at about 400 nm for the *C. pasteurianum* spectrum while the crossover point for *D. vulgaris* flavodoxin is about 440 nm. With respect to the latter two properties, the CD spectrum of *D. vulgaris* flavodoxin corresponds closely to those of *R. rubrum* flavodoxin and the Shethna flavoprotein. On the other hand, the CD spectrum of *C. pasteurianum* flavodoxin is similar to the spectra of *Clostridial* MP and *P. elsdenii* flavodoxins (Edmondson and Tollin, 1971a). The spectra of these two classes of flavoprotein dehydrogenases have been described in terms of a gaussian curve analysis of the CD and electronic absorptions (Edmondson and Tollin, 1971a). In terms of that model, the observed differences can be attributed to a change in sign of the CD rotational strength of the third vibronic band in the lowest energy electronic transition. This sign change was interpreted as resulting from specific but undetermined differences in interaction between FMN and the apoprotein. Henceforth, we shall refer to those flavoproteins having ellipticity crossover points in the visible CD spectra at 400 nm as *pasteurianum* type. Those with the longer wavelength ellipticity crossover point shall be referred to as *rubrum*-type flavoproteins (see below).

Far-Uv CD Spectra. The far-uv CD spectra of the holo- and apoproteins of four flavodoxins are given in Figure 2. There are several observations that need to be considered. Firstly, the ellipticity values of the holoprotein from *C. pasteurianum* are quite large.[1] Based on studies with model polypeptide systems (Greenfield and Fasman, 1969; Saxena and Wetlaufer, 1971), one would expect such large ellipticity values only if the protein backbone was 100% in the α-helical configuration. However, the spectral shape and the magnitude of the ellipticity at 190 nm are not typical of an α helix. We cannot offer a sound theoretical explanation of this, except to point out that the models used may not be completely relevant. It seems reasonable to conclude, however, that *C. pasteurianum* flavodoxin is a highly structured protein.

Secondly, the flavodoxins as a group exhibit significant changes in rotational strengths in going from the holo to apo form. Reconstitution of the *P. elsdenii* holoprotein by mixing apoprotein and FMN restored approximately 80% of the ellipticity (Figure 2b). This degree of restoration is consistent with our binding studies using fluorescence quenching (see below). These results can be interpreted as indicating large secondary structural changes in the protein upon FMN binding. However, we must consider the presence of the flavin chromophore and its possible contributions in the far-uv region. In the free state (aqueous solution), FMN has several CD bands in the uv (Miles and Urry, 1968). Unfortunately, these bands are obscured in the protein and their signs and rotational strengths are not known. However,

[1] Spectra were measured from two different batches of protein and cellulose acetate electrophoresis gave a single band.

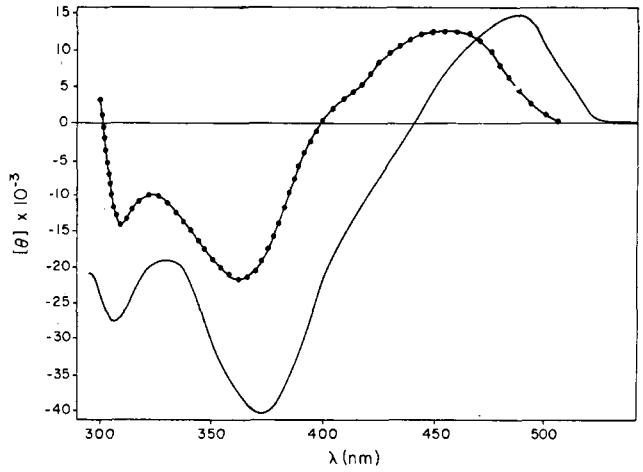

FIGURE 1: Visible CD spectra of *D. vulgaris* (—) and *C. pasteurianum* (●) flavodoxins.

in the visible region, the FMN CD bands are inverted in sign and increased in ellipticity by a factor of about 10 when the coenzyme is bound to protein. If one considers that similar increases in ellipticity occur in the far-uv region, bound FMN could contribute average ellipticities per residue of the order of 0.6×10^{-3} to 0.7×10^{-3} (deg cm^2) per dmole for small proteins of about 150 amino acid residues. As the ratio of protein to flavin increases, one would expect to see decreased contributions in the far-uv region. Based on these considerations, and the values of the ellipticities observed for the apo- and holoproteins (Figure 2), one would expect to have some flavin contributions in the uv region, but hardly enough to account for the changes observed upon binding FMN to the flavodoxin apoproteins. Hence, we interpret the CD spectra as reflecting significant loss of secondary structure (increase in random coil) accompanying flavin removal from the apoprotein. This appears to be reversible, at least in the case of the *P. elsdenii* flavodoxin. With the Shethna flavoprotein (Edmondson and Tollin, 1971b), a small reversible increase in secondary structure was noted upon flavin removal. Veeger *et al.* (1971) observed a large increase in ellipticity at 220 nm upon removal of FAD from lipoamide dehydrogenase. No change in the CD spectrum below 250 nm occurs upon removal of FMN from yeast L-lactate dehydrogenase (Tsong and Sturtevant, 1969).

Fluorescence. The maximum wavelengths of apoprotein fluorescence emission, riboflavin binding constants and second order rate constants for protein and/or flavin fluorescence quenching upon FMN binding to the apoproteins are given in Table I. Protein fluorescence of the holoproteins of *C. pasteurianum*, *P. elsdenii*, and *D. vulgaris* flavodoxins is effectively quenched and thus the spectra could not be measured. Protein fluorescence is only 40% quenched in *R. rubrum* flavodoxin, and the fluorescence spectrum is very similar to that of the apoprotein. Note that the fluorescence maxima of the several apoproteins follow the same pattern as do the visible CD spectra of the holoproteins.

Although there is considerable variation in the rate constants among the flavodoxins (up to 60-fold), in all cases both protein and flavin fluorescence quenching obey the same second-order rate law. Riboflavin binds more strongly ($\geq$4 kcal/mole) to apoproteins derived from the *rubrum* type of flavodoxin than with those from the *pasteurianum* type. Degrees of riboflavin fluorescence quenching comparable

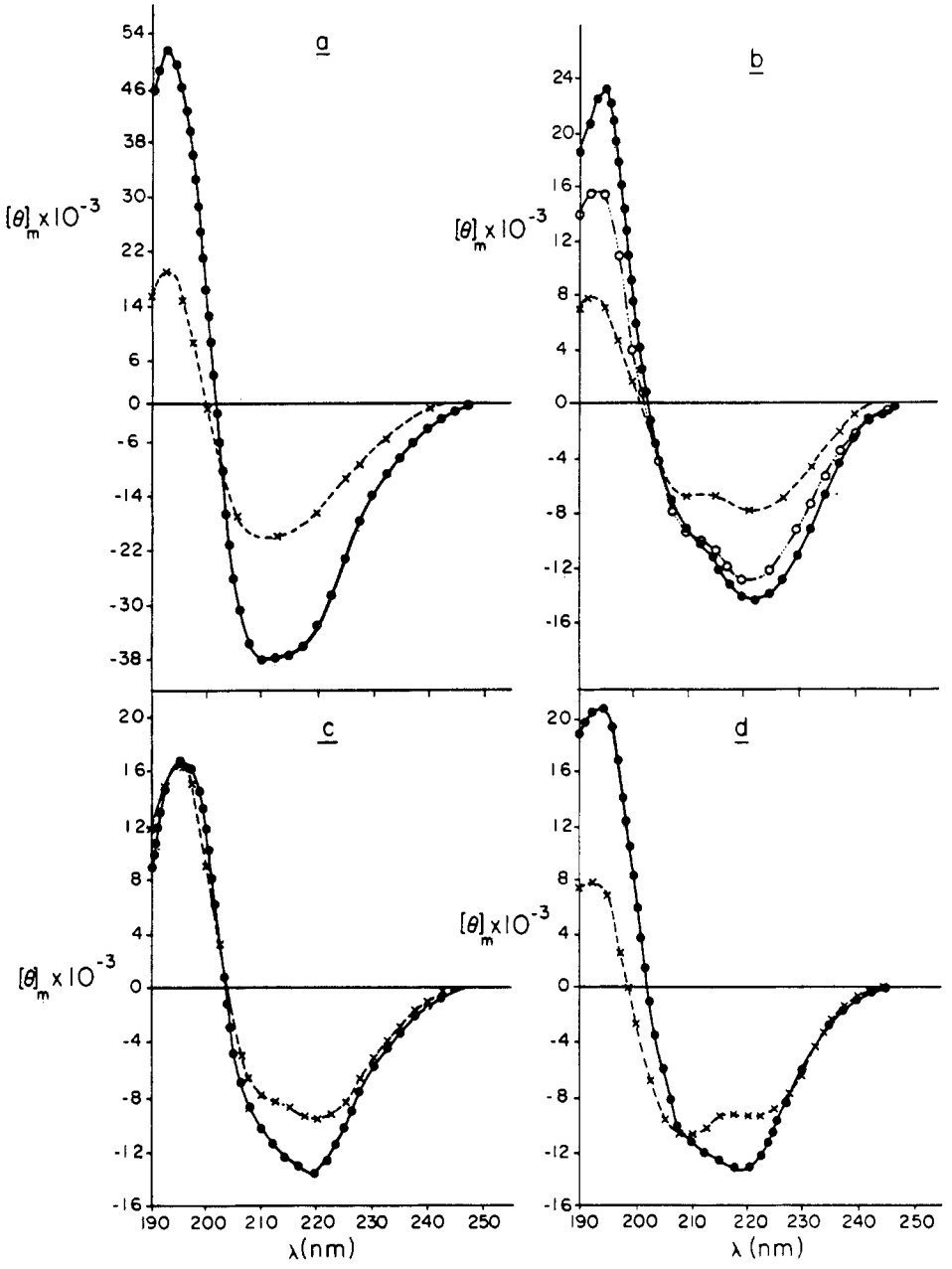

FIGURE 2: Far-uv CD spectra of flavodoxin holo- (●) and apoproteins (×). The CD spectrum of reconstituted *P. elsdenii* flavodoxin is designated as (○) in part b. θ is reported in terms of a mean residue ellipticity. (a) *C. pasteurianum*, based on 148 residues; (b) *P. elsdenii*, based on 143 residues; (c) *D. vulgaris*, based on 152 residues; (d) *R. rubrum* based on 210 residues.

to those obtained with FMN are observed for the *rubrum* type proteins. As with the Shethna protein, quenching ($t_{1/2} \leq 1$ sec) is faster than the resolving time of the instrument used in this work. However, temperature-jump fluorescence studies carried out in these laboratories (Barman and Tollin, 1971)[2] have shown that riboflavin binding to the Shethna apoprotein has a second-order rate constant of 8.5×10^5 M^{-1} sec^{-1}, which is about four times faster than with FMN. Similar increases in rate appear to hold for riboflavin binding to the *D. vulgaris* and *R. rubrum* apoproteins. In all cases, flavin–protein interaction is the rate-limiting step for both flavin and protein fluorescence quenching. No indications of slower first-order processes were noted.

Several trends among the flavodoxins are noteworthy. Firstly, the rate of FMN binding increases with increasing wavelength of the absorption maxima for the first flavin electronic transitions (Tables I and II). It also appears that those flavoproteins that bind riboflavin strongly bind FMN faster than those that do not. The latter trend suggests that the binding sites in the apoproteins vary in accessibility and/or the necessity for a cooperative type of interaction of protein with flavin which acts to modify the nature of the site (induced fit). As was pointed out by Mayhew (1971), the ribityl phosphate group is essential to flavin binding in *C. pasteurianum*, *Clostridial* MP, and *P. elsdenii* flavodoxins. Thus, this functional group probably helps to trigger a protein rearrangement which is responsible for modification of the flavin binding site in these proteins and which is reflected in the large spectral differences in the far-uv CD observed between the apo- and holoproteins. In fact, it is difficult to

[2] Unpublished results.

TABLE I: Apoprotein Fluorescence Maxima, Riboflavin Equilibrium Binding Constants, Fluorescence Quenching Rate Constants upon FMN Binding, and Classification of the Holoprotein Based upon the Visible CD Spectra.

Flavoprotein	λ_{max}	K_b (Riboflavin) (M^{-1})	k (M^{-1} sec^{-1}) $\times 10^{-5}$	Visible[a] CD Type
C. pasteurianum	353	$\leq 10^{3}$ [b]	0.3	*pas.*
P. elsdenii	352	$\leq 10^{3}$ [b]	1.5	*pas.*
Shethna	343	1.8×10^{6} [c]	1.6	*rub.*
D. vulgaris	347	1.3×10^{6}	7.0[d]	*rub.*
R. rubrum	345	1.5×10^{6}	17.0	*rub.*

[a] *pas.* denotes the *pasteurianum* type of visible CD spectrum while *rub.* denotes the *rubrum* type of visible CD spectrum (see Figure 1). [b] Mayhew (1971). [c] Edmondson and Tollin (1971b). [d] Dr. M. Dubourdieu (private communication) has obtained a value of approximately 10^6 M^{-1} sec^{-1} at pH 9 for this rate constant.

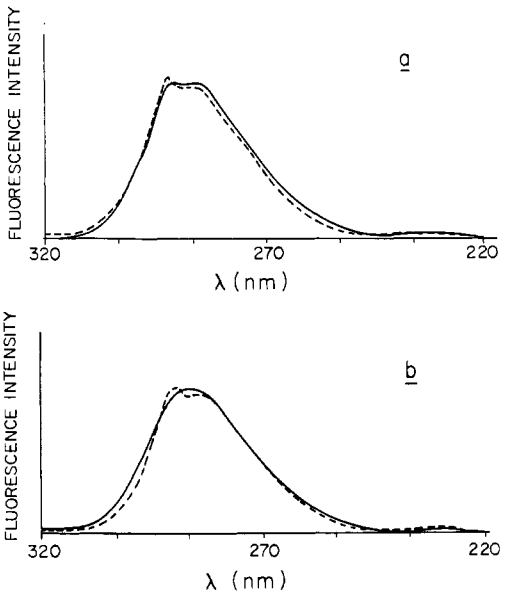

FIGURE 3: Fluorescence excitation spectra of flavodoxin apoproteins: (a) *D. vulgaris* (—) and *C. pasteurianum* (----); (b) *R. rubrum* (— —) and *P. elsdenii* (----). Intensities are not comparable.

conceive of any other mechanism by which removal of the ribityl phosphate could lead to such large differences in binding energy (≃7 kcal/mole) between FMN and riboflavin. Consistent with this concept is the observation that the ellipticity changes upon flavin removal are smaller for *D. vulgaris* and *R. rubrum* flavodoxins (Figure 2c,d) and the Shethna flavoprotein (Edmondson and Tollin, 1971b), all of which bind riboflavin effectively (Table I). However, it should be pointed out that various other types of measurements strongly indicate that a phosphate-triggered protein rearrangement does occur in the Shethna flavoprotein (Edmondson and Tollin, 1971b,c). Thus, it would appear that the different CD and flavin binding properties of this group of flavoproteins are probably a consequence, at least in part, of a difference in the extent of the conformation change which occurs upon protein–flavin contact.

A second aspect of the data which relates to the above hypothesis is the general trend observed with the flavodoxins in the fluorescence maxima of the apoproteins (the Shethna flavoprotein seems to be an exception, but it does not have flavodoxin activity). As judged by this criterion, the tryptophan side chains of those apoproteins which bind FMN slowly are relatively more accessible to solvent (Eisinger, 1969; Longworth, 1968). The fact that protein fluorescence is quenched upon coenzyme binding indicates either that the aromatic amino acids become incorporated into the protein interior due to the conformation change (in actual molecular orbital overlap with other residues) or that they are in close proximity (≤ 10 Å) to the flavin so that quenching may occur by energy transfer (D'Anna and Tollin, 1971).

Fluorescence excitation spectra of the apoproteins are shown in Figure 3. The *pasteurianum* type of apoprotein gives a red-shifted tryptophan-like excitation spectrum while the *rubrum*-type apoproteins have excitation spectra which are reminiscent of that of the Shethna apoprotein. In the latter material, energy transfer from tyrosine to tryptophan occurs and is interrupted by flavin binding (D'Anna and Tollin, 1971). It is more likely that energy transfer would occur in the *rubrum* type of apoproteins inasmuch as their tyrosine to tryptophan ratios are higher than for the *pasteurianum* type (Table III). This difference in tyrosine:tryptophan ratio could be significant to the class distinction. It is worth noting in this connection that tyrosyl pK's are appreciably changed upon flavin

TABLE II: Absorption Band Maxima and Extinction Coefficients for Flavodoxins and the Shethna Protein.

Flavoprotein	λ_{max}[e]	ϵ (l. $mole^{-1}$ cm^{-1}) $\times 10^{-3}$	λ_{max}[e]	ϵ (l. $mole^{-1}$ cm^{-1}) $\times 10^{-3}$
C. pasteurianum	443.5	9.1[a]	373.5	7.9[a]
P. elsdenii	446	10.2[b]	377.5	8.7[b]
Shethna	450	10.6[c]	371	9.5[c]
D. gigas	456.5[d]	10.2[d]	374[d]	8.2[d]
D. vulgaris	458	10.7[d]	376[d]	8.7[d]
R. rubrum	461	11.2	377	11.1

[a] Knight and Hardy (1966). [b] Mayhew and Massey (1969). [c] Hinkson and Bulen (1967). [d] Dubourdieu and LeGall (1970). [e] As measured in our spectrometer unless otherwise referenced.

TABLE III: Number of Tyrosine and Tryptophan Residues for Several Flavodoxins and the Shethna Flavoprotein.

Flavoprotein	Tryptophan	Tyrosine
C. pasteurianum[a]	4	2
P. elsdenii[b]	4	2
Shethna[c]	4	5
D. vulgaris[d]	2	5
R. rubrum[e]	4	9

[a] Knight and Hardy (1966). [b] Mayhew and Massey (1969). [c] Edmondson and Tollin (1971b). [d] Dubourdieu and LeGall (1970). [e] Cusanovich and Edmondson (1971).

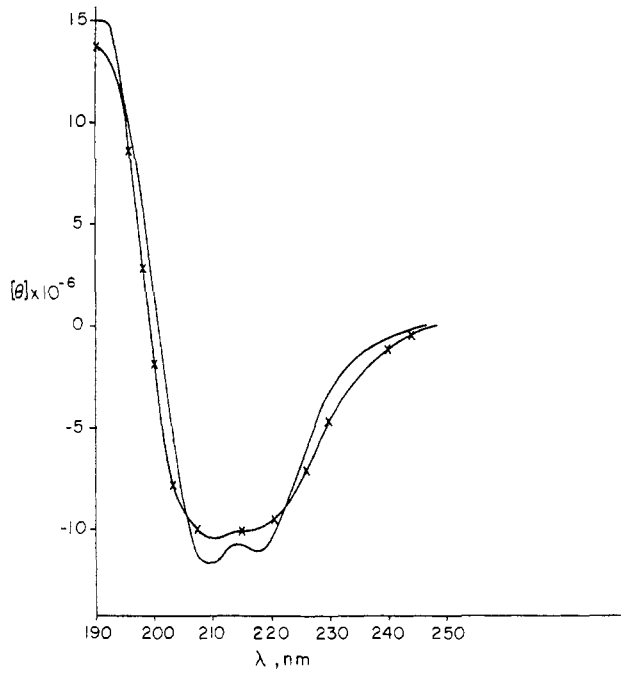

FIGURE 4: Far-uv CD spectra of glucose oxidase apo- (×) and holoproteins (—). θ is reported per mole of apoprotein rather than per mole of amino acid residue.

binding to the Shethna apoprotein (Edmondson and Tollin, 1971c). It would be of interest to determine the tyrosyl titration behavior of the flavodoxin apo- and holoproteins. In the case of *R. rubrum* flavodoxin, which retains appreciable protein fluorescence, the excitation spectrum of the holoprotein is similar in shape to tryptophan, which is consistent with an interruption of energy transfer upon FMN binding.

Other Flavodoxin Correlations. Several other similarities and differences among the flavodoxins and the Shethna flavoprotein emerge from the present experiments and the available literature. Generally, increases in extinction coefficient accompany increases in wavelength of the band maxima of the first electronic transitions of the flavoproteins (Table II). Although band maxima are used instead of 0–0 transitions, the similarities in the overall shape of the spectra indicate that the maxima are probably reasonable indicators of energy shifts. Except for the *C. pasteurianum* and *P. elsdenii* flavodoxins, the band maxima are red shifted relative to the band maximum for FMN in H_2O (445 nm). Although there are no clear cut trends in extinction coefficients for the second electronic transition, all of the maxima occur at wavelengths greater than 370 nm. Among the *rubrum* type of flavodoxin, red shifts of the maxima of the second electronic transition accompany red shifts of the first electronic transition. In terms of solvent–solute interactions as elucidated in model systems (Harbury *et al.*, 1959; Kotaki *et al.*, 1970), the flavin chromophore is probably involved in hydrogen bonding in all of the flavoproteins considered (as deduced from the fact that the position of the second band is >370 nm). It also appears that the general polarity of the flavin environment increases (as indicated by the first electronic transition maxima) in going from *C. pasteurianum* through *R. rubrum* flavodoxins. Solvent perturbation effects on the flavin chromophore in flavoproteins (D'Anna and Tollin, 1971) could provide more information about the relative exposures of the flavin group.

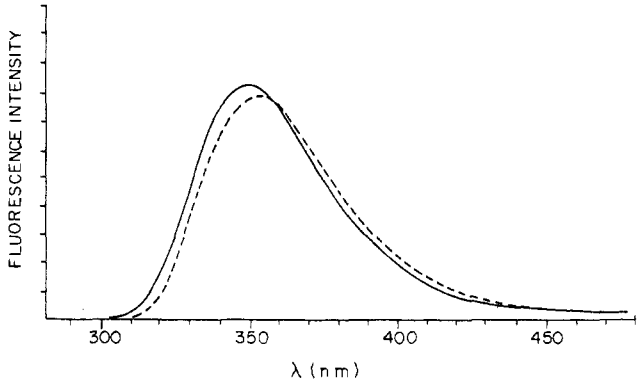

FIGURE 5: Fluorescence spectra of glucose oxidase holoprotein (—) and glucose oxidase apoprotein (----). The concentration of FAD is 3×10^{-6} M in the holoprotein. The spectrum has been corrected for flavin "inner filter" effects (Wu *et al.*, 1970).

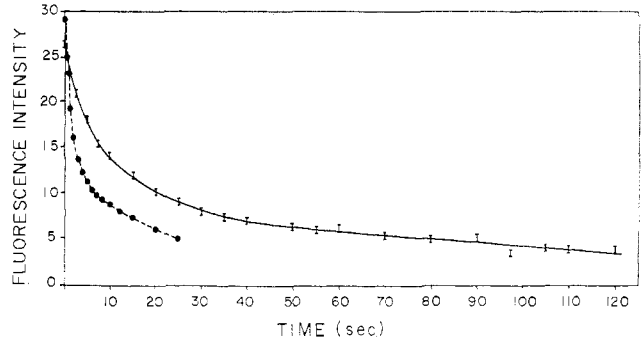

FIGURE 6: Fluorescence quenching accompanying FAD–apoprotein binding: (†) Flavin fluorescence and (●) protein fluorescence. Fluorescence is reported in arbitrary units.

Glucose Oxidase. Far-uv CD spectra of glucose oxidase apo- and holoprotein are given in Figure 4. The similarities between the spectra indicate that although some structural differences exist, these are not of the same magnitude as occur in the flavodoxins.

Fluorescence spectra of glucose oxidase apo- and holoprotein are shown in Figure 5. The protein fluorescence is ≃85% quenched by FAD binding and the fluorescence maximum is blue shifted in the holoprotein, indicating a decrease in the polarity of the tryptophyl chromophores (or an increase in tyrosine emission).

The kinetics of protein and flavin fluorescence quenching are complex. In Figure 6, rate curves are shown for 4.3×10^{-7} M apoprotein and 8.6×10^{-7} M FAD. These concentrations of apoprotein and FAD were chosen so that a flavin molecule would be available for each of the two binding sites per protein molecule. It is evident that the major portion of protein fluorescence quenching occurs prior to flavin quenching; the half-life of the protein fluorescence quenching is about 2.5 sec while the half-life of flavin fluorescence quenching is about 12 sec. Both rate curves do not give straight lines for either first- or second-order plots. Thus, the binding process is mechanistically complex. The second-order plot of the decay of the protein fluorescence curves downward, suggesting that a first-order component may be involved. Interestingly, the slope of a straight line drawn through the points of the second-order plot of protein fluorescence quenching gives an apparent second-order rate constant of 3.5×10^5 M^{-1} sec^{-1}. Such a constant would be in general agreement with the rates observed by Swoboda (1969a) for flavin polarization changes

upon binding ($\simeq$80% of the final polarization in 5 sec for 1.0 $\times$ 10^{-6} M apo and FAD) and is also comparable to what is found for the flavodoxins and the Shethna protein. Flavin fluorescence quenching may be resolved into two first-order components (k_1 = 0.10 sec^{-1} and k_2 = 0.008 sec^{-1}) in general agreement with the results of Swoboda (1969a).

In contrast to the protein fluorescence quenching observed upon mixing FAD and apoprotein, no such quenching was observed upon addition of ADP (8-fold molar excess) to the apoprotein. Furthermore, the apoprotein fluorescence spectrum was not altered. After allowing 5-min exposure of the apoprotein to ADP, FAD was added. Protein fluorescence was quenched under these conditions, but the half-life was several minutes as compared to a few seconds for apoprotein and FAD alone. The relatively slow quenching in the presence of ADP demonstrated that this compound was bound to the apoprotein. Thus, although ADP interacts with the apoprotein it does not cause protein fluorescence quenching. The far-uv CD spectrum of apoprotein plus a 50% excess (per binding site) of ADP was not noticeably different from that of the apoprotein alone. However, we do not know how much of the ADP is actually bound at those concentrations.

Previously, Swoboda (1969a) reported that the intensity and polarization of flavin fluorescence were not altered by the addition of FMN, riboflavin, or lumiflavin to the ADP–apoprotein complex. He also reported that simultaneous addition of ADP and riboflavin or FMN and AMP does not regenerate enzymic activity. We have found that neither addition of ADP to solutions containing riboflavin and apoprotein nor addition of AMP to solutions containing FMN and apoprotein results in protein fluorescence quenching.

The fact that ADP does not cause protein fluorescence quenching could be a consequence of one of the following possibilities—(1) ADP causes a conformational change in the apoprotein (*cf.* ultracentrifuge studies by Swoboda, 1969b), but the resulting conformation is not the same as that of the FAD–apoprotein complex, (2) the conformation of the two complexes is the same but the isoalloxazine ring of the flavin is essential in the protein fluorescence quenching process, and (3) the determining factor is not protein conformation but the fact that ADP does not have energy levels available for transfer of energy from excited tryptophan residues.

The CD spectrum of the apoprotein–ADP complex would indicate that the overall apoprotein secondary structure is not altered by ADP binding and thus is not the same as that of the FAD–apoprotein complex. The inability of FAD to displace ADP after 1-hr incubation at room temperature (Swoboda, 1969b) suggests that ADP binding causes localized changes which prevent the formation of the conformation required to allow FAD binding. In effect, the interaction of apoprotein with ADP could produce a "denatured" complex. It is also reasonable to suppose that the absence of suitable energy levels in ADP plays a role in the lack of quenching.

It is possible to account for the faster quenching of protein fluorescence in the formation of holoprotein in terms of energy transfer from aromatic amino acids to flavin if, in the initial FAD–protein contact, the nonflavin portion of the coenzyme binds more completely than does the isoalloxazine ring. An initial direct molecular orbital interaction of flavin and protein residues would be expected to yield *mutual* flavin–protein fluorescence quenching (Wu and McCormick, 1971; D'Anna and Tollin, 1971) and thus similar kinetics (as is observed with dehydrogenases). The absence of flavin fluorescence quenching on the same time scale as protein fluorescence quenching would thus suggest that the longer range energy-transfer process is operative. Following this initial partial binding process, the isoalloxazine portion of the coenzyme could be more slowly bound resulting in flavin fluorescence quenching (the biphasic nature of this process (Swoboda, 1969a) suggests that at least two steps are involved). The lack of protein quenching in the presence of ADP plus riboflavin or AMP plus FMN emphasizes the cooperative nature of the binding of FAD, *i.e.*, both portions of the coenzyme apparently must bind simultaneously.

Conclusions

Trends and correlations have been observed among several physical and enzymic properties of flavodoxins and the Shethna flavoprotein. The physical properties include visible electronic absorption spectra, CD spectra, fluorescence spectra, flavin–protein equilibrium constants, and fluorescence quenching kinetics accompanying flavin–protein binding. The relationships which have been found serve to emphasize the significance of protein structure in determining flavin physical properties and enzymic function. The fact that such regularities exist suggests that a coherent physicochemical rationale can eventually be developed when more structural information becomes available.

Glucose oxidase illustrates the first example, to our knowledge, of a flavoprotein in which protein fluorescence quenching precedes flavin quenching during coenzyme binding. The overall binding process is evidently complex, and its complete characterization deserves further experimentation. It is of interest to note that studies of flavin fluorescence quenching upon coenzyme binding to the apoprotein of D-amino acid oxidase (Massey and Curti, 1966) also indicate mechanistic complexity. It may well be that this is a characteristic feature of the oxidase type of flavoprotein, as opposed to the apparent simplicity which has been observed for the dehydrogenases in this and previous work.

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Fluorescence Studies on the Interaction of Pepsin with Its Substrates†

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ABSTRACT: A series of pepsin substrates having an amino-terminal dansyl (Dns) group has been synthesized, and measurement of the kinetic parameters of their enzymic hydrolysis has given additional evidence for the importance of secondary enzyme–substrate interactions in determining the catalytic efficiency of pepsin. Steady-state fluorescence measurements of the binding of the dansyl group by the protein have provided estimates of the dissociation constants of the enzyme–substrate complex. Whereas, under the conditions of this study, dansylamide is bound only weakly, the dansyl group of substrates such as Dns-Gly-Gly-Phe-Phe-OEt is bound strongly, with a major contribution to the binding energy coming from the interaction of the Phe-Phe unit (the sole site of enzymic hydrolysis). During the activation of pepsinogen to pepsin in the presence of a dansyl peptide ester, the enhancement of fluorescence of the dansyl group is increased.

It has been shown that, in the action of pepsin on synthetic substrates related to Z-Gly-Gly-Phe-Phe-OP4P,[1] where the Phe–Phe bond is the only sensitive linkage, the replacement of Gly-Gly by other dipeptidyl units leads to very large changes in the catalytic efficiency of the enzyme (as measured by k_{cat}[2]) without marked changes in K_M (Sachdev and Fruton, 1970). Together with other findings on the effect of the removal of the Z group, or its replacement by the less hydrophobic Moc group, this result was taken to indicate that the Z-dipeptidyl group of the substrate interacts with the enzyme as a unit, and that the benzyl portion of the benzyloxycarbonyl group participates importantly in this interaction. The possibility was considered that the large effects observed upon changing the structure of the Z-dipeptidyl unit are a consequence of the induction, by such "secondary" interactions, of conformational changes at the catalytic site of the enzyme (Fruton, 1970). Moreover, it may be expected that the conformation of the peptide in the productive enzyme–substrate complex will be influenced by such secondary interactions so as to make the Phe–Phe bond more susceptible to enzymic cleavage. Since the three-dimensional structure of pepsin has not yet been elucidated, model building cannot be used to define the limits of conformational change in either the enzyme or the substrate when the two interact productively. For this reason, we have attempted to approach this problem by the use of the fluorescent-probe technique (Edelman and McClure, 1968; Stryer, 1968). In this communication, we report data on the interaction, with pepsin, of peptides bearing an amino-terminal dansyl group as a fluorescent probe for secondary en-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 2485 (1966), are: Dns, dansyl or 1-dimethylaminonaphthalenesulfonyl; OP4P, 3-(4-pyridyl)propyl-1-oxy; Z, benzyloxycarbonyl; Moc, methyloxycarbonyl; ANS, 1-anilinonaphthalene-8-sulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; TPDM, tosyl-L-phenylalanyldiazomethane. Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

[2] The kinetic parameters mentioned in this paper are defined by the equation $v = V_m(S)/[K_M + (S)]$ for the process

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P_1 + P_2$$

where v = initial velocity, the maximal velocity $V_{max} = k_{cat} \times$ total enzyme concentration (E), (S) = initial substrate concentration, $K_M = (k_{cat} + k_{-1})/k_1$, and $K_S = k_{-1}/k_1$.