Protein Fluorescence and Solvent Perturbation Spectra as Probes of Flavin–Protein Interactions in the Shethna Flavoprotein*

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ABSTRACT: Fluorescence emission and excitation spectra of the Shethna apoprotein and the oxidized and semiquinone forms of the holoprotein have been measured. Singlet–singlet energy transfer from tyrosine to tryptophan is shown to occur in the apoprotein. This energy transfer is interrupted, and a large fraction of the direct tryptophan fluorescence intensity is quenched, in the two redox forms of the holoprotein. Solvent perturbation absorption difference spectra in 20% ethylene glycol and fluorescence emission spectra have shown that the extent of exposure of tryptophan side chains to solvent is similar in the apo- and holoproteins. Values for "exposed" tyrosines and tryptophans are calculated. It is also shown that protein-bound FMN is probably not exposed to solvent inasmuch as its visible absorption spectrum is not perturbed by the ethylene glycol. Binding of flavin to protein, as determined from both protein fluorescence and flavin fluorescence quenching, follows second-order kinetics. The extent of flavin quenching parallels the extent of protein quenching for several flavin analogs. The results are discussed in terms of the nature of the flavin–protein interaction.

The Shethna flavoprotein from *Azotobacter vinelandii* (Shethna *et al.*, 1965; Hinkson and Bulen, 1967; Hinkson, 1968) has two properties which make it a very useful object of study: it can be reduced to a free radical which is quite stable to oxidation by oxygen and it is easily and reversibly separated into free FMN and a stable apoprotein. These desirable features have led to several investigations of the chemical and physical properties of this protein and of the energetics and mechanism of flavin binding to the apoprotein (Hinkson and Bulen, 1967; Hinkson, 1968; Edmondson and Tollin, 1971a–c). Utilizing flavin fluorescence quenching, Hinkson (1968) and Edmondson and Tollin (1971b) have determined binding constants and second-order rate constants for the flavin–protein interaction.

Although the fluorescence of the apo- and holoproteins has been the subject of some cursory study (Hinkson and Bulen, 1967; Hinkson, 1968), it has not been investigated in any depth. Such a study would be of interest since preliminary evidence (Hinkson, 1968) has indicated that nitration of tyrosines inhibits flavin binding to the apoprotein. Additionally, recent work by McCormick (1970) on a related protein, flavodoxin, has suggested the possible importance of tryptophan in FMN binding. Of particular significance would be an investigation of the effect of flavin binding on the wavelength of protein fluorescence, on fluorescence excitation spectra and on protein fluorescence quantum yields. Changes in these parameters would provide further insight into the nature of the flavin–protein interaction. Kinetics of such changes would relate to mechanisms of flavin binding to the protein.

In this study, the fluorescence emission and excitation spectra of the Shethna apoprotein (P), oxidized holoprotein (PF), and semiquinone holoprotein (PFH·) have been measured. Additionally, the kinetics and extent of apoprotein fluorescence quenching have been determined for the binding of several flavin analogs. Solvent perturbation of the protein absorption spectra has also been utilized to further characterize the tyrosine and tryptophan environments in the apo- and holoproteins.

Experimental Section

Materials

The Shethna flavoprotein (PF) was isolated by the method of Hinkson and Bulen (1967). Apoprotein was prepared from PF by the procedure of Edmondson and Tollin (1971b). Flavoprotein free radical was prepared according to Massy and Palmer (1966). Because of the slow reaction of PFH· with oxygen at atmospheric pressure ($k = 1 \times 10^{-4}$ min^{-1}, Edmondson and Tollin, 1971c), no precautions were taken to remove air from the samples, since all of the measurements were performed within a few minutes after removal of the semiquinone species from the desalting column. Crystallized bovine serum albumin was purchased from Pentex Inc. Chymotrypsinogen (three-times recrystallized) was obtained from Worthington Biochemical Corp. L-Tryptophan, L-tyrosine, and L-phenylalanine (all A grade), obtained from Calbiochem, were used without further purification. Flavin derivatives were obtained and purified as reported by Edmondson and Tollin (1971a–c). Mallinckrodt A. R. reagent urea was recrystallized from 95% ethanol. Ethylene glycol was commercial reagent grade.

Methods

Fluorescence spectra were measured with instrumentation assembled in these laboratories. Excitation was provided by a PEK 110-W high-pressure mercury arc lamp. The light from this source was passed through either a chemical filter solution (5 cm of solution: 240 g/l. of $NiSO_4 \cdot 6H_2O$, 45 g/l. of $CuSO_4 \cdot 7H_2O$, and 5×10^{-5} mole/l. of *p*-nitrophenol) which isolated the 255-nm mercury spectral line, or a Jarrel-Ash Model 82-410 grating monochromator or a Bausch and Lomb 600-groove/mm grating monochromator. The exciting light was mechanically chopped at 240 Hz. Fluorescence emission was analyzed at right angles to the sample (con-

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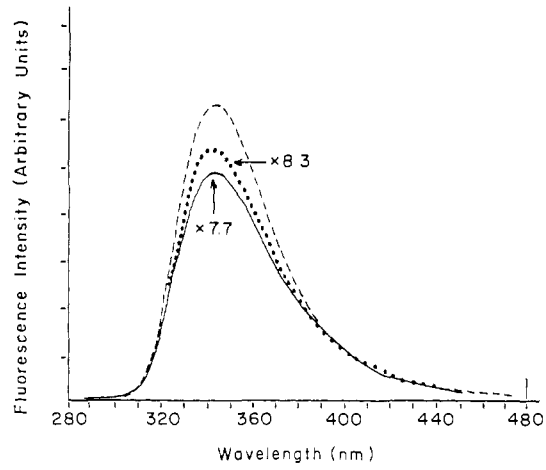

FIGURE 1: Fluorescence emission spectra of P (– – –), PF (—), and PFH· (· · · ·) in 0.025 M phosphate buffer (pH 7.0). The excitation source was a mercury arc lamp (255-nm region); protein concentrations are 3.0×10^{-6} M.

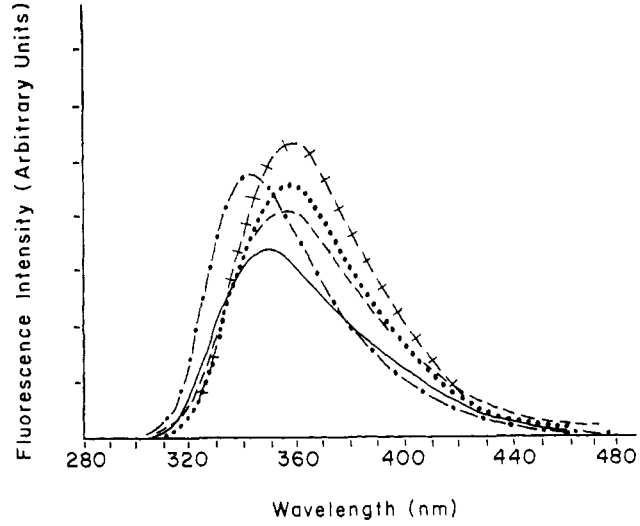

FIGURE 2: Effect of urea on the fluorescence emission spectra of apo- and holoprotein. PF: in phosphate buffer (pH 7.0), (— · —) in 4.9 M urea (—) in 9 M urea (– – –); P: in 5 M urea (· · · ·); tryptophan: in phosphate buffer (pH 7.0) (–|– –|–). Relative intensities are not comparable.

tained in a 1-cm² quartz cuvet) using another Bausch and Lomb 600-groove/mm grating monochromator. The monochromator was modified for continuous scanning at 0.83 nm/sec. Fluorescence emission was detected with a quartz window photomultiplier, amplified and rectified by an EMC Model RJB lock-in amplifier, and recorded with a Heath recorder. For most fluorescence measurements, a spectral resolution of 6 nm was achieved with the slit setting on the Bausch and Lomb monochromator. Fluorescence excitation spectra were obtained by utilizing a Hanovia 150-W xenon arc lamp in conjunction with the modified Bausch and Lomb monochromator. A Corning 7-37 band-pass filter or a second Bausch and Lomb monochromator were used to monitor fluorescence intensity. Again the lock-in detection system was utilized. For very weak fluorescence excitation spectra, a Varian computer of average transients (CAT) was used to improve signal-to-noise.

Fluorescence quenching kinetics were measured with the previously described spectrometer system except that a Sanborn 151 recorder was used for data readout. A 20-μl sample of flavin solution was added to 2 ml of apoprotein solution in the spectrophotometer cell compartment by means of a plastic dip stick. We estimate a 20% random error due to concentration inaccuracies, diffusion effects (because of incomplete mixing) and noise in data readout. Measurements were performed at ambient temperature, *i.e.*, 23 ± 2°.

In all cases, emission spectra were not corrected to compensate for phototube response. Excitation spectra were also not corrected for the wavelength variation in photon flux. However, all fluorescence emission and excitation spectra were measured at concentrations of $1.0–3.0 \times 10^{-6}$ M, so that corrections for total absorption by the sample are small or negligible. Thus, the various spectra can be compared to each other. For weakly fluorescing samples, and for those samples containing urea, it was necessary to correct for cell emission and light scattering by the sample.

Solvent perturbation absorption difference spectra were measured according to the method of Herskovits and Sorensen (1968a,b). A Cary 14R spectrophotometer (dynode voltage setting 2) was used for the experiments.

Circular dichroism spectra were obtained with a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment.

Determination of the concentration of apoprotein solutions by absorption measurements (ϵ_{280} 3.2×10^4) should be done with care due to the high apoprotein fluorescence. Because of the sample phototube arrangement, the Cary 14R spectrometer is suitable for these determinations (Donovan, 1969). Single-beam spectrophotometers which have the phototube very close to the sample will give too low an absorbance value. This error may be as much as 5–7% in a single-beam instrument such as the Gilford Model 240 UV-VIS spectrophotometer.

Results and Discussion

Protein Fluorescence Emission. Protein fluorescence of P, PF, and PFH· was excited by isolating the 255-nm spectral region of a high-pressure mercury arc lamp. The resulting emission spectra (Figure 1) of the three materials are very similar in shape and in the wavelength of the maximum fluorescence intensity. Solutions of P and PF in urea give fluorescence red shifts of varying amounts (Figure 2). Urea (5 M) apparently totally denatures the apoprotein, inasmuch as the fluorescence maximum is red shifted nearly to that of free tryptophan. Comparison of the absorption spectrum to "mock protein", a mixture of amino acids in the proper proportions (Edmondson and Tollin, 1971b), also suggests total unfolding. On the other hand, 5 M urea caused only a partial red shift in the fluorescence maximum of PF, whereas 9 M urea brought about a red shift to the region of free tryptophan emission (Figure 2). Thus, as was pointed out by Hinkson (1968), flavin binding contributes significantly to protein stability.[1]

In Figure 3, the fluorescence emission spectrum of P is compared to those of chymotrypsinogen, bovine serum albumin, tyrosine, and tryptophan. The results obtained

[1] This is also seen in the pH dependence of the circular dichroism spectra in the ultraviolet region (D. E. Edmondson and G. Tollin, unpublished data).

with urea-treated samples (Figure 2) and a comparison of the apoprotein fluorescence spectrum to the other fluorescence spectra suggest that the tryptophan residues in the Shethna apoprotein are only partly accessible to solvent (Eisinger, 1969a; Longworth, 1968). This assumes that tyrosine makes a negligible contribution to the fluorescence emission; we shall see below that this is true. The accessibility of the emitting tryptophans to solvent in PF and PFH· is indicated to be the same as in P by the identity of the fluorescence spectral maxima. This also suggests that (1) the bound flavin does not interact strongly enough with the emitting tryptophan residue(s) to perturb its energy levels and (2) any change in protein structure which may result upon flavin binding does not modify the environment of emitting tryptophans. These conclusions are consistent with results of circular dichroism measurements (Edmondson and Tollin, 1971c).

In contrast to the similarity of the fluorescence emission maxima in P, PF, and PFH·, the tryptophan emission quantum yield is significantly reduced (≃90%) in going from P to either PF or PFH· (Figure 1). Hence, the presence of oxidized or half-reduced FMN in the coenzyme binding site severely quenches protein fluorescence. This has also been reported by Hinkson (1968).

In order to provide an estimate of the absolute fluorescence yields of P, PF, and PFH·, the emission intensities of P and chymotrypsinogen were compared. Because fluorescence spectra were not corrected for phototube response, it was necessary to compare the fluorescence intensity of P to a standard which has a very similar fluorescence spectrum. The emission of chymotrypsinogen is superimposable with that of P (see Figure 3). Also, tryptophan residues of chymotrypsinogen are ≃40% exposed to solvent (Williams *et al.*, 1965). As we shall see (next section), the exposure to solvent of the tryptophans in P are similar in extent. This would imply that the absorption coefficients of the tryptophan transitions in chymotrypsinogen and in P should also be very similar. Samples of the two proteins (each 4×10^{-6} M in tryptophan) were excited with 280-nm radiation and their fluorescence was passed through a Corning 7-37 band-pass filter to the photomultiplier. Apoprotein fluorescence was found to be 4.5 times as intense as that of chymotrypsinogen. On a protein molar basis, the fluorescence of P in very dilute solution has about twice the intensity of the chymotrypsinogen emission. Teale (1960) has made tabulations of the quantum yields of several proteins including chymotrypsinogen. Thus, a comparison of Shethna apoprotein fluorescence intensity to that of other proteins may be obtained from this reference. Using Teale's "tryptophan quantum yield" for chymotrypsinogen (assuming that the fluorescence is due to tryptophan only and that no energy transfer to tryptophan occurs from tyrosine) and his quantum yield value for tryptophan (Teale and Weber, 1957),[2] it is found that the "tryptophan quantum yield" of P is approximately 1.5 times that of free tryptophan in aqueous solution. The relative tryptophan quantum yields of PF and PFH· are only about 0.15 that of tryptophan in solution.

Solvent Perturbation Absorption Difference Spectra. Solvent perturbation difference spectroscopy verifies the partly buried nature of the tryptophans in P and PF. The protein solutions were initially dissolved in 0.025 M phosphate buffer (pH 7.0) and ethylene glycol (20%, v/v) was used as the perturbing solvent.

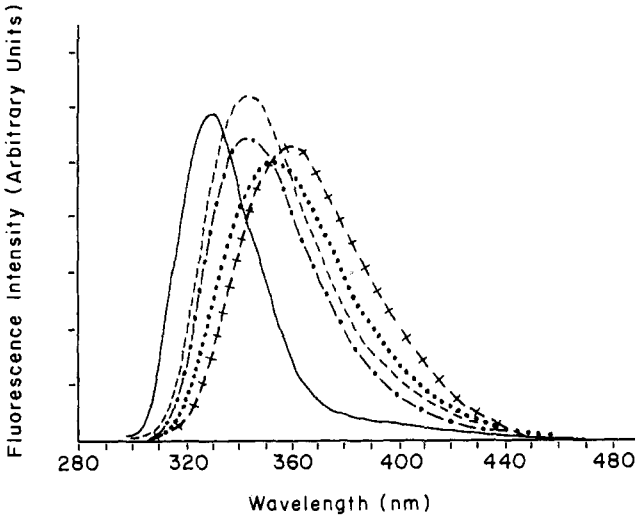

FIGURE 3: Comparison of the apoprotein fluorescence emission spectrum (– – –) with the fluorescence emission spectra of chymotrypsinogen (–·–), bovine serum albumin (· · · ·), tryptophan (–|– –|–), and tyrosine (——). All spectra are in 0.025 M phosphate buffer (pH 7.0). Relative intensities are not comparable.

The presence of 20% ethylene glycol has no affect on the overall structure of P or PF, as far as can be detected by circular dichroism measurements in the near and far-ultraviolet region. Also, the circular dichroism spectrum of PF is unaltered by ethylene glycol concentrations as high as 50% (D. E. Edmondson, unpublished data, 1971). In these measurements, no circular dichroism spectral changes were observed in either position or ellipticity. The fluorescence emission maxima of the proteins are *not* shifted in position in the presence of ethylene glycol. It should be pointed out, however, that L-tryptophan in 0.025 M phosphate (pH 7) does not show a change in fluorescence emission maximum in 20% ethylene glycol at the monochromator slit widths used in these measurements. Weinryb and Steiner (1970) have also noticed the absence of a fluorescence solvent shift for papain in 20% ethylene glycol solution.

Values for the molar absorptivity differences caused by 20% ethylene glycol were obtained from the data of Herskovits and Sorenson (1968a,b) for acetyl-L-tryptophan ethyl ester and for acetyl-L-tyrosine ethyl ester. The equations which relate the exposure of the aromatic side chains to the absorptivity difference for the two maxima are

$$\Delta\epsilon_{292.5} = 13.4x + 305y \quad (1)$$

$$\Delta\epsilon_{284.5} = 82x + 189y \quad (2)$$

in which x is the number of exposed tyrosines, y is the number of exposed tryptophans, and $\Delta\epsilon_\lambda$ is difference in molar absorption coefficient at wavelength λ.

Solvent perturbation ultraviolet spectra of P and PF are shown in Figure 4. Exposed tyrosine and tryptophan values, calculated *directly* from the spectra using eq 1 and 2, are shown in Table I. The difference spectrum of P is typical of that for proteins containing tyrosine and tryptophan residues. Peaks are observed at 292.5, 284.5, and 278.0 nm (Herskovits and Sorenson, 1968b). In the case of PF, the perturbation difference spectrum exhibits an anomalously

[2] Although Eisinger (1969c) has reported lower tryptophan quantum yield values (0.14) than Teale and Weber (1957) (0.19), the protein–tryptophan *relative* quantum yields of Teale are still good values. Only absolute quantum yield values are indicated to be in error.

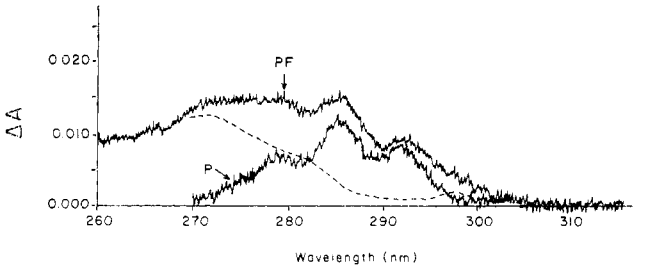

FIGURE 4: Solvent perturbation absorption difference spectra of apoprotein (P) and holoprotein (PF) in the ultraviolet region. The difference of the two difference spectra is given by the broken curve. The perturbing solvent is 20% ethylene glycol. P is 2.7×10^{-5} M and PF is 2.2×10^{-5} M.

TABLE I: Exposure of Tyrosine and Tryptophan Residues in P and PF Calculated from Solvent Perturbation Difference Sepctra.

Protein	$\Delta\epsilon_{292.5}$	$\Delta\epsilon_{284.5}$	No. of Exposed[b] Tryptophans	No. of Exposed[b] Tyrosines
PF[a]	424	625	1.1	4.7
P	311	416	0.85	2.9

[a] Calculated directly from the PF difference spectrum (see text). [b] There are four tryptophans and five tyrosines in the Shethna protein.

broad region of relatively high intensity between 260 and 280 nm. Since FMN is present in PF, perhaps the flavin is also perturbed by the ethylene glycol and contributes to the solvent perturbation difference spectrum. The perturbation spectrum of free FMN in 20% ethylene glycol (Figure 5) exhibits a positive deviation in the ultraviolet region. This deviation is similar in shape to the spectrum (dotted line in Figure 4) generated by subtraction of the apo- and holoprotein difference spectra. However, free FMN shows several perturbations in the visible region which are not observed in the holoprotein difference spectrum (Figure 5). This suggests that the increased absorbance in the 260–280-nm region in the perturbation spectrum of the holoprotein is not solely due to flavin. On the other hand, the shape of this increased absorbance is not what would be expected for a pure tyrosine perturbation (Herskovits and Sorensen, 1968b). Thus, it is possible that the effect is a consequence of direct flavin–tyrosine interaction in the holoprotein or of flavin-induced changes in protein side-chain interactions. Hence, we are unable to unambiguously determine the exposure of tyrosines in the holoprotein and the value given in Table I is probably not correct. Protein modification and other experiments are currently in progress; hopefully they will help answer these questions regarding flavin and tyrosine.

Tryptophan exposure in the holoprotein does not present so formidable a problem. The shape of the holoprotein difference spectrum is quite similar to that of the apoprotein in the region of the first tryptophan perturbation peak (292.5 nm). The fact that the 292.5-nm peak is well resolved indicates definite tryptophan exposure (it is estimated that a minimum of 0.3 exposed tryptophan would be necessary to effect resolution of the band). As we have seen (Table I), the difference spectrum yields a value of 1.1 exposed tryptophans in the holoprotein. These arguments tend to restrict the tryptophan exposure to between 0.3 and 1.1. Within the limitations of the solvent perturbation method, it appears that the degree of tryptophan exposure is not significantly different in the apo- and holoproteins and that the bound FMN is probably not exposed to solvent to any great extent.

Fluorescence Excitation Spectra. In an effort to elucidate the reason for the high quantum yield of "tryptophan fluorescence" of the apoprotein, the fluorescence excitation spectra of P, PF, and PFH· were measured. Appropriate slit widths were used to give 3-nm resolution in the exciting monochromator, and a Corning 7-37 secondary filter was utilized. The fluorescence spectra (Figure 6) show marked differences. The high-energy peak (285 nm) of the apoprotein fluorescence excitation is more intense than is the peak at 293 nm. Interestingly, the 285-nm peak corresponds closely to the first maximum in the tyrosine absorption spectrum, while the peak at 293 nm corresponds to the ^1L_b transition of tryptophan. In PF and PFH·, the high-energy peak is depressed relative to the 293-nm peak, although it is still more pronounced than in the excitation spectra of tryptophan or of mock apoprotein or mock holoprotein solutions (see Figure 7). This peak is reduced to a shoulder in those excitation spectra. This difference in the mock proteins can be attributed to the relative fluorescence intensities of the free tryptophans and tyrosines.

To ensure that simple light absorption by the protein-bound FMN did not cause the depression of the peak at 285 nm, the fluorescence excitation spectrum of P in the presence of unbound 3-methylriboflavin (Edmondson and Tollin, 1971b) was measured. Although the intensity was slightly reduced (1–3%) in the presence of the flavin, the shape of the fluorescence excitation spectrum was unaltered. Hence, the present data indicate that binding of flavin to the protein results in (1) decreased tyrosine fluorescence and/or (2) interrupted energy transfer from tyrosine to tryptophan.

Two sets of experiments were performed to determine the amount of tyrosine fluorescence present in the emission of the apoprotein: (1) fluorescence difference spectra (Weber and Young, 1964) and (2) fluorescence excitation spectra of P monitored at various wavelength regions of the fluorescence apoprotein For the fluorescence difference experiments, the spectrum. fluorescence was excited by irradiation at 280, 285, and 295 nm. Rather large slit widths (6 nm) had to be used to compensate for the low light intensity of the excitation source. Comparison of the resulting fluorescence emission spectra did not reveal detectable differences which could be attributed to tyrosine fluorescence.

In the second approach (Weber, 1961), the slit widths of the exciting monochromator were adjusted to give 3-nm resolution, while the secondary monochromator had slit widths corresponding to 20-nm resolution. Even with the poor resolution of the secondary monochromator, significantly different portions of the fluorescence spectrum could be isolated. Fluorescence was monitored at 330, 352, and 375 nm, and a CAT was used to enhance signal-to-noise ratios (10–12 scans). As can be seen from Figure 8, no significant differences in the fluorescence excitation spectra are observed. Since tyrosine normally has an emission maximum at 330 nm (as measured in our spectrometer), a large tyrosine fluorescence contribution is not indicated. Thus, the shape of the protein fluorescence excitation spectrum of P, the

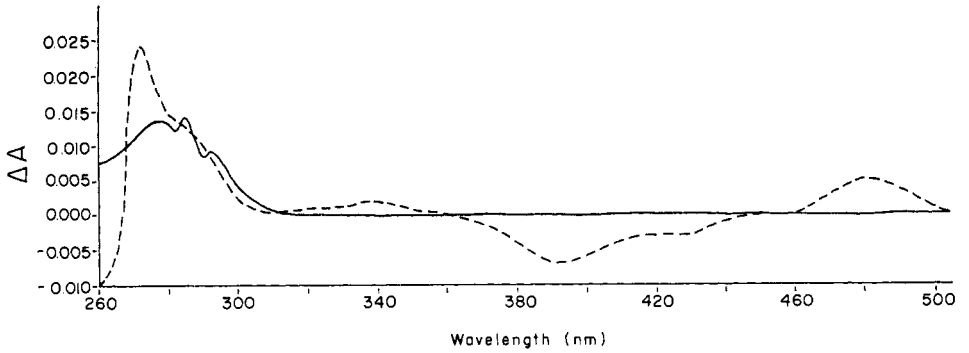

FIGURE 5: Solvent perturbation absorption difference spectra of PF (—) and FMN (- - -). The perturbing solvent is 20% ethylene glycol. PF and FMN are both 2.2×10^{-5} M.

invariance of the fluorescence emission spectrum of P with exciting wavelength, and the similarity of the fluorescence excitation spectra of P as a function of fluorescence monitoring wavelength lead us to conclude that energy transfer from tyrosine to tryptophan occurs in the Shethna apoprotein. Based on the data of Eisinger (1969a), and the fact that there are five tyrosine and four tryptophan residues in the Shethna protein, a maximum increase in tryptophan fluorescence due to energy transfer from tyrosine would be about 20–25%. Thus, energy transfer can account for only part of the high quantum yield of tryptophan emission in the apoprotein. The remaining enhancement must be due to the greater intrinsic efficiency of tryptophan fluorescence in the protein. These experiments were not performed with PF or PFH· because of their low fluorescence quantum yields. The data also indicate that tyrosine–tryptophan energy transfer is reduced by flavin binding. It is not conceivable, however, that interruption of energy transfer from tyrosine could completely account for the 90% fluorescence quenching which accompanies flavin binding. The remainder of the quenching is most probably due to other types of interaction of the protein with flavin. Measurements of fluorescence emission and excitation spectra of lipoamide dehydrogenase and its apoprotein (Visser and Veeger, 1970) suggest that a similar pattern also exists for that protein.

Protein Fluorescence Quenching Kinetics. Apoprotein fluorescence quenching was measured as a function of time for the binding of several flavin derivatives[3] (FMN, 3-MeFMN, isoFMN, dFMN, riboflavin, and *N*-10-ω-carboxybutylisoalloxazine). Because of the near invariance of the position and shape of the protein fluorescence of the apo- and holoproteins, the fluorescence intensity was monitored through a Corning 7-37 filter. It is presupposed that no appreciable change in shape or position of the protein fluorescence occurs *during* the quenching process. Use of exciting radiation of 280, 285, or 255 nm (bandwidth ≃6 nm) did not affect the quenching kinetics. Equimolar concentrations of flavin and protein (1.0×10^{-6} and/or 2.0×10^{-6} M) were used in the experiments. Appreciably lower concentrations lead to incomplete flavin–protein binding (equilibrium effects), and higher concentrations make quenching too fast for our measuring technique.

Protein fluorescence quenching gave linear second-order plots (representative curves are shown in Figure 9); the kinetics were assumed to be first order in protein and first

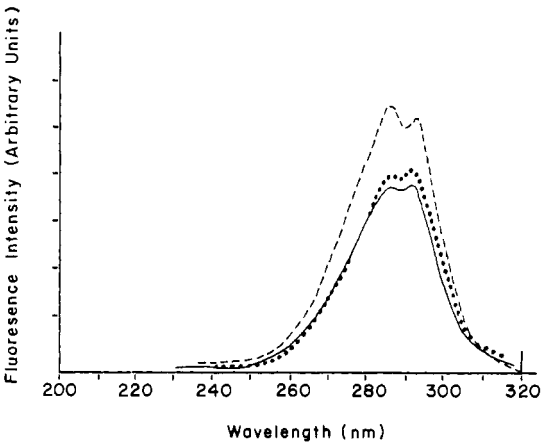

FIGURE 6: Fluorescence excitation spectra of P (- - -), PF (——), and PFH· (· · · ·). Concentrations are 1.0×10^{-6} M in 0.025 M phosphate buffer (pH 7.0). Spectral resolution is 3 nm. Relative intensities are not comparable.

order in flavin.[4] The second-order rate constants (Table II) of the various analogs are in the same relative order as those obtained by Edmondson and Tollin (1971b) by monitoring flavin fluorescence quenching accompanying binding. However, the present values are somewhat smaller. To check the consistency of the results, flavin fluorescence was also measured after the addition of flavin to protein solution. An excitation band centered at 345 nm was used to selectively excite the flavin. Since there was no fluorescence until flavin was added to the apoprotein solution, it was necessary to extrapolate to zero time to get F_0, the initial fluorescence. It was found that, within experimental error, the second-order rate constants obtained from either flavin or protein fluorescence quenching were the same. Thus, the discrepancy

[3] Abbreviations used are: isoFMN, 5,7-dimethyl analog of FMN; dFMN, 2,'3',4'-deoxy analog of FMN.

[4] For equal initial concentrations of flavin and protein, a_0, the second-order rate equation is given by

$$\frac{x}{a_0 - x} = a_0 k t$$

in which x is the concentration of flavoprotein at time t, and k is the second-order rate constant. In terms of the protein fluorescence, the equation may be rewritten as

$$\frac{F_0 - F}{F - F_\infty} = a_0 k t$$

in which F_0 is the initial fluorescence of the apoprotein at $t = 0$, F is the fluorescence at time t, and F_∞ is the final fluorescence at $t = \infty$.

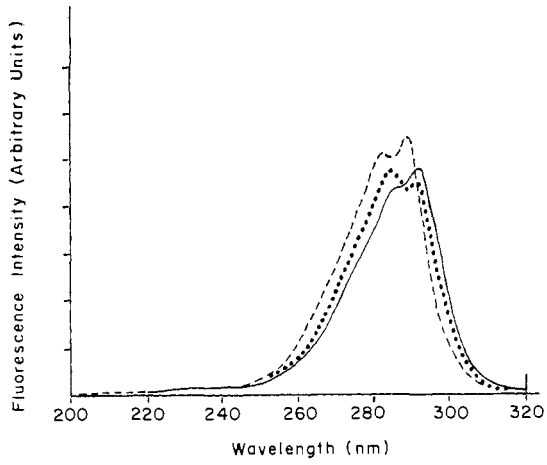

FIGURE 7: Fluorescence excitation spectra of P (····), chymotrypsinogen (——), and tryptophan (– · –). Solvent is 0.025 M phosphate buffer (pH 7.0). Mock apo- and holoprotein excitation spectra were identical with that of tryptophan.

between the two sets of results may be attributed to systematic errors in the measurements. Protein fluorescence quenching accompanying binding of riboflavin and *N*-10-ω-carboxybutylisoalloxazine was faster than the resolution time of our measuring device.

During the course of the measurements, it was found that the rate of flavin binding was reduced by harsh treatment of the apoprotein. For example, if concentrated (6×10^{-5} M) apoprotein in 0.025 M phosphate buffer (pH 7) was left at room temperature overnight, the binding rate constants were reduced by a factor of about 2. At 5°, concentrated apoprotein was found to be stable for several days, but at 10^{-6} M the protein seemed to denature within a matter of hours.

Degree of Protein Fluorescence Quenching. Binding of all of the FMN analogs resulted in 90–95% quenching of protein fluorescence. This indicates that the modifications of the FMN molecule used here do not alter protein quenching mechanisms. On the other hand, riboflavin binding results

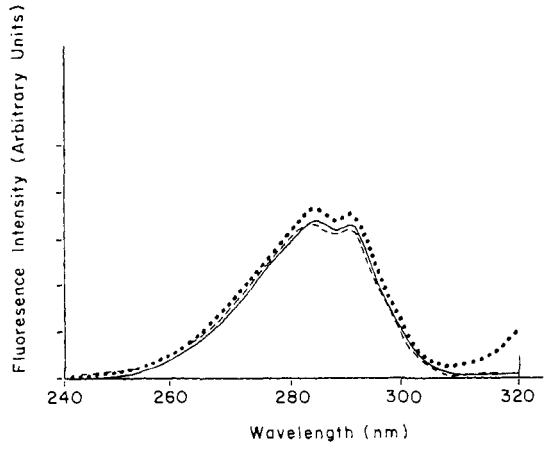

FIGURE 8: Fluorescence excitation spectra of P for which the fluorescence was monitored at 330 (····), 352 (– – –), and 375 nm (—). Concentrations of 2.0×10^{-6} M rather than 1.0×10^{-6} M were used for these experiments; hence, the increased absorption of the sample at the higher concentration results in a slight reduction in the relative fluorescence excitation intensity at 285 nm compared to 293 nm (see Figure 7 for the excitation spectrum at 1.0×10^{-6} M).

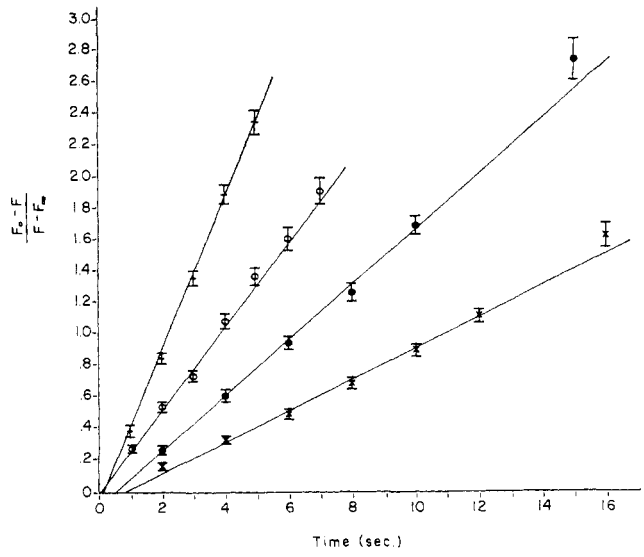

FIGURE 9: Representative second-order plots of protein fluorescence quenching accompanying flavin binding to the apoprotein. Flavins: FMN (●), dFMN (—), 3-MeFMN (×), and isoFMN (○).

in ≃70% protein fluorescence quenching, while binding of *N*-10-ω-carboxybutylisoalloxazine causes only about 30% quenching. These patterns are particularly interesting because the degree of protein fluorescence quenching approximately parallels the degree of flavin fluorescence quenching obtained by Edmondson and Tollin (1971b). This result, coupled with the similarities in the relative magnitudes of the quenching rate constants for the various analogs measured with either protein or flavin fluorescence, suggests a direct relationship between the processes involved in protein and flavin fluorescence quenching.

Possible Mechanisms of Quenching. Under the conditions used in the kinetic experiments, the protein fluorescence quenching appears to be governed by the initial contact of flavin and protein. As was shown earlier (Edmondson and Tollin, 1971b) and confirmed in these experiments, the rate of the flavin–protein interaction is slowed by the presence of a phosphate group and hydroxyl groups on the flavin side chain. It was also shown earlier, and reconfirmed in this work, that the substitution of a carboxyl group in the side

TABLE II: Second-Order Rate Constants for Protein Fluorescence Quenching by Flavin Analogs.

Flavin	$k \times 10^{-5}$ (l. mole^{-1} sec^{-1})	Deg of Protein Fluorescence Quenching (%)
FMN	1.6 ± 0.4	95
3-MeFMN	1.1 ± 0.2	95
IsoFMN	2.5 ± 0.3	95
dFMN	4.0 ± 0.7	95
Riboflavin	Fast	70
N-10-ω-Carboxybutyl-isoalloxazine	Fast	30

TABLE III: Calculated Förster Distances for Energy Transfer.

Donor	Donor Environment	Acceptor	Acceptor Environment	$J'_{AD} \times 10^{14}$ (M^{-1} cm^6)	R_0 $\phi = 0.05$	$\phi = 0.10$	$\phi = 0.20$
Tryptophan	Solution	FMN	Solution	1.58	21.2	23.8	26.7
Tryptophan	Apoprotein	FMN	Holoprotein	1.12	19.9	22.3	25.1
Tyrosine	Solution	FMN	Holoprotein	0.892	19.3	21.7	24.3

chain does not retard the binding rate as compared to riboflavin. These results imply a specific and rate-limiting interaction of the ribityl phosphate group with the protein during the binding process.

The facts that (1) the protein and flavin fluorescence quenching follow second-order kinetics and have the same rate constants, and (2) the degree of protein quenching parallels the flavin quenching, suggest that these two effects are the result of the same process. There are several possible quenching mechanisms that can be considered: rearrangement of the protein leading to an increased probability of radiationless energy dissipation, direct molecular overlap between aromatic side-chain groups and flavin, Förster energy transfer to flavin.

Förster energy transfer would probably be operative over relatively large distances within the protein. In order to make this quantitative, we have calculated Förster critical distances[5] for transfer of energy from tyrosine and tryptophan to flavin. These are in the vicinity of 20 Å (see Table III). Thus, the long-range nature of this type of interaction would suggest that considerable protein fluorescence quenching should occur for any flavin analog bound to the protein, unless there were mutual orientation effects of the transition dipoles which decreased the transfer efficiency. Energy which is transferred to the flavin could be degraded by collisional or molecular orbital overlap interactions with other portions of the protein. MacKenzie *et al.* (1969) have demonstrated flavin fluorescence quenching in flavinyl peptides of tyrosine and tryptophan. Although one might expect a mutual fluorescence quenching of the amino acid side chain in these systems, this was not reported in the MacKenzie paper. While some excitation energy transfer from tyrosine and tryptophan to flavin undoubtedly occurs in the Shethna protein, the fact that some bound flavin analogs (those without side-chain phosphate and hydroxyl groups) show only partial quenching suggests that this is not the only process involved in fluorescence quenching (assuming no effects due to mutual orientation; this assumption would be consistent with the similar circular dichroism spectra in the flavin absorption region shown by all of the bound analogs (Edmondson and Tollin, 1971a,b)).

A direct molecular orbital overlap interaction of flavin with all of the tyrosines (5) and tryptophans (4) does not seem feasible on steric grounds. Additionally, the circular dichroism work of Edmondson and Tollin (1971b) has shown no differences in the tryptophan dichroism of the apo- and holoproteins.[6]

The occurrence of a rapid rearrangement following the initial protein–flavin contact is a possibility, inasmuch as the circular dichroism spectra of the apo- and holoproteins are somewhat different in the far-ultraviolet region (Edmondson and Tollin, 1971b). Such a rearrangement, however, cannot significantly alter the tryptophan environment (*e.g.*, solvent exposure) and must be fast relative to the rate-determining step at the concentrations used in the kinetic experiments. That extensive and rapid rearrangements in proteins are possible has been shown by recent experiments of Schechter *et al.* (1970). These workers followed the renaturation of acid-denatured staphylococcal nuclease by monitoring tryptophan fluorescence. Using the stopped-flow technique, they obtained a first-order rate constant of 12.1 sec^{-1}. Such a rapid protein structural rearrangement can be contrasted to the relatively slow first-order quenching of protein fluorescence ($k = 0.217$ min^{-1}) which accompanies FAD binding to the apoprotein of D-amino acid oxidase (Massey and Curti, 1966). This difference in behavior may relate to the fact that the oxidase has subunit structure, whereas both the nuclease and the Shethna protein consist of single polypeptide chains. Thus, it is conceivable that the initial contact of flavin with protein causes a (first-order) rearrangement of the protein structure around the binding site, and that interactions following this change determine the degree of flavin and protein quenching. Structural modifications of the flavin may result in varying degrees of rearrangement which, as shown by Edmondson and Tollin (1971b,c), can cause changes in many of the properties of the flavoprotein. It should be pointed out that even if such a protein structural change takes place, the two previous mechanisms may still be operative in fluorescence quenching.

An unequivocal decision regarding the occurrence of a rapid protein rearrangement step requires further kinetic evidence, *e.g.*, using relaxation techniques. Such experiments are currently under way in this laboratory. Also, it would be desirable to more firmly establish quenching mechanisms, and the possibility of energy transfer from tyrosine and tryptophan to flavin, by further study of model systems.

[5] The Förster critical distance, R_0, for energy transfer (Förster, 1959, 1965) was calculated using the relationship

$$R_0^6 = 8.8 \times 10^{-25} \phi_D K^2 n^{-4} J'_{AD}$$

in which ϕ_D is the fluorescence quantum yield of the donor, K^2 is a transition moment orientation factor, and n is the refractive index of the medium. J'_{AD} is equal to

$$\int \frac{F(\nu)\epsilon(\nu)d\nu}{\nu^4}$$

in which $F(\nu)$ is the normalized frequency distribution of the donor molecule, $\epsilon(\nu)$ is the extinction coefficient of the acceptor, and ν is the frequency in cm^{-1}. For purposes of comparing our values to those of Eisinger (1969b) for energy transfer among aromatic amino acids in proteins, values of $K^2 = 2/3$ and $n = 1.5$ were used in the calculations.

[6] It is, of course, possible that not all of the tryptophan residues contribute to the optical activity.

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Specific Oxidation of Copper Binding Sites in Copper(II)–Oligopeptide Complexes*

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ABSTRACT: The specific oxidation of the copper(II) binding sites by chloroiridate in two peptide–copper complexes is described.

The octapeptide and the 20-peptide derived from the amino terminal of RNase were used to prepare the 1:1 copper complexes. In both cases the copper was found to bind to the α-amino end of the molecule, using the chloroiridate oxidation technique. The possibility of using this or similar reactions for locating copper binding sites in proteins is discussed.

Recently it has been shown that $IrCl_6^{2-}$ oxidizes Cu(II)-tetrapeptide complexes *via* the formation of a Cu(III) intermediate (Levitzki *et al.*, 1967) the final step being the modification of two of the metal ligands. Thus it was of interest to investigate the interaction of $IrCl_6^{2-}$ with copper complexes of larger peptides in an attempt to find whether the chloroiridate oxidation can be used as a method for the location of copper in both synthetic copper–protein complexes and in natural occurring copper proteins.

In the present investigation the reaction of $IrCl_6^{2-}$ with the copper complexes of RNase fragments was undertaken.

Experimental Section

Materials. The N-terminal octapeptide (P_{1-8}) and 20-peptide (S-peptide) were kindly donated by Mr. S. Levit from our department. The octapeptide was prepared by chymotryptic digestion of the 20-peptide obtained by Nagarse digestion of RNase (S. Levit and A. Berger, in preparation). DNP-amino acids were the product of Mann. $Na_2IrCl_6 \cdot H_2O$ was obtained from Alfa Inorganic Chemicals.

Methods. The electrophoresis at pH 6.5 and 1.4 was performed as described earlier (Levitzki *et al.*, 1967). The determination of $IrCl_6^{2-}$ and ketoacyl peptides, and total amino acid analyses were performed as described previously (Levitzki *et al.*, 1967).

Dinitrophenylation. The copper-free peptide solution was brought to 0.2 M $NaHCO_3$ (pH 8.0) and an excess of FDNB[1] was added. The reaction mixture was stirred overnight at room temperature. The excess FDNB was extracted with ether several times and then the aqueous solution was lyophilized; 1.0 ml of 6 N HCl was added to the lyophilized material in a hydrolysis tube which was vacuum sealed and incubated at 120° for 12 hr. After hydrolysis the HCl was removed over KOH *in vacuo*. The residue was dissolved in DMF and the mixture was analyzed for DNP-amino acids on thin-layer

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[1] Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNPH, 2,4-dinitrophenylhydrazine; DMF, dimethylformamide.