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Flavine–Protein Interactions in Flavoenzymes. pH Dependence of the Binding of Flavine Mononucleotide and Riboflavine to *Azotobacter* Flavodoxin†

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ABSTRACT: In order to specify the ionization state of the ribityl phosphate group in the bound flavine mononucleotide (FMN), and also to determine whether any ionizable protein side-chain groups are involved directly or indirectly in flavine binding, we have studied the pH dependence of the interaction between *Azotobacter* apoflavodoxin and FMN and riboflavine. Both kinetics and equilibria have been investigated. Below pH 3.5, FMN is reversibly released from the holoprotein. This follows a two-proton dependence with a pK of 3 (suggestive of protein side-chain carboxyls). Circular dichroism (CD) spectra demonstrate a change in protein conformation accompanying this release. The second-order rate constants for FMN binding to the apoprotein increase by approximately a factor of 40 between pH 8 and 3.7. Riboflavine shows a much smaller increase in this region. At least two pK values are required to fit the FMN data. The equilibrium constants for both FMN and riboflavine binding do not change appreciably between pH 8 and pH 5. Below pH 5, both analogs show a decrease in binding. FMN follows a curve with a midpoint of about 3.5. Riboflavine displays a more complex behavior involving a rapid fluorescence decrease followed by a slower increase; the latter corresponds to a pK of about 4.5. These results are interpreted as follows. FMN can bind to the apoprotein as both the dianion and the monoanion (referring to the phosphate group). The dianion is released from the protein about 10 times more slowly than is the monoanion. This raises the possibility that positively charged protein side-chain groups are involved in an interaction with the negatively charged phosphate group. Two protein side-chain carboxyls are required to be in their negatively charged forms in order for effective binding to occur. The pK of these carboxyls is ~4 in the apoprotein and is shifted to about 3 in the holoprotein.

Interest in the flavodoxins has been heightened by the recent publication of medium resolution X-ray structures (Watenpaugh *et al.*, 1972; Andersen *et al.*, 1972) for two of these proteins which show the polypeptide chain folding pattern and the orientation of the FMN molecule in the binding site. Although all of the protein side-chain groups which interact with the flavine have not yet been identified, among the features which are clearly specified are the following: (a) the phosphate binding site is located very near to the N terminus of the protein; (b) the entire ribityl phosphate side chain is largely buried within the protein. The latter point is consistent with a variety of kinetic and thermodynamic measurements of flavine binding to several apoflavodoxins (Edmondson and Tollin, 1971c; Barman and Tollin, 1972a). Furthermore, a high degree of homology has been found to exist in the N-terminal region of four of these proteins, specifically those obtained from *Peptostreptococcus elsdenii* (Tanaka *et al.*, 1971), *Desulfovibrio vulgaris* (Dubourdieu *et al.*, 1973), *Clostridium pasteurianum* (Fox *et al.*, 1972; J. L. Fox, personal communication), and *Azotobacter vinelandii* (M. L. MacKnight, W. L. Gray, and G. Tollin, manuscript in preparation).

In order to specify the ionization state of the ribityl phosphate group in the bound cofactor, and also to determine whether any ionizable protein side-chain groups are involved directly or indirectly in flavine binding, we have studied the pH dependence of the interaction between *Azotobacter* apoflavodoxin and FMN and riboflavine. This flavodoxin is of particular interest because of its highly stable free-radical form (Hinkson and Bulen, 1967; Edmondson and Tollin, 1971c), its function as the electron donor to nitrogenase in nitrogen fixation (Yates, 1972), and its unusually high first reduction potential (Barman and Tollin, 1972b). Three previous investigations of the effect of pH on flavine binding to a flavoprotein have been reported, *P. elsdenii* flavodoxin (Mayhew, 1971a), *Azotobacter* flavodoxin (Hinkson, 1968), and old yellow enzyme (Theorell and Nygaard, 1954). Only in the case of the old yellow enzyme was the study extensive enough to establish the ionization state of the FMN phosphate on the protein and to provide evidence for the involvement of ionizable protein functional groups in binding.

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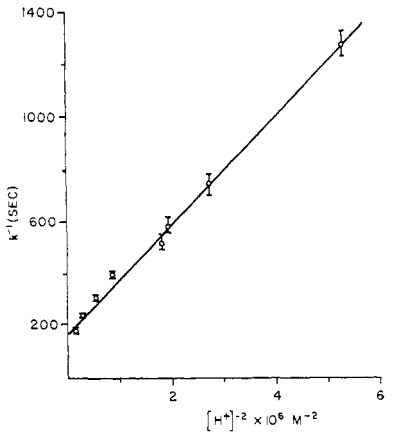

FIGURE 1: pH dependence of the rate of flavine release from *Azotobacter* holoprotein; phosphate–acetate buffer (0.025 M in each); protein concentration was 1×10^{-6} M. Each point represents an average of three–five experiments.

Experimental Section

Materials. Riboflavine and FMN were purchased from Calbiochem, Los Angeles, Calif. FMN was purified by DEAE-cellulose column chromatography at pH 7.0; riboflavine was used without further purification. DEAE-cellulose was obtained from Calbiochem, Los Angeles, Calif.

Azotobacter flavodoxin (Shethna flavoprotein) was isolated from *Azotobacter vinelandii*, strain Wisconsin "O," according to the procedure of Hinkson and Bulen (1967), as modified by Edmondson (1970). The flavine-free apoprotein was prepared as described by Edmondson and Tollin (1971b).

Published extinction coefficients (Beinert, 1960; Yagi, 1971; Hinkson and Bulen, 1967; Barman and Tollin, 1972a) were used in the spectrophotometric determination of flavine and protein concentrations.

Methods. A Cary Model 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment was used for measuring circular dichroism (CD) spectra, as previously described (Edmondson and Tollin, 1971a,b; D'Anna and Tollin, 1971). Cylindrical quartz cells of 10 mm (for the near-ultraviolet (uv) wavelength region) and 1 mm (far-uv) path length were used. Protein concentrations were adjusted such that the maximum absorbance was 0.8 for both the near- and far-uv wavelength regions.

A Radiometer pH meter (Model 26) was used for measurements of pH. Fluorescence quenching was measured using a fluorimeter built in this laboratory and described previously (Edmondson and Tollin, 1971b; D'Anna and Tollin, 1971). The excitation beam from a PEK 110-W high-pressure mercury arc lamp was mechanically chopped and the emission signal was detected utilizing a lock-in amplifier. The sample was excited at 360 nm, and the fluorescence emission was set at the 530-nm fluorescence maximum of the flavine, using Bausch and Lomb monochromators.

Dissociation kinetics were determined by adding a microliter quantity of the holoprotein to 2 ml of 0.025 M phosphate–acetate buffer at a given pH (pH 2.6–3.6) and recording the fluorescence increase upon dissociation of the FMN from the protein. Similarly, measurements of equilibrium binding of FMN and riboflavine were made by adding 25 μl of apoprotein to 2 ml of 1×10^{-6} M flavine solution in 0.05 M phosphate–acetate buffer, and recording the residual flavine fluorescence at equilibrium.

The kinetics of FMN binding were measured by two methods. At higher pH values, where the rate was relatively slow, a 20-μl sample of apoprotein was added to 2 ml of FMN solution (0.05 M phosphate–acetate buffer at a given pH) in the fluorimeter cell compartment by means of a plastic dipstick and the fluorescence quenching was followed on a Sanborn 151 recorder. In all cases, the final protein and flavine concentrations were 1×10^{-6} M. At lower pH values, as well as for riboflavine binding at all pH values, a Gibson-Durrum stopped-flow spectrophotometer was used, which had been modified for fluorescence detection by replacing the normal observation cell with a fluorescence cell (Barman and Tollin, 1972a).

A least-squares fitting procedure was used to calculate the best computer fit to the experimental data points for FMN binding. The kinetic scheme and parameters used in this procedure are discussed later.

Results and Discussion

Release of Flavine at Low pH. When *Azotobacter* flavodoxin was placed into a buffered solution of pH lower than 3.5, the FMN cofactor was released from the protein. This was shown by an increase in flavine fluorescence (measured at 530 nm) and a change in the visible CD spectrum to that of free FMN. These changes were reversible upon increasing the pH of the solution to neutrality. The rate of dissociation of FMN increased with decreasing pH and was first order at all pH values studied. As shown in Figure 1, a plot of the reciprocal of the rate constant for FMN release is linear with respect to $1/[H^+]^2$, indicating that two protons are involved in the mechanism. Assuming that the two protonations of the holoprotein which lead to flavine release have the same pK (or closely similar pK values), a value of 2.9 can be calculated from the slope and intercept in Figure 1 (a least-squares fit was used to obtain these values). A pK in this range can be attributed to perturbed protein side-chain carboxyl groups and thus suggests that two such groups are involved in maintaining the integrity of the binding site of the FMN cofactor.[1] The limiting rate constant was calculated from the intercept in Figure 1 to be 5.9×10^{-3} sec^{-1}.

The dissociation of FMN was accompanied by significant conformational changes in the protein, as shown by a comparison of the far-uv circular dichroism spectra of the holoprotein at pH 7 and 2 (Figure 2). The increase in the negative dichroism at 210 nm and decrease of the 195-nm dichroic band demonstrate that changes have occurred in the folding of the polypeptide chain. The near-uv CD spectra (Figure 3) indicate that the low pH induced conformational change also caused alterations in the environment of the aromatic residues of the protein (particularly tryptophan side chains). Both the positive dichroic band at 290 nm and the negative 270-nm band are virtually eliminated at pH 2.

As shown in both Figures 2 and 3, the conformational change was largely reversible. When the pH of the holoprotein solution was readjusted to 7, the CD spectra in both the near- and far-uv wavelength regions returned very nearly to those

[1] The protein contains no histidine residues (Edmondson and Tollin, 1971b) and the tyrosyl ionizations occur above pH 9 (Edmondson and Tollin, 1971c). The possibility that protonation of the bound FMN phosphate group is involved here is made unlikely by the facts that monoprotonated FMN binds more rapidly to the apoprotein than does the dianion and that bound riboflavine (which does not have a phosphate ester group) shows a similar pH-induced increase in fluorescence (see below for details).

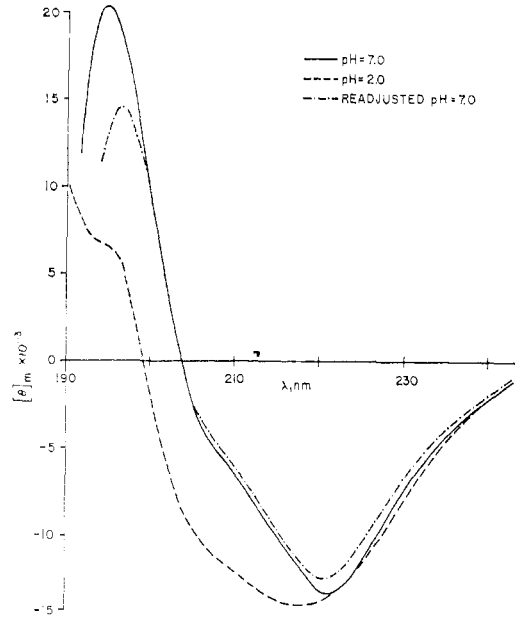

FIGURE 2: Far-uv circular dichroism spectra of *Azotobacter* flavodoxin at neutral and acid pH; phosphate–acetate buffer (0.025 M in each). A mean residue weight of 130 was used to calculate the mean residue ellipticity.

of the original pH 7 solution. The lack of complete recovery of the CD spectra[2] probably is due to loss of some of the protein through precipitation which occurred as the pH of the solution passed through the protein isoelectric point (around pH 4).

Kinetics of Flavine Binding as a Function of pH. Using stopped-flow and rapid mixing techniques, the second-order rate constants for the binding of FMN and riboflavine to *Azotobacter* apoprotein, as measured by quenching of flavine fluorescence, were determined as a function of pH. Some representative kinetic plots for FMN at low pH are shown in Figure 4. Second-order behavior was found to be obeyed down to pH 3.70. Below this pH, the dissociation rates became too high for accurate kinetic data to be obtained.

The complete results for the two flavines are shown in Figure 5. The rate of riboflavine binding to apoprotein was faster than that for FMN at pH 7 by about a factor of ten and increased only slightly with increasing H^+ concentration. The rate of binding of FMN, however, increased approximately 40-fold as the pH was lowered from 8 to 3.7. At low pH, the binding rates of the two analogs were approximately the same. It was not possible to fit the data for FMN binding by a single pK value. Thus, more than one protonation must be involved in controlling the rate of the coenzyme–protein interaction. The faster rate of binding (as well as an ~100-fold lower association constant) of riboflavine as compared with FMN at neutral pH has been reported previously (Edmondson and Tollin, 1971b; Barman and Tollin, 1972a); the values of $1.4 \pm 0.1 \times 10^6$ and $1.6 \pm 0.4 \times 10^5$ M^{-1} sec^{-1} for these analogs at pH 7 are in reasonable agreement with the earlier results. The difference in the pH dependence of the binding rates of the two flavines must be a consequence of the presence of the ribityl phosphate group in FMN and its

[2] The apparent lesser recovery below 200 nm can be ascribed to a greater uncertainty in the spectrum in this region due to a poorer signal-to-noise ratio. It is unlikely that conformational differences are involved inasmuch as the redox properties of the protein have been found to be invariant to low pH treatment (Edmondson and Tollin, 1971b,c).

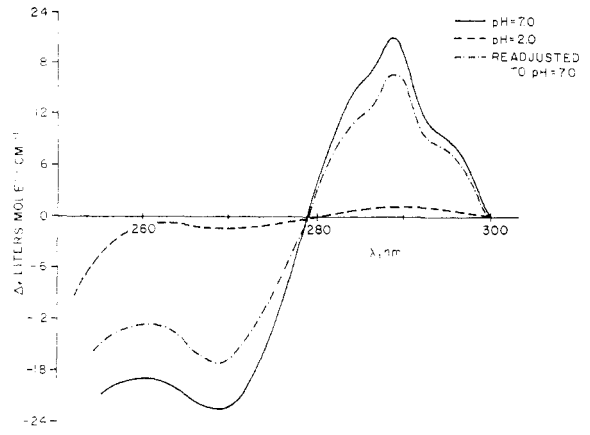

FIGURE 3: Near-uv circular dichroism spectra of *Azotobacter* flavodoxin at neutral and acid pH; phosphate–acetate buffer (0.025 M in each).

absence in riboflavine. Thus, the number of negative charges on this group, and perhaps also an interaction between this charge and the protein, must affect the rate-determining step in the binding process.

Equilibrium Binding Studies. Because of the competing rates of flavine binding and dissociation at lower pH values, it was hoped that an investigation of the pH dependence of the extent of binding at equilibrium could be used to estimate the pK values which affect the overall process. These experiments were done by measuring the intensity of flavine fluorescence at equilibrium after the apoprotein had been added to a flavine solution buffered at a given pH. It was assumed that complete binding of flavine did not occur and that the flavine–protein complex was negligibly fluorescent at each pH, *i.e.* that the residual 530-nm fluorescence was due to unbound flavine. This was known to be valid from previous studies (Edmondson and Tollin, 1971b) in the pH range above 6 and was found also to be true in the lower pH region, inasmuch as repeated additions of apoprotein continued to quench the residual fluorescence proportionally (see, for example, points representing consecutive additions of apoprotein to FMN at pH 4.5 shown in Figure 6). The fluorimeter was adjusted to full scale deflection for the free flavine solution at each pH; the extent of fluorescence quenching was measured as a percentage of this value. Because photobleaching of the unbound flavine in the fluorimeter could alter the equilibrium fluorescence value, solutions were allowed to come to equilibrium in the dark before the final fluorescence level was recorded. The results of these studies are shown in Figure 6. As can be seen, the binding of FMN to apoprotein is markedly diminished by a protonation occurring in the region of pH 3–4. From the shape of this curve, it is possible to conclude that more than one proton is involved here (probably two or three) and that the pK is in the range of 3.5–4.0. This is again suggestive of multiple carboxyl group ionization. Note that the decrease in extent of FMN binding occurred in the same pH region in which the binding rate increase was maximal (Figure 5). This will be discussed more fully below.

The pH dependence of riboflavine fluorescence quenching was more complex than with FMN. In the range from pH 3 to 5, an initial rapid fluorescence quenching was observed, followed by a slower increase in fluorescence[3] which resulted

[3] No indications that FMN displayed this type of behavior on a more rapid time scale were obtained from the stopped-flow measurements of binding kinetics.

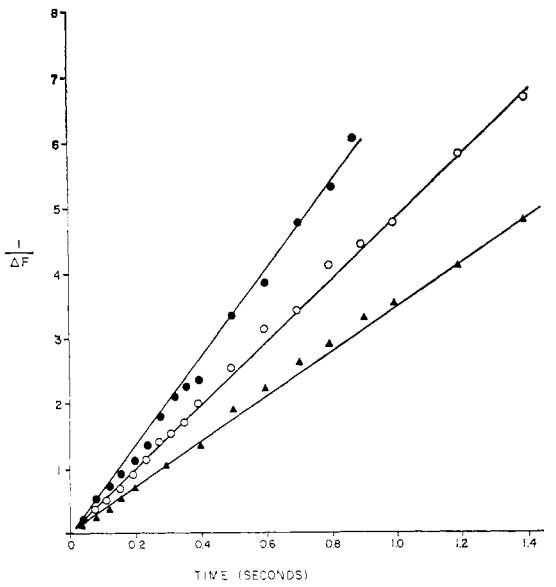

FIGURE 4: Second-order plots for FMN binding to *Azotobacter* flavodoxin; phosphate–acetate buffer (0.05 M in each); flavine and protein concentrations were 1×10^{-6} M; (●) pH 3.70; (○) pH 4.06; (▲) pH 4.20.

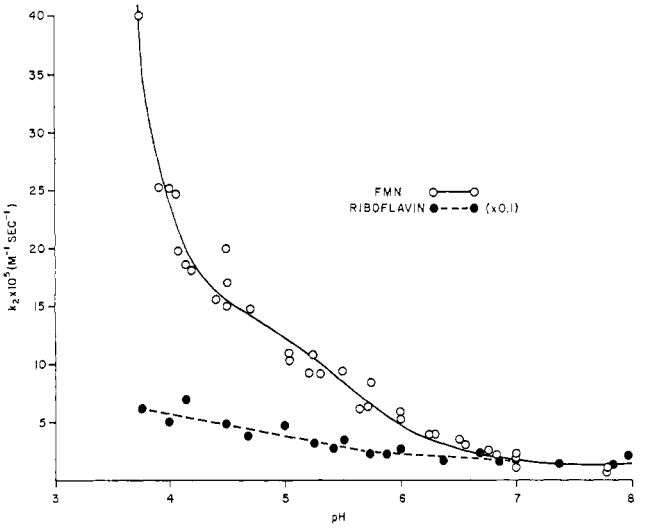

FIGURE 5: pH dependence of rate of flavine binding to *Azotobacter* apoflavodoxin; phosphate–acetate buffer (0.05 M in each); flavine and protein concentrations were 1×10^{-6} M. Note change in scale for riboflavine values. Solid line through FMN points is a least-squares computer fit using the mechanism given in Figure 7. In order to confirm that the fit was unique, a range of values of the starting parameters was used. pK_2 was varied from 3.3 to 3.7, k_2 from 5×10^5 to 10×10^5, and k_3 from 110×10^5 to 150×10^5. The final computed values for $pK_2 = 3.3$ were $k_1 = 1.57 \times 10^5$, $k_2 = 16.6 \times 10^5$, $k_3 = 219 \times 10^5$, and $pK_1 = 5.4$; with $pK_2 = 3.7$, $k_1 = 1.23 \times 10^5$, $k_2 = 15.0 \times 10^5$, $k_3 = 65.9 \times 10^5$, and $pK_1 = 5.6$. Varying the starting values of k_2 and k_3 did not significantly alter the final values. In all cases, the fit to the data points was satisfactory. The least-squares error value for the calculation using $pK_2 = 3.5$ was 67.27.

in a final equilibrium fluorescence level that was higher than the initial value (Figure 7). As shown in Figure 6, the final fluorescence intensities reached at equilibrium for riboflavine binding fit a curve with a pK in the range of 4.5–5.0 and a slope which is indicative of multiple protonation.

The slow increase in riboflavine fluorescence mentioned above followed first-order kinetics. The rate of this process increased with decreasing pH. This is demonstrated by the traces of fluorescence intensity *vs.* time at three pH values in Figure 7. These results demonstrate that, at low pH, the mechanism of riboflavine binding involves a minimum of two steps. The slow increase in riboflavine fluorescence following the initial binding can be interpreted as being due to the same type of protonation reaction as is the release of FMN from the holoprotein shown in Figure 1. However, the ionizations which control the former process occur at about 1 pH unit higher than is the case with the normal enzyme. This is still within the range of carboxyl group protonations.

Conclusions

A set of reactions which can account for the pH dependence of the binding of FMN to *Azotobacter* flavodoxin is shown in Figure 8. The known pK values for free phosphoric acid are: $pK_1 = 2.12$, $pK_2 = 7.21$, $pK_3 = 12.67$. In FMN, pK_3 is eliminated due to the ester linkage of the phosphate to the ribityl side chain. The value of pK_1 has been shown to be less than 2 (Cerletti and Rossi-Fanelli, 1958) and a value for pK_2 of 5.9 has been determined by titration at high ionic strength (Theorell and Nygaard, 1954). In the pH range used in the present work, only pK_2 would be expected to influence the binding kinetics. As the pH is lowered from 7, a proton is added to the FMN phosphate which is thereby converted from a doubly charged into a singly charged species. This process is labeled by K_1 in Figure 8. Inasmuch as the binding rate of FMN is still appreciable at pH 8, we may conclude that both ionic forms are capable of being bound to the apoprotein. Furthermore, the equilibrium studies indicated that protein groups which affect binding are also protonated as the pH is lowered still further. If we argue, on the basis of the two-proton dependence of the release of FMN from the holoprotein, that two protons with the same pK are involved in this process as well, we obtain the equilibrium labeled by K_2 in Figure 8. From the pH dependence of the FMN binding constant, we know that $k_1 < k_2 < k_3$, and that $K \simeq K' \gg K''$ or $k_1/k_{-2} \simeq k_2/k_{-2} \gg k_3/k_{-3}$. Thus, it follows that $k_{-1} < k_{-2} \ll k_{-3}$. Using this scheme and assigning $pK_2 = 3.5$ [from the curve obtained in the equilibrium studies (Figure 6)], a theoretical curve was generated for the FMN kinetic data. This is shown as the solid line in Figure 5. As is apparent, the agreement is satisfactory. Inasmuch as the binding rates follow second-order kinetics at each pH, the variation with hydrogen ion concentration must reflect the protonation of the free FMN and apoprotein. The fact that a pK of 3.5 is compatible with both the kinetic and the equilibrium results indicates that it is the apoprotein which is being titrated in the experiments on FMN binding shown in Figure 6. The computer fit yields $pK_1 = 5.5$, $k_1 = 1.46 \times 10^5$ M^{-1} sec^{-1}, $k_2 = 16.1 \times 10^5$ M^{-1} sec^{-1}, and $k_3 = 110 \times 10^5$ M^{-1} sec^{-1}. The value of $pK_1 = 5.5$ is in reasonable agreement with the previously mentioned value for the pK of the FMN phosphate (5.9). From the value of the association constant at pH 7 (2.2×10^8 M^{-1}; Edmondson and Tollin, 1971b), we can calculate k_{-1} to be 6.7×10^{-4} sec^{-1} and k_{-2} to be 7.3×10^{-3} sec^{-1}. The present data do not permit a reliable value for k_{-3} to be calculated.

According to this interpretation, protonation of the FMN phosphate has little effect on the overall equilibrium constant for binding, although it does affect the individual rate constants. Specifically, when the doubly charged FMN is protonated to form the singly charged species, the rate of binding is increased. This could be a consequence of a decrease in the strength of the solvent–phosphate interaction upon reducing the charge on the oxygen atoms. Such an interpretation is

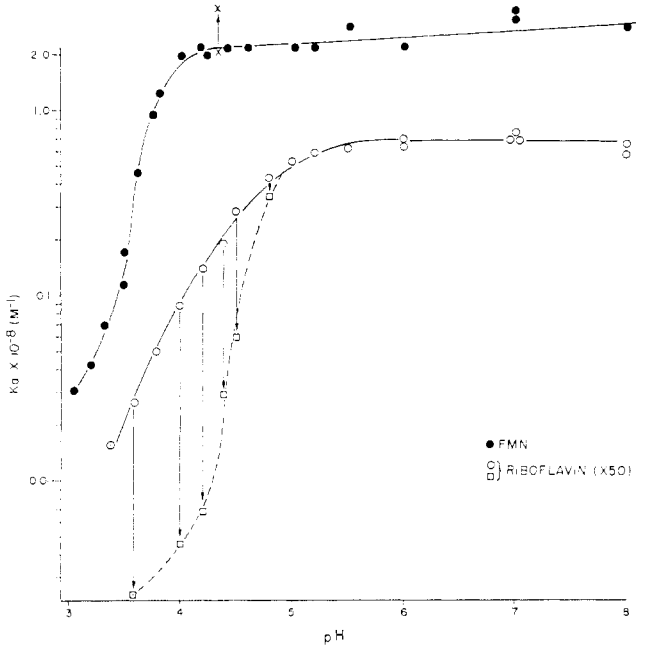

FIGURE 6: pH dependence of association constant for flavine binding to *Azotobacter* apoflavodoxin; phosphate–acetate buffer (0.05 M in each); flavine and protein concentrations were 1×10^{-6} M; (×) successive additions of apoprotein to FMN solution; (□) fluorescence levels reached after initial rapid binding of riboflavine followed by slow release (see text).

consistent with previous kinetic experiments (Barman and Tollin, 1972a). The equilibrium results (Figure 6) demonstrate that the dissociation rate is increased to approximately the same extent as the binding rate upon protonation of the phosphate. Thus, doubly charged FMN is released from the protein more slowly than is the singly charged species. In view of this, one must consider the possibility that there are positively charged amino acid side-chain groups located near the phosphate binding site which interact with the negative charges on the phosphate. It is perhaps significant that lysine residues are found to be present at positions 13, 16, and 19, and arginine at position 15, in the sequence of *Azotobacter* flavodoxin (M. L. MacKnight, W. L. Gray, and G. Tollin, manuscript in preparation). This portion of the polypeptide chain is known to be involved in the FMN phosphate binding site in the three-dimensional structures of *D. vulgaris* and *Clostridium MP* flavodoxins (Watenpaugh *et al.*, 1972; Andersen *et al.*, 1972). Inasmuch as an appreciable degree of homology exists in this region among the flavodoxins (see introductory statement), it is likely that the phosphate binding site lies near the N terminus in the *Azotobacter* protein as well. More detailed structural information is obviously required in order to test the validity of these ideas.

It seems reasonable to assume that the same two protein side-chain groups (probably carboxyls) are being titrated in both the experiments involving FMN release from the holoprotein (Figure 1) and the equilibrium constant for FMN binding (Figure 6). Thus, a shift in the pK of these groups of about 1 pH unit must occur when FMN is bound to the apoprotein. An alternative possibility is that only one protein group is being titrated in the equilibrium fluorescence experiments. In that case, since the binding constant is decreased by about a factor of 100 from pH 4 to 3, a pK shift of two units could be occurring. This effect can be correlated with a phosphate-induced protein conformational change which has been observed (Barman and Tollin, 1972a) to result from the

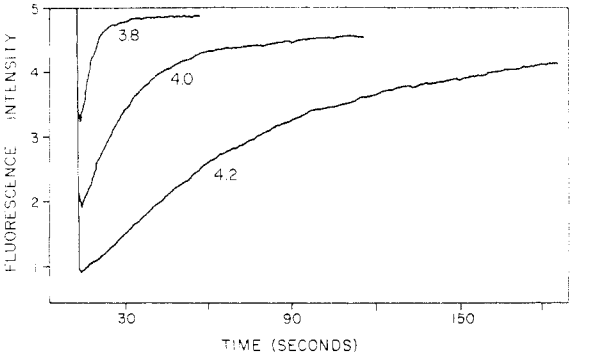

FIGURE 7: Kinetics of riboflavine binding to *Azotobacter* apoflavodoxin at various pH values; phosphate–acetate buffer (0.05 M in each); flavine and protein concentrations were 1×10^{-6} M.

protein–FMN interaction. Significantly, such a conformational step was not detected in the kinetics of riboflavine binding (see below for further discussion). A rearrangement of the protein structure could cause a change in the environment of these carboxyl groups which leads to a stabilization of their negatively charged form and thus a lowering of their pK values.

The increase in FMN binding rate which occurred upon protonation of the two protein carboxyl groups (Figure 5) suggests that the coenzyme binding site is modified in some way by the neutralization of the negative charges so as to make the binding of the ribityl phosphate side chain more rapid. We have previously shown (Edmondson and Tollin, 1971b; Barman and Tollin, 1972a) that the rate-determining step in the coenzyme binding process is the protein–ribityl phosphate interaction. A possible explanation of this is as follows. If the two carboxyls are involved in intramolecular ion pairs with two positively charged protein side-chain groups, and these are disrupted upon protonation, some protein rearrangement would be expected to result.[4] In fact, the uv and CD spectra (Figures 2 and 3) indicate that such a conformational change does indeed occur. This pH-induced conformational rearrangement could be related to the one which is induced as a consequence of the phosphate–protein interaction (Barman and Tollin, 1972a). Thus, the two negative charges on the FMN phosphate could act to replace the negatively charged carboxyls and hence cause disruption of the intramolecular ion pairs. This could in turn lead to a protein rearrangement which allows the FMN to bind more tightly (Barman and Tollin, 1972a). Inasmuch as the riboflavine binding rate does not increase to nearly the same extent as does that for FMN in the pH range 4.5 to 3.5, the protonation of the side-chain carboxyls certainly affects the portion of the binding site which interacts with the ribityl phosphate. This is consistent with the above interpretation.

The increase in riboflavine fluorescence which occurred subsequent to binding in the pH range 5 to 3 (Figure 6) can be interpreted as follows. Riboflavine also induces a change in the protein structure upon binding. This causes a shift from 3.5 to about 4.5 in the pK value of the protein side-chain carboxyls which are involved in maintaining the binding site. Note that this shift is in the opposite direction to that ob-

[4] An alternative possibility is a decrease in electrostatic repulsion between the carboxyl groups and the negatively charged phosphate. If this were the case, a protein conformational change need not be involved in the mechanism. However, this would not explain the change in the mode of binding of riboflavine which results from the protonation of these carboxyls (see below).

tained for FMN, and thus the nature of the riboflavine-induced protein change is necessarily quite different from that caused by FMN binding. Such a shift to higher pK must be due to a modification of the environment of the two carboxyl groups such that their negatively charged forms are destabilized. Thus, if the pH is below 5, rapid protonation will occur. This causes the binding site to be loosened and riboflavine to undergo an increase in fluorescence yield. This may be represented as follows

$$P + R \underset{\text{low fluorescence yield}}{\overset{\text{fast}}{\rightleftharpoons}} (PR) \underset{\text{high fluorescence yield}}{\overset{\text{slow}}{\rightleftharpoons}} (PR)'$$

The fact that a riboflavine-induced protein conformational change was not observed in temperature-jump relaxation experiments (Barman and Tollin, 1972a) may indicate that the effect is too small or too fast to be detected. It would be of interest to investigate the relaxation behavior of riboflavine at low pH values.

It is important to point out that the fluorescence levels reached shortly after mixing riboflavine and apoprotein (see upper riboflavine curve in Figure 6) do not represent equilibrium values and thus do not define a thermodynamic pK. In fact, if the pK shift to 4.5 did not occur (or if it were possible to measure "instantaneous" fluorescence levels), the riboflavine curve in Figure 6 would be expected to have the same midpoint as does the FMN curve.

The flavodoxins obtained from *P. elsdenii* and *Clostridium MP*, in contrast to the *Azotobacter* and *D. vulgaris* flavodoxins, do not bind riboflavine at all (Mayhew, 1971b; D'Anna and Tollin, 1972). Thus, it is possible that in the former flavoproteins side-chain carboxyl groups in the apoprotein are not important in maintaining structure and that the phosphate alone acts to cause the formation and stabilization of the coenzyme binding site. It is of interest in this connection that the large increase in FMN binding rate observed in the present studies between pH 5 and 3.5 does not seem to be occurring in the *P. elsdenii* flavodoxin (Mayhew, 1971a).

The results of the present study can be compared with those of Theorell and Nygaard (1954), who investigated the pH dependence of the rates of binding and dissociation of FMN and riboflavine with yeast old yellow enzyme. Qualitatively, the same patterns as observed here were obtained for the dissociation rates of the two flavines, *i.e.* an increasing rate of dissociation at lower pH values and a more rapid dissociation rate for riboflavine than for FMN. Furthermore, as with *Azotobacter* flavodoxin, riboflavine was not bound as strongly as was FMN. The binding kinetics, however, for the two proteins are quite different. Although the rate of riboflavine binding was only slightly affected by changes in pH, as we have observed for *Azotobacter* flavodoxin, the rate constants for riboflavine binding to old yellow enzyme were smaller than those for FMN binding, in contrast to the *Azotobacter* protein. FMN was found to bind more rapidly to old yellow enzyme in the dianion form than as the monoanion, since the rate of binding increased with increasing pH with a pK corresponding to the protonation of the ribityl phosphate (pK = 6.0). This is in sharp contrast to what we have observed for *Azotobacter* flavodoxin and what Mayhew (1971a) has found for *P. elsdenii* flavodoxin. The pH dependence of the FMN-binding rate at high pH values indicated that two positively charged protein groups of old yellow enzyme were involved in the binding, perhaps interacting directly with the two negative charges on the FMN. Although we have not investigated the high pH region thoroughly, FMN remains bound to *Azotobacter* flavodoxin up to pH 11. No evidence for protein carboxyl group involvement was obtained with old yellow enzyme.

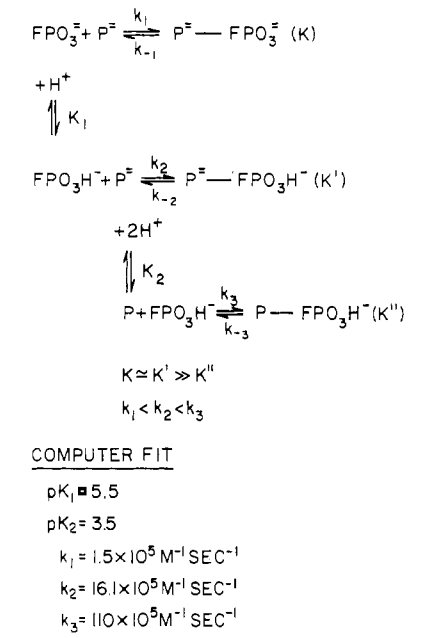

FIGURE 8: Scheme depicting equilibria involved in FMN binding to *Azotobacter* apoflavodoxin as a function of pH. The protonation equilibrium involved in FMN release from the holoprotein is not included. Furthermore, any equilibria involving protein conformational changes subsequent to binding or FMN phosphate ionization occurring in the bound coenzyme are ignored.

In view of the differences in coenzyme binding properties which exist between the flavodoxins and old yellow enzyme, it is interesting to note that they differ in other ways as well. For example, the visible CD spectra (due to the bound flavine) for the two types of proteins are quite different (Edmondson and Tollin, 1971a; V. Massey, personal communication), indicating that the flavine environments are not alike. Also, old yellow enzyme generates the anionic flavine radical form upon reduction (Massey *et al.*, 1969), in contrast to the flavodoxins which produce the neutral radical. The difference which exists between the *Azotobacter* and *P. elsdenii* flavodoxins (see above) may be correlated with the facts that these proteins do have somewhat different visible CD spectra (Edmondson and Tollin, 1971a; D'Anna and Tollin, 1972) and that the *P. elsdenii* apoprotein does not bind riboflavine (Mayhew, 1971b).

While structural conclusions such as the ones presented here must be verified by determinations of the three-dimensional structures of the flavoproteins, it is apparent that protein carboxyl groups play an important role in the coenzyme binding process. Further work is necessary in order to clarify the detailed mechanisms involved and to obtain more accurate values for the pK values of these groups.

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Characterization of Modified Myosin at Low Ionic Strength. Enzymatic and Spin-Label Studies†

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ABSTRACT: In order to gain insight into the nature of the actin-induced acceleration of the myosin-catalyzed hydrolysis of ATP several chemical modifiers of myosin have been studied at the low ionic strength conditions where actin is most effective. From determinations of myosin catalytic activity under a variety of conditions it was found that actin and chemical modifiers (*e.g.*, sulfhydryl reagents, butanol, substitution of ITP for ATP) share common properties in a low ionic strength medium. Modification results in activation of the myosin catalytic activity if Mg^{2+} is present; below a certain critical Mg^{2+} concentration inhibition occurs. This Mg^{2+}-moderated activation varies with the pH of the assay medium; maximal activation by the modifiers occurs at pH 7 (*i.e.*, at the pH of minimal activity for the unmodified myosin). The effect of modification on the nucleotide-induced conformational change was investigated with a spin-labeled preparation of heavy meromyosin. The magnitude of the ADP-induced mobilization of spin labels attached to the SH_1 groups of heavy meromyosin was reduced by modification. For three vastly different modifiers (actin, butanol, substitution of inosine nucleotide for adenine nucleotide) the potency of activation of the myosin catalytic activity was inversely related to the concentration of Mg^{2+} required to support activation and the magnitude of the nucleoside diphosphate induced spectral change. These findings suggest that the modifiers impair the nucleoside diphosphate induced conformational change in myosin and that such impairment may be associated with acceleration of the catalytic activity.

A central question for the mechanism of muscle contraction is how the chemical energy of ATP is converted into the mechanical energy responsible for the relative translation of the thin and thick filaments. Hydrolysis of ATP is essential for contraction and this process is catalyzed by the thick filament protein, myosin. Under physiological conditions (*i.e.*, at low ionic strength and in the presence of Mg^{2+}) the catalytic activity of myosin alone is poor. However the thin filament protein, actin, enhances this activity to levels sufficient for contraction. Attempts to study the unique properties of the actin-modified myosin ATPase have been thwarted by the fact that myosin is insoluble in the ionic conditions which support actin activation. Attention has therefore been focused on a number of chemical reagents (*e.g.*, a variety of alcohols, sulfhydryl reagents, and substitution of ITP for ATP) which activate the Ca^{2+}-moderated ATPase of myosin at high ionic strength (*i.e.*, conditions where myosin can be studied in solution). Recent studies with molecular probes (Duke *et al.*, 1966; Cheung and Morales, 1969; Cheung, 1969;

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