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Flavine–Protein Interactions in Flavoenzymes. Effects of Aggregation of the Apoprotein of *Azotobacter* Flavodoxin on Coenzyme Binding†

Kiyoshi Shiga and Gordon Tollin*

ABSTRACT: The effects of apoprotein concentration and buffer pH, concentration, and type on the kinetics and thermodynamics of the binding of flavine analogs to *Azotobacter* apoflavodoxin have been studied. The affinities of 3-methylflavine mononucleotide (3-MeFMN) and riboflavine were found to increase approximately tenfold when the concentration of apoprotein was decreased from 10^{-6} to 5×10^{-8} M. The shapes of the pH-affinity curves were also observed to be dependent on apoprotein concentration. At high apoprotein concentrations, the affinity of 3-MeFMN was the same in both phosphate-acetate and pyrophosphate buffers, whereas at low concentrations, the affinity in pyrophosphate buffer was higher than it was in phosphate-acetate. In order to explain the above results, it is proposed that the apoprotein exists in an equilibrium between monomer and polymer and that the affinity of flavine derivatives to monomer is higher than to polymer. The extent of polymerization is assumed to be a function of pH and buffer type. We have also observed that the binding velocity of 3-MeFMN to apoprotein was unaffected by the protein concentration. Thus, it can be concluded that the binding rates of flavine to monomer and to polymer are the same. The primary effect of apoprotein polymerization must therefore be on the rate constant for the release of flavine from the holoprotein. However, the binding velocities were found to be a function of buffer type and concentration. This must be due to a direct modification of the apoprotein-flavine interaction and not to a change in the equilibrium constant for the monomer-polymer reaction.

Previous work in this laboratory (Edmondson and Tollin, 1971a,b; Edmondson *et al.*, 1972; Barman and Tollin, 1972; D'Anna and Tollin, 1971, 1972; MacKnight *et al.*, 1973) has provided information on the kinetics, thermodynamics and pH dependence of the binding of flavine analogs to the apoprotein of various flavodoxins, particularly that derived from *Azotobacter*. However, very little information is available concerning the possibility of protein-protein interactions in these enzymes. In the earlier work, it was assumed that the flavodoxins have no subunit structure, mainly based on hydrodynamic measurements of the holoprotein of *Azotobacter* flavodoxin (Edmond-

† From the Department of Chemistry, University of Arizona, Tucson, Arizona 85721. *Received January 21, 1974.* This work was supported by a National Institutes of Health Research Grant (AM 15057).

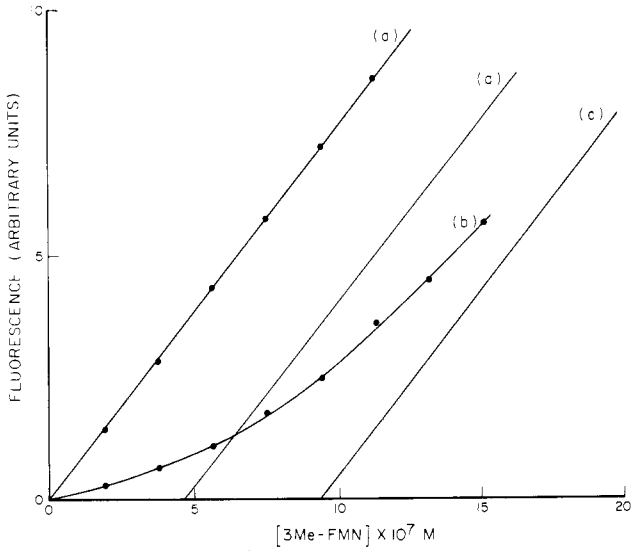

FIGURE 1: Titration of *Azotobacter* apoflavodoxin with 3-MeFMN. Fluorescence was excited at 365 nm and observed at 530 nm: (a) fluorescence data obtained in the absence of apoprotein; (b) results in the presence of 9.2×10^{-7} M apoprotein; (c) limiting line which (b) reaches when the protein is saturated with 3-MeFMN; (d) line drawn parallel to (a) and (c) and midway between them. Experiments were carried out in 0.05 M phosphate–acetate buffer at pH 7.0 and 25°.

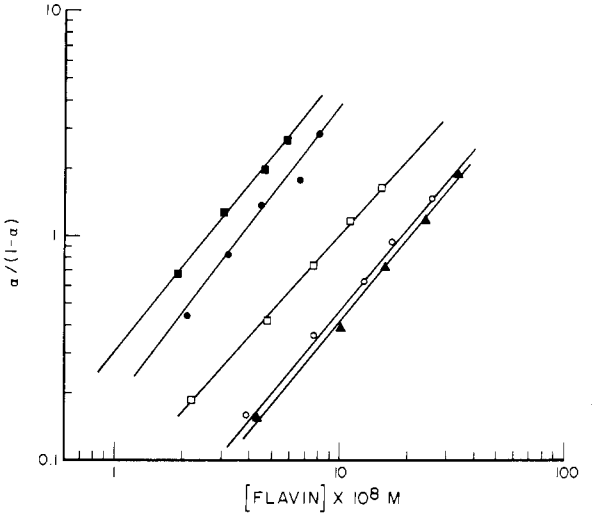

FIGURE 2: Hill plots of titrations of *Azotobacter* apoflavodoxin with 3-MeFMN. The experimental conditions were the same as those in Figure 1 except for the concentration of the apoprotein. α is the fraction saturation of protein with 3-MeFMN. [F] is the concentration of free 3-MeFMN. The concentrations (M) of protein used were as follows: (■) 5.2×10^{-8}; (●) 1.0×10^{-7}; (□) 2.5×10^{-7}; (○) 5.0×10^{-7}; (▲) 9.3×10^{-7}.

son and Tollin, 1971a) which showed that it existed as a monomer at concentrations up to 3×4^{-4} M. These experiments did not, however, rule out the possibility of polymerization of the apoprotein. In fact, it has been observed that this species is more stable at high concentrations than at low concentrations (D'Anna and Tollin, 1971), which suggests the possibility that protein–protein interactions occur.

In the present paper, we report studies of the effect of protein concentrations and buffer composition, concentration, and pH on the affinity and the binding velocity of flavine analogs to *Azotobacter* apoflavodoxin. These experiments clearly show the existence of protein–protein interactions in the apoflavodoxin which affect the flavine binding properties.

Materials and Methods

The apoprotein of *Azotobacter* flavodoxin (Shethna flavoprotein) was prepared as described by Edmondson and Tollin (1971a). All other chemicals were the same as previously reported (Edmondson and Tollin, 1971a). Published extinction coefficients were used in the spectrophotometric determination of 3-MeFMN,[1] riboflavine, and protein concentrations. A Gilford Model 240 spectrophotometer was used for single wavelength absorbance measurements.

All experiments, unless otherwise stated, were carried out in 0.05 M phosphate–acetate buffer at 25°.

Circular dichroism (CD) spectra were measured using a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment. A 1-mm cell was used at the high protein concentrations and a 5-cm cell at the low concentrations.

Titration curves of apoprotein with 3-MeFMN and riboflavine at various concentrations of protein were obtained using a fluorimeter as described previously (Edmondson and Tollin, 1971a). In calculating the binding constant, it was assumed that the apoprotein–flavine complex was nonfluorescent. We have determined that residual fluorescence amounted to less than 3% with 3-MeFMN and less than 5% with riboflavine (*cf.* also Tollin and Edmondson, 1971). In the present experiments, the protein concentration was held constant and aliquots of flavine solution were added. This is the inverse of the method used in the previous work. 3-MeFMN was used in these studies rather than FMN because its binding constant is somewhat smaller and is thus more accurately measurable. 3-MeFMN binding rate constants at various concentrations of apoprotein were obtained by measuring the second-order kinetics of the quenching of flavine fluorescence (Edmondson and Tollin, 1971a).

Results

Figure 1 shows a typical titration curve obtained with 3-MeFMN at a protein concentration of 9.3×10^{-7} M. In this figure, the difference (a) minus (b) gives the quantity of protein bound by 3-MeFMN. The concentration of free 3-MeFMN at the cross-over point of lines (b) and (d) gives a value of 2.0×10^{-7} M. This is equal to the 3-MeFMN concentration which results in 50% saturation of the protein ($S_{0.5}$). The inverse of this value is the association constant of 3-MeFMN to the apoprotein.

In order to analyze similar titration curves obtained with 3-MeFMN at various apoprotein concentrations, the Hill equation (Hill, 1910) was used. In this expression, α is the fraction

$$\alpha = K'/(1 + K'[\mathrm{F}]^{N_H})$$

saturation of protein with 3-MeFMN, [F] is the concentration of free 3-MeFMN, N_H is the Hill coefficient, and K' is a constant. From this equation, $\log [\alpha/(1 - \alpha)] = N_H \log [\mathrm{F}] + \log K'$. Figure 2 shows the relation obtained between $\log [\alpha/(1 - \alpha)]$ and log [F] (Hill plots) for various concentrations of apoprotein. It is seen that the Hill plots were linear at all protein concentrations and that each line moved in a parallel manner to the right when the concentration of the protein was increased. From this, we can conclude that the affinity of 3-MeFMN to the apoprotein decreases when the protein concentration increases. The Hill coefficient, N_H, which was obtained from the slope of the plots, is essentially independent of the protein concentration. From Figure 2, values of $S_{0.5}$ (which is the concentration of free 3-MeFMN at $\alpha = 0.5$) and of N_H were obtained and are shown in Table I and Figure 3.

[1] Abbreviation used is: 3-MeFMN = 3-methylflavine mononucleotide.

TABLE I:[a] Hill Coefficients (N_H) and Half-Saturating Concentrations ($S_{0.5}$) for 3-MeFMN Binding to Various Concentrations of *Azotobacter* Apoflavodoxin.

Apoprotein Concn (M)	$S_{0.5}$ (M)	N_H
5.2×10^{-8}	2.4×10^{-8}	1.23
1.0×10^{-7}	3.6×10^{-8}	1.33
2.5×10^{-7}	9.6×10^{-8}	1.16
5.0×10^{-7}	1.8×10^{-7}	1.24
9.3×10^{-7}	2.0×10^{-7}	1.24

[a] Data obtained from Figure 2.

In contrast to the behavior of N_H, the value of $S_{0.5}$ changes greatly with protein concentration (this quantity is about ten times higher at high protein concentrations than at low concentrations and tends to level off as the protein concentration increases (*cf.* Figure 3)). These results indicate the possibility that protein-protein interactions affect the binding of 3-MeFMN to apoprotein. This will be further discussed below.

Figure 4 shows the relation between pH and $1/S_{0.5}$ at high and low concentrations of protein. These results also show the concentration dependence of the binding affinity, *i.e.*, at all pH's the $S_{0.5}$ values were larger at the higher protein concentration. Furthermore, the pH profiles were found to be different at the two concentrations.

In Figure 4, the points labeled (●) and (x) show values of $1/S_{0.5}$ obtained in 0.05 M pyrophosphate buffer (pH 8.0). It is apparent that the affinity of 3-MeFMN at low protein concentrations is greater than it is at high concentrations in this buffer as well as in phosphate-acetate buffer. However, at high protein concentrations the affinity in pyrophosphate buffer is the same as in the phosphate-acetate buffer, whereas at low concentrations the affinity is greater.

We have also observed an increase of affinity with a decrease in protein concentration with riboflavine as well as with 3-MeFMN. This is shown in Table II.[2] Thus, we can conclude that the ribityl phosphate group does not play an essential role in the affinity changes which occur as a function of the protein concentration. This is somewhat unexpected in view of the crucial role of the phosphate group in the kinetics and thermodynamics of the binding process (Edmondson and Tollin, 1971a; Barman and Tollin, 1972).

In view of the above results, it was of interest to determine the dependence of the flavine-binding rate constant on the protein concentration. Figure 5 shows the results of such experiments. It is seen that the kinetic constants for binding are essentially independent of the concentration of protein. Thus, these results and the data shown in Figure 3 demonstrate that

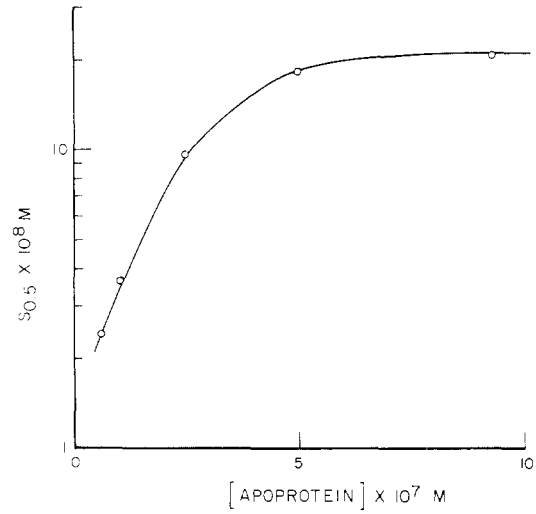

FIGURE 3: Relationship between half-saturating concentration of 3-MeFMN ($S_{0.5}$) and apoprotein concentration. The experimental conditions were the same as those in Figure 2.

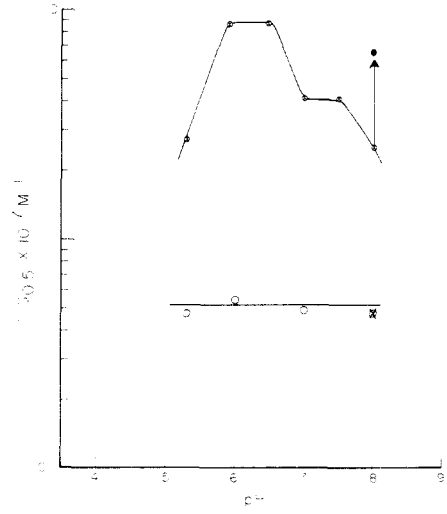

FIGURE 4: Relationship between $1/S_{0.5}$ of 3-MeFMN and pH. The concentrations (M) of apoprotein were: (⊗) 5.2×10^{-8}; (○) 9.3×10^{-7}, in phosphate-acetate buffer (0.05 M, 25°); (●) 5.2×10^{-8}; (X) 9.3×10^{-7}, in pyrophosphate buffer (0.05 M, 25°).

TABLE II:[a] Half-Saturating Concentrations ($S_{0.5}$) for Riboflavine Binding to Various Concentrations of *Azotobacter* Apoflavodoxin.

Apoprotein Concn (M)	$S_{0.5}$ (M)
1.0×10^{-7}	4.5×10^{-8}
8.5×10^{-7}	3.4×10^{-7}
9.4×10^{-7}	4.0×10^{-7}

[a] pH was 7.0 in 0.05 M phosphate-acetate buffer at 25°.

the overall rate constant for release of 3-MeFMN from the protein must significantly increase when the concentration of the protein increases. Calculated values for the off-rate constant are also shown plotted in Figure 5.

Although the binding rate constants do not change as a function of protein concentration, these values clearly depend on the kinds of buffer solutions used and on their concentrations. This is shown in Figure 6. The rate constants are larger in pyrophosphate buffer than in phosphate-acetate buffer. Interme-

[2] Previously obtained values of dissociation constants between the *Azotobacter* apoprotein and 3-MeFMN and riboflavine were 2.7×10^{-8} and 5.6×10^{-7} M, respectively (Edmondson and Tollin, 1971a). These were determined by titrating a fixed amount of flavine with varying amounts of apoprotein. This method is simple and precise when the affinity of the flavine analog is independent of the apoprotein concentration. However, as shown in the present study, this is not the case. Even so, the agreement between these results and those shown in Tables I and II of the present paper is not unreasonable, when one takes into account the fact that the earlier riboflavine titrations were done at relatively high concentrations of apoprotein. It is also worth noting that the titration plots in the earlier work did not pass through the origin. This is most likely due to the concentration dependence of the dissociation constant (see below for further discussion), although it was not so noted at that time.

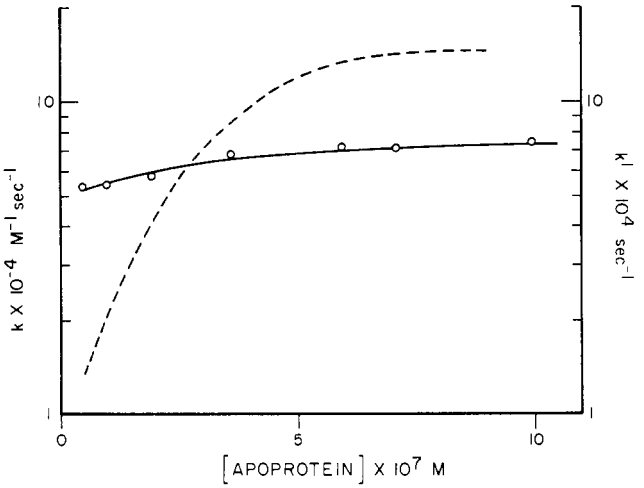

FIGURE 5: Relationship between the second-order rate constants for binding (k,—) and for release (k',- - -) of 3-MeFMN and apoprotein concentration. The concentrations of 3-MeFMN and apoprotein were equal; k was obtained from second-order plots and k' was calculated using the equation $k' = S_{0.5}k$ and values obtained from the solid lines in Figure 3 and 5. pH was 7.0 in 0.05 M phosphate-acetate buffer, 25°.

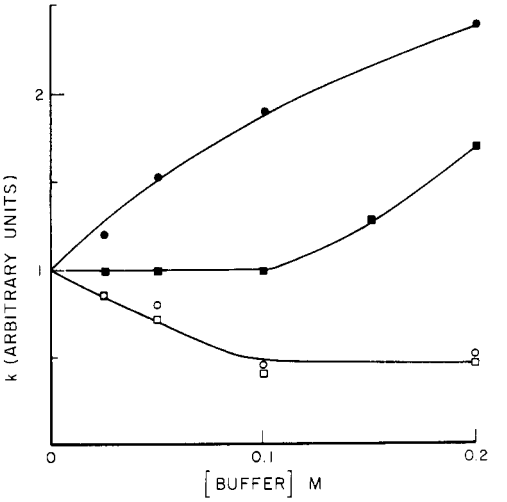

FIGURE 6: Relationship between the second-order rate constants for binding of 3-MeFMN to apoflavodoxin and buffer composition and concentration. pH was 8.0 at 25°: (●) pyrophosphate, (■) Tris, (□) phosphate, (○) phosphate-acetate.

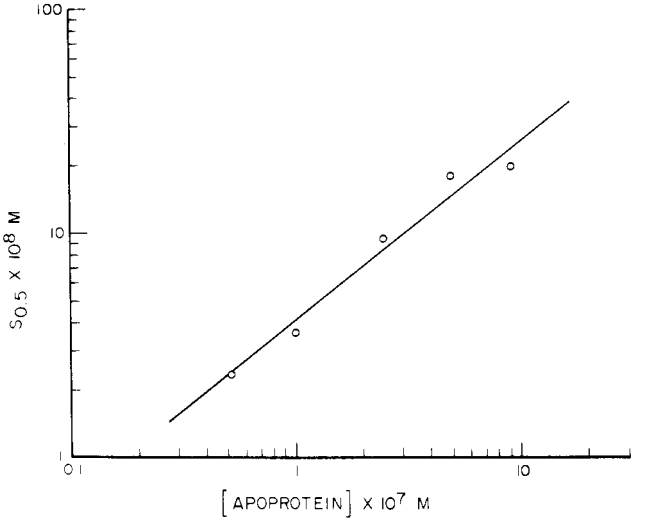

FIGURE 7: Relationship between log $S_{0.5}$ and log apoprotein concentration for binding of 3-MeFMN to *Azotobacter* apoflavodoxin. The data were obtained from Figure 3.

diate values are obtained in Tris buffer. The same values are observed in phosphate buffer as in phosphate–acetate buffer.

In order to determine the possible existence of conformational changes in the apoprotein as a function of protein concentration, far-ultraviolet circular dichroism spectra were measured (at 30° in 0.02 M pyrophosphate buffer (pH 7)) at two concentrations of apoprotein, 2.3×10^{-5} and 1.4×10^{-7} M. No significant difference was found between these spectra. Thus, there is probably no large change in the secondary structure of the protein between the forms which exist at high and low concentrations. However, in view of the large difference in the thermodynamic binding constants between these forms, there might be some difference in a small but critical region of the protein conformation. Another alternative is that protein–protein interactions prevent the apoprotein from assuming a proper conformation for flavine binding (Barman and Tollin, 1972).

Discussion

As shown above (Figure 3), *Azotobacter* flavodoxin exhibits large decreases in the thermodynamic affinity for flavine as the apoprotein concentration is increased. This strongly suggests that the apoprotein exists in a monomer–polymer equilibrium. The ultracentrifuge study on the *Azotobacter* holoprotein by Edmondson and Tollin (1971a) clearly showed that the holoprotein exists as a monomer, even at rather high concentrations (3×10^{-4} M) of the protein. Thus, we can conclude that only the apoprotein monomer can combine with flavine to give an appreciable amount of flavoprotein at equilibrium (even if the polymerized apoprotein can bind flavine, the affinity must either be much lower than is that of the monomer or depolymerization must occur subsequent to binding). Stated another way, the holoprotein exists chiefly as a monomer and, upon release of flavine, the apoprotein tends to polymerize.[3] These considerations imply that the k' values shown in Figure 5 do not represent true kinetic constants.

The model described above can be represented as

$$n(\mathrm{AP}) \rightleftarrows (\mathrm{AP})_n$$
$$(\mathrm{AP}) + \mathrm{F} \rightleftarrows \mathrm{holo}$$

Then, according to the linked function theory of Wyman (1964)

$$\partial(\ln S_{0.5})/\partial(\ln C) = (n - 1)/n$$

In this equation, C is the total concentration of protein and $S_{0.5}$ is as previously defined. The theory assumes that the fraction saturation of the polymer is zero and that of the monomer is unity. If this relationship holds, a plot of log $S_{0.5}$ *vs.* log C should be linear and the slope should give the value of $(n - 1)/n$. Figure 7 shows such a plot. It is seen that a fairly good straight line is obtained; the slope of the line is 0.8 (using a least-square fit). Thus, the value of n is calculated to be 5.[4]

[3] This circumstance is reversed with another flavoprotein, D-amino acid oxidase (Massey *et al.*, 1966; Yagi *et al.*, 1967; Fonda and Anderson, 1968; Miyake *et al.*, 1971; Shiga *et al.*, 1973). In this case the apoprotein exists in a equilibrium between monomer and dimer, and FAD has a much larger affinity to the dimer than to the monomer. Therefore, the equilibrium in the holoprotein is shifted much more to dimer than is the case with the apoprotein.

[4] When $n = 4$, the slope is 0.75; when $n = 3$, the slope is 0.67. Our data are probably not precise enough to distinguish between these values.

These considerations provide strong support for the contention that *Azotobacter* apoflavodoxin exists in a monomer–polymer equilibrium, because if the protein does not polymerize, n should be unity and the slope should be zero.

In the above mentioned model, the Hill coefficient N_H is given as

$$N_H = \left\{\frac{\partial \log [\alpha/(1-\alpha)]}{\partial \ln (\text{flavine(free)})}\right\}_{\alpha=1/2} = \frac{2n}{n+1}$$

This equation expresses the fact that the value of N_H should be independent of the protein concentration. This is consistent with our data in which an almost parallel movement of the Hill plots was observed as the concentration of protein was increased (*cf.* Figure 2). However, for n values from 3 to 5, N_H varies from 1.5 to 1.7. Our data give an N_H value somewhat below this range (*cf.* Table I). This may be an indication that the present model is not completely correct. For example, more than one polymeric species could exist in equilibrium with the monomeric apoprotein.

The relation between pH and $1/S_{0.5}$ (Figure 4) can be explained as follows. If the affinity of flavine to monomer depends on pH, the pH profile should be the same at high and low concentration of protein, inasmuch as only the monomer binds flavine in appreciable amounts. Since this is not the case, the concentration dependence of the pH profile must be due to changes in the proportion of monomer and polymer as a function of pH. This is supported by the fact that at high concentrations of protein, the affinity is constant with pH, a result which is in agreement with previous work (MacKnight *et al.*, 1973). Furthermore, as shown in Figure 3, the $S_{0.5}$ value at high apoprotein concentrations becomes fairly constant. This demonstrates that most of the protein exists as a polymer under these conditions. Therefore, at high protein concentrations, in spite of the pH dependence of the monomer–polymer equilibrium constant, the distribution between monomer and polymer would be essentially unaffected and the affinity would remain constant.

The shape of the pH–affinity curve obtained at low concentrations of protein (Figure 3) shows that both release and uptake of protons are occurring in the polymerization reaction. We can distinguish the following three processes: (a) at pH 5–6, protons are released; (b) at pH 6.5–7, protons are taken up; (c) at pH values above 7.5, proton uptake occurs again. The fact that this pH dependence is not obtained at high protein concentrations indicates that the ionizations involved occur in the apoprotein and not in the flavine. A possible assignment of these proton equilibria to specific amino acid residues can be made. In the pH range 5–7, carboxyl group ionizations (aspartic acid or glutamic acid) may be involved (the *Azotobacter* protein contains no histidine (Edmondson and Tollin, 1971a)). These would have to have abnormally high pK's resulting from their being buried in nonpolar regions of the protein (for example, the pK of Glu-35 in lysozyme is 6.1 (Banerjee and Rupley, 1973)). Above pH 7.5, the α-NH_2 group may be considered. Obviously, such assignments are tentative and must be confirmed by further experimentation.

We have also shown that the binding rate constants are essentially independent of protein concentration (*cf.* Figure 5). From this and the data of Figure 3, the apparent rate constant for release of 3-MeFMN from protein must significantly increase in the polymer (*cf.* Figure 5). However, it should be noted that the fact that the affinity does not change through a dependence of the monomer–flavine interaction on pH does not mean that pH has no effect on this interaction. In fact, previous work (MacKnight *et al.*, 1973) has shown that the binding rate constants for FMN change greatly with pH. We have also observed this with 3-MeFMN in the present study. This pH dependence must be due to a direct effect on the protein–flavine interaction, inasmuch as the binding rate constants are the same in both monomer and polymer (*cf.* Figure 5). Our earlier interpretation (MacKnight *et al.*, 1973) of the pH effects on kinetic and equilibrium constants was that the rate constants for both binding and release are changed by similar magnitudes by pH changes which affect the state of protonation of the ribityl phosphate group of FMN. The present work is consistent with this.

Similar considerations apply to the changes of affinity and binding rate constants for 3-MeFMN caused by different buffer compositions. As shown in Figure 4, different values of $S_{0.5}$ for pyrophosphate and phosphate–acetate buffers were obtained at low protein concentrations, but the same values were observed at high protein concentrations. Thus, it is reasonable to conclude (as was done for the pH dependence) that the protein polymerization constant varies with the buffer type. According to this view, different buffer solutions do not change the affinity of 3-MeFMN directly through the monomer–flavine interaction, but through a shift in the monomer–polymer equilibrium. The data of Figure 4 show that this equilibrium tends to shift more to the monomer in pyrophosphate buffer than in phosphate–acetate buffer. On the other hand, the binding rate constants in different buffer solutions are not the same (*cf.* Figure 6). This phenomenon must be due to a change in the direct protein–flavine interaction caused by differences in the protein–buffer interaction, again because the binding rate constants of monomer and polymer are the same (*cf.* Figure 5). It follows from this that changes in the magnitude of the rate constant for flavine release from the protein caused by different buffers must compensate the change of binding rate constants, so that the affinity remains the same at high protein concentrations.

The increase in binding rate observed in Tris and pyrophosphate buffers as the ionic strength increases (Figure 6) is most likely due to electrostatic shielding effects. This would be consistent with the fact that FMN binding is slower than that of riboflavine (Edmondson and Tollin, 1971a) and with our previous temperature-jump study of flavine binding (Barman and Tollin, 1972). The opposite result found for phosphate-containing buffers could be a consequence of competition between the buffer and the ribityl phosphate for the coenzyme binding site on the apoprotein.

As is evident from the above considerations, the assumption of polymerization of the apoprotein gives a reasonably consistent explanation of our present data. However, it would be valuable to obtain more precise measurements of $S_{0.5}$ over a wider range of protein concentration, to determine N_H values at various values of α and to carry out a hydrodynamic study on the apoprotein. Even so, our present data clearly show that the *Azotobacter* apoflavodoxin exhibits a fairly complex protein–protein interaction pattern which has a large effect on the protein–flavine interaction. This of course means that the reciprocal relationship is also true, namely that the protein–flavine interaction exerts a strong influence of the protein–protein interaction properties of the flavodoxin.

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A Hydrogen-Exchange Study of Lysozyme Conformation Changes Induced by Inhibitor Binding†

R. R. Wickett,* G. J. Ide, and A. Rosenberg

ABSTRACT: Precise data on lysozyme hydrogen-exchange kinetics have been obtained at pH 6.8 in presence and absence of the lysozyme inhibitor *N*-acetyl-D-glucosamine. The action of ligand resembles that of temperature influencing the whole exchange curve, which is the sum of individual rates, uniformly. An analysis of the apparent activation energy profile of the exchange demonstrates that the inhibitor binding effects most, if not all, of the observable exchange rates. The influence of the bound inhibitor molecule is thus propagated throughout the protein structure.

Hydrogen-isotope-exchange kinetics of polypeptides and proteins are extremely dependent upon conformation (Hvidt and Nielsen, 1966; Woodward and Rosenberg, 1971a,b; Hvidt and Wallevik, 1972). Hydrogen-exchange techniques are sufficiently sensitive to observe changes occurring when proteins bind metal ions (Emery, 1969) or ligand (Di Sabato and Ottesen, 1965; Englander and Mauel, 1972; Nakanishi *et al.*, 1973). Unfortunately, the analysis of such changes is greatly complicated by the wide distribution of exchange rates. Even oxidized ribonuclease in 6 M guandine-HCl has exchange rates distributed over at least two orders of magnitude (Woodward and Rosenberg, 1970) and the distribution in native proteins is much broader.

Because rate constants cannot be obtained specifically, the interpretation of differences in exchange curves is difficult. A difference between exchange curves in the presence and absence of ligand may result from large changes in a few rates (Englander and Mauel, 1972; Englander and Rolf, 1973) or small changes in many or all rates (Benson *et al.*, 1973).

We have obtained very precise data on hydrogen–tritium exchange from lysozyme at a variety of temperatures. The precision of the data has allowed us to obtain an activation energy profile for exchange from the lysozyme molecule. In the presence of *N*-acetyl-D-glucosamine (AcGlcn)[1] this profile is altered over the entire range of observable hydrogens. Furthermore, the rank order[2] of the exchange does not change upon inhibitor binding. These facts allow us to conclude that most of the observable exchange rates change when inhibitor is bound.

Experimental Section

Materials. Medium grade G-25 Sephadex was supplied by A. B. Pharmacia. Tritiated water was supplied by International Chemical and Nuclear Corporation. A Beckman Model L 200 scintillation counter was used. Sigma Chemical Company Grade I lysozyme and Worthington LYSF, salt-free, lysozyme were used interchangeably with no difference in results noted. *N*-Acetyl-D-glucosamine was obtained from Sigma. Protein concentrations were determined at 280 nm with a Cary 118C spectrophotometer. Scintillation fluid was prepared according to the method outlined by Bray.

In-Exchange of Protein. Protein was dissolved in pH 8, 0.05 M tricine buffer to form a solution of concentration 20 mg/ml. An equal volume of tritiated water (0.5 Ci/l.) was added to the protein solution. The resulting solution was incubated for 16 hr at 40°. The level of in-exchange represents the maximal obtainable with pH, temperature, and time as variables.

Out-Exchange. The two-column separation described by Englander (1963) was used with some modifications.

The in-exchanged protein solution (cooled to 1°) (4 ml) was filtered on a column of known dead volume equilibrated to pH

† From the Department of Laboratory Medicine and Pathology, Medical School, and the Laboratory for Biophysical Chemistry, Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received March 7, 1974.* Supported by National Science Foundation Grant GB 25795 and National Institutes of Health Grant HL-06314-13. This is publication 92 from the Laboratory for Biophysical Chemistry.

[1] Abbreviation used is: AcGlcn, *N*-acetyl-D-glucosamine.

[2] The rank order of the exchange is determined by the relative magnitudes of the rate constants, $k_1 \ldots k_n$, associated with hydrogens $H_1 \ldots H_n$. Given a set of experimental conditions under which $k_1 > k_2 > k_{n-1} > k_n$ and a change in those conditions, the rank order is invarient to the change if $k_1' > k_2' \ldots k_{n-1}' > k_n'$ still holds for the new rate constants $k_1' \ldots k_n'$.