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Kinetics and Equilibria in Flavoprotein Systems

II. The Effects of pH, Anions and Temperature on the Dissociation and Reassociation of the Old Yellow Enzyme

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The effects of pH, anions and temperature on the dissociation of the old yellow enzyme (O.Y.E.) and its resynthesis from FMN and protein have been investigated. The results suggest that primary amino groups of the protein may serve as binding sites for FMN. Only doubly anionic FMN couples rapidly with the protein.

With respect to the binding of FMN, the apoprotein of O.Y.E. can be compared to an anionic exchanger of the weakly basic type. Thus, only anions of strong acids are able to dissociate the enzyme. Multivalent anions strongly decrease the association velocity of FMN and apoprotein. This effect is counteracted by univalent anions like chloride or acetate.

A study of the reactions between riboflavin and the old yellow apoenzyme has also been carried out. The association reaction of the riboflavin-protein complex is roughly one-tenth as rapid, and the dissociation one hundred thousand times as rapid as the corresponding reactions in the FMN-protein system.

The possible physiological role of anions in regulating the activity of coenzyme-protein systems has been discussed.

The fluorimetric technique developed in previous studies [1,2] of the dissociation and association velocities in the system

$$\text{FMN} + \text{apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{FMN-Protein (O.Y.E.)}$$

offered a possibility of making a systematic and detailed study of the kinetics and equilibria in this system under varied conditions. The effect of pH and temperature on the dissociation and association of O.Y.E. has yielded information regarding the chemical groups involved in the binding of FMN to the apoenzyme, and our observation that riboflavin combines with this apoenzyme gave us the opportunity to investigate the role that the phosphoric ester group of FMN plays in this binding. The discovery, previously reported, that

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O.Y.E. was readily dissociated by chloride ions led to a more complete investigation of the effect of different anions on the dissociation of the enzyme, and it was found that anions also influence the association reaction. We have reason to believe that anions may play a general role in the regulation of the activity of coenzyme-protein systems.

MATERIALS AND METHODS

O.Y.E. was prepared as described previously[2]. Using this procedure, Dr. C. S. Vestling in this laboratory was able to prepare an approximately 90 % pure product without making use of electrophoresis. The fluorescence of this preparation was 1 % of the fluorescence of an equimolar solution of FMN, whereas the preparations which were 25 % pure showed 4 % fluorescence. Neither the fluorescence of the enzyme nor the apoenzyme was quenched by chloride or by alkali, as is the fluorescence of FMN. This small residual fluorescence of the enzyme is in all probability due to impurities.

A commercial preparation of FMN can be purified by paper electrophoresis, as shown in a previous paper [3]. A more convenient method, which allows purification on a larger scale, is to adsorb the commercial preparation on Dowex 2, 200—400 mesh, and elute with formic acid and ammonium formate. Fraction 1 [2], which may be riboflavin or some other non-phosphorylated flavin, can be eluted with water or 1 *M* formic acid, FMN is eluted with 4 *M* formic acid, and the diphosphate fraction, which is inhomogeneous, can be eluted with 4 *M* formic acid plus 2 *M* anmonium formate.

Riboflavin was recrystallized from a commercial preparation. A fresh solution was made each day.

At the time of publication of previous papers [1,2] we had not yet succeeded in preparing FMN which was pure enough to entirely loose its fluorescence on coupling with apoprotein. We have now found that FMN, prepared from O.Y.E. by splitting with HCl in the absence of light, gives a rest fluorescence of only 2 % after combination with apoenzyme. We conclude that pure apoprotein does not fluoresce at all and that it extinguishes completely the fluorescence of an equivalent amount of FMN.

FMN was titrated in the alkaline range by following the disappearance of the fluorescence. The pK' was found to be 10.2 at 23.5°, the same as for riboflavin [3]. At 13.5° we found pK = 10.29. 0.4 *M* sodium chloride did not affect the pK'-values to any appreciable degree.

In the association experiment described in detail in a previous paper, FMN was added to a solution of apoenzyme. The concentration of apoenzyme was relatively high (1.57×10^{-6} *M*), and there was no serious deviation from a second order reaction. However, when the concentration of apoenzyme is of the order of 0.2×10^{-6} *M* or less, it is rather unstable and should be added to a solution of FMN in the cuvette in order to minimize the time for inactivation in the dilute condition. Otherwise deviation from a second order reaction and lower titration values for the apoenzyme may occur. This procedure also has the advantage that the level of deflection for free FMN at $t = 0$ is determined in advance, and has to be corrected only for the small fluorescence of the apoenzyme itself. If, in contrast, FMN is added to a solution of apoenzyme, small errors in the pipetting of FMN may cause appreciable errors when the reaction is very rapid.

With this modification, the association velocity curves in hundreds of experiments were found to coincide strictly with the requirements for a second order reaction.

The inactivation of the apoenzyme in very dilute solutions can be diminished by the addition of serum albumin to the solution (0.3 mg/ml). Serum albumin did not disturb the reaction between FMN and apoenzyme. The inactivation of dilute solutions of apoenzyme has also been frequently noted in dissociation reactions, especially when the dissociation proceeds slowly. The inactivation is observed as a fluorescence drift, no definite equilibrium being reached.

The inactivation of the apoenzyme in high dilutions is probably not due to metal ions or to other SH-inhibitors, since the addition of Cu^{2+} ions (3×10^{-5} *M*) or *p*-chloromercuribenzoate (6×10^{-5} *M*) did not cause any inactivation.

RESULTS

Dissociation of the old yellow enzyme

The rate of dissociation of the old yellow enzyme in acetate and glycine buffers between pH 3.7 and pH 11 is very slow; the rate constant, k_2, is less than 10^{-5} sec.$^{-1}$. In the presence of certain anions the enzyme dissociates readily. The effect of different anions appears to be related to the ionization constant of the acid formed by each ion. Only the anions of strong acids have a strong dissociating effect. Some results are shown in Table 1.

Table 1. Dissociation of O.Y.E. by different anions at pH 4.40 23.5° C.

Salt	Conc. *M*	$k_2 \times 10^4$ sec^{-1}
Na formate	0.1	0
Na acetate	0.1	0
Na F	0.1	0
NaH_2PO_4	0.1	1
Na_2SO_4	0.1	9
NaCl	0.1	33
$NaNO_3$	0.1	63
NaBr	0.1	99

The effect of pH on k_2 in the presence of 0.4 *M* NaCl is shown in Fig. 1. In the acidic range, between pH 4.7 and 6, k_2 is proportional both to the hydrogen and the chloride ion concentration. Thus at 23.5°, $k_2 = 1\,000\,[H^+][Cl^-]$.* Below pH 4.7, some inactivation may take place, resulting in a k_2, which is somewhat higher than expected from the formula. k_2 increases roughly four-fold from 13.5° to 23.5° over the whole pH range, and the energy of activa-

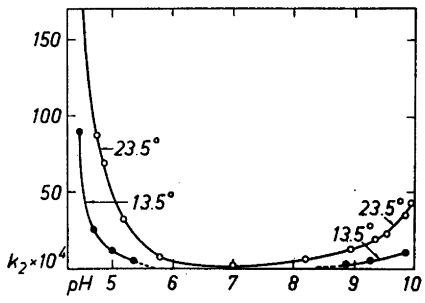


Fig. 1. k_2 *for O.Y.E. as a function of pH in 0.4* M *NaCl.*

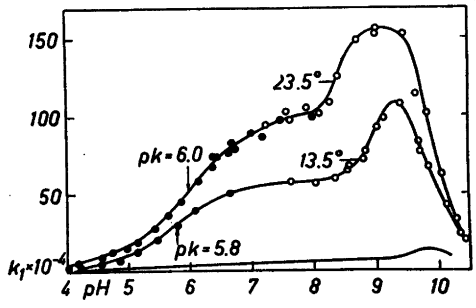


Fig. 2. k_1 *for FMN + protein as a function of pH.* ●●● *in 0.1* M *acetate buffer* ○○○ *in 0.1* M *glycine buffer. The lower curve, experimental points omitted, is* k_1 *for riboflavin + protein in 0.1* M *acetate at 13.5°.*

* One of us (H. T.) dissociated the O.Y.E. reversibly for the first time by dialysis against weak HCl. As indicated by the formula, this was a fortunate choice.

tion, μ, may be calculated to be $\mu = 23\,400$ cal. k_2 is proportional to the chloride concentration also in the alkaline range. This is shown in Tables 2 and 3.

Table 2. Effect of varied [NaCl] *on* k_2 *at pH = 4.93. 0.1* M *sodium acetate buffer, 23.5° C.*

[NaCl]	Q.f.	$k_2 \times 10^4$ sec.$^{-1}$	$\frac{k_2}{[NaCl]}$
0.1	1.06	10.5	105
0.4	1.22	48	120
1.0	1.55	104	104
2.0	2.11	222	111

Association of FMN and apoprotein

In Fig. 2, k_1, the association velocity constant, is plotted as a function of pH, at 23.5° and 13.5°. Acetate and glycine buffers were used in these experiments, since they did not interfere with the reaction velocity. In the alkaline range (glycine buffer), k_1 has a maximum at pH 9.1 at 23.5° and 9.3 at 13.5°, and falls rapidly at higher pH values. This is not due to denaturation of the apoenzyme. Correct titration values of the apoenzyme were obtained between pH 4.6 and 10.5. Below pH 7, k_1 coincides with the dissociation curves of a monovalent acid of $pK' = 6.0$ (23.5°) and $pK' = 5.8$ (13.5°). Since the second dissociation constant of many phosphoric acid esters is usually around $pK' = 6.2$, it was of interest to carry out an acid-base titration of FMN in this region. The results, which are given in Table 4, are somewhat uncertain because of the low solubility of FMN.

In Fig. 3, k_1 is plotted as a function of pH in the presence of 0.4 *M* NaCl. As can be seen from the figure, the maximum of k_1 in the alkaline range does not appear in 0.4 *M* chloride. Instead, the curve drops continuously above pH 7 with increasing pH following a dissociation curve of a monovalent base of p*K* 9.1. As can be seen from the figures, k_1 increases twofold between 13.5° and 23.5°, giving $\mu = 11\,700$ cal/mole. In the pH region around 6 it is seen that k_1 is somewhat higher in glycine than in acetate. In the presence of chloride the negatively charged acetate ions cause an inhibition of k_1: this inhibition does not occur with glycine which is a Zwitterion at this pH.

From the data in Figs. 2 and 3 it may be seen that chloride decreases k_1 in the alkaline range, has no effect around pH 7, and a slightly increasing effect in the acidic range.

Table 3. Effect of varied [NaCl] *on* k_2 *at pH 9.45. 0.05* M *glycine buffer, 23.5° C.*

[NaCl]	Q.f.	$k_2 \times 10^4$ sec^{-1}	$\frac{k_2}{[NaCl]}$
0.1	1.43	4.0	40
0.4	1.61	15	37.4
1.0	2.20	35	35

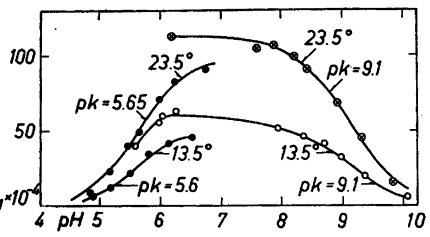


Fig. 3. k_1 *as a function of pH in 0.4* M *NaCl.* ● ● ● *0.1* M *acetate buffer* ⊙ ⊙ ⊙ *and* ○ ○ ○, *0.1* M *glycine buffer.*

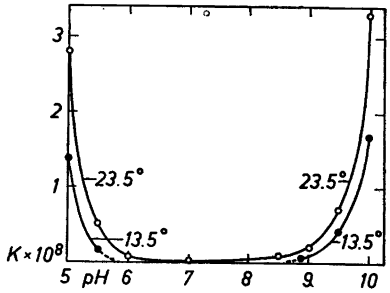


Fig. 4. Dissociation constant, K, for O.Y.E. as a function of pH in 0.4 M *NaCl.*

In Fig. 4, the dissociation constant, K, in 0.4 M NaCl, is plotted as a function of pH at 23.5° and 13.5°. The values are calculated from the results shown in Figs. 1 and 3. The heat of dissociation

$$\triangle H = -4.57 \cdot \log \frac{K_2}{K_1} \left(\frac{T_1 \times T_2}{T_2 - T_1} \right)$$

is approximately —11 700 cal over the entire pH range.

The association reaction is strongly inhibited by polyvalent anions. Some results are shown in Table 5. Monovalent ions generally have no effect or a very slight one on the association reaction. The effect of monovalent anions as compared to the effect of polyvalent anions, is illustrated in the following examples: Whereas secondary phosphate, pH 8.2, $\Gamma/2 = 0.1$, decreased k_1 about 4 fold, there was no measurable effect of primary phosphate at pH 4.23. Divalent anions of stronger acids, like sulfuric acid, however, were found still to inhibit in the acidic range. In the alkaline range, 0.1 M bicarbonate at pH 8.48 decreased k_1 from 125×10^4 M^{-1} sec^{-1} to 95×10^4 M^{-1} sec^{-1}. At pH 9.79, where a greater percentage of the salt is in the form of carbonate ions, it decreased k_1 from 106×10^4 to 25.3×10^4.

The inhibiting effect of polyvalent anions is partially counteracted by monovalent anions. In the presence of polyvalent anions, monovalent anions thus have an effect of increasing the association velocity see Table V.

Table 4. The second dissociation constant of the phosphoric acid group of FMN under different conditions.

Temp. °C	No salt added pK'	In 0.4 M NaCl pK'
23.5	6.2	5.9
13.5	6.2	6.0

Table 5. The inhibition of k_1 *by polyvalent anions and its counteraction by monovalent anions. pH = 7.0. 23.5° C.*

Salts		$k_1 \times 10^{-4}$ M^{-1} sec^{-1}
None		90
Na-phosphate, $\Gamma/2 = 0.1$		14
—»—	+ 0.4 *M* NaCl	64
0.033 *M* Na_2SO_4		50
0.05 *M* Na citrate		10
—»—	+ 0.4 *M* NaCl	40
—»—	+ 0.4 *M* Na acetate	25
0.01 *M* Na versene		19
—»—	+ 0.4 *M* NaCl	63

The reaction between riboflavin and apoenzyme

Riboflavin has been shown to be able to substitute for FMN to some degree [4]. In the system: O.Y.E. apoenzyme, TPN, Zwischenferment and glucose-6-phosphate, a large excess of riboflavin gave a considerable oxygen uptake. It was, therefore, of interest to investigate whether riboflavin could form a nonfluorescent compound with the apoprotein. This was found to be the case, and the rate of the reaction followed the equation

$$-dc/dt = k_1 \text{ [Riboflavin] [Apoenzyme]}$$

A plot of k_1 as a function of pH is included in Fig. 2. k_1 was calculated using the formulas for a second order association reaction with an opposing first order dissociation reaction [2]. As in the FMN-apoenzyme system, a maximum of k_1 was observed in the alkaline range, although the peak in this system was 0.5 pH unit higher, namely pH 9.8. Although k_1 decreased with decreasing pH below pH 7, it did not decrease as rapidly as did k_1 for the FMN-protein system.

The kinetic data for riboflavin-protein could not be measured with the same degree of accuracy as for the FMN-protein system. It may suffice to mention, that k_2 in 0.1 *M* acetate at pH 7 is around 10^{-1} sec^{-1}, that is at least one hundred thousand times higher than k_2 for the old yellow enzyme. Since k_1 for riboflavin + protein is around one tenth of k_1 for O.Y.E. (see Fig. 2), the dissociation constant of riboflavin-protein is at least one million times greater than for O.Y.E. in the absence of dissociating anions.

We have not investigated in detail the effect of anions on the riboflavin-protein system. The dissociation constant, K, seems to be sensitive to small changes in ionic strength of the medium in dilute solutions. For example, in water it is roughly twice as high as in 0.1 *M* acetate. In 0.1 *M* acetate, polyvalent anions like sulfate and phosphate were found to have practically no effect on k_1 and k_2. Chloride ions had some dissociating effect. At pH 6 and 9, 0.4 *M* sodium chloride increased K by about twofold.

DISCUSSION

With respect to binding the negatively charged FMN the protein of the O.Y.E. may be compared to an anion exchanger. Thus, the dissociation of O.Y.E. by anions may be considered to be a displacement of FMN from the protein. The positive charges of the protein are of the weakly basic type; and it is, therefore, to be expected that anions of weak acids are not able to dissociate the enzyme. The strong inhibitory effect of polyvalent anions on the association velocity of FMN and protein is likely to be due to a tendency of these anions to interact with the positive groups of the protein which are essential for the attachment of FMN. Polyvalent anions are known to be bound more strongly to proteins than are monovalent anions [5]. The counteraction of the inhibitory effect of polyvalent anions on k_1 by chloride and other monovalent anions, could be due to a displacement of the polyvalent anions from their binding sites, just as FMN itself is displaced from the protein by for example chloride ions.

In regard to the fact that polyvalent anions are more strongly attached to the protein than monovalent anions, it must be remembered that FMN itself is a divalent anion at pH-values from 6 to 10. It combines rapidly with the protein in this pH-region. The combination velocity decreases with pH down to practically zero around pH 4, where FMN is a monovalent anion. The direct titration of FMN gave a pK' of 6.2 for the second dissociation constant of the phosphoric acid group. The combination velocity constant decreased along a dissociation curve with pK' = 6.0 (at 23.5°), that is, very close to the dissociation curve of the phosphoric acid group. This indicates that the association velocity is essentially proportional to the fraction of FMN which carries a double negative charge. The fact that 0.4 *M* NaCl displaced both curves by 0.2—0.3 pH units (see Figs. 2 and 3), supports the same conclusion. The 0.2 pH units difference in the titration curve and the velocity curve, if significant, remains to be explained.

Riboflavin, which has no phosphoric acid residue, shows a gradual rise in k_1 from pH 4 to 8. In contrast to FMN, riboflavin gives a k_1 curve that is far too flat to be interpreted as a simple dissociation curve.

Considering the fact that only FMN with two negative charges combines rapidly with the protein, it is tempting to suggest that the phosphoric acid residue might be bound to *two* positively charged groups on the protein. This binding would require the two positive groups to be close to one another in the protein molecule, for instance at the same side of a "helix" in two consecutive turns. Such a coincidence would be rare enough to account at least partly for the specific binding of only one molecule of FMN to the large protein molecule and for the high affinity of FMN for the apoprotein. In addition, there must be required a binding site for the imino group of the isoalloxazine, or a hydroxyl group of a corresponding enol form, at a suitable distance from the binding sites for the phosphoric acid.

The fact that k_1 drops sharply at pH-values above 9.3 could be caused by a discharge of the coupling positive groups of the protein. Terminal amino groups and lysine ε-amino groups have their dissociation constants located in this pH region. In the presence of chloride this descending branch of the k_1-

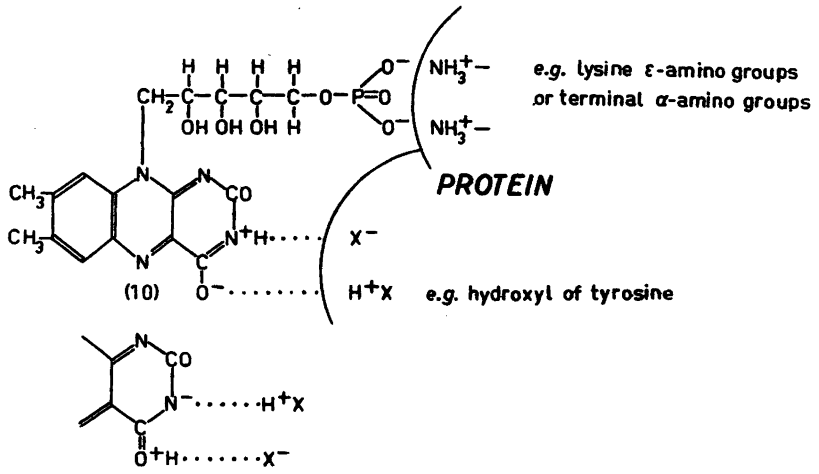
curve on the alkaline side is displaced towards lower pH-values, which might be expected from the fact that salts are known to displace pK'-values towards the isoionic point of the protein [6]. The fact that the pK' = 10.2 of FMN is not influenced by 0.4 M NaCl strongly indicates that the decrease of k_1 above pH 9.1 is not caused by the dissociation of the NH (3) group of FMN. Lowering the temperature from 23.5° to 13.5° C displaces the alkaline part of the k_1-curve towards higher pH-values, by 0.2—0.3 pH units per 10°, see Fig. 2. Primary amino groups are displaced in the same direction by 0.3 pH units per 10°. Thus we have reasons to believe that the phosphoric acid residue in FMN is coupled to one, or more probably, two primary amino groups in the protein.

The increase of k_1 from pH 8.5 to 9 now remains to be explained. In strongly alkaline solution FMN acquires one extra negative charge through the dissociation of the imino group (3) with pK = 10.2. This means that 2 % of the FMN molecules at pH 8.5, 9 % at pH 9.2 are trivalent. If we assume that the association velocity is many times higher for the trivalent FMN molecules than for the divalent ones, this would result in an increase in association velocity in this pH-region. At still higher pH the discharge of the positive protein groups must prevent the association reaction. In the presence of sodium chloride the maximum disappears because the pK':s of the protein amino groups are considerably lowered, whereas the pK' of the isoalloxazine imino group is not. Further experimental investigation must determine whether this tentative explanation is satisfactory or not.

It is interesting to note that Drs. O. and E. Walaas [7] in their measurements of the D-amino acid oxidase activity found a maximum at pH 9.2, that disappeared on the addition of sodium chloride, similar to the curves obtained for k_1 in the FMN-protein system. The maximum of association velocity between protein and flavin nucleotides near pH 9 thus could be a general phenomenon for flavoproteins.

Our data show that riboflavin, as determined by the quenching effect of the protein on the fluorescence, is bound much less firmly than FMN. In the pH range where there is no acid dissociation of the isoalloxazine nucleus (below pH 8), a slow rise of k_1 for riboflavin + protein occurs with increasing pH. The reason for this is not yet clear. The fact that even high concentrations of polyvalent anions did not affect k_1 suggests that cationic group of the protein may not be involved in the binding of riboflavin. The rapid rise in k_1 above pH 9 can perhaps, as for FMN, be understood in terms of increased concentration of the anionic form of isoalloxazine. The decrease above pH 10 could be due to the formation of a negative group on the protein, *e.g.* the dissociation of a tyrosine hydroxyl group. Weber [8] has suggested that the fluorescence might be quenched by tyrosine hydroxyl group. With both riboflavin and the binding site on the protein negatively charged, they would repel each other.

Between pH 4.3 and 9, the linkage between isoalloxazine and the protein could involve hydrogen bonding. As suggested in the formulae below, there are several possibilities for the formation of hydrogen bonds. Geissman [9] has suggested that a hydrogen bond from the protein to nitrogen atom 10 might exist in the leuco form of the isoalloxazine. This extra bond could explain why the reduced complex must be still less dissociated than the oxidized,



as suggested from the oxidation-reduction potential which is higher for O.Y.E. than for riboflavin.

Since phosphate decreases the association velocity, whereas chloride increases the dissociation velocity, it would be possible to prepare two solutions of O.Y.E., one in phosphate, the other in chloride, with the same dissociation constant for the FMN-protein. However, in phosphate with low values for both k_1 and k_2, the equilibrium state would be reached much more slowly than in chloride, where both k_1 and k_2 are high. This difference could be of great importance in such coenzyme-enzyme systems in which the coenzyme is "mobile", thus oxidized when bound to one protein, reduced when bound to another. Under such conditions the anions present in the solution will have influence on the overall reaction velocity. Furthermore, when the anionic influence on the oxidized and reduced complexes are not of equal magnitude, the anions will change the oxidation-reduction potential [10]. Examples of such effects will be given in forthcoming papers.

Finally it might be worth while to point out the interesting anion effects to be expected at the cell membranes, because chloride is often present outside, but not inside the cells. All factors, may it be hormones or others, which change the structure and permeability of the cell membranes will thus be able to influence enzyme reactions through anion effects.

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