On the Chemical Nature of the FMN-binding Groups in the Old Yellow Enzyme

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The observed effects of pH, anions and temperature on the kinetics of the old yellow enzyme system have suggested that the phosphoric acid ester group of FMN is attached to primary amino groups of the protein [1]. It was therefore of interest to study the effect of formaldehyde on this system. Although a great variety of reactions may take place between proteins and formaldehyde, the addition product formed instantaneously at low temperature and low concentrations of the reagent is likely to involve amino groups only [2]. We have now found that low concentrations of formaldehyde instantaneously affect both the dissociation (k_2, sec^{-1}) and reassociation (k_1, M^{-1} sec^{-1}) of the old yellow enzyme. Formaldehyde in water did not dissociate the enzyme. However, in the presence of sodium chloride it increased the rate of dissociation without previous incubation. For example, 0.12 *M* formaldehyde roughly doubled the dissociating effect (k_2) of 0.4 *M* sodium chloride above pH 7. In the neutral and acidic range, 0.12 *M* formaldehyde had no dissociating effect. The apoenzyme was affected by still lower concentrations of formaldehyde. The apoenzyme was added to solutions of FMN and formaldehyde, and k_1 determined at $t \simeq 0$. 0.02 *M* formaldehyde was found to decrease the association velocity constant (k_1) 21 % at pH 8.0, 50 % at pH 8.6, 87 % at pH 10. In the neutral and acidic range stronger formaldehyde (0.12 *M*) decreased k_1 about 25 %. Formaldehyde also decreased the FMN combining capacity of the protein in the alkaline range, but not in the neutral or acidic range. Also acetic anhydride, added to an aqueous solution of apoenzyme, and phenyl*iso*cyanate were found to inhibit k_1 and to decrease the FMN combining capacity.

The association reaction between riboflavin and apoenzyme was found to be far less sensitive to formaldehyde than was the reaction between FMN and apoenzyme. Thus, with our experimental procedure even 0.25 *M* formaldehyde at pH 10 did not inhibit this reaction.

These observations give additional evidence to the previous indications that primary amino groups are essential for the attachment of the phosphoric acid ester group of FMN to the old yellow apoenzyme.

Weber [3] has suggested that aromatic groups in the protein (tyrosine) are the more likely to act as fluorescence quenchers for FMN or FAD. Some of our kinetic data for the riboflavin–apoenzyme system could be interpreted on this basis [1]. The "old yellow" apoenzyme gives a negative nitroprusside test [4], and –SH inhibitors like $Cu^{\cdot\cdot}$, $Fe^{\cdot\cdot\cdot}$, *p*-chloromercuribenzoate or H_2O_2 do not inhibit the reaction between FMN and protein. Since unmasked –SH groups therefore are probably not present in the apoenzyme, iodine could be expected to react only with tyrosyl groups [5]. It was found that incubation of 0.3×10^{-6} *M* apoenzyme with 20×10^{-6} *M* iodine for 2 minutes at 25° and pH 7.4 completely destroyed the ability of the apoenzyme to combine both with FMN and riboflavin. The tyrosine content of the protein, measured by the Millon-Lugg test, decreased by an amount corresponding roughly to the iodine introduced into the protein. The coupling of the apoenzyme with azobenzene sulfonic acid likewise was found to decrease k_1 and the FMN combining capacity.

These observations suggest that a tyrosine residue of the apoenzyme is involved in the quenching of the fluorescence of FMN; perhaps by forming a hydrogen bond with the imino group (3) of the *iso*alloxazine nucleus [1].

The results briefly presented here are another example of the usefulness of kinetic measurements in revealing the nature of the chemical bonds between coenzymes and apoproteins [1].

A detailed report on this work will be presented in this journal.

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Received September 17, 1954.

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