Kinetic Studies on Coenzyme Binding and Coenzyme Dissociation in Tryptophanase Immobilized on Sepharose†

Sei-ichiro Ikeda, Yutaka Sumi, and Saburo Fukui*

ABSTRACT: The binding rate of pyridoxal 5′-phosphate (Pxa-P) to apotryptophanase and the dissociation rate of the coenzyme from holotryptophanase were able to be determined by following the enzyme activity in continuous flow reactions on a column of immobilized tryptophanase. When the enzyme activity was assayed continuously in the flow system in the absence of coenzyme added to the reaction mixture, immobilized holotryptophanase lost gradually its initial activity owing to dissociation of coenzyme. The coenzyme dissociation at a given concentration of substrate (tryptophan) followed first-order kinetics. In a low substrate concentration range below the K_m value, a more decreased rate constant was obtained for the coenzyme dissociation. This indicates that the coenzyme is more dissociable from the apoenzyme–coenzyme–substrate complex (ECS complex) rather than from the apoenzyme–coenzyme complex (holoenzyme). Immobilized tryptophanase freed of coenzyme restored rapidly its original activity, when the assay mixture containing a given concentration of substrate and Pxa-P was passed through the immobilized enzyme column. The coenzyme binding at a given coenzyme concentration followed first-order kinetics, but the rate was not first order in regard to the coenzyme concentration. A plot of the reciprocal of the first-order rate constant obtained vs. the reciprocal of the coenzyme concentration was linear. This implies that the coenzyme binding occurs in a two-step fashion; the first step is rapid and the second step is rate determining. Both the dissociation constant for the first step and the rate constant for the second step were shown to be independent of the substrate concentration. This means that Schiff base formation between Pxa-P and tryptophan in the assay mixture has no effect on the binding of Pxa-P to apoenzyme. The coenzyme dissociation constant at a given substrate concentration was calculated from both the rate constant of the coenzyme binding and the rate constant of the coenzyme dissociation. The values obtained by this method at different substrate concentrations were almost identical with those measured at the corresponding substrate concentrations directly by an ordinary method.

Immobilization of enzymes has been shown to provide new approaches for studies on the structures and functions of enzymes. The microenvironmental effects on the function of enzyme of the localized concentration of enzyme-generated product(s) or proton(s) were investigated by using an immobilized enzyme or an immobilized multienzyme system (Katchalski et al., 1971; Srere et al., 1973; Gestrelius et al., 1973). In the cases of some immobilized enzymes, it has been shown that the charge on a carrier itself may change their pH-activity profiles (Katchalski et al., 1971). These studies would be available as model systems for enzymes located in vivo in organella or on membranes, or for enzymes which constitute a multienzyme system. Furthermore, catalytic properties of the subunits of some enzymes were conveniently investigated by immobilizing them covalently on a rigid matrix and thus preventing them from reassociation (Chan, 1970; Feldmann et al., 1972; Ikeda and Fukui, 1974).

In the course of our studies on an immobilized preparation of tryptophanase, it has been found that the immobilized holotryptophanase loses gradually its original activity when used repeatedly in a batch system or continuously in a flow system in the absence of added coenzyme in the reaction mixture and that supplement of coenzyme to the reaction mixture restores its initial activity quickly (Fukui et al., 1975a). These phenomena seem to be closely related to dissociation of coenzyme from immobilized holoenzyme and rebinding of coenzyme to immobilized apoenzyme. According to the proposed mechanism of tryptophanase reaction (Morino and Snell, 1967a), the coenzyme moiety pyridoxal 5′-phosphate (Pxa-P)[1] recycles in the bound state during enzymatic reaction. However, the affinity of apotryptophanase for Pxa-P is fairly low (2×10^{-6} *M*, Newton et al., 1965; 1.6×10^{-6} *M*, Groman et al., 1972; 0.71×10^{-6} *M*, Morino and Snell, 1967c) and it has been observed that coenzyme dissociates partially from holoenzyme by extensive dialysis (Newton et al., 1965). These results and our finding in the immobilized tryptophanase reaction strongly suggest that the coenzyme moiety of holotryptophanase dissociates during enzymatic reaction in the absence of added coenzyme, although the rate of coenzyme dissociation is considered to be much slower than that of enzyme reaction itself. In spite of extensive studies on the structure and kinetic properties of tryptophanase carried out by Morino and Snell (1967a–c), little is known about the rate of coenzyme dissociation and coenzyme binding. The present paper describes the detailed kinetic studies on immobilized tryptophanase, proposing a new and convenient method for determination of the rate constant of coenzyme dissociation as well as the rate constant of coenzyme binding by using the flow reaction with the immobilized enzyme.

Experimental Section

Chemicals. Pyridoxal 5′-phosphate (Pxa-P) was a generous gift from Yamanouchi Pharmaceutical Co., Tokyo. Determination of Pxa-P concentrations was based on the

† From the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Sakyo-Ku, Kyoto, Japan. *Received September 17, 1974.*

[1] Abbreviation used is: Pxa-P, pyridoxal 5′-phosphate.

molar extinction coefficient at 388 nm at pH 7.0, $4.90 \times 10^3\ M^{-1}\ cm^{-1}$ (Peterson and Sober, 1954). All other chemical reagents were obtained from commercial sources and were used without purification. A molar extinction coefficient, $5.25 \times 10^3\ M^{-1}\ cm^{-1}$ at 280.5 nm (in 0.1 *N* NaOH), was used for the estimation of tryptophan.

Enzyme Preparation. Tryptophanase was prepared according to the method of Newton et al. (1965) from *Escherichia coli* B/1t 7-A. This strain was kindly donated by Dr. H. Wada and Dr. Y. Morino, Osaka University School of Medicine. The specific activity of the enzyme used was about 6.0 enzyme units/mg of protein (37°). Resolution of coenzyme from holotryptophanase was carried out principally under the procedure described by Morino and Snell (1967b) as follows. To a soluble tryptophanase solution (ca. 1.0 mg/ml of 0.1 *M* potassium phosphate buffer, pH 7.0), DL-penicillamine (10 μmol/ml) was added and the mixture was incubated for 15 min at 37°. Then the enzyme solution was dialyzed for 12 hr twice against a 500-fold volume of 0.02 *M* potassium phosphate buffer (pH 7.0) containing 5 m*M* mercaptoethanol. In the case of immobilized tryptophanase, Sepharose gel was incubated with DL-penicillamine in a similar manner as described above and then washed with a 20-fold volume of 0.02 *M* potassium phosphate buffer (pH 7.0) containing 5 m*M* mercaptoethanol. The obtained soluble or immobilized apotryptophanase preparation, having no actual activity unless Pxa-P was added, was used for measurement of the apparent K_m value for Pxa-P or for measurement of the rate of coenzyme binding.

Immobilization of Tryptophanase. Among several covalent-binding procedures used for immobilization of tryptophanase, the coupling of apoenzyme to Sepharose-bound Pxa-P, followed by reduction with $NaBH_4$ to fix the aldimine linkage between Pxa-P and the ε-amino group of a lysine residue at one subunit, yields the most active immobilized enzyme preparation (Ikeda and Fukui, 1973; Fukui et al., 1975a,b). This immobilized tryptophanase preparation, however, seems unsuitable for this study because the active site of one subunit of tetrameric apotryptophanase is blocked with Sepharose-bound Pxa-P and becomes inactive by the following reduction treatment. On the contrary, the immobilized tryptophanase prepared by direct coupling to CNBr-activated Sepharose is considered to retain its four active sites as they are, although its activity is fairly low as compared to the above-mentioned preparation (Ikeda and Fukui, 1973; Fukui et al., 1975a,b). Hence, the latter immobilized enzyme preparation was used in this study. The immobilization of tryptophanase with CNBr-activated Sepharose was carried out in a similar way to that described by Axén and Ernback (1971). Sepharose 4B (10 ml) activated with CNBr (250 mg/ml of Sepharose) was mixed with 1.0 mg of apotryptophanase dissolved in 10 ml of 0.1 *M* potassium phosphate buffer (pH 7.0) containing 40 μ*M* Pxa-P. After the coupling reaction was allowed to proceed for 16 hr at 4°, Sepharose gel was thoroughly washed with 0.1 *M* potassium phosphate buffer at pH 9.0 and 5.5, alternatively. By this method, about 84% of the initial amount of protein used was immobilized on Sepharose (84 μg of protein/ml of Sepharose). The amount of protein immobilized on Sepharose was determined by the difference between the initial amount of enzyme used and that of free enzyme remaining in the supernatant after the immobilization process. Protein concentrations were determined by the method of Lowry et al. (1951).

Enzyme Assays. Soluble tryptophanase activity was assayed under the procedures mentioned by Newton and Snell (1964), with some modifications as follows. To a 20-ml test tube were added 0.10 ml of 400 μ*M* Pxa-P, 0.10 ml of 0.05 *M* mercaptoethanol, 0.10 ml of 1.0 *M* potassium phosphate buffer (pH 8.0), enzyme solution (1.0 μg of tryptophanase), and sufficient distilled water to give a final volume of 0.50 ml. This solution was layered with 5.0 ml of toluene and incubated for 10 min at 37°. The reaction was started by adding 0.5 ml of 12.5 m*M* tryptophan solution. The incubation was carried out with gentle shaking for 10 min at 37°. The reaction was stopped by the addition of 5 ml of a freshly mixed acid–Ehrlich reagent (5 vol of 5% *p*-dimethylaminobenzaldehyde in 95% ethanol plus 12 vol of 5% sulfuric acid in 1-butanol). After 20 min, the color intensity was measured with a Shimadzu spectrophotometer SP-88. The assay was linear up to about 0.15 μml of indole.

Activity measurement of immobilized tryptophanase by a batch method was carried out principally under the same conditions as described above. To a 10-ml glass funnel equipped with a sintered glass filter were added immobilized enzyme gel containing 3–4 μg of tryptophanase and the other components of the asay mixture described above. In this case, toluene was not used in order to prevent the aggregation of Sepharose gel. The assay mixture was preincubated with gentle shaking for 10 min at 37°. The reaction was started by addition of tryptophan and allowed to proceed with gentle shaking for 10 min at 37°. The reaction was stopped by separation of enzyme catalyst from other components of the reaction mixture by filtration. Indole formed in the filtrate was extracted with 5 ml of toluene and determined with 5 ml of acid–Ehrlich reagent. The assay was linear up to about 0.040 μmol of indole.

Activity Measurement of Immobilized Tryptophanase by a Flow Method. Immobilized tryptophanase (0.50 ml of Sepharose containing 42 μg of tryptophanase) was placed into a small column (4.0 mm diameter) and maintained at 37°. A sufficient amount of 0.1 *M* potassium phosphate buffer (pH 8.0) containing 40 μ*M* Pxa-P and 5 m*M* mercaptoethanol was kept in the column for 10 min. Then an assay mixture containing 6.25 m*M* tryptophan, 40 μ*M* Pxa-P, 5 m*M* mercaptoethanol, and 0.1 *M* potassium phosphate buffer (pH 8.0) preheated at 37° was passed upward through the column at a given constant flow rate. Fractions of eluate (each 0.5 ml) were collected and the amount of indole formed in each fraction was determined after extraction with 2.5 ml of toluene. After about 1 min, a steady state was attained. The time (Δt) of exposure of substrate to enzyme in the column changed with variation in the flow rate (v) with a microtube pump: Δt (minutes) = column volume (milliliters)/flow rate (milliliters/minute) = 0.50/v. A plot of the amount of indole in the effluent (0.50 ml) against Δt gives the rate of the enzymatic reaction. From the slope of the linear part, the activity of the immobilized enzyme was calculated (Figure 1A, curve 1). When the flow rate was decreased below v = 2.0 ml/min, the linear relationship was broken, probably due to product inhibition by the indole formed. Hence, the flow rate of v = 2.0 ml/min was employed hereafter in this study. When the substrate concentration was decreased to 0.08 m*M*, a similar linear relationship between the amount of product and Δt was observed at a flow rate above v = 2.0 ml/min (Figure 1A, curves 2–4). From the reciprocal plots of the slopes of these lines, the apparent K_m value between immobilized tryptophanase and tryptophan, 0.33 m*M*, was obtained

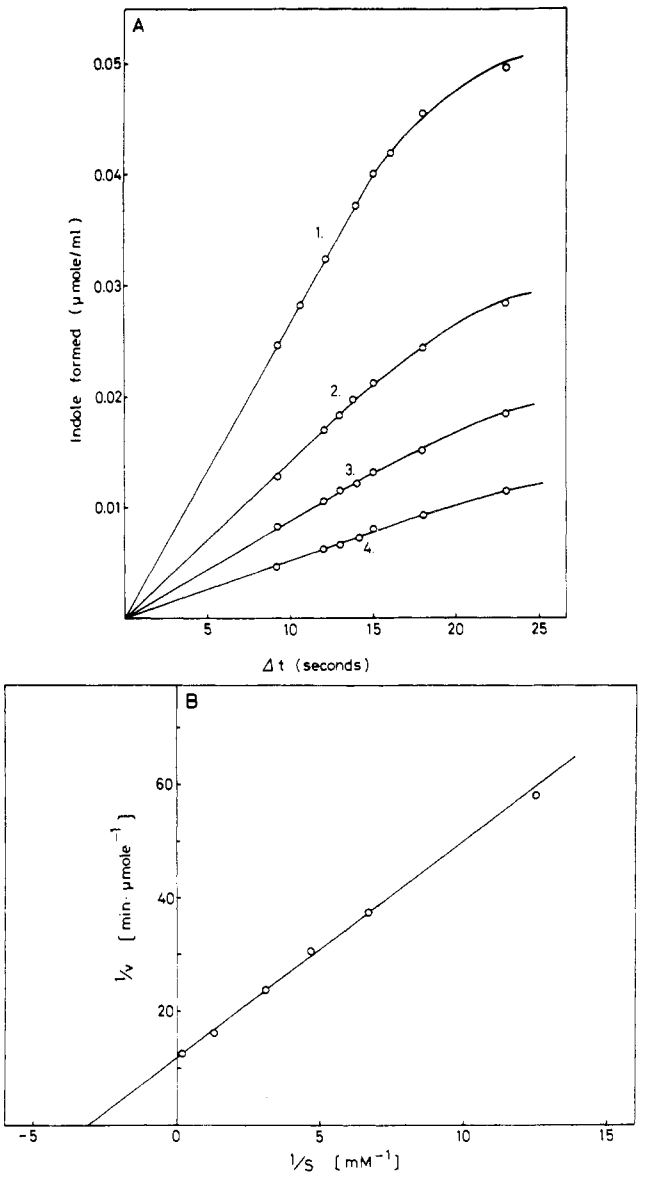


FIGURE 1: (A) Activity measurement of immobilized tryptophanase by a flow method at different substrate concentrations: curve 1, 6.25 mM Trp; curve 2, 0.33 mM Trp; curve 3, 0.15 mM Trp; curve 4, 0.08 mM Trp. (B) Apparent K_m of immobilized tryptophanase for Pxa-P. Activities were measured by the flow method described in (A) with a flow rate of v = 2.0 ml/min.

(Figure 1B).

Measurement of the Rate of Coenzyme Dissociation. By using the above-mentioned immobilized tryptophanase column used for the assay, the rate of coenzyme dissociation from immobilized holotryptophanase was measured as follows. After the enzyme in the column was reconstituted for 10 min with 40 μM Pxa-P, an assay mixture containing a given concentration of tryptophan (6.25–0.08 mM), 40 μM Pxa-P, 5 mM mercaptoethanol, and 0.1 M potassium phosphate buffer (pH 8.0) was passed through the column at the flow rate of v = 2.0 ml/min. Fractions of eluate (each 0.5 ml) were collected and the amount of indole formed in each fraction was determined. After the steady state was reached (A_0), 40 μM Pxa-P was omitted from the assay mixture and the activity (A_t) was determined continuously. From eq 1, the first-order rate constant of coenzyme dissociation at a given substrate concentration (k_{-2}) was calculated.

$$\ln A_t/A_0 = -k_{-2}t \qquad (1)$$

Measurement of the Rate of Coenzyme Binding. After the coenzyme was completely resolved from the column of immobilized tryptophanase as described above, an assay mixture containing a given concentration of tryptophan (0.08–6.25 mM), 5 mM mercaptoethanol, and 0.1 M potassium phosphate buffer (pH 8.0) was passed through the column at the flow rate of v = 2.0 ml/min. Fractions of eluate (each 0.5 ml) were collected and the amount of indole formed in each fraction was determined. After the steady state was reached (A_0, which was actually zero), a given concentration of Pxa-P (2–40 μM) was added to the above-mentioned assay mixture and the activity (A_t) was determined continuously. After reaction for 15–60 min, the time length depending on the concentration of Pxa-P added, the steady state was reached (A_∞). From eq 2, the apparent first-order rate constant (k) of coenzyme binding at a given concentration of Pxa-P and a given concentration of tryptophan was calculated.

$$\ln A_\infty/(A_\infty - A) = kt \qquad (2)$$

Measurement of Schiff Base Formation. Schiff base formation between Pxa-P and tryptophan in the assay mixture was estimated spectrophotometrically by the procedure described by Matsuo (1957), with some modifications. Pxa-P has two absorption maxima at neutral or alkaline pH, one at 330 nm and the other at 388 nm. In the presence of various amino compounds, a new maximum appears at 278 nm, and the 388-nm peak moves to a higher wavelength (400 to 415 nm). The Schiff base formation between Pxa-P and an amino compound, therefore, has been able to be followed spectrophotometrically at 278 nm (Matsuo, 1957). However, this procedure cannot be used directly in this study, due to the strong ultraviolet absorption of tryptophan. Hence, the Schiff base formation between Pxa-P and tryptophan was estimated by measuring an increase in the absorbance at 415 nm. At this wavelength, the Schiff base between Pxa-P and tryptophan showed a molar extinction coefficient (E_{415}) of approximately 5460 (at pH 8.0), while E_{415} of Pxa-P was 3000. To obtain this molar extinction coefficient of the Schiff base, 0.4 μmol of Pxa-P, 460 μmol of tryptophan, and 0.1 M potassium phosphate buffer (pH 8.0) in a total volume of 10.0 ml were allowed to stand for 20 min at room temperature to complete the maximal formation of Schiff base. The absorbance at 415 nm was measured using a Hitachi spectrophotometer 124. By using the value thus obtained E_{415} was calculated of the Schiff base between Pxa-P and tryptophan. Under the experimental conditions, the great excess of tryptophan over Pxa-P (1150:1) seemed to push the reaction toward completion. Mixtures of known concentrations of Pxa-P and tryptophan were then prepared and the optical densities of these solutions were determined. The concentration of the Schiff base in the test mixture was calculated by direct application of Beer's law. When 40 μM Pxa-P was mixed with 6.25, 0.15, and 0.08 mM tryptophan, the extents of the Schiff base formation between Pxa-P and tryptophan were ca. 75, 19, and 9% of the initial amount of Pxa-P, respectively.

Results

Kinetic Properties of Immobilized Tryptophanase. Upon immobilization, kinetic properties of enzymes have been shown to be fairly altered in some cases (Katchalski et al., 1971). The changes in the catalytic properties would result from a conformational alteration of enzyme protein, interaction between the enzyme and insoluble support, or limited

Table I: Comparison of the Kinetic Properties of Soluble and Immobilized Tryptophanase.[a]

Kinetic Properties	Soluble Tryptophanase	Immobilized Tryptophanase[b] Batch Method	Immobilized Tryptophanase[b] Flow Method
Rel sp act.[c] (%)	100	42	38
K_m for Pxa-P[d] (μM)	1.1	1.2	1.3
K_m for Trp[e] (mM)	0.33	0.34	0.33

[a] All assays were carried out in 0.1 M potassium phosphate buffer (pH 8.0) as described in detail in the Experimental Section. [b] Immobilized tryptophanase was assayed by two different methods, i.e., (a) by a batch assay method and (b) by a flow assay method. [c] Activity was measured with 6.25 mM Trp after the preincubation with 40 μM Pxa-P for 10 min and expressed as the relative value to the specific activity of soluble enzyme. [d] Activity was measured with 6.25 mM Trp after the preincubation with various concentrations of Pxa-P for 10 min. [e] Activity was measured with various concentrations of Trp after the preincubation with 40 μM Pxa-P for 10 min.

diffusion rates of reactants. To study the influence of immobilization upon the function of enzyme, kinetic properties of immobilized tryptophanase were examined by a batch assay method or by a flow assay method as compared with those of soluble tryptophanase (Table I).

As described in our previous papers (Ikeda and Fukui, 1973; Fukui et al., 1975a), the immobilized tryptophanase prepared with CNBr-activated Sepharose showed ca. 40% specific activity of soluble enzyme when assayed by a batch method (Table I). The same extent of activity of the immobilized tryptophanase was obtained also when assayed by a flow method. The K_m values of the immobilized tryptophanase for coenzyme and substrate, obtained by a batch assay method, were almost identical with those of soluble enzyme. Furthermore, the saturation curve of the immobilized enzyme for substrate or coenzyme was a normal hyperbolic one similar to that of soluble enzyme. These results are analogous to the case of another kind of immobilized tryptophanase prepared with Pxa-P bound Sepharose (Fukui et al., 1975a), suggesting that the steric environment around the active centers is not influenced by the immobilization to an appreciable extent.

The K_m values of the immobilized tryptophanase for coenzyme and substrate obtained by a flow assay method were almost similar to the above-mentioned values obtained by a batch assay method (Table I). In some immobilized enzymes (Tosa et al., 1971; Lilly et al., 1966), it has been observed that the apparent K_m value for substrate obtained by a flow assay method becomes lower with the increase of flow rate and approaches the value obtained by a batch assay method. This phenomenon has been explained by regarding each immobilized enzyme particle as being surrounded by a diffusion layer. The transfer of substrate would be inversely related to the thickness of the layer, which is inversely related to the flow rate of substrate solution. In our case of immobilized tryptophanase flow reaction, such a diffusion layer seems not to be present owing to the adequately high flow rate employed (v greater than 2.0 ml/min), because the K_m value for substrate in the flow system was almost similar to that in a batch system. These kinetic properties obtained on the flow reaction of immobilized tryptophanase seem to indicate that the phenomena observed in the flow system would be closely correlated to those of native soluble enzyme.

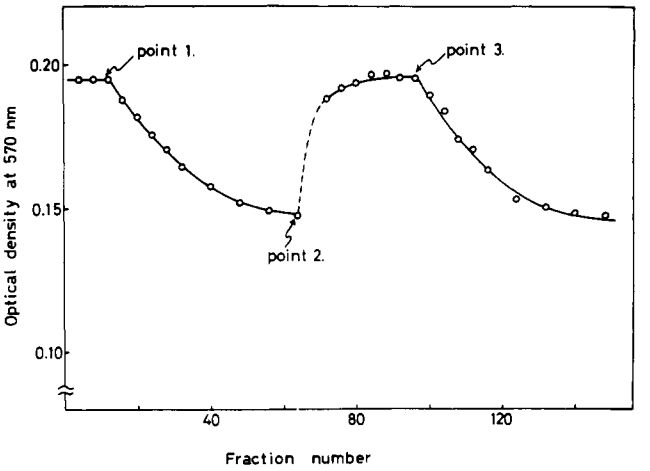


FIGURE 2: Coenzyme dissociation and coenzyme binding during the flow reaction of immobilized tryptophanase. After the immobilized tryptophanase column (see Experimental Section) was reconstituted with 40 μM Pxa-P for 10 min, the assay mixture containing 6.25 mM Trp, 40 μM Pxa-P. 5 mM mercaptoethanol, and 0.1 M potassium phosphate buffer (pH 8.0) was passed through the column at the flow rate of v = 2.0 ml/min and the eluate (each fraction 5 ml) was collected. Then, at point 1, Pxa-P was omitted from the assay mixture, at point 2 Pxa-P was again added, and at point 3 Pxa-P was omitted again. The amount of indole in each fraction is expressed as the optical density at 570 nm of the color developed with acid-Ehrlich reagent. Other conditions are mentioned in the Experimental Section.

Coenzyme Dissociation from Immobilized Holotryptophanase. The immobilized tryptophanase previously reconstituted with Pxa-P retained the constant level of activity when the flow reaction was run in the presence of Pxa-P (40 μM) in an assay mixture containing 6.25 mM tryptophan (Figure 2). Omission of Pxa-P from the assay mixture, however, resulted in a gradual decrease in the activity. Supplementing the assay mixture system with Pxa-P rapidly restored its initial activity. These phenomena are closely related to those observed in the repeated batch reactions of immobilized tryptophanase (Fukui et al., 1975a). In general, the extent of functional binding of coenzyme to apoenzyme, or the extent of coenzyme dissociation, can be measured by assaying the enzyme activity in the presence and absence of added coenzyme (Litwack and Cleland, 1968). Hence, the decrease in activity occurring in the flow reaction in the absence of added Pxa-P is considered to be caused by the dissociation of coenzyme from the immobilized holotryptophanase. In the continuous flow reaction system, the coenzyme dissociated should be quickly removed from the vicinity of the enzyme molecule bound to insoluble carrier. Accordingly, a highly exact value would be obtained regarding the rate of coenzyme dissociation. As shown in Figure 3, curve 1, this decrease in activity obeyed good first-order kinetics. The rate constant of coenzyme dissociation, k_{-2}, was calculated from the slope of the line (Table II). When the substrate concentration in the flow reaction mixture was decreased from 6.25 mM to that below the K_m value (0.33 mM), the rate constants of coenzyme dissociation were fairly lower than the case with 6.25 mM tryptophan (Figure 3, curves 2 and 3; Table II). In general, enzyme is considered to be present mainly as an apoenzyme–coenzyme–substrate-complex (ECS complex) when the concentration of substrate is significantly higher than the K_m value, assuming that the step in which products are formed from the ECS complex is rate determining in a total enzymatic reaction. On the contrary, enzyme is assumed to be present mainly as

Table II: Effect of Substrate Concentrations on the Kinetic Parameters of Immobilized Tryptophanase.[a]

Trp (mM)	K_1 (M)[b]	k_2 (sec^{-1})[b]	k_{-2} (sec^{-1})[b]	K_m for Pxa-P (M)	
				Calcd[c]	Obsd[d]
6.25	4.5×10^{-6}	0.84×10^{-2}	5.8×10^{-4}	0.31×10^{-6}	1.3×10^{-6}
0.15	4.5×10^{-6}	0.84×10^{-2}	3.7×10^{-4}	0.20×10^{-6}	0.81×10^{-6}
0.08	4.5×10^{-6}	0.84×10^{-2}	3.1×10^{-4}	0.17×10^{-6}	0.43×10^{-6}

[a] All assays were carried out by the flow method described in the text. [b] K_1 represents the dissociation constant for the first binding step, and k_2 and k_{-2} represent the rate constants of the forward and reverse reactions for the second binding (conformational change step), respectively (see eq 3). [c] Coenzyme dissociation constant (K_m for Pxa-P) calculated from eq 4. [d] Concentration of coenzyme for the half-maximal activation of the apoenzyme. Assays were carried out with three different concentrations of substrate after the preincubation with various concentrations of coenzyme for 10 min. When the preincubation time was elongated to 60 min, the K_m value for Pxa-P became close to the calculated one, e.g., 0.40×10^{-6} at 6.25 mM Trp.

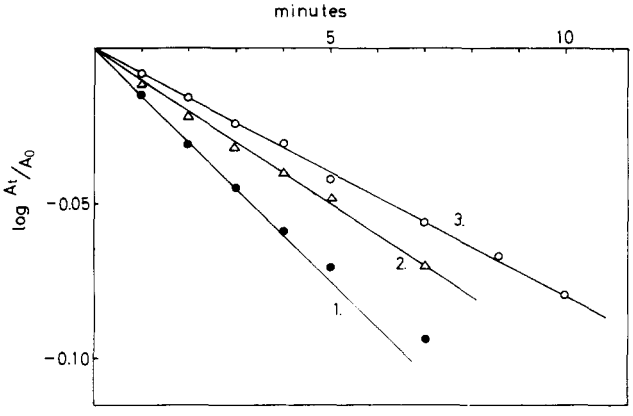


FIGURE 3: Coenzyme dissociation from immobilized holotryptophanase during the flow reactions at three different substrate concentrations. The tryptophan concentrations in the respective assay mixtures were: curve 1, 6.25 mM; curve 2, 0.15 mM; curve 3, 0.08 mM. Other experimental conditions are described in the Experimental Section.

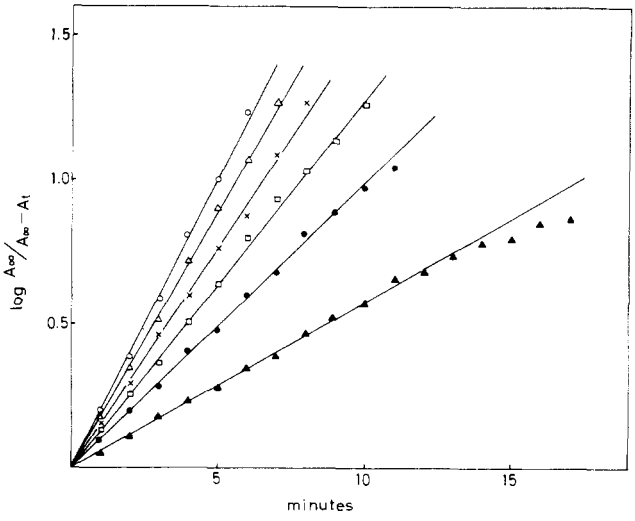


FIGURE 4: Coenzyme binding to immobilized apotryptophanase during the flow reactions at different coenzyme concentrations. The Pxa-P concentration in an assay mixture was: (○) 40 μM; (△) 20 μM; (×) 10 μM; (□) 6.4 μM; (●) 4 μM; (▲) 2 μM. The tryptophan concentration in the assay mixture was 6.25 mM in all cases. Other conditions are described in the Experimental Section.

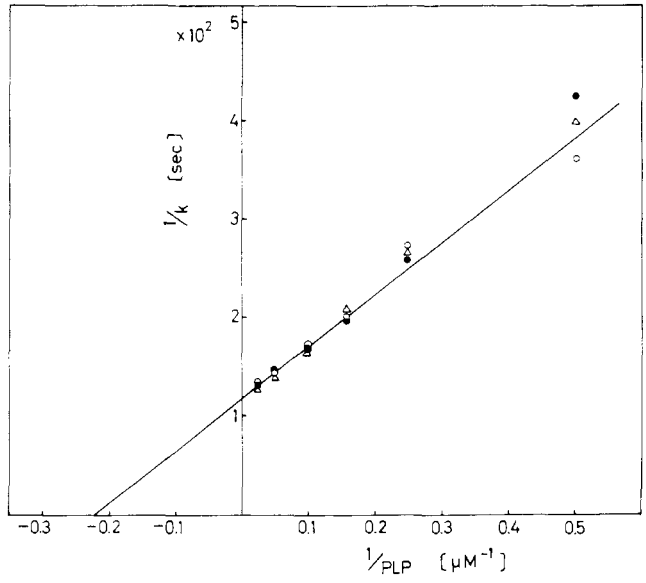


FIGURE 5: Double reciprocal plots of first-order rate constants for binding of various concentrations of Pxa-P to immobilized apotryptophanase. The tryptophan concentration in an assay mixture was: (●) 6.25 mM; (△) 0.15 mM; (○) 0.08 mM. Other conditions are given in the Experimental Section.

holoenzyme (EC complex) when the substrate concentration is somewhat lower than the K_m value. The results on the rate of coenzyme dissociation from immobilized holotryptophanase obtained by the flow reaction with different concentrations of substrate indicate that the coenzyme is more dissociable from the ECS complex rather than from the EC complex.

Coenzyme Binding to Immobilized Apotryptophanase. As shown in Figure 2, the immobilized tryptophanase, partially liberating the coenzyme, rapidly restored its full activity when the coenzyme was supplied to the assay mixture. To estimate the rate of coenzyme binding to apoenzyme, the appearance of tryptophanase activity in the flow system was followed when the reaction mixture containing Pxa-P was passed through the immobilized apotryptophanase column. With 6.25 mM tryptophan and a given concentration of Pxa-P (2–40 μM), the coenzyme binding process followed the good first-order kinetics (Figure 4). A plot of the reciprocal of the observed rate constant (k) for Pxa-P binding vs. the reciprocal of the Pxa-P concentration gave a straight line with a finite y intercept (Figure 5). Such a linear plot indicates that the binding does not occur in one step but occurs in a two-step fashion in a similar way to the case of glutamate decarboxylase (O'Leary and Malik, 1972), in which the first step is rapid and the second step is rate determining. The kinetic parameters for this mechanism, i.e., the dissociation constant for the first rapid step (K_1) and the rate constant for the second slow binding step (k_2), were calculated from the x intercept and the slope of the

plot (Table II). These two kinetic parameters were shown not to be changed with the tryptophan concentration lower than the K_m value (Figure 5). The Schiff base of Pxa-P and a certain amino acid are formed very rapidly in the absence of enzyme or metal (Matsuo, 1957). As described in the Experimental Section, it has been observed that different extents of Schiff base formation (75–9% of the initial amount of Pxa-P) were observed in the assay mixture containing different concentrations of tryptophan (6.25–0.08 mM) and a constant level of Pxa-P (40 μM). It is assumed that the Schiff base formation between Pxa-P and tryptophan in the assay mixture has no apparent effect on the kinetics of coenzyme binding to apoenzyme, as judged from the fact that kinetic parameters for coenzyme binding are independent of the substrate concentration.

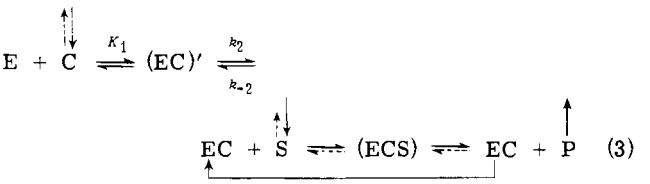
Discussion

It has been elucidated that holotryptophanase is composed of four identical subunits and the apoenzyme exists also as a tetramer (mol wt 220,000) at room temperature under ordinary conditions (Morino and Snell, 1967b). Binding of 4 mol of Pxa-P per 2.2×10^5 g of protein has been shown by equilibrium dialysis (Newton et al., 1965), spectrophotometric titration (Newton et al., 1965), and ultracentrifugal titration (Morino and Snell, 1967b). The concentration of the coenzyme necessary for the half-maximum activation of the apoenzyme (K_m for Pxa-P) was shown to be 2×10^{-6} M (Newton et al., 1965), 1.6×10^{-6} M (Groman et al., 1972), or 0.71×10^{-6} M (Morino and Snell, 1967c), being somewhat different depending on the conditions of reconstitution. However, little is known about the rates of coenzyme dissociation and coenzyme binding.

The strength of binding of coenzyme to apoenzyme has been investigated in several other Pxa-P dependent enzymes by using gel filtration, dilution, dialysis, or activated charcoal treatment (Litwack and Cleland, 1968; Litwack and Rosenfield, 1973). The results show that the coenzyme of aspartate aminotransferase or alanine aminotransferase is almost nondissociable and that the coenzyme of tyrosine aminotransferase or ornithine aminotransferase is more dissociable. In the meantime, the kinetics of binding of Pxa-P to apoaspartate aminotransferase (Churchich and Farrelly, 1968; Arrio-Dupont, 1969) or to apoglutamate decarboxylase (O'Leary and Malik, 1972) has been studied by absorption spectroscopy, fluorescence, circular dichroism, or enzyme activity.

In this paper, a new approach is presented that the rate of coenzyme binding to apotryptophanase as well as the rate of coenzyme dissociation from the holoenzyme can be conveniently determined by using the flow reaction of immobilized tryptophanase. When the enzyme activity was assayed continuously in the flow system in the absence of coenzyme added to the reaction mixture, immobilized holotryptophanase gradually lost its initial activity (see Figures 2 and 3). On the other hand, immobilized tryptophanase freed of coenzyme rapidly restored its original activity, when the assay mixture containing a given concentration of substrate and Pxa-P was passed through the immobilized enzyme column (see Figures 2 and 4). These phenomena are considered to be directly related to the processes of coenzyme dissociation and coenzyme binding, if the next two assumptions are applicable: (1) the rate of diffusion of coenzyme, substrate, or product from the vicinity of immobilized enzyme is much higher compared to the rate of coenzyme dissociation or coenzyme binding; (2) the rate of coenzyme dissociation or coenzyme binding is much slower than the rate of enzyme reaction itself. The first assumption is supported by the fact that the K_m value of immobilized tryptophanase for coenzyme or substrate measured by the flow reaction system was almost identical with that obtained by the batch system, both of them being similar to that observed in the soluble enzyme system (see Table I). If a diffusion layer would be present around the immobilized enzyme particle and the rate of diffusion of coenzyme or substrate would be appreciably slower, a larger K_m value would be obtained in the flow system. When the reaction mixture containing adequate concentrations of substrate and coenzyme was allowed to flow onto the column of immobilized holotryptophanase, the maximum activity was reached within 15 sec. This result indicates that the rate of diffusion of substrate is very rapid, supporting the assumption mentioned above, provided that the rate of diffusion of coenzyme or product would be as high as the case of substrate. The second assumption would be reasonably supported by the fact that the binding of Pxa-P to apotryptophanase to yield active holotryptophanase species (EC) requires several minutes (see Figure 4). As shown in eq 3, the resulting EC seems to recycle rapidly according to the generally recognized mechanism of Pxa-P dependent enzymatic reactions (Morino and Snell, 1967a).

The data presented above show that the coenzyme binding process to immobilized apotryptophanase consists of two steps, in which the first step is rapid and the second step is rate determining. It can be assumed that the first step is the binding of Pxa-P to the catalytic sites of the enzyme and the second one is concerned with a conformational change of the enzyme protein. The catalytically active conformation of the holoenzyme would be achieved in the latter step.



(3)

In eq 3, E and C represent apotryptophanase (immobilized) and Pxa-P, respectively. (EC)′ and EC represent the inactive intermediate complex of coenzyme and apoenzyme and the active holoenzyme, respectively. S is the substrate and P is the product. The dissociation constant for the first rapid step (K_1) and the rate constant for the second slow step (probably the conformational change step) (k_2) were obtained by measuring the rates of binding of various concentrations of Pxa-P to immobilized apotryptophanase (see Figures 4 and 5). Similar two-step mechanisms have been observed also on the coenzyme binding processes to apoaspartate aminotransferase (Churchich and Farrelly, 1968) and apoglutamate decarboxylase (O'Leary and Malik, 1972). In these cases, the results have been deduced from spectroscopic or chemical studies. In our case, on the contrary, the coenzyme binding process was able to be followed directly by measuring the enzyme activity in the flow reactions.

Although the presence of substrate is unavoidable in our measurement procedure of the coenzyme binding rate, the data presented above indicated that the presence of substrate in the assay mixture, even though the Schiff base was formed between the substrate and Pxa-P, had no appreciable effect on the coenzyme binding rate. This phenomenon could be explained by one of the following three reasons. (1)

The Schiff base formed between Pxa-P and tryptophan in the reaction mixture can serve as the substrate and can bind directly to the apoenzyme. (2) The Schiff base can react with the apoenzyme to form the holoenzyme through transaldimination as fast as free Pxa-P can. (3) As the apoenzyme reacts with a small amount of free Pxa-P in an equilibrium state with the Schiff base formed between Pxa-P and the substrate in the assay system, the equilibrium is shifted to liberate Pxa-P. A similar phenomenon has been observed in tyrosine aminotransferase reaction (Litwack and Cleland, 1968). In this case, the appearance of the enzyme activity was not influenced under the conditions where all of the Pxa-P added to the reaction mixture was present as the Schiff base with tyrosine.

On the other hand, it has been shown that the rate of coenzyme dissociation from immobilized holotryptophanase is dependent on the substrate concentration in the flow assay mixture, i.e., the coenzyme is more readily dissociated from the holoenzyme in the presence of the substrate rather than in the absence of the substrate (see Figure 3). In the cases of almost all the Pxa-P dependent enzymes, the binding process of Pxa-P to apoenzyme involves many factors. Formation of the Schiff base between the aldehyde group of Pxa-P and the ϵ-amino group of the lysine residue at the active center of the enzyme is considered to be the most important and critical of these factors. In the presence of substrate, this covalent binding of the internal Schiff base is severed by transaldimination to form an apoenzyme–coenzyme–substrate complex. This may contribute to the abovementioned phenomenon that the coenzyme was more dissociable from the ECS complex of immobilized tryptophanase than from the EC complex.

In eq 3, the rate constant of the reverse reaction of the first step is presumed to be so large that the observed rate constant of coenzyme dissociation represents the rate constant of the reverse reaction of the second step (k_{-2}). From the kinetic parameters (K_1, k_2, and k_{-2}) obtained independently, the dissociation constant of holotryptophanase was calculated using eq 4.

$$K_m \text{ for Pxa-P} = K_1(k_{-2}/k_2) \qquad (4)$$

This calculated dissociation constant was shown to be dependent on the substrate concentration used for the measurements of the coenzyme binding rate and coenzyme dissociation rate, obviously because of the substrate dependency of k_{-2} (see Table II). In agreement with these results, the apparent K_m for Pxa-P measured directly was similarly dependent on the concentration of substrate employed (the last column of Table II). The difference between the calculated and measured K_m values for Pxa-P may be caused by the fact that the time for reconstitution of holoenzyme (10 min) employed for the direct measurement of K_m for Pxa-P was too short to complete the whole coenzyme binding process, especially at a low concentration region of coenzyme.

For the measurement of the coenzyme binding rate, at least 30–60 min incubation was necessary to complete the coenzyme binding process in the low Pxa-P concentration region employed (see Figure 4). Actually, when the longer reconstitution time (60 min) was employed, a fairly low K_m value for Pxa-P close to the calculated one was obtained (see the footnotes to Table II). Consequently, the calculated dissociation constants became analogous to the measured ones. This indicates that our new method presented in this paper—that is, application of flow reaction with an immobilized enzyme column—is fairly reliable for measurements of the coenzyme binding rate as well as the coenzyme dissociation rate.

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References

Arrio-Dupont, M. (1969), *Biochem. Biophys. Res. Commun. 36,* 306–311.

Axén, R., and Ernback, S. (1971), *Eur. J. Biochem. 18,* 351–360.

Chan, W. W.-C. (1970), *Biochem. Biophys. Res. Commun. 41,* 1198–1204.

Churchich, J. E., and Farrelly, J. G. (1968), *Biochem. Biophys. Res. Commun. 31,* 316–321.

Feldmann, K., Zeisel, H., and Helmreich, E. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2278–2282.

Fukui, S., Ikeda, S., Fujimura, M., Yamada, H., and Kumagai, H. (1975a), *Eur. J. Appl. Microbiol. 1,* 25–39.

Fukui, S., Ikeda, S., Hara, H., Yamada, H., and Kumagai, H. (1975b), *Eur. J. Biochem.* (in press).

Gestrelius, S., Mattiasson, B., and Mosbach, K. (1973), *Eur. J. Biochem. 36,* 89–96.

Groman, E., Huang, Y. Z., Watanabe, T., and Snell, E. E. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3297–3300.

Ikeda, S., and Fukui, S. (1973), *Biochem. Biophys. Res. Commun. 52,* 482–488.

Ikeda, S., and Fukui, S. (1974), *Eur. J. Biochem. 46,* 553–558.

Katchalski, E., Silman, I., and Goldman, R. (1971), *Adv. Enzymol. 34,* 445–536.

Lilly, M. D., Hornby, W. E., and Grook, E. M. (1966), *Biochem. J. 100,* 718–723.

Litwack, G., and Cleland, W. W. (1968), *Biochemistry 7,* 2072–2079.

Litwack, G., and Rosenfield, S. (1973), *Biochem. Biophys. Res. Commun. 52,* 181–188.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Matsuo, Y. (1957), *J. Am. Chem. Soc. 79,* 2011–2015.

Morino, Y., and Snell, E. E. (1967a), *J. Biol. Chem. 242,* 2793–2799; 2800–2809.

Morino, Y., and Snell, E. E. (1967b), *J. Biol. Chem. 242,* 5591–5601; 5602–5610.

Morino, Y., and Snell, E. E. (1967c), *Proc. Natl. Acad. Sci. U.S.A. 57,* 1692–1699.

Newton, W. A., and Snell, E. E. (1964), *Proc. Natl. Acad. Sci. U.S.A. 51,* 382–389.

Newton, A. W., Morino, Y., and Snell, E. E. (1965), *J. Biol. Chem. 240,* 1211–1218.

O'Leary, M. H., and Malik, J. M. (1972), *J. Biol. Chem. 247,* 7097–7105.

Peterson, E. A., and Sober, H. A. (1954), *J. Am. Chem. Soc. 76,* 169–175.

Srere, P. A., Mattiason, B., and Mosbach, K. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2534–2538.

Tosa, T., Mori, T., and Chibata, I. (1971), *J. Ferment. Technol. 49,* 522–528.