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Pyridoxamine-Pyruvate Transaminase. 2. Temperature-Jump and Stopped-Flow Kinetic Investigation of the Rates and Mechanism of the Reaction of 5′-Deoxypyridoxal with the Enzyme†

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ABSTRACT: The kinetics and mechanism of enzymatic Schiff base formation and hydrolysis were investigated by rapid reactions measurements of 5′-deoxypyridoxal with pyridoxamine-pyruvate transaminase (EC 2.6.1.30). The dissociation rate constant, k_{off}, was determined as a function of pH over the range pH 7–9 by a stopped-flow method in which the nascent free enzyme was trapped by the potent bisubstrate analogue inhibitor, *N*-pyridoxyl-L-alanine. The values of k_{off} increase with pH and are dependent upon a pK_a (app) of 8.35 which is assigned to the pyridine nitrogen of the Schiff base formed between 5′-deoxypyridoxal and an ϵ-amino group of the active site lysine. The rate-determining step in the dissociation reaction is assigned to the separation of the components of the Michaelis complex by diffusion. A temperature-jump investigation of the pH dependence of the association rate constant, k_{on}, showed a maximum at pH 8.15. This is engendered by a mechanism involving formation of a productive Michaelis complex only when the active site ϵ-amino group is unprotonated and 5′-deoxypyridoxal is in its neutral zwitterionic form. The pK_a of the lysine ϵ-amino group has a kinetically determined pK_a of 8.2. Analysis of the amplitudes of the temperature-jump experiments confirms that the enzyme has 4 active sites per tetramer and gives values of −14.4 kcal/mol and −24.2 eu for the enthalpy and entropy of the association reaction, respectively.

The wealth of spectral detail accompanying transformations between the various intermediates involved in pyridoxal phosphate dependent enzyme catalyzed transamination has stimulated important efforts to elucidate the mechanistic details of this reaction by the temperature-jump technique. The prototypic enzyme for these investigations has been aspartate aminotransferase studied by Hammes and Haslam (1968, 1969), Giannini et al. (1975), Fasella and Hammes (1967), and Czerlinski and Malkewitz (1965). This enzyme catalyzes the reactions shown in eq 1.

$$\text{L-aspartate} + \text{PLP} \rightleftarrows \text{oxaloacetate} + \text{PMP} \qquad (1)$$

$$\text{PMP} + \alpha\text{-ketoglutarate} \rightleftarrows \text{PLP} + \text{L-glutamate}$$

Each of the two equations shown in eq 1 is itself composed of several steps which include carbinolamine or *gem*-diamine formation and decomposition, and the interconversion of aldimine and ketimine forms of the amino acid-B-6 adducts (Snell and DiMari, 1970). These are reflected, for example, in the 11 relaxation times observed for the interaction of the

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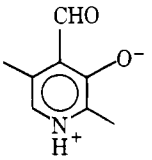
PLP[1] form of this enzyme with *erythro*-β-hydroxyaspartic acid (Hammes and Haslam, 1969).

Because of the regenerative nature of the two steps in eq 1, the pyridoxyl phosphate moiety is not consumed and does not normally dissociate from the enzyme during the course of the catalyzed reaction. Indeed studies on the reconstitution of the holoenzyme, combining apoenzyme with PMP or PLP, have shown that this reaction is much slower than the overall catalyzed reaction (Snell, 1970; Fonda, 1971). The reconstitution of glutamate decarboxylase from apoenzyme and PLP is also much slower than the catalyzed decarboxylation (O'Leary and Malik, 1971).

Contrastingly PL, or PM in the reverse reaction, is a substrate rather than a cofactor for the reaction catalyzed by pyridoxamine-pyruvate transaminase (EC 2.6.1.30) (PPT) (eq 2).

$$\text{L-alanine} + \text{PL} \rightleftarrows \text{pyruvate} + \text{PM} \qquad (2)$$

This enzyme thus provides a tool for the direct study of an enzyme catalyzed Schiff base formation between PL or its analogues and PPT. The foundations for the work reported here were laid by the steady-state and stopped-flow investigations of Ayling and Snell (1968a,b) which showed that the catalyzed reaction proceeds by a kinetically ordered mechanism in which the pyridoxyl moiety binds to the enzyme before the 3-carbon acid. Of particular value was their discovery that the PL analogue, 5′-deoxypyridoxal (5′-deoxy-PL), functions about as well in transamination as does PL (Ayling and Snell,



1968b), because the use of this analogue eliminates the enzyme-independent hemiacetal formation reaction of PL which occurs within a reaction time which considerably overlaps that characteristic of the PPT–PL association reaction in the accessible concentration range (Ahrens et al., 1970). The only significant side reaction for the 5′-deoxy-PL–PPT system is the much slower hydration reaction of 5′-deoxy-PL which does not seriously interfere with the enzymatic studies. A preliminary account of this research has been presented (Kury and Kirsch, 1973).

Experimental Section

Materials and Instrumentation. Those materials and procedures not described herein are given in the previous paper (Gilmer et al., 1977). NPA was synthesized by the method of Ikawa (1967). The recrystallized product, which was dried over phosphorus pentoxide at the temperature of boiling acetone in an Abderhalden vessel, was assumed to have the molecular weight of the monohydrate (Ikawa, 1967) in calculations for the experimentally determined extinction coefficient (ϵ = 7300 at 308 nm in 0.1 N sodium hydroxide). The temperature-jump experiments were performed in a 1.5-mL cell (7-mm pathlength) supplied with the single beam instrument manufactured by Messenlagen Studiengesellschaft (Göttingen, West Germany). A Durrum-Gibson stopped-flow spectrophotometer with a 20-mm pathlength cell was used for the stopped-flow experiments.

Temperature-Jump Measurements. The magnitude of the temperature-jump was calibrated by the use of an indicator, phenolpthalein, in a buffer whose pK_a is very sensitive to temperature (0.1 M Tris, ionic strength 1.1). It was so determined that the solutions were raised from 17.5 to 23 °C by a 30-kV discharge. The relaxation rate was routinely monitored at 430 nm, the largest peak in the difference spectrum, (Gilmer et al., 1977) where the substrate hydration relaxation amplitude is relatively small (maximum change in absorbance at 390 nm) and the enzyme–substrate relaxation is relatively large in amplitude (maximum change in absorbance at 410 nm). Normally the PPT–5′-deoxy-PL relaxation amplitude was 10 to 15 times larger than that due to the hydration of aldehyde, but, under unfavorable conditions such as at $([S_t]/[E_t]) = 2.25$ at pH 9.0, the enzyme–substrate relaxation displayed an amplitude only twice as large as that due to 5′-deoxy-PL hydration. The amplitude of the hydration relaxation process increases markedly as the pH is lowered from 9 to 7 making it more difficult to determine the relaxation time of the enzyme–5′-deoxy-PL interaction at neutral pH.

The absorbance at 430 nm of the enzyme–5′-deoxy-PL solution increases, following each addition of 5′-deoxy-PL so that at constant light intensity less light is received by the photomultiplier. The optimal response of the photomultiplier is maintained after successive substrate additions by adjusting either the lamp intensity or the slit opening so that the total light on–light off voltage detected by the photomultiplier remains constant (i.e., a constant change in voltage is maintained between the % T of the solution and 0% T). Repeated temperature jumps did not produce a significant change in enzyme activity (e.g., 66 temperature jumps with one enzyme–5′-deoxy-PL solution resulted in only a 1.5% loss in enzyme activity).

Correction of Calculated Substrate Concentration for Depletion by the Slow Reaction. In a temperature-jump experiment with PPT a certain amount of the total 5′-deoxy-PL in solution is bound at nonactive site lysine residues under conditions of $[S_t] > [E_t]$. These sites equilibrate in a time range which is much slower than that of the active site (Gilmer et al., 1977) resulting in a reduced concentration of free 5′-deoxy-PL. A correction must therefore be applied in order to calculate the [5′-deoxy-PL] available to react at the active sites. This is accomplished by calculating the concentration of E′S, from the following coupled equilibria

$$\text{E'S} \overset{\text{E'}}{\rightleftarrows} \text{S} \overset{\text{E}}{\rightleftarrows} \text{ES} \qquad (3)$$

where the primed forms represent the non-active site lysine residues. The equilibrium constants for binding 5′-deoxy-PL at the active and non-active sites were taken from Gilmer et al. (1977).

Recording and Analysis of Temperature-Jump Traces. Relaxation traces were recorded on 35-mm film. The amplitudes and relaxation times were obtained either by enlarging the negative and hand-plotting the log transmittance vs. time or by superimposing a synthetic trace of a single exponential on the oscilloscope over the experimental recording. (We thank Mr. Peter Lovely for the construction of the exponential generator.)

Data Analysis. A general nonlinear regression program was used to fit the data in Figures 1 and 4. The slopes of the lines in Figure 3 were fit by linear regression with the ordinate fixed as the values of k_{off} determined in the stopped-flow experiments.

Results

Stopped-Flow Experiments. The enzyme–5′-deoxy-PL

[1] Abbreviations used are: 5′-deoxy-PL, 5′-deoxypyridoxal; NPA, *N*-pyridoxyl-L-alanine; PPT, pyridoxamine-pyruvate transaminase; HPA, 3-hydroxypyridine-4-aldehyde; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PL, pyridoxal; PM, pyridoxamine.

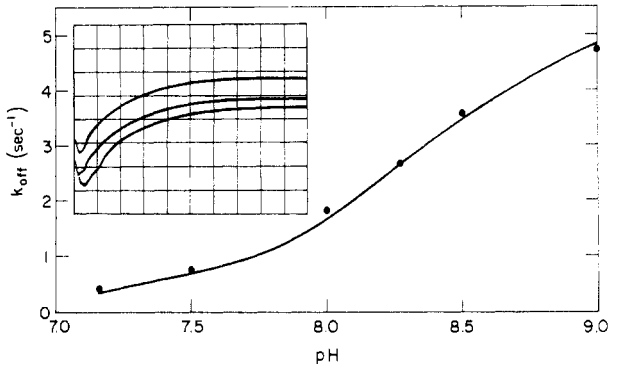


FIGURE 1: Rate constants for dissociation of the PPT–5′-deoxy-PL complex, k_{off}, plotted as a function of pH as determined in stopped-flow experiments. Conditions: 0.05 M potassium pyrophosphate buffer, μ = 0.5 at 25 °C. The curve is theoretical based on eq 11 or 13 with the constants given in the text. (Insert) Spectrophotometric records of a typical experiment at 430 nm. (Conditions) Syringe A contained 5′-deoxy-PL (13 μM) and PPT (26 μM) and syringe B contained NPA (390 μM), pH 8.17. Each horizontal division corresponds to 200 ms and each vertical division to 3.4% of the total transmittance of the sample after reaction. The +Y direction corresponds to a decrease in absorbance.

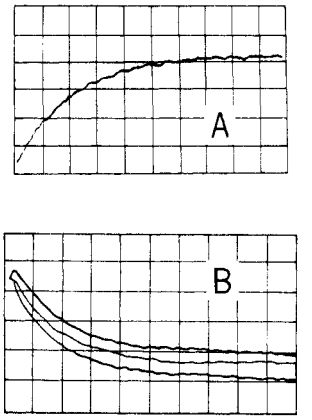


FIGURE 2: Spectrophotometric records of the temperature-jump perturbations of the following systems at 23 °C. The +Y direction corresponds to an increase in absorbance. (A) Hydration of 5′-deoxy-PL (100 μM) at 390 nm, pH 8.85, in 0.05 M sodium pyrophosphate buffer, μ = 1.1. Each horizontal division corresponds to 100 ms and each vertical division to 0.28% of the total transmittance of the sample. (B) Interaction of 5′-deoxy-PL (100 μM) and PPT (70 μM) at 430 nm in 0.05 M potassium pyrophosphate buffer, pH 9.03, μ = 0.5. Each horizontal division corresponds to 10 ms and each vertical division to 0.63% of the total transmittance of the sample.

dissociation rate constants are too slow to be accurately determined by the temperature-jump method under the accessible conditions; therefore they were measured by a stopped-flow method. In these experiments a mixture of PPT–5′-deoxy-PL complex in one syringe was mixed with NPA in the other in order to trap the newly released enzyme and thus prevent the reverse reaction (eq 4).

$$\text{PPT-5′-deoxy-PL} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{5′-deoxy-PL} + \text{PPT} \qquad (4)$$

$$\text{5′-deoxy-PL} \xrightarrow{\text{fast, NPA}} \text{PPT-NPA}$$

The conditions were designed so that virtually all of the 5′-deoxy-PL was bound to the active site of the enzyme before mixing; i.e., [PPT] $\geq 10K_s$ and [5′-deoxy-PL] = ½[PPT]. The concentration of NPA was in large excess over that of total 5′-deoxy-PL so that the rate of combination of NPA with the free enzyme was fast relative to the rate of the reversible reaction of eq 4. The observed first-order rate constants for release of 5′-deoxy-PL were unaffected by varying the concentration of NPA over a fourfold range confirming that the trapping was completely effective. The reactions were followed at 430 nm at 25 °C. The plot of k_{off} vs. pH shows that the rate constants decrease as the pH is lowered (Figure 1).

Temperature-Jump Relaxation Measurements. These experiments were usually done by measuring relaxation times after each addition of an aliquot of a concentrated 5′-deoxy-PL solution to a highly concentrated (50–120 μM) solution of PPT in the temperature-jump cell. The high concentration of enzyme served to resolve in time the PPT–5′-deoxy-PL relaxation from the slower one due to the enzyme independent hydration of 5′-deoxy-PL ($1/\tau$ = 2–7.5 s^{-1} between pH 7 and 9; Ahrens et al., 1970) (Figures 2A and B). Measurements were made at pH values of 9.03, 8.82, 8.48, 8.06, and 7.84.

All experiments were carried out with the 5′-deoxy-PL concentration no greater than 2.5 times that of the enzyme in order to minimize the reaction of the aldehyde substrate at the non-active site lysines. At pH values greater than 8.1, the total 5′-deoxy-PL concentrations (at $[S_t] > [E_t]$) were corrected for the amount depleted by reaction at the non-active site lysines ($\leq$15%, see Experimental Section).

This bimolecular relaxation process is not detectable when NPA is present in a concentration greater than that of the enzyme. Since NPA is a competitive inhibitor of PL and of pyridoxamine (Dempsey and Snell, 1963), this indicates that the relaxation seen in the absence of NPA is associated with the active site.

In some experiments enzyme aliquots were added to a solution of 5′-deoxy-PL, and the concentration of the latter was kept at a low value of about 35 μM in order to minimize the binding of the aldehyde to the non-active site lysines since the aldehyde concentration was initially in excess of that of the enzyme. Measurements at pH 8.06, 8.48, 8.82, and 9.03 employed this procedure.

The inverse relaxation time, $1/\tau$, for a simple bimolecular association reaction is directly proportional to the sum of the equilibrium concentrations of free enzyme and of free substrate ($[\bar{E}] + [\bar{S}]$) (Eigen and De Maeyer, 1963):

$$1/\tau = k_{on}([\bar{E}] + [\bar{S}]) + k_{off} \qquad (5a)$$

$$\text{for } E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES$$

where

$$K_e = k_{off}/k_{on} \qquad (5b)$$

The equation can be rewritten for the case of the enzyme combining only with the aldehyde form of the free substrate (Eigen and De Maeyer, 1963) when the bimolecular association reaction equilibrates much faster than the substrate hydration reaction.

$$1/\tau = k_{on}([\bar{E}] + [\bar{S}_A]) + k_{off} \qquad (6a)$$

$$\text{for } E + S_A \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES$$

$$\text{slow} \downarrow\uparrow K_H$$

$$S_H$$

where

$$K_s = k_{off}/k_{on} \qquad (6b)$$

The raw data are plotted according to eq 6a in Figure 3. The ordinate intercept, k_{off}, was fixed at the value of the rate con-

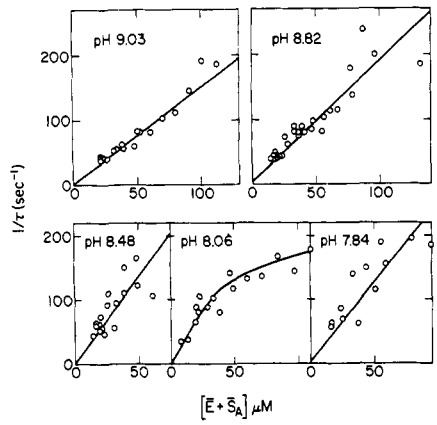


FIGURE 3: Reciprocal relaxation time at 23 °C plotted as a function of $(\bar{E}) + (\bar{S}_A)$ as calculated from the following values of K_d and K_H at each pH [pH, $K_d(\mu M)$, K_H]: 9.03, 5.8, 0.130; 8.82, 4.1, 0.138; 8.47, 2.1, 0.160; 8.06, 1.05, 0.196; 7.84, 0.71, 0.216. Calculations of $[E_t]$ allowed for 10–15% inactive protein. The buffers were 0.05 M potassium pyrophosphate, μ = 0.35–0.5.

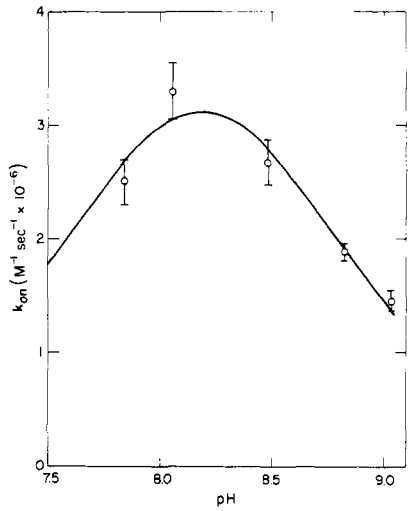


FIGURE 4: Dependence of k_{on}, as determined by temperature-jump kinetics, on pH. All experiments were performed in 0.05 M potassium pyrophosphate buffer, μ = 0.35–0.5 at 23 °C. The solid line represents the least-squares fit to eq 14 (see Discussion section).

stant determined independently in the stopped-flow experiments. The values of $([\bar{E}] + [\bar{S}_A])$ were calculated from the independent determinations of K_d and K_H (Gilmer et al., 1977) where $K_d = [\bar{E}][5'\text{-deoxy-PL}]/[\text{E-5'-deoxy-PL}]$ and $K_H = [\bar{S}_H]/[\bar{S}_A]$. A linear dependence of $1/\tau$ vs. $([\bar{E}] + [\bar{S}_A])$ was observed at pH values 8.48, 8.82, and 9.03. A leveling off of $1/\tau$ at high values of $([\bar{E}] + [\bar{S}_A])$ was noted at pH 8.06. This might indicate the presence of an intermediate preceding the Schiff base. However, at the lowest pH value examined, 7.84, it was not possible to utilize high enough concentrations of $([\bar{E}] + [\bar{S}_A])$ to detect any possible nonlinearity in the plots because of the smaller amplitudes of the PPT–5'-deoxy-PL relaxation at the lower pH values.

The value of k_{on} at each pH value was determined from the slopes in Figure 3. A plot of k_{on} vs. pH indicates that this parameter has a maximal value at pH 8.1 (Figure 4).

Temperature-Jump Amplitudes. There are two basic types of information that can be obtained from temperature-jump kinetic experiments. One is the relaxation time, τ, and the other is the amplitude of the relaxation, δI_{12}.

The enthalpy of association of 5'-deoxy-PL with PPT can be obtained from a plot of the relaxation amplitude according to eq 7 (Guillain and Thusius, 1970)

$$\delta I_{12}/I_0 = \frac{-(2.30\Delta\epsilon_{12}\Delta H_{12}dT)}{RT^2}\Gamma_{12} \qquad (7)$$

where $\delta I_{12}/I_0$ is the experimental relaxation amplitude, $\Delta\epsilon_{12} = (\epsilon_{SB} - \epsilon_{S_A})$, $\Delta H_{12} = H_{SB} - H_{S_A} - H_E$, dT is the temperature change, and Γ_{12}, the amplitude factor. The definition of the amplitude factor depends on the mechanism (Winkler, 1969; Thusius, 1972). The amplitude factor for the mechanism shown in eq 6b is given in eq 8.

$$\Gamma_{12} = \frac{(Q - \sqrt{Q^2 - 4P})(1 + K_H)}{(1/K_s)(\sqrt{Q^2 - 4P}(2 + K_H) + K_H(2[E_t] - Q))} \qquad (8)$$

where $Q = [E_t] + [S_t] + (1 + K_H)(K_S)$, $P = [E_t][S_t]$, and $K_H = [\bar{S}_H]/[\bar{S}_A] = 0.13$ at pH 9.03. A plot of δI_{12} (at constant I_0) vs. Γ_{12} should be linear and pass through the origin. A linear dependence is observed in this plot when the enzyme concentration is corrected for 15% inactive protein (Figure 5).

From the slope of Figure 5 one can calculate from eq 7 that the ΔH_{12} for the reaction is −14.4 kcal/mol where the experimental slope is $(3.12 \pm 0.11) \times 10^6$ mV M^{-1}, I_0 = 800 mV, $\Delta\epsilon_{12} = \epsilon_{SB} - \epsilon_{S_A} = 5350 - 1600 = 3750$, dT = 5.5 °C, T = 298 K. The value of ΔH_{12} together with that of K_s leads to the values of ΔG_{12} and ΔS_{12} of −7.2 kcal/mol and −24.2 eu, respectively.

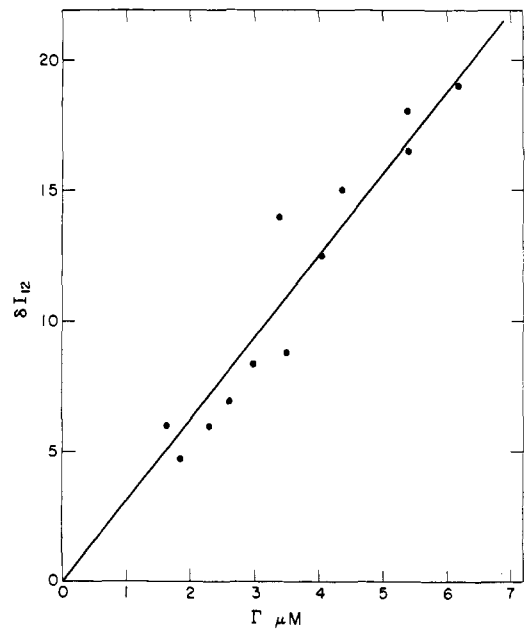


FIGURE 5: Determination of the enthalpy of the PPT–5'-deoxy-PL reaction from amplitudes of relaxation experiments at pH 9.03, plotted according to eq 7. Conditions as in Figure 6. The value of K_s at this pH is 3.5 μM and allowance was made for 15% inactive enzyme.

The number of enzyme binding sites can be determined from the concentration dependence of the relaxation amplitude values in a titration experiment of enzyme with substrate. Under conditions where $[E_t] \gg K_s$, the maximum amplitude for a bimolecular association reaction is found when $[E_t] = [S_t]$ (Winkler, 1969).

Amplitude data from the temperature-jump titration experiment at pH 9.03 is plotted together with the theoretical curve calculated from eq 7 and 8, as $\delta I_{12}/[E_t]$ (at constant I_0) vs. $\log([S_t]/[E_t])$ (Winkler, 1969) where I_0 is the intensity of the transmitted light and $[E_t]$ is the total enzyme monomer concentration (Figure 6). It was necessary to correct the en-

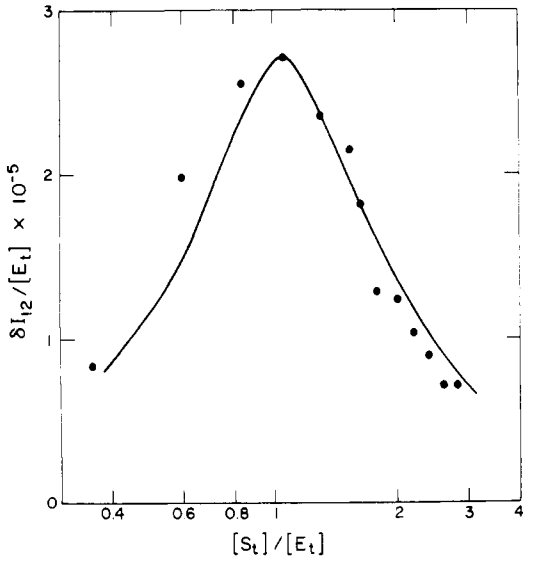


FIGURE 6: Amplitudes of temperature-jump relaxations for the 5′-deoxy-PL-PPT system at pH 9.03. Conditions: 0.05 M potassium pyrophosphate, μ = 0.5, 23 °C. Reactions were monitored at 430 nm with I_0 constant at 800 mV.

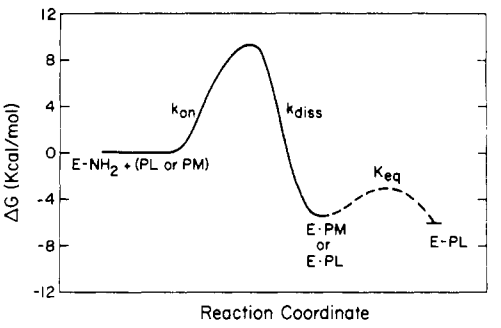
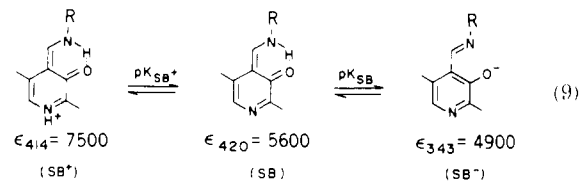


FIGURE 7: Free energy vs. reaction coordinate (1 M standard state) diagram for the reaction of PL and PM with pyridoxamine-pyruvate transaminase calculated from the steady-state data of Ayling and Snell (1968a) at pH 8.85. The rate constants are calculated for the total of the ionic forms of each species represented. Assignments of the reacting ionic species are made in the text. The observed rate constants for the combination of PL or PM with the enzyme (k_{on}) are nearly identical. An additional 700 cal/mol of stability at this pH is provided to the E·PL complex by formation of a covalent schiff base (E-PL). Covalent bond formation contributes more significantly to the stability at lower values of pH. The height of the latter kinetic barrier (dashed curve) is unknown, but must be smaller than that for the formation of the Michaelis complex. Thus the rate constant for the dissociation of PL or its analogue 5′-deoxy-PL from the enzyme is given by $k_{off} = k_{diss}K_{eq}$.

zyme concentration for 15% inactive protein[2] or else the curve would have had a maximum at $[S_t]/[E_t] = 0.9$ which is theoretically impossible. With this correction the maximum amplitude is found where PPT monomer concentration is nearly equal to that of 5′-deoxy-PL thus providing additional evidence for four binding sites per tetramer.

Discussion

Spectral Properties and pK_a's of Schiff Bases. The prototropic equilibria involving the Schiff bases formed from 5′-deoxy-PL and amines are shown in eq 9



(9)

Johnson and Metzler (1970) give pK_{SB^+} = 6.5 and pK_{SB} = 11.7 for the aldimine formed from 5′-deoxy-PL and leucine.

The spectrum of the aldimine formed between PPT and 5′-deoxy-PL has a peak at 410–420 nm and exhibits no significant absorption at 340–360 nm from pH 7 to 9 (Gilmer et al., 1977). This indicates by comparison with the models that there is little of the SB$^-$ species present.

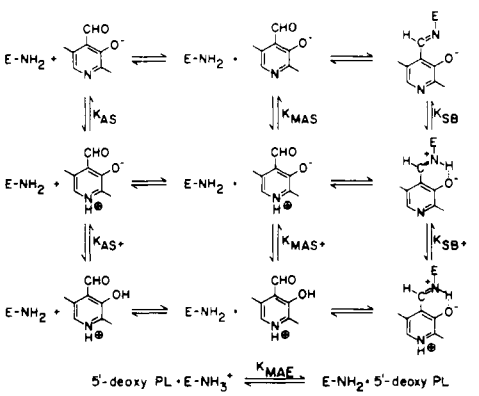
Dissociation of 5′-Deoxy-PL from the 5′-Deoxy-PL-PPT Complex. The observation that PM and PL combine and dissociate from PPT with nearly the same rate constants at pH 8.85 (Ayling and Snell, 1968a) suggests that Michaelis complex formation and decomposition is rate determining and that subsequent Schiff base formation from the aldehyde derivative contributes only about 700 cal/mol to the stability of the enzyme-PL complex at this pH. These data are shown in a free energy vs. reaction coordinate diagram in Figure 7. The pH dependence for the rates of reaction of PM with this enzyme has been followed by the fluorescence temperature-jump technique (J. F. Kirsch and H. Winkler, unpublished results). It was observed that k_{off} is pH independent between pH 7 and 9, while k_{on} decreases with increasing pH and is dependent upon a pK_a of 8.1 which is assigned to the pyridine nitrogen of PM. This implies that the only form of PM capable of combining with the enzyme is that in which the pyridine nitrogen is protonated, and that the pK_a of this group is raised to a value considerably greater than 9 in the enzyme-PM complex.

Assuming that PM, PL, and 5′-deoxy-PL form essentially the same noncovalent Michaelis complex, any differences in the pH dependence of the dissociation rate constants result from the pH dependence of the equilibrium constant, K_{eq}, for the Schiff base formed from *enzyme bound PL or 5′-deoxy-PL*, since $k_{off} = k_{diss}K_{eq}$ (Figure 7).

Auld and Bruice (1967) have published the results of a valuable study on the pH dependence for the formation of Schiff bases from HPA and alanine which provide a framework in which to analyze the equilibrium depicted in Figure 7. Chart I is adapted from their publication and the pH dependence of the association constant, K_{pH}, is given in eq 10

$$K_{pH} = K_{eq}^{-1} = \frac{K\left[1 + \left(\frac{a_H}{K_{SB}}\right)\left(1 + \frac{a_H}{K_{SB^+}}\right)\right]}{\left[1 + \left(\frac{a_H}{K_{MAS}}\right)\left(1 + \frac{a_H}{K_{MAS^+}}\right)\right]\left(1 + \frac{a_H}{K_{MAE}}\right)} \quad (10)$$

CHART I



[2] This may in part be a result of an undetected complex formed from the enzyme and the hydrate form of 5′-deoxy-PL.

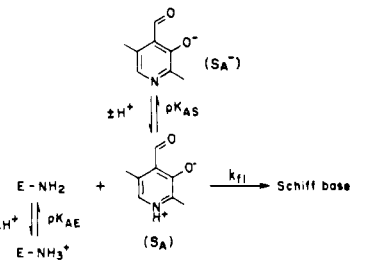
The values of pK_{SB} and pK_{SB^+} are equal to 11.7 and 6.5, respectively, for the nonenzyme bound aldimine formed from 5′-deoxy-PL and leucine, and pK_{AS} and pK_{AS^+} for free 5′-deoxy-PL equal 8.1 and 3.8, respectively (Johnson and Metzler, 1970). Normal values of protein ϵ-amino groups range between 9.4 and 10.6 (Dixon and Webb, 1964). These values are not necessarily representative of those for enzyme-bound species and of the active site lysine which are the important ones in the equilibrium under consideration; and in the absence of additional information, eq 10 could not be further analyzed to provide assignments for the pK_a values of enzyme bound species. We noted, however, that the pH dependences of the observed values of k_{off} are fitted extremely well by nonlinear regression to eq 11

$$k_{off} = \frac{k_{off}^{lim} K_1}{a_H + K_1} \tag{11}$$

with the limiting values of $k_{off}^{lim} = 5.9\ s^{-1}$ and $pK_1 = 8.35$ (Figure 1). This observation suggests that all of the ionizable groups identified in eq 10 save one titrate well outside of the investigated pH range of 7.15–9.0, or that they demonically vary in such a way that their combined effects cancel. Assuming the former, tractable situation the problem reduces to one of the assignment of the group in eq 10 which has the pK_a of 8.35. Fortunately, sufficient additional information is available to permit this assignment to K_{SB^+} with reasonable confidence.

As discussed above the pH independence of the rate constant for the dissociation of PM from the enzyme–PM complex requires that the pK_a of the pyridine nitrogen of PM be raised to a value above the pH range investigated. Since it is very likely that a similar Michaelis complex, at least with respect to the pyridine ring, is formed with PL, 5′-deoxy-PL, and PM (see above) we may conclude that pK_{MAS} for enzyme-bound 5′-deoxy-PL is also well above 9. If this is true, then pK_{MAS^+} will likely also be increased above the value of that for free 5′-deoxy-PL (3.8), but it is improbable that the shift could be as much as the nearly 5 pK units necessary to account for the observed pH dependence of k_{off}. We assume, therefore, $pK_{MAS^+} < 6$. As discussed in the next section, the dependence of the rate constant for the association of 5′-deoxy-PL with PPT is best accommodated by a model in which the ϵ-amino group of the active site lysine is assigned a value of 8.2 in the free enzyme and is further reduced on association with 5′-deoxy-PL. We, therefore, assign a value of $pK_{MAE} < 6.5$ for the ϵ-amino group in the Michaelis complex. The remaining quantities in eq 10 to be considered are pK_{SB} and pK_{SB^+}. The value of the former is 11.7 for the model compound discussed above (Johnson and Metzler, 1970), and since the pK_a of the pyridine nitrogen atom of PM is raised in the Michaelis complex, it is likely that pK_{SB} would at least not be lowered thereby. Thus by elimination we assign the observed pK_a of 8.35 to pK_{SB^+}. This can be compared with the value of 6.5 assigned by Johnson and Metzler (1970) to the aldimine formed from 5′-deoxy-PL and leucine. Direct spectral evidence supporting this assignment is available from the active site titration experiment reported in the previous paper (Gilmer et al., 1977). When the end-point absorbance representing $[\epsilon_{SB}(\text{total}) - \epsilon_{5'\text{-deoxy-PL}}]_{430nm}$ was plotted as a function of pH, it was noted that a decrease in extinction coefficient occurred at about pH 8.3. The species present below this pH has an apparent $\Delta\epsilon_{430}$ of 5000 at 430 nm while that existing above pH 8.3 has an apparent $\Delta\epsilon_{430}$ of 3750. These figures may be compared with those given for SB^+ and SB in eq 9 which show a similar decrease upon deprotonation of the pyridine nitrogen atom (Nagano and Metzler, 1967). The increase in pK_a upon binding to the protein is consistent with the increase in the pK_a of the pyridine nitrogen atom of PM.

SCHEME I



With the assignments derived above eq 10 reduces to eq 12

$$K_{pH} = \frac{KK_{MAS}\left(1 + \frac{a_H}{K_{SB^+}}\right)}{K_{SB}} = \frac{k_{diss}}{k_{off}} \tag{12}$$

because $(1 + a_H/K_{MAE})$ and $(1 + a_H/K_{MAS^+}) \approx 1$ over the pH range 7–9, and a_H/K_{SB} and a_H/K_{MAS} are both $\gg 1$, therefore

$$k_{off} = \frac{k_{off}^{lim} K_{SB^+}}{K_{SB^+} + a_H} \tag{13}$$

where $k_{off}^{lim} = k_{diss}K_{SB}/K_{MAS}K$, and K_1 of eq 11 is K_{SB^+} of eq 13.

Schiff Base Formation. It is known from model studies that only the free base form of the active site lysine can react with the aldehyde (Auld and Bruice, 1967). In addition it is necessary that the enzyme catalyze Schiff base formation only from the neutral zwitterionic form of 5′-deoxy-PL, S_A, in order to account for the pH dependence of the forward rate constant (Scheme I).

The rate expression derived for this mechanism is given in eq 14

$$k_{on} = \frac{k_{fl}}{(1 + a_H/K_{AE})(1 + K_{AS}/a_H)(1 + K_H)} \tag{14}$$

where $K_H = [S_H]/[S_A]$. The value of pK_{AS} is taken as 8.08 (Johnson and Metzler, 1970), and the best fit to the experimental data was obtained with $pK_{AE} = 8.2 \pm 0.1$ and $k_{fl} = 1.79 \pm 0.17 \times 10^7\ M^{-1}\ s^{-1}$. The theoretical curve for this mechanism is the solid line in Figure 5. The rate-determining step in Schiff base formation is thus the combination of the neutral ϵ-amino form of the enzyme with the zwitterionic 5′-deoxy-PL to form the Michaelis complex.

The pK_a of the active site lysine of the free enzyme is tentatively assigned the value 8.2 on the basis of the proposed mechanism. The pK_a of a normal ϵ-amino group of a lysine on a protein is in the range 9.4–10.6 (Dixon and Webb, 1964). An abnormally low pK_a of 5.9 for the essential lysine in acetoacetate decarboxylate has been reported (Kokesh and Westheimer, 1971). The pK_a of the essential lysine of aspartate aminotransferase has recently been estimated to be 7.98 ± 0.08 from the pH dependence of the rates of reaction with KNCO (Slebe and Martinez-Carrion, 1976). If the pK_a of the active site lysine of PPT were that of a normal lysine on a protein, then the observed rate constant for imine formation, k_{on}, would not decrease with increasing pH in the range studied (pH 8–9). The fact that the association reaction for 5′-deoxy-PL with the enzyme, which does not chemically involve the ϵ-amino group of the active site lysine, is dependent upon the ϵ-amino group being in the free base form, taken together with the fact that the association of PM with the enzyme shows no such dependence (Kirsch and Winkler, unpublished) argues that the

TABLE I: Rate Constants for Combination of 5′-Deoxy-PL with PPT at pH 8.85.

Method	$k_{on} \times 10^{-6}$ (M^{-1} s^{-1})	k_{off} (s^{-1})
Steady-state kinetic measurements (Ayling and Snell, 1968b)[a]	0.89	17
Stopped-flow kinetics (Ayling and Snell, 1968b)[a]	0.72	6.5
Temperature-jump and stopped-flow kinetics (this study)[b]	1.35	4.5

[a] Sodium pyrophosphate buffers, $\mu = 0.5$, 25 °C. [b] Potassium pyrophosphate buffers, $\mu = 0.5$, 23 °C.

ϵ-amino group in the Michaelis complex formed from 5′-deoxy-PL is not in a mobile protonic equilibrium. If it were, k_{on} should not show a dependence on the lysine pK_a. For this reason and for those discussed in the previous section, pK_{MAE} is assigned a value <6.5.

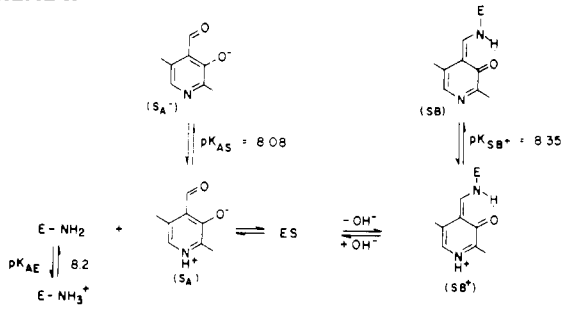
We can compare the rate constants obtained in this study with those determined by Ayling and Snell (1968b) at pH 8.85 (Table I). The agreement is substantial.

Comparison of Binding at the Active Site and the Nonactive Site. The fast relaxation due to imine formation at the active site and the slow reaction of B-6 aldehydes at the nonactive sites exhibit differing pH rate dependencies and optima for the dissociation constants. The affinity of the substrate for the active site is greatest near pH 7. This is due primarily to the decreased values of k_{off} at neutral pH (Figure 2). In contrast, binding of 5′-deoxy-PL at nonactive sites shows little variation with pH (Gilmer et al., 1977).

There are two lines of evidence that indicate that the reaction of 5′-deoxy-PL at nonactive site lysines does not effect enzymatic activity: (1) Michaelis–Menten kinetics was observed in the steady-state analysis of the enzyme when [5′-deoxy-PL] was as high as 100 μM (the range of concentrations where non-active site lysines are modified (Ayling and Snell, 1968b)); (2) the temperature-jump data indicate the same association and dissociation rate constants for the bimolecular reaction at the active site under conditions when only the active site is modified and when both the active and nonactive sites are modified by aldimine formation. These two types of kinetic behavior support the idea that the nonactive site reactions do not effect the reactivity at the active site.

In conclusion it has been demonstrated that the rate of formation of the Schiff base for 5′-deoxy-PL and PPT is controlled by an ionizable group of pK_a of ca. 8.2 on the enzyme which is presumably the active site lysine and by the pK_a of 8.1 of the pyridine nitrogen atom of 5′-deoxy-PL. Any enzyme–substrate complexes preceding the aldimine must be relatively unpopulated because intermediates cannot be detected by the temperature-jump experiments except possibly at pH 8.06 (by the nonlinearity in Figure 3 at high values of ($[\bar{E}] + [\bar{S}_A]$). The limiting rate constant of 1.79×10^7 M^{-1} s^{-1}, although below the diffusion-controlled limit, is about equal to that observed for the reaction of PM with PPT (Kirsch and Winkler, unpublished results) where no covalent interactions follow the formation of the Michaelis complex. This similarity of values of k_{f1} for PM and 5′-deoxy-PL thus suggests that the rate-determining step in aldimine formation is formation of the ES complex, and from the law of microscopic reversibility it follows that dissociation of PL or 5′-deoxy-PL from the Michaelis complex is rate determining in the reverse direction (Figure 7).

SCHEME II



Scheme II summarizes the resolvable ionizations involved in the association of 5′-deoxy-PL with PPT. The value of the dissociation constant for the aldehyde form of 5′-deoxy-PL (S_A) from the 5′-deoxy-PL–PPT complex is given in eq 15

$$K_s = \frac{[\bar{E}][\bar{S}_A]}{[\text{E-5′-deoxy-PL}]} = \frac{k_{off}}{k_{on}} \qquad (15)$$

The spectrophotometrically determined values are compared with the ratio of the rate constants k_{off}/k_{on} obtained by rapid reaction kinetics in Figure 6 of the previous paper (Gilmer et al., 1977). The agreement is generally within experimental error.

Acknowledgment

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Activation of Human Factor X (Stuart Factor) by a Protease from Russell's Viper Venom†

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ABSTRACT: Human Factor X (Stuart factor) is a plasma protein (mol wt 59 000) that participates in the middle phase of blood coagulation. It is composed of a heavy and a light chain held together by a disulfide bond(s). Factor X is readily converted to an enzyme, factor X_a, by a protease from Russell's viper venom. In this reaction, a specific arginyl-isoleucine bond is cleaved in the amino-terminal region of the heavy chain giving rise to an activation peptide and factor X_a. This results in the formation of an Ile-Val-Gly-Gly-Gln-Glu-Cys-Lys-Asp-Gly-Glu-Cys-Pro-Thr-Gln-Ala-Leu- sequence in the heavy chain of the enzyme. No change was observed in the light chain of factor X during the activation reaction. The heavy chain of human factor X_a also contains the active site sequence of Phe-Cys-Ala-Gly-Tyr-Asp-Thr-Lys-Gln-Glu-Asp-Ala-Cys-Gln-Gly-Asp-SER-Gly-Gly-Pro-His-Val-Thr-Arg-Phe-. The amino-terminal and active site sequences of the heavy chain are homologous with the corresponding amino-terminal and active site sequences of bovine factor X_a and a number of other plasma serine proteases. Human factor X_a was rapidly inhibited by antithrombin III and formed a stable one-to-one molar complex with the inhibitor. These data indicate that the mechanism of activation and inhibition of human factor X are essentially identical with that previously described for bovine factor X.

Factor X (Stuart factor)[1] is one of four known coagulation factors present in plasma that requires vitamin K for its biosynthesis. During the coagulation process, factor X is converted to an enzyme, factor X_a, which in turn converts prothrombin to thrombin [see Davie and Fujikawa (1975) and Suttie and Jackson (1977) for reviews].

In recent years, human factor X has been purified and characterized in several different laboratories (Aronson et al., 1969; Rosenberg et al., 1975; Kosow, 1976; Vician and Tishkoff, 1976; Di Scipio et al., 1977). It is a glycoprotein composed of a heavy and a light chain held together by a disulfide bond(s). In the present article, we describe the mechanism of activation of human factor X by a protease from Russell's viper venom. In this reaction, factor X is converted to factor X_a by the cleavage of a specific arginyl-isoleucine bond in the amino-terminal portion of the heavy chain of the precursor molecule. This mechanism of activation is essentially identical with that previously observed for bovine factor X when it is activated by (a) the protease from Russell's viper venom, (b) trypsin, (c) factor IX_a and factor VIII, or (d) factor VII and tissue factor (Fujikawa et al., 1972a, 1974, 1975; Jesty and Esnouf, 1973; Jesty and Nemerson, 1974).

Experimental Section

Materials

Human factor X was purified to homogeneity by a slight modification of the method of Di Scipio et al. (1977). In the present experiments, a single 0–40% ammonium sulfate precipitation was substituted for the sequential 0–10% and 10–40% ammonium sulfate precipitation. This preparation does not contain two forms of factor X similar to bovine factor X_1 and factor X_2 (Fujikawa et al., 1972b). The protease from Russell's viper venom that activates factor X (RVV-X)[2] was purified from crude *Vipera russeli* venom by the method of Schiffman et al. (1969) or Kisiel et al. (1976).

Barbital, urethane, imidazole (grade I), morpholinoethanesulfonic acid (Mes), *N*-acetylneuraminic acid, Coomassie brilliant blue R, galactosamine, dithiothreitol, aldolase, bovine serum albumin, bovine carbonic anhydrase, and myoglobin were obtained from Sigma Chemical Co., St. Louis, Mo. Sodium sulfate, sodium arsenate, and 4-vinylpyridine were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. The 4-vinylpyridine was vacuum distilled before use. Periodic acid was a product of Frederick Smith Chemical Co., Columbus, Ohio. 2-Thiobarbituric acid, 2-mercaptoethanol, *N,N*-methylenebisacrylamide, iodoacetic acid, and *N,N,N,N'*-tetraethylenediamine were purchased from Eastman Kodak Co., Rochester, N.Y. Agarose was a product of

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[1] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] Abbreviations used: RVV-X, protease from Russell's viper venom that activates factor X; DFP, diisopropyl phosphorofluoridate; Tris, tris(hydroxymethyl)aminomethane; Mes, morpholinoethanesulfonic acid.