The Interaction of Glutamic–Aspartic Transaminase with Pseudo Substrates[1]

BY GORDON G. HAMMES AND PAOLO FASELLA[2]

RECEIVED JULY 25, 1963

Equilibrium and kinetic studies have been made of the interaction of the aldehydic form (E_L) of glutamic–aspartic transaminase (aspartate amino transferase) with hydrogen ion, hydroxylamine, ketoglutarate, and oxalacetate. Spectrophotometric methods and the temperature jump technique were primarily employed. A pK of 6.25 was measured for the transformation of the enzyme between a yellow and colorless form. The rate constant for the protolytic association is abnormally small, indicating the solvent structure around the acidic group in question is considerably perturbed. Hydroxylamine forms a complex, presumably an oxime, with both forms of the enzyme, the binding constant for the interaction with the protonated enzyme being much larger. The rate constants for this process are similar to those of the enzyme–substrate reactions. The keto acids, ketoglutarate and oxalacetate, also form complexes with both forms of the enzyme. Again the protonated enzyme interacts much more strongly with the pseudo substrates. However, the binding constants are considerably smaller than those for Schiff base formation with substrates and hydroxylamine. The formation of these keto acid complexes is essentially diffusion controlled. The relevance of these findings to the enzymatic mechanism is discussed.

Introduction

In a previous publication, the results of a kinetic investigation of the enzyme glutamic–aspartic transaminase were reported.[3] The experimental method employed, namely the temperature jump,[4] permitted the use of high enzyme concentrations ($>10^{-5}$ M) so that reaction intermediates could be directly observed. At pH 8.0 (0.16 M phosphate buffer) and 25° the minimal mechanism, together with the values of the rate constants consistent with all of the data, is

$$E_L + As \underset{>5 \times 10^3\ sec.^{-1}}{\overset{>10^7\ M^{-1}\ sec.^{-1}}{\rightleftharpoons}} X_1 \underset{26\ sec.^{-1}}{\overset{80\ sec.^{-1}}{\rightleftharpoons}} X_2 \underset{7 \times 10^7\ M^{-1}\ sec.^{-1}}{\overset{1.4 \times 10^2\ sec.^{-1}}{\rightleftharpoons}} E_M + Oa \quad (1)$$

$$E_M + Kg \underset{70\ sec.^{-1}}{\overset{2.1 \times 10^7\ M^{-1}\ sec.^{-1}}{\rightleftharpoons}} Y_2 \underset{61\ sec.^{-1}}{\overset{30\ sec.^{-1}}{\rightleftharpoons}}$$

$$Y_1 \underset{3.3 \times 10^7\ M^{-1}\ sec.^{-1}}{\overset{2.8 \times 10^3\ sec.^{-1}}{\rightleftharpoons}} E_L + Gm$$
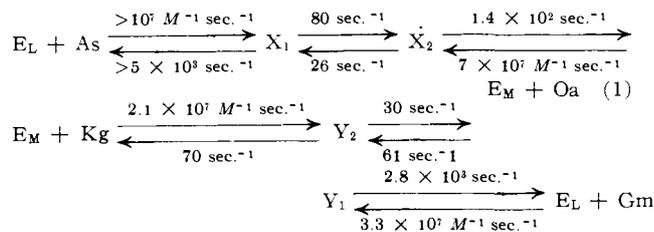
Here E_L designates the pyridoxal form of the enzyme, E_M is the pyridoxamine form, As is aspartate, Oa is oxalacetate, Kg is ketoglutarate, Gm is glutamate, and the X's and Y's are intermediate complexes. The spectral characteristics of the reaction intermediates are consistent with their being Schiff bases.

In order to shed further light on this mechanism, the interaction of the aldehydic form of glutamic–aspartic transaminase (E_L) with several pseudo substrates, $H^+(aq)$, hydroxylamine, ketoglutarate, and oxalacetate, has been investigated and the results are reported here. Although several workers have described the equilibrium properties of these systems previously,[5–8] a complete quantitative interpretation of the pH dependence was not presented. This information is necessary if a correlation with the enzymatic mechanism is to be attempted. Kinetic studies of these systems have not been carried out previously. The results obtained permit some speculations to be made concerning the details of the reaction mechanism.

Experimental

Materials.—The preparation of pure glutamic–aspartic transaminase was exactly as previously described.[3,9] The keto acids

(1) This work was supported in part by the U. S. Army Signal Corps, Air Force Office of Scientific Research, and Office of Naval Research, and in part by the National Institutes of Health. A preliminary report of this work was presented at the I. U. B. Symposium on Pyridoxal Catalysis, October, 1962, Rome, Italy.

(2) Istituto di Chimica Biologica, University of Rome, Rome, Italy.

(3) G. G. Hammes and P. Fasella, *J. Am. Chem. Soc.*, **84**, 4644 (1962).

(4) G. Czerlinski and M. Eigen, *Z. Elektrochem.*, **63**, 652 (1959).

(5) W. T. Jenkins and I. W. Sizer, *J. Am. Chem. Soc.*, **79**, 2655 (1957).

(6) W. T. Jenkins and I. W. Sizer, *J. Biol. Chem.*, **234**, 1179 (1959).

(7) S. F. Velick and J. Vavra, *ibid.*, **237**, 2109 (1962).

(8) O. L. Polyanovskii and Yu. M. Torchinskii, *Dokl. Akad. Nauk SSSR (Biochem.)*, **141**, 488 (1961).

were obtained from Calbiochem and hydroxylamine from Eastman Kodak Co. All other chemicals were standard reagent grade.

Determination of Binding Constants.—All of the binding constants were determined spectrophotometrically using a Beckman DU or DK2 spectrophotometer with the cell compartment being thermostated at $25.0 \pm 0.1°$. In the case of the interaction between E_L and $H^+(aq)$ the change in absorbancy at 430 and 362 mμ as a function of pH was used to determine the ionization constant. In this case $E_L H^+$ has a maximum absorption at 430 mμ and E_L at 362 mμ. The interaction of keto acids with enzyme can be studied by measuring the absorption spectra of the system as a function of pH and keto acid (KA) concentration. By saturating the enzyme with keto acid at different pH values, it can be shown that the spectrum of $E_L KA$ is essentially the same as that of E_L, while that of $E_L HKA^+$ is quite similar to that of $E_L H^+$: the i osbestic point for the change caused by keto acid binding to the protonated enzyme is at 415 mμ for the enzyme–oxalacetate system and at 406 mμ for the enzyme–ketoglutarate system. The presence of hydroxylamine produces changes in the spectrum of the enzyme.[7,10] The complex formed has a spectral peak at 370 mμ which can be used to measure the extent of complex formation. The total enzyme concentrations employed ranged from 6×10^{-5} to 1.2×10^{-4} M, the total hydroxylamine concentrations from 1×10^{-6} to 1×10^{-3} M, the ketoglutarate concentrations from 1×10^{-4} to 1×10^{-2} M, and the oxalacetate concentrations from 4.3×10^{-4} to 4.4×10^{-2} M.

The pH measurements were made on a Radiometer TTT1 pH meter and the hydrogen ion concentration was calculated, when necessary, by using the average value, 0.79, of known activity coefficients at the appropriate ionic strength.

Kinetic Measurements.—All of the systems under consideration were also studied with the temperature jump method. The experimental procedures and methods of analyzing the data have already been presented.[3] The experimental error in the relaxation times is about $\pm 10\%$.

Results and Calculations

A spectrophotometric pH titration at 430 and 362 mμ in 0.1 M KNO$_3$ at 25° permitted determination of the ionization constant

$$K_A = (E_L H^+)/(E_L)(H^+) = 10^{6.25} \quad (2)$$

Temperature jump experiments in the pH range 5.6–6.7 revealed a single relaxation time strongly dependent on pH, but independent of enzyme concentration. An example of the relaxation effect observed is shown in Fig. 1. When the solution is strongly buffered, rapid proton-transfer reactions cause the relaxation effect to occur too fast for measurement of the relaxation time. If the reaction mechanism is depicted as

$$E_L + H^+ \underset{k_{-H^+}}{\overset{k_{H^+}}{\rightleftharpoons}} E_L H^+ \quad (3)$$
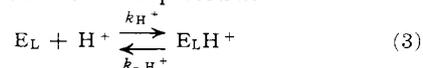
then the reciprocal relaxation time, $1/\tau$, is[3,11]

$$1/\tau = k_{H^+} \bar{C}_{H^+} + k_{-H^+} \quad (4)$$

(This equation is valid when $\bar{C}_{H^+} \ll \bar{C}_{E_L}$ and $\bar{C}_{E_L H^+}$,

(9) H. Lis, *Biochim. Biophys. Acta*, **28**, 191 (1958).

(10) I. W. Sizer and W. T. Jenkins, "Methods of Enzymology," S. P. Colowick and N. Kaplan, Ed., Vol. 5, Academic Press, Inc., New York, N. Y., 1962, p. 677.

(11) M. Eigen, *Z. Elektrochem.*, **64**, 115 (1960).
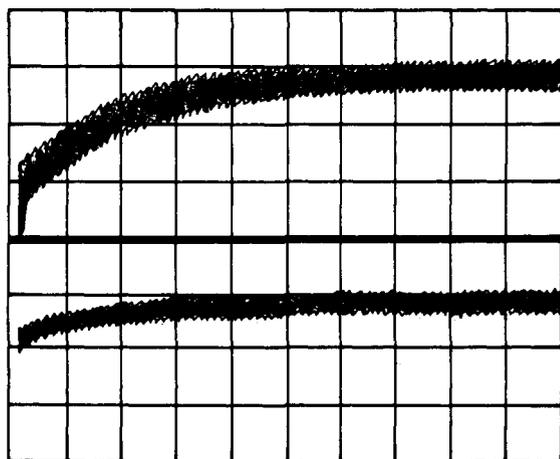
Fig. 1.—Tracings of oscilloscope displays of relaxation effects. Top: interaction of H^+ and E_L, pH 6.25, ΣE_L 2.07 $\times$ 10^{-5} M, 0.1 M KNO_3, 25°; the horizontal scale is 1 msec./division; the vertical scale is in arbitrary units proportional to the absorbancy; λ = 430 mμ, τ = 1.6 msec. Bottom: interaction of NH_2OH and E_L, pH 7.9, ΣE_L 9.6 $\times$ 10^{-6} M, ΣNH_2OH 8 $\times$ 10^{-6} M, 0.16 M phosphate buffer, 25°; the horizontal scale is 10 msec./ division; the vertical scale is in arbitrary units proportional to the absorbancy; λ = 410 mμ, τ = 12 msec.

i.e., some buffering occurs due to other protein acid–base groups so that the over-all hydrogen ion concentration remains almost constant.) The bar designates equilibrium concentrations. In Fig. 2 the observed relaxation time is plotted *vs.* $\bar{C}_{H^+}$, and from this graph, which is linear in conformity with eq. 4, the following rate constants ($\pm 25\%$) can be obtained.

$$k_{H^+} = 5.4 \times 10^8 \; M^{-1} \, \text{sec.}^{-1}$$
$$k_{-H^+} = 240 \; \text{sec.}^{-1}$$

The ratio of these rate constants gives a pK_A of 6.35 which is in satisfactory agreement with the equilibrium measurements.

In the case of the keto acid–E_L interactions, the minimal mechanism consistent with the spectrophotometric titrations of enzyme with keto acid in the pH range 5–8 is

$$H^+ + E_L + KA \underset{}{\overset{K_1}{\rightleftarrows}} E_LKA + H^+ \qquad (5)$$
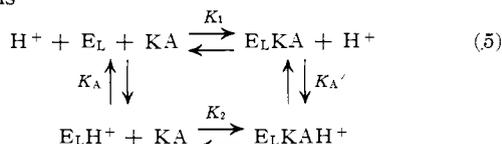
$$K_A \updownarrow \qquad\qquad\qquad \updownarrow K_A{}'$$

$$E_LH^+ + KA \underset{}{\overset{K_2}{\rightleftarrows}} E_LKAH^+$$

where KA designates either ketoglutarate or oxalacetate and

$$K_1 = (E_LKA)/(E_L)(KA) \qquad (6)$$
$$K_2 = (E_LKAH^+)/(E_LH^+)(KA)$$
$$K_A{}' = (E_LKAH^+)/(E_LKA)(H^+)$$

The absorbancy, a, at the E_LH^+–E_LHKA^+ isosbestic point, i, is $a_i = [(E_LH^+) + (E_LHKA^+)]\epsilon_{E_LH^+} + [(E_L) + (E_LKA)]\epsilon_{E_L}$ where the ϵ's are extinction coefficients which can be directly determined. Here $\epsilon_{E_LH^+} = \epsilon_{E_LHKA^+}$ and $\epsilon_{E_L} = \epsilon_{E_LKA}$ within experimental error. (The latter extinction coefficients are quite small.) Also mass conservation requires that

$$(E)_{Total} = (E_L) + (E_LKA) + (E_LH^+) + (E_LHKA^+)$$

By solving the above equations simultaneously the ratio R can be calculated

$$R = \frac{(E_LH^+) + (E_LHKA^+)}{(E_L) + (E_LKA)} = \frac{(E_LH^+)[1 + K_2(KA)]}{(E_L)[1 + K_1(KA)]} =$$
$$K_A(H^+) \frac{1 + K_2(KA)}{1 + K_1(KA)}$$

An additional check on the calculation of R can be made in a similar way by measurement of the absorbancy at 362 mμ.
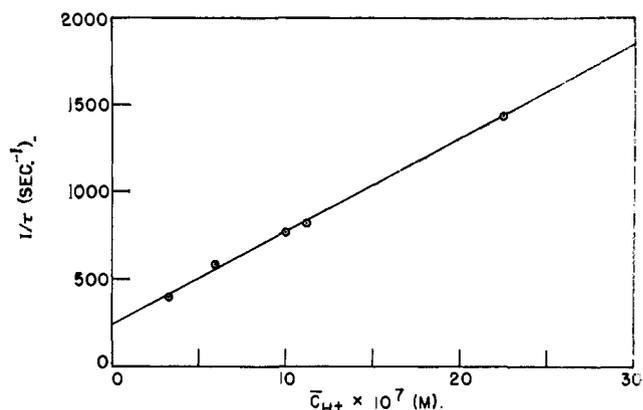
Fig. 2.—Plot of the reciprocal relaxation time *vs.* $\bar{C}_{H^+}$ for the H^+–E_L interaction. See text for experimental conditions.

If K_A, (H^+), and (KA) can all be determined independently, which is usually the case, K_1 and K_2 can be determined by studying R as a function of keto acid concentration. This assumes $(KA) \gg (E)_{total}$; if this approximation is not valid, a method of successive approximations can be used to obtain K_1, K_2, and (KA). Once K_1, K_2 and K_A are known, $K_A{}'$ can be calculated since $K_A{}' = K_AK_2/K_1$. At least nine values of R were determined for each keto acid. All permutations of these R values were solved simultaneously to obtain the binding constants. In order to min.mize buffer effects, R was usually determined at a constant pH of 6.8. However, in the case of ketoglutarate, four different pH's were investigated; no systematic changes in the constants at different pH's were observed. The average constants obtained over the pH range 5–8 at 25°, 0.16 M phosphate buffer, are assembled in Table I. The constants K_1 are quite small relative to K_2; therefore these values should be regarded as upper bounds only. Although the average deviations were from 5 to 10%, the keto acid binding constants are probably only accurate to $\pm 20\%$ if one takes into account possible errors in the extinction coefficients and the enzyme ionization constant.

Temperature jump experiments were then attempted to measure the rate constants characterizing the interactions between keto acids and E_L. Buffered solutions were used so that the protonic equilibria were adjusted rapidly ($\tau < 10^{-6}$ sec.). Surprisingly, no concentration-dependent relaxation effects were observed over a considerable range of keto acid concentrations ($\sim 10^{-4}$ to 10^{-2} M). A concentration independent relaxation effect was observed at high oxalacetate concentrations ($>6 \times 10^{-3}$), but this is probably related to the oxalacetate keto–enol equilibrium since no such effects were found with ketoglutarate.

The determination of the binding constant between hydroxylamine and E_L is rather difficult because of the large value of the binding constant. The apparent binding constant is defined as

$$K_{app} = \frac{(E_LNH_2OH) + (E_LHNH_2OH^+)}{(E_L)(NH_2OH)}$$

at high pH's (>8) and as

$$K_{app} = \frac{(E_LHNH_2OH^+)}{(E_LH^+)[(NH_2OH) + (NH_3OH^+)]}$$

at low pH's (<5.5).

The enzyme was titrated spectrophotometrically with hydroxylamine by measuring the absorbancy change at either 390 (high pH's) or 370 and 430 mμ (low pH's).[7] The binding constant was first calculated assuming the free hydroxylamine concentrations were equal to the total hydroxylamine concentrations. A method of successive approximations was then used to arrive at

TABLE I
ENZYME–KETO ACID BINDING CONSTANTS[a]

	Ketoglutarate	Oxalacetate
K_A (M^{-1})	1.8×10^6	1.8×10^6
K_A' (M^{-1})	$\geq 3.6 \times 10^7$	$\geq 2.1 \times 10^7$
K_1 (M^{-1})	$\leq 1.0 \times 10^2$	$\leq 7.0 \times 10^1$
K_2 (M^{-1})	2.0×10^3	8.0×10^2

[a] $25°$, 0.16 M phosphate buffer.

final values for the free hydroxylamine concentrations and the binding constants. In order to obtain a true measure of the relative binding strength of E_L and E_LH^+, the fact that hydroxylamine becomes protonated at lower pH's and that the complex is protonated at relatively high pH's must be taken into account. The pK of hydroxylamine at $25°$, 0.2 M KNO$_3$, measured by pH titration is 6.10. Assuming only the neutral species of hydroxylamine is reactive, and calculating the constant at high pH's in terms of the nonprotonated species only, a constant K_{act}, independent of pH, can be determined. All of the pertinent constants are assembled in Table II. The maximum error in the constants is estimated as $\pm 20\%$.

TABLE II
ENZYME–HYDROXYLAMINE BINDING CONSTANTS

Enzyme species	pH	K_{app} (M^{-1})	K_{act} (M^{-1})
E_L	8.00^a	9.0×10^4	6.0×10^4
E_LH^+	5.00^b	1.1×10^6	1.5×10^6
E_LH^+	5.37^b	2.3×10^6	1.5×10^6

[a] $25°$, 0.16 M phosphate buffer. [b] $25°$, 0.2 M acetate buffer.

A concentration-dependent relaxation time was found at $25°$, pH 7.9, 0.16 M phosphate buffer, in the hydroxylamine–enzyme system. A sample oscilloscope tracing of the relaxation effect is given in Fig. 1.

Assuming the mechanism

$$H^+ + E_L + NH_2OH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_LNH_2OH + H^+ \qquad (7)$$
$$\uparrow \downarrow$$
$$E_LH NH_2OH^+$$
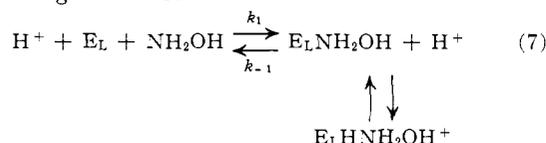
and that the hydrogen ion concentration is buffered, the relaxation time can be evaluated[10] and is

$$1/\tau = k_1[(\bar{E}_L) + (\overline{NH_2OH})] + k_{-1}/[1 + K_A'(\bar{H}^+)] \qquad (8)$$

where

$$K_A' = (E_LH NH_2OH^+)/(E_LNH_2OH)(H^+) = 4.0 \times 10^7$$

The equilibrium concentrations can be calculated using the binding constant determined spectrophotometrically. A plot of $1/\tau$ vs. $[(\bar{E}_L) + (\overline{NH_2OH})]$ is shown in Fig. 3 and from this plot the rate constants can be determined

$$k_1 = 3.7 \times 10^6 \ M^{-1} \ sec.^{-1}$$
$$k_{-1}/[1 + K_A'(H^+)] = 38 \ sec.^{-1}$$
$$k_{-1} = 62 \ sec.^{-1}$$

These rate constants are probably precise to about $\pm 25\%$ and their ratio is consistent with the equilibrium measurements within experimental error. Unfortunately, a kinetic study of this reaction could not be carried out at low pH's because of rapid decomposition of either NH$_2$OH or the E_L–NH$_2$OH complex in the temperature jump cell, probably catalyzed by the platinum electrodes.

Mechanistic Implications.—Before attempting to correlate the above results with available data concerning the enzymatic mechanism, it should be pointed out that these "model" systems may be completely unrelated to the enzymatic mechanism so that the conclusions reached must be viewed with considerable caution.

The combination of E_L with a proton is characterized by a second-order rate constant considerably smaller than the value of $\sim 10^{10}$ M^{-1} sec.$^{-1}$ found for normal
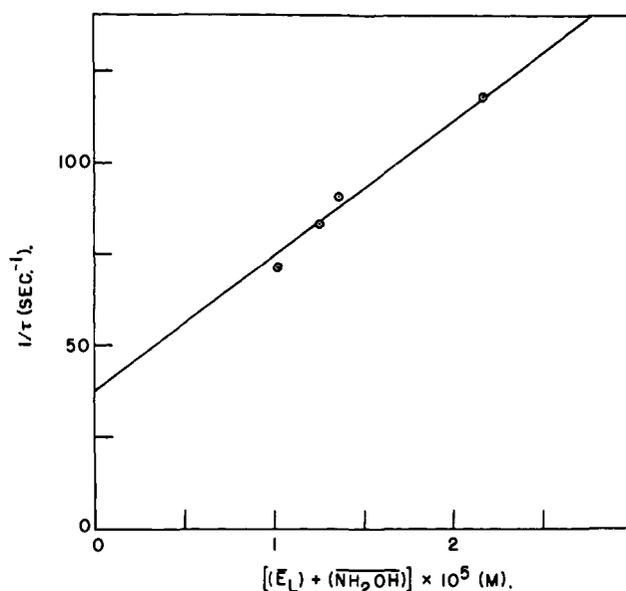
Fig. 3.—Plot of the reciprocal relaxation time vs. $\bar{E}_L + \overline{NH_2OH}$ for the NH$_2$OH–E_L interaction. The total enzyme concentration was equal to 9.6×10^{-6} M for all points; the total hydroxylamine concentration was 4.0×10^{-6}, 8.0×10^{-6}, 1.2×10^{-5}, and 2.4×10^{-5} M, respectively, for each point proceeding from left to right on the graph. See text for other experimental conditions.

acids.[10] In model systems, abnormally small protolytic rates are observed if the base is stabilized in some manner, for example by hydrogen bonding, or if the solvent structure around the base is substantially perturbed from that of liquid water, for example by the presence of a number of charged groups.[12] Another possibility in the case of a macromolecule is that a conformational change is the rate-determining step in the over-all process under consideration. At the present time the various possibilities cannot be distinguished, but all of these explanations indicate that the structure around the active site is quite rigidly fixed and/or the solvent structure is appreciably different from that of the bulk phase.

The identification of the acidic group under consideration is not certain. Initially it was identified as the phenolic group on pyridoxal phosphate[5]; however, more recent evidence[13-15] from model systems suggests that the ring and imine nitrogens are also likely sites for protonation in the same pH range. In addition, a protein group which strongly interacts with the coenzyme is a possibility. At the present time, a definite answer to this problem cannot be given. Perhaps the pH dependence of fluorescence, which should reflect the state of the ring nitrogen, will clarify this issue.[16]

The binding of the keto acids to the enzyme has several interesting aspects. The fact that the observed relaxation time for the E_L–oxalacetate system is independent of oxalacetate concentration means that it cannot be related to a bimolecular step in the mechanism of complex formation. However, the possibility does exist that this relaxation time is related to a unimolecular step in the mechanism of complex formation or that a protein conformation change is being measured. The fact that a similar effect was not observed with ketoglutarate makes this possibility unlikely. Two explanations for the absence of a concentration-

(12) M. Eigen, *Angew. Chem.*, **75**, 489 (1963).
(13) C. C. Johnston, H. G. Brooks, J. P. Albert, and D. E. Metzler "I. U. B. Symposium on Pyridoxal Catalysis," Pergamon Press, in press.
(14) A. E. Martell, *ibid.*
(15) I. W. Sizer and W. T. Jenkins, *ibid.*
(16) P. Fasella, in preparation.

dependent relaxation effect exist: (1) the effect is too small to observe or (2) the relaxation time is too short to be measured. Although an unequivocal choice cannot be made, the first explanation seems improbable because a large change in optical density was observed due to either protolytic reactions or complex formation or both ($\tau < 10^{-6}$ sec.). The amplitude of the change was dependent on keto acid concentration; moreover, such a perturbation of protolytic equilibria should produce an observable relaxation effect for the complex formation. Assuming the second alternative, a lower bound for the second-order rate constant can be calculated from the conventional relaxation time

$$k > 1/\tau[(KA) + 1/K_2]$$

Here the constant K_2 has been used since the greatest lower bound can be estimated for low pH's. Conservative estimates of τ (<2 μsec.) and $[(KA) + 1/K_2]$ ($<10^{-3}$ M) yield a value of k greater than 5×10^8 M^{-1} sec.$^{-1}$. This is quite close to the rate constant expected for a diffusion-controlled reaction ($\sim 10^9$ M^{-1} sec.$^{-1}$).[17,18]

The keto acids bind much more strongly to the protonated form of the enzyme than to the nonprotonated form.[6] However, even with the protonated enzyme, the binding constants for the keto acids are considerably smaller than the corresponding constants for Schiff base formation between them and the aminic form of the enzyme, these latter constants being about 10^5 M^{-1}. Coupling the above evidence with the fact that the rates of E_L–keto acid complex formation are essentially diffusion controlled suggests that these complexes may represent intermediate steps in the mechanism between the state consisting of free enzyme and substrates and that of Schiff bases. These intermediate complexes probably represent protein–substrate interactions, as contrasted to substantial coenzyme–substrate interaction in the case of Schiff bases. Recent rotary dispersion measurements support this idea.[19] In the actual transamination, the protein–substrate complexes would be present at concentrations small compared to those of the Schiff base intermediates and therefore could not be directly observed.

The results obtained with hydroxylamine as a pseudo substrate indicate quite clearly that both E_L and E_LH^+ form Schiff bases, and, in fact, E_LH^+ forms a much more stable Schiff base. The possible generality of this conclusion is strengthened by the results of Jenkins, Orlowski, and Sizer, who found that isonicotinic acid hydrazine formed a more stable hydrazone with E_LH^+ than with E_L by a factor of more than ten.[20] Note that the rate constant for Schiff base formation is only slightly smaller than those for the actual substrates.

The paramount question now is what happens when the amino acid is involved in Schiff base formation? In model systems, an amino acid with a neutral amino group is probably the reactive species since it is difficult to see how any chemistry can happen without the unpaired electrons on the nitrogen being free. However, in the case of Schiff base formation with an en-

zyme, the possibility exists that the protonated amino acid reacts with the enzyme and that a rapid intramolecular proton transfer occurs before Schiff base formation. Deciding unequivocally which form of the amino acid reacts to give the initial enzyme–substrate complex is virtually impossible at the present time. However, the steady state study of Velick and Vavra[7] suggests that the amino acid with a protonated amino group may be the reactive species. The Michaelis constant for glutamic acid (calculated on the basis of the RNH_3^+ concentration) varied only fourfold in the pH range 5–9. If RNH_2 were the reactive species, a variation of about 10^4 would be obtained unless a fortuitous cancellation from some ionizable groups in the enzyme occurred. However, in the case of ketoglutarate, where this complication is missing, the Michaelis constant only varies a factor of seven over this same pH range. If a comparison between the amino acids and hydroxylamine is valid, the presence of a protonated amino group on the substrate lowers the binding constant considerably.

Assuming either the protonated or nonprotonated amino group (but not both) to be the reactive species, two plausible mechanisms for each half reaction can be written on the basis of the available data

(1)
$$E_L + AA \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons X_3 \rightleftharpoons X_4 \rightleftharpoons E_M + KA$$
$$\updownarrow \qquad\qquad \updownarrow \qquad \updownarrow$$
$$E_LH^+ + AA \rightleftharpoons X_1H^+ \rightleftharpoons X_2H^+$$

(2)
$$E_L + AA \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons X_3 \rightleftharpoons X_4 \rightleftharpoons E_M + KA$$
$$\updownarrow$$
$$E_LH^+$$
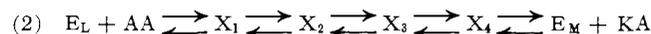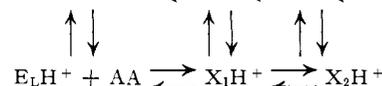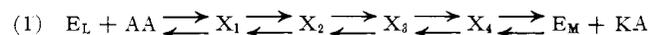
Here X_1, X_1H^+, and X_4 represent protein–substrate interactions, in the sense previously discussed, X_2 and X_3 are Schiff bases, AA is the amino acid, and KA is the keto acid. The first mechanism attributes the apparent lack of catalytic activity of E_LH^+ to its inability to catalyze the interconversion of Schiff bases, while the second mechanism attributes this inactivity to the inability of E_LH^+ and AA to form a Schiff base. (Actually the possibility also exists that the apparent inactivity of E_LH^+ is related to the keto acid inhibition which is much greater for E_LH^+ than for E_L.) To our knowledge, Schiff base formation between E_LH^+ and an initially protonated amino group has not been demonstrated. Although such a reaction was not noted for E_LH^+ and NH_3OH^+, this cannot be taken as positive evidence against Schiff base formation since if the binding constant were much less than that for Schiff base formation between E_LH^+ and NH_2OH, it would not be detected.

At first glance, one might expect that the pH dependence of the steady-state kinetic parameters would be different for the two mechanisms proposed. Actually the observed pH dependence for the two mechanisms can be identical providing certain not unreasonable inequalities exist among the rate constants which prevail in mechanism 1. However, this ambiguity will not prevail in nonsteady-state experiments and experiments are now under way that should decide which of the proposed mechanisms best represents the actual situation.

(17) P. Debye, *Trans. Electrochem. Soc.*, **82**, 265 (1942).
(18) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).
(19) P. Fasella and G. G. Hammes, *Biochem.*, in press.
(20) W. T. Jenkins, S. Orlowski, and I. W. Sizer, *J. Biol. Chem.*, **234**, 2657 (1959).