A Temperature Jump Study of Aspartate Aminotransferase. A Reinvestigation*

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ABSTRACT: The interaction of the α subform of aspartate aminotransferase with its substrates has been studied with the temperature jump technique. Three relaxation times are associated with the reaction of the enzyme and each amino–keto acid pair, glutamate–ketoglutarate and aspartate–oxalacetate. This implies at least two reaction intermediates exist in each half-reaction; since spectral evidence suggests even more than two intermediates are present, the kinetic results are discussed in terms of a mechanism involving an arbitrary number of intermediates. The concentration dependence of the longest relaxation time for each half-reaction permits determination of specific rate and binding constants. Individual rate constants could not be obtained from the other relaxation times, but lower bounds of 10^7–10^8 M^{-1} sec^{-1} and 10^5–10^6 sec^{-1} can be estimated for the rate constants associated with the bimolecular and dissociation processes involving amino acids; the corresponding lower bounds for the keto acids are 10^8 M^{-1} sec^{-1} and 10^4 sec^{-1}. The wavelength dependence of the amplitudes of the relaxation effects over the range 300–500 mμ was also investigated. The results obtained suggest intermediates with spectral peaks at 360, 430, and 490 mμ occur on one side of the slowest step in each half-reaction, while a spectral peak at 330 mμ is associated with intermediates on the other side.

The data obtained with the temperature jump method are consistent with equilibrium constants measured spectrophotometrically and with the results of steady-state kinetic studies.

Several years ago (Hammes and Fasella, 1962) we presented the results of a kinetic study of the transamination reaction catalyzed by aspartate aminotransferase, primarily by use of the temperature jump method. The mechanism proposed was

$$\begin{aligned} E_L + As &\rightleftarrows X_1 \rightleftarrows X_2 \rightleftarrows E_M + Oa \\ Kg + E_M &\rightleftarrows Y_2 \rightleftarrows Y_1 \rightleftarrows E_L + Gm \end{aligned} \qquad (1)$$

where E_L is the pyridoxal form of the enzyme, E_M is the pyridoxamine form, As is aspartate, Gm is glutamate, Oa is oxalacetate, and Kg is ketoglutarate. Many of the individual rate constants in the above mechanism were derived from the six relaxation times observed and the results were correlated with stopped-flow and equilibrium spectral measurements. Unfortunately since that time, it has become apparent that what was thought to be a pure enzyme by all available criteria, actually consists of several subforms (Martinez-Carrion *et al.*, 1965). Also the temperature jump apparatus has been greatly improved. The over-all sensitivity is greater and a considerably wider range in the wavelength of the light used for detecting concentration changes is possible. Therefore, it was decided to reinvestigate the kinetics of the transaminase reaction using the pure α enzyme (the most active subform) and an improved temperature jump apparatus. The results obtained are qualitatively similar to those of our earlier work in that at least six relaxation processes are observed. However, the values of the individual rate constants are considerably greater than those previously reported. Also the wavelength dependence of the amplitude of the relaxation processes indicates that eq 1 is too simple to be consistent with all of the experimental observations.

Experimental Section

The temperature jump apparatus used was essentially that previously described (Hammes and Steinfeld, 1962). However, the optics has been improved and a tungsten–iodide lamp with a quartz envelope (General Electric Airport Lamp) was employed so that the reaction could be monitered at wavelengths as short as 300 mμ.

The α subform of cytoplasmatic aspartate aminotransferase (EC 2.6.1.1.) was prepared as previously described (Martinez-Carrion *et al.*, 1965). Amino and keto acids were obtained from Calbiochem. All other reagents were standard analytical grade.

The concentration of the pyridoxal enzyme was calculated from the pyridoxal phosphate absorbancy at 388 mμ in 0.1 N NaOH using a molar extinction coefficient of 6550 M^{-1} cm^{-1}. This procedure is valid because the enzyme has never been treated with pyridoxal phosphate. The aminic form of the enzyme was

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TABLE I: Summary of Temperature Jump Results.[a]

Initial Reaction Mixture	Relaxation Times (msec)	Sign of Absorbancy Change 330 mμ	360 mμ	430 mμ	490 mμ	Tentative Interpretation
E_L + AA	<0.1	None	−	+	+	$E_L + AA \rightleftarrows X_1 \ldots \rightleftarrows X_{l-1}$
	1–10	+	−	−	−	$X_{l-1} \rightleftarrows X_l$
E_L + AA (constant) + KA (varied)	<0.1	None	−	+	+	$E_L + AA \rightleftarrows X_1 \ldots \rightleftarrows X_{l-1}$
	1–10	+	−	−	−	$X_{l-1} \rightleftarrows X_l$
	0.1–0.7	None	+	−		$X_n \rightleftarrows E_M + KA$ and/or $E_L + KA \rightleftarrows E_LKA$
E_L + KA	<0.3	None	+	−		$E_L + KA \rightleftarrows E_LKA$
E_M + KA	1–10(?)	None	−(?)	−(?)		$X_{l-1} \rightleftarrows X_l$

[a] Abbreviations used: E_L, pyridoxal enzyme; E_M, pyridoxamine enzyme; AA, amino acid; KA, keto acid; X, reaction intermediates.

TABLE II: Initial Concentrations and Relaxation Times.

10^4 E_L (M)	10^4 As (M)	10^5 Oa (M)	τ (msec) Obsd	τ (msec) Calcd	10^4 E_L (M)	10^4 Gm (M)	10^4 Kg (M)	τ (msec) Obsd	τ (msec) Calcd
0.98	2.60	—	3.4	3.2	1.0	2.57	—	8.8	8.8
1.19	6.67	—	2.1	2.0	1.0	5.38	—	6.1	6.3
0.96	25.0	—	1.7	1.5	1.18	10.2	—	3.8	4.3
1.18	75.6	—	1.2	1.1	1.0	26.0	—	2.2	2.5
0.475	127	—	1.2	1.2	0.508	50.5	—	1.8	2.1
0.95	255	—	1.1	0.98	1.16	110.0	—	1.2	1.1
0.86	500	—	0.90	0.97	1.0	53.0	—	2.0	1.8
0.80	62.5	—	1.3	1.3	0.80	53.0	1.10	1.7	1.6
0.80	62.5	3.34	0.93	1.1	0.80	53.0	2.27	1.2	1.4
0.80	62.5	6.68	1.0	1.1	0.915	516	—	0.94	0.52
0.80	62.5	14.2	0.85	0.89					

obtained as described by Jenkins and D'Ari (1966).

The general procedure was to mix the aldimine or the pyridoxamine form of the enzyme with varying amounts of the substrates in pyrophosphate buffer. The pyrophosphate buffer was made by titrating tetrasodium pyrophosphate with HCl. The final buffer concentration and pH in the enzyme–substrate solutions were 0.2 M and pH 8.0, respectively. The equilibrium mixtures containing the various forms of the enzyme and substrates were thermostatted at 17° and temperature jumps of 8° were applied to the solution. About 3 min was allowed to elapse between pulses in order to reestablish thermal equilibrium in the system. Each relaxation effect was photographed several times and the relaxation times were evaluated from the slopes of plots of the logarithm of the amplitude *vs.* time. The amplitudes of the relaxation processes were calculated by extrapolating the logarithmic plot to zero time and dividing the zero-time amplitude by the signal change in going from 0 to 100% transmission; this is equal to 2.303 times the change in absorbancy for such small changes. The absorption spectrum of each reaction mixture before and after the temperature jump experiments was determined with a Beckman DK2A recording spectrophotometer provided with a cell holder thermostatted at 25°. The spectra of various enzyme–substrate mixtures essentially corresponded to those published in the literature (Jenkins and D'Ari, 1966; Jenkins and Taylor, 1965).

Results

As in the previous investigation, the over-all transamination reaction could be divided into the two half-

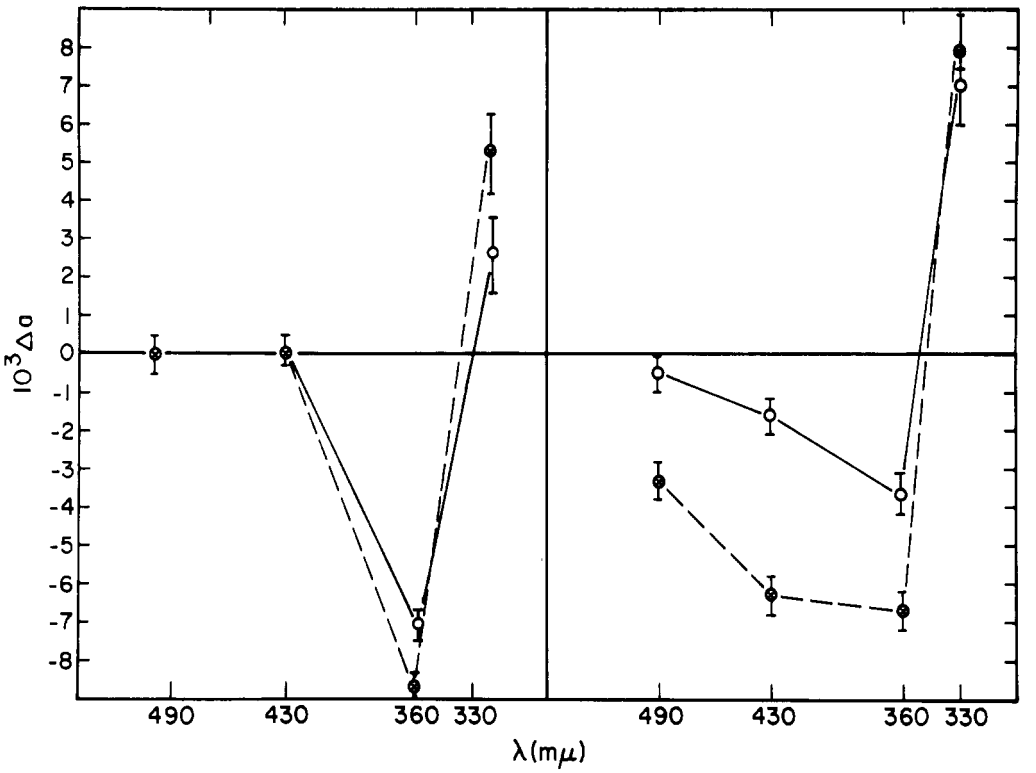

FIGURE 1: Plot of the amplitude at zero time of the slowest relaxation process (Δa) *vs.* wavelength (λ) for aspartate–oxalacetate (right) and glutamate–ketoglutarate (left). The total enzyme concentration was $\sim 1 \times 10^{-4}$ M. For aspartate–oxalacetate, the initial aspartate concentration was 2.6×10^{-4} (O) and 5.0×10^{-2} M (⊗). For glutamate–ketoglutarate, the initial glutamate concentration was 1.0×10^{-3} (O) and 1.10×10^{-2} M (⊗); no effects could be detected at 430 and 490 mμ at these concentrations. This amplitude corresponds to that associated with the slowest process and is considerably smaller than the *total* change in absorbance at a given wavelength; in some cases it is even of the opposite sign.

reactions of eq 1 and the interaction of each amino acid–keto acid pair with the enzyme was studied separately. No relaxation effects were observed in the absence of substrates with either the pyridoxal or pyridoxamine enzyme. Relaxation times were measured by monitoring the reaction with light of wavelengths 330, 360, 370, 430, and 490 mμ. A qualitative summary of the results is given in Table I. When amino acid was added to pyridoxal enzyme, two relaxation processes could be discerned. One was associated with a relaxation time of less than 100 μsec, but the concentration and wavelength dependence of this process could not be quantitatively measured. The relaxation time of the second process became progressively shorter as the amino acid concentration was raised (at constant enzyme concentration) until a limiting value was reached. The measured relaxation times associated with the second process are assembled in Table II along with the total enzyme and substrate concentrations employed. The values of the relaxation times were the same at all wavelengths; however, the amplitude of the relaxation process did depend on the particular wavelength used for observation. Typical results for the wavelength dependence of the amplitude are shown in Figure 1. In addition it was noted with aspartate that a fast initial change in absorbance was observed which was of opposite signs for light of wavelengths 360 and 430 mμ (Table I, line 1). Experiments were also carried out where the amino acid concentration was kept constant and the corresponding keto acid concentration was varied. As keto acid was added a new relaxation process could be discerned which was associated with a shorter relaxation time. This relaxation time could not be measured with great precision because of the difficulty in separating it from the larger longer effect, but it ranged from several hundred to about one hundred microseconds, becoming progressively shorter as the keto acid concentration was raised. The change in absorption was of opposite signs when the reaction progress was monitered with light of wavelengths 430 and 360 mμ (Table I, line 5). The longer relaxation time appeared to be reaching a limiting value at high keto acid concentrations; however, comprehensive measurements could not be made because the interaction of the keto acid with the pyridoxal enzyme at high keto acid concentrations (Fasella and Hammes, 1963) obscured the relaxation process. The results obtained are included in Table II. Although the interaction of keto acid with enzyme is quite rapid at

low pH values (Fasella and Hammes, 1963) measurements on a solution containing only pyridoxal enzyme and keto acid at pH 8.0 in 0.16 M pyrophosphate buffer indicate that a process associated with a relaxation time of about 10^{-4} sec occurs. The relaxation times associated with the transamination process were not evaluated under conditions where the interaction between keto acid and pyridoxal enzyme was appreciable (keto acid concentration $>\sim 10^{-4}$ M). The estimated error in the relaxation times given in Table II is about $\pm 15\%$.

Temperature jump experiments were also carried out on solutions of pyridoxamine enzyme with successive additions of keto acids. However no usable results were obtained, apparently because a very small amount of keto acid shifts the equilibrium almost entirely to the pyridoxal side. The amino acid concentration is then so low that it is not bound appreciably to the pyridoxal enzyme. At sufficiently high keto acid concentrations, the keto acid–pyridoxal enzyme interaction again becomes of importance. A summary of the range of relaxation times observed and the signs of the associated amplitudes is given in Table I.

Discussion

We first consider the slowest relaxation time observed for each half-reaction. This must be related to the rate-controlling step in the mechanism since otherwise this reaction would serve as an indicator for slower processes. Spectral evidence suggests that reaction intermediates with spectral peaks at 430, 360, 490, and 330 mμ are probably involved in each half-reaction (*cf.* Fasella *et al.*, 1966). The wavelength dependence of the amplitude of the relaxation process, namely the sign of the absorbance change is positive at 330 mμ and negative at 360, 430, and 490 mμ (Figure 1), suggests that the intermediates with spectral peaks at 430, 360, and 490 mμ are on one side of the rate-controlling step, while intermediates with spectral peaks at 330 mμ are on the other side. It is impossible to ascertain whether or not more than one intermediate is associated with the different spectral peaks. A quantitative analysis of the amplitudes is not possible because of the large experimental errors, the unknown extinction coefficients of the intermediates, and the probable overlap of the spectral bands of the intermediates. In particular, the absorption bands of the species absorbing at high wavelengths overlap appreciably with each other and with the absorption band at 330 mμ whereas the converse is not true. Therefore, if the extinction coefficients of the intermediates are similar the amplitude of the relaxation process at 330 mμ would be expected to be less than the sum of the amplitudes observed at 490, 430, and 360 mμ. This is in agreement with the observed results (*cf.* Figure 1). The equilibrium spectra indicate that for the aspartate–oxalacetate system an appreciable amount of intermediates absorbing at 430 and 490 mμ are present, whereas very small amounts of these intermediates are present for the glutamate–ketoglutarate system (Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966). This is in agreement with the amplitudes shown in Figure 1. The amplitude is appreciable at 430 and 490 mμ for the aspartate–oxalacetate system whereas for glutamate–ketoglutarate the amplitudes were usually too small to measure at these wavelengths.

Strong evidence exists for the occurrence of intermediates with spectral peaks at 490, 430, and 360 mμ on one side of the rate-limiting step. The substrate analogs, α-methylaspartate and α-methylglutamate, which can form Schiff bases with the enzyme but cannot transaminate, form a complex with the enzyme which has absorption peaks at 430 and 360 mμ (Fasella *et al.*, 1966; P. Fasella, A. Giartosio, G. G. Hammes, and D. L. Miller, submitted for publication). In the case of α-methylaspartate the ratio of the absorption at 430 and 360 mμ is approximately one, whereas for α-methylglutamate the absorption at 360 mμ is considerably greater than that at 430 mμ. This behavior parallels that found for the real substrates, as described above. Evidence for the existence of an intermediate having an absorption peak at 490 mμ comes from the work of Jenkins (1961). When β-hydroxyaspartate is added to the pyridoxal enzyme, an intermediate with a spectral peak at 490 mμ is rapidly formed which decomposes slowly to give the keto acid and the pyridoxamine enzyme. An intermediate with a spectral peak at 330 mμ is present with the natural substrates (Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966). The wavelength dependence of the amplitude suggests that very little of an intermediate with an absorption peak at 330 mμ, if any, is present on the same side of the rate-limiting step as the intermediates having absorption peaks at 490, 430, and 360 mμ.

The fact that the relaxation time approaches a limiting value at high amino and keto acid concentrations at constant enzyme concentration (*cf.* Table I) indicates that the relaxation process is intramolecular. If the reaction involved second-order or higher order reactions, the relaxation time would become progressively shorter as the amino and keto acid concentrations are raised. A general mechanism for the half-reaction is

$$E_L + AA \rightleftarrows X_1 \rightleftarrows \cdots X_{l-1} \underset{k_{-l}}{\overset{k_l}{\rightleftarrows}} X_l \cdots \rightleftarrows X_n \rightleftarrows E_M + KA \quad (2)$$

where the rate constants associated with the rate-limiting step are k_l and k_{-l} and the number of reaction intermediates on each side of the rate-limiting step is arbitrary. If the reaction $X_{l-1} \rightleftarrows X_l$ is slow relative to all other reactions (as implied by the data) the reciprocal of the slowest relaxation time is given by eq 3 where the K_i's are equilibrium constants: $K_1 = (X_1)/(E_L)\cdot(AA)$, $K_2 = (X_2)/(X_1)$, etc. This equation can be rewritten to give eq 4 where the starred constants are defined by comparison of eq 3 and 4. If $n = 2$, $k_l^* = k_l$, $k_{-l}^* = k_{-l}$, $K_{AA}^* = 1/K_1$, and $K_{KA}^* = K_3$; thus if only two intermediates are present, the rate constants for the slow step and the binding constants for the amino and keto acids can be

$$1/\tau = \frac{k_l \Big/ \left(1 + \sum_{j=2i=j}^{l-1\,l-1} \prod 1/K_i\right)}{1 + \dfrac{\left(\prod_{i=1}^{l-1} 1/K_i\right) \Big/ \left(1 + \sum_{j=2i=j}^{l-1\,l-1} \prod 1/K_i\right)}{(E_L) + (AA)}} + \frac{k_{-l} \Big/ \left(1 + \sum_{j=l+1i=l+1}^{n\quad j} \prod K_i\right)}{1 + \dfrac{\left(\prod_{i=l+1}^{n+1} K_i\right) \Big/ \left(1 + \sum_{j=l+1i=l+1}^{n\quad j} \prod K_i\right)}{(E_M) + (KA)}} \quad (3)$$

$$1/\tau = \frac{k_l^*}{1 + \dfrac{K_{AA}^*}{(E_L) + (AA)}} + \frac{k_{-l}^*}{1 + \dfrac{K_{KA}^*}{(E_M) + (KA)}} \quad (4)$$

determined. If $n > 2$, the starred rate constants are lower bounds for the actual rate constants associated with the slow step, $K_{AA}^* = (E_L)(AA)/\sum_{i=1}^{l-1}(X_i)$ and $K_{KA}^* = (E_M)(KA)/\sum_{i=l}^{n}(X_i)$. Thus the concentration dependence of the relaxation times should in principle, uniquely determine four independent constants. However, a number of auxilary data are available, which considerably restrict the values of the constants. In particular the constants must be consistent with the over-all binding constants for the amino and keto acids (Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966) and the ratio of the steady-state maximum velocities (Velick and Vavra, 1962; Banks *et al.*, 1963). The equilibrium dissociation constants reported by Jenkins *et al.* (1965, 1966) are defined as $(E_L)(AA)/\sum_{i=1}^{n}(X_i)$ and $(E_M)(KA)/\sum_{i=1}^{n}(X_i)$ in terms of the mechanism of eq 2 and can be used to calculate the equilibrium concentrations of pyridoxal enzyme, pyridoxamine enzyme, amino acid, keto acid, and the sum of the reaction intermediates for all reaction mixtures. The values of the dissociation constants used are 3×10^{-3}, 3×10^{-5}, 1×10^{-2}, and 5×10^{-4} M for aspartate, oxalacetate, glutamate, and ketoglutarate, respectively. (Jenkins and co-workers determined these constants at somewhat different ionic strengths than employed in this study; however, we have performed spectral titrations which indicate the binding constants do not vary significantly with pyrophosphate concentration, 20–50% maximally between 0.05 and 0.2 M pyrophosphate. Therefore we have used the rounded off values given above.) With these constants the number of independent constants is reduced from four to two for each half-reaction. The ratio of the steady-state maximal velocities, $V_f:V_r$, is about 2.5 if glutamate is the starting amino acid (Velick and Vavra, 1962; Banks *et al.*, 1963). The absolute magnitudes of the turnover numbers cannot be used as criteria since these numbers have not been precisely determined for the pure α subform of the enzyme. With the above restrictions, only three independent constants are left to fit the concentration dependence of the relaxation times for both halves of the reaction. The best values of the constants were determined by a trial and error procedure so as to minimize the deviation between experimentally determined relaxation times and those calculated by use of the assumed constants. The best values of the constants are assembled in Table III and the relaxation times calculated with these constants are included in Table II. The agreement between the calculated and experimental relaxation times is within experimental error (±15%) with the exception of the relaxation time at the highest glutamate concentration. No obvious reason for this single discrepancy can be given and this point was not used in the final fitting of the data, although inclusion of this point would not significantly alter the values of the constants given in Table III. The experimental error in these constants is estimated as about ±25%. The turnover numbers calculated from the constants of Table III are 920 and 370 sec^{-1} for the reaction starting with glutamate and aspartate, respectively. These numbers are higher than any previously reported values (Velick and Vavra, 1962; Banks *et al.*, 1963) which is not too surprising since all of the steady-state work was done with an enzyme containing an inactive subform. The results reported here are in reasonable accord with the results of experiments reported by Banks (1965) which employed amino acids with deuterium in the α position. Although the starred rate constants are only lower bounds for the rate constants associated with the rate-determining step, they are among the largest found for rate-determining steps in enzymatic reactions (Eigen and Hammes, 1964). Finally the relative amounts of the reaction intermediates are qualitatively consistent with the spectral data discussed earlier.

TABLE III: Summary of Kinetic Parameters.[a]

	Aspartate–Oxalacetate	Glutamate–Ketoglutarate
k_l^* (sec^{-1})	530	2940
k_{-l}^* (sec^{-1})	1330	1170
K_{AA}^* (M)	4.2×10^{-3}	3.5×10^{-2}
K_{KA}^* (M)	1.05×10^{-4}	7.0×10^{-4}

[a] Temperature 25°, 0.2 M sodium pyrophosphate, pH 8.0.

The existence of at least two other relaxation processes was detected. One of these, observed at low amino acid concentrations, may be related to the binding of amino acid to enzyme but could not be quantitatively measured. At relatively high amino acid concentrations

($>10^{-3}$ M) a very fast change in absorbancy ($\tau < 10$ μsec) can be seen which goes in opposite directions at 360 and 430 mμ (Table I). This may be related to the biomolecular reaction of amino acid and enzyme or to the interconversion of intermediates. In any event it permits the establishment of lower bounds for the second-order rate constants, namely these rate constants must be greater than 10^7–10^8 M^{-1} sec^{-1}; the dissociation rate constants must be greater than 10^5–10^6 sec^{-1}. (These bounds are obtained by assuming the reciprocal relaxation time for the bimolecular process is 10^5 $sec^{-1} < 1/\tau = (kC_{AA} + k')$ and $k/k' = 10^1$–10^2 M^{-1}, where k is the bimolecular rate constant, k' is the dissociation rate constant, and $C_{AA} = 10^{-3}$ M.) Furthermore, since the relaxation times associated with all intramolecular steps to the left of the rate-determining step of eq 2 must be less than about 10^{-5} sec, the individual rate constants are probably greater than 10^4 sec^{-1}. (This estimate depends on the equilibrium constants of the various reactions, and in fact, is somewhat conservative.) The exact nature of the one or more relaxation processes observed when keto acid is added to the reaction mixture is obscured by the fact that the binding of keto acid to the pyridoxal enzyme causes a detectable relaxation effect. The wavelength dependence of the amplitude suggests keto acid binding to pyridoxal enzyme. Alternatively the observed relaxation process may be related to the interaction of keto acid with the pyridoxamine enzyme. If the former is the case, the relaxation time associated with the reaction between pyridoxamine enzyme and keto acid must be shorter than the observed relaxation time since otherwise the process being observed would serve as an indicator for the pyridoxamine–keto acid interaction. Thus in either case, lower bounds for the bimolecular and dissociation rate constants associated with the binding of keto acid to pyridoxamine enzyme can be estimated by use of the procedure outlined above for the amino acid–pyridoxal enzyme reaction. Since relaxation times of approximately 10^{-4} sec are observed at keto acid concentrations of about 10^{-4} M, the second-order rate constants must be greater than 10^8 M^{-1} sec^{-1} and the dissociation rate constants must be greater than about 10^4 sec^{-1} for oxalacetate and ketoglutarate. Presently no way seems to exist for reliably determining the individual rate constants. However, the lower bounds for the second-order rate constants already approach the maximum possible value of approximately 10^9 M^{-1} sec^{-1} (Alberty and Hammes, 1958). A summary of the mechanistic interpretations of the various relaxation processes observed is included in Table I.

The rate constants (and lower bounds) are considerably larger than reported in our earlier work (Hammes and Fasella, 1962). This is due to a number of different factors. A nonhomogeneous enzyme was used in the earlier work; this can give rise to a spectrum of closely overlapping relaxation times and would be expected on the average to give rise to somewhat slower apparent rates. The sensitivity of the temperature jump apparatus has been improved by approximately fourfold over that of the instrument used in the earlier study (primarily by use of a better light source). This permits better resolution of overlapping relaxation times and allows a wider range of substrate concentrations to be investigated. In the earlier work, phosphate buffer was used which interacts with the enzyme–substrate mixtures to produce relaxation effects which obscure those due to enzyme–substrate interactions. Because of the improved resolution, the fastest two relaxation processes detected in the earlier work have been shown to consist of several processes; moreover the wavelength dependence of the amplitudes, which could not be determined previously, and the concentration dependence of the relaxation times indicates these steps are not simply related to the bimolecular steps in the mechanism as previously assumed. Earlier attempts to study the interaction of keto acids with the pyridoxal enzyme were mainly carried out at low pH values where the binding constants are more favorable and the rates were too fast to observe (Fasella and Hammes, 1963). In the present work considerably lower keto acid concentrations could be employed at pH 8 (in a different buffer) and a small relaxation effect was observed. In view of the low binding constants, it is rather surprising that a relaxation effect could be detected at keto acid concentrations as small as 10^{-4} M and this system probably warrants further investigation. For the reasons cited above the previous temperature jump investigation of the mechanism of action of aspartate aminotransferase is superceded by the results reported here.

The results presented here are consistent with the detailed chemical mechanism previously proposed (Fasella *et al.*, 1966). However, the occurrence of a primary isotope effect in the rate-controlling step when deuterium is substituted for hydrogen on the α carbon of the amino acid reported by Banks (1965) is difficult to reconcile with this mechanism. It is apparent that the mechanism of enzymic transamination is extremely complex and the elementary steps are very rapid. The temperature jump results indicate at least two reaction intermediates (and almost certainly more) are involved in each half-reaction, *i.e.*, the reaction given by eq 2. Also the wavelength dependence of the amplitudes establishes which spectral peaks are to be associated with reaction intermediates on each side of the rate-determining step. All existing equilibrium and kinetic data are now reasonably consistent. Unfortunately much of the older data were obtained with a nonhomogeneous enzyme so only semiquantitative agreement can be expected.

The most profitable approach for obtaining further mechanistic information appears to be kinetic studies of the interaction of pseudo substrates with aspartate aminotransferase since the reaction rates are considerably slower than with the natural substrates. Such an investigation with β-hydroxyaspartate has already been reported (Czerlinski and Malkewitz, 1964), but unfortunately the enzyme used was nonhomogeneous and the wavelength dependence of the absorption changes was not investigated. A kinetic study employing α-methylaspartate as substrate is now in progress. The precise role of ionizable groups and the protein in the enzymic catalysis remain to be assessed.

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