A Kinetic Investigation of the Interaction of *erythro*-β-Hydroxyaspartic Acid with Aspartate Aminotransferase*

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ABSTRACT: Temperature-jump, stopped-flow, and spectrophotometric techniques have been used to study the kinetics of the interaction of *erythro*-β-hydroxyaspartic acid with aspartate aminotransferase. Eleven relaxation times were observed in a time range of <10 μsec–>1 day.

Experiments were monitored at 492, 460, 430, 362, 332, and 310 mμ in order to determine the wavelength dependence of each step. Eight of the relaxation times are inferred to be directly related to the transamination reaction, two are probably characteristic of product decomposition, and one is believed to be associated with a nonproductive side reaction. A simple sequential reaction mechanism is proposed involving seven different intermediates, and the associated rate constants have been calculated by use of a computer analysis of the data. The spectral and structural features of some of the intermediates are inferred from the data. The proposed mechanism involves multiple conformational states of aldimine, quinoid, and ketimine structures. The quinoid intermediate has an extinction coefficient at 490 mμ greater than 10^5 M^{-1} cm^{-1}. The rate-determining step is tentatively assigned to the hydrolysis or a conformational change of the ketimine. These results imply that the mechanism of enzymatic transamination involves at least 16 discrete steps. The occurrence of many reaction intermediates may be an important characteristic of enzymatic catalysis.

The mechanism of action of aspartate aminotransferase has been intensively studied by a number of investigators utilizing many different techniques (*cf.* Fasella, 1967). An understanding of the reaction mechanism requires information about the number and nature of significant reaction intermediates. Such information can be obtained by kinetic investigations at high enough enzyme concentrations so that the reaction intermediates can be detected directly. A temperature-jump investigation of the interaction of the natural substrates with aspartate aminotransferase indicated that the interconversion of reaction intermediates occurs very rapidly (Fasella and Hammes, 1967). For the reaction of aspartate or glutamate with the pyridoxal enzyme to give oxalacetate or ketoglutarate and pyridoxamine enzyme, a single kinetic process occurs in times longer than 10^{-4} sec; although a number of reaction intermediates are implied to be present from spectral data (Fasella *et al.*, 1966), apparently the interconversion of many of the intermediates occurs in times less than 10^{-4} sec. The difficulty of measuring the kinetic properties of such short-lived reaction intermediates can be alleviated by studying the interaction of the enzyme with modified substrates which react at considerably slower rates than the natural substrates. Groups in either the α or β positions of amino acid substrates have marked effects on the rates and types of reactions catalyzed by the enzyme (Jenkins, 1964; Fasella *et al.*, 1966; Hammes and Haslam, 1968; Manning *et al.*, 1968). For example, α-methylaspartic acid reacts with the pyridoxal enzyme to form a Schiff base, but transamination cannot occur because of the methyl group in the α position. A kinetic study of this reaction has shown that at least three distinct enzyme–substrate complexes are involved in Schiff base formation, and the associated rate constants have been obtained (Hammes and Haslam, 1968).

The substance *erythro*-β-hydroxyaspartic acid serves as a substrate for aspartate aminotransferase although the rate of transamination is considerably slower than that of the natural substrates (Jenkins, 1961). The equilibrium characteristics of the interaction between the pyridoxal enzyme and *erythro*-β-hydroxyaspartic acid have been extensively studied by Jenkins (1961, 1964); a temperature-jump investigation of this system has also been made (Czerlinski and Malkewitz, 1964). However, the temperature-jump study was carried out with a nonhomogeneous enzyme and the reaction progress was only monitored at a single wavelength, 505 mμ. In this work, a kinetic study of the interaction between the α subform of aspartate aminotransferase and *erythro*-β-hydroxyaspartic acid has been carried out using temperature-jump, stopped-flow, and spectrophotometric techniques. The reaction progress was monitored by following absorption changes at many wavelengths in the spectral region 300–500 mμ. Eleven relaxation processes have been observed and the associated relaxation times for ten of these have been measured as a function of enzyme and substrate concentrations. Eight of these relaxation processes appear

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to be directly related to the enzymatic reaction; one probably is associated with a reaction not on the catalytic pathway; and two are associated with product decomposition.

A quantitative analysis of the data according to a simple sequential mechanism containing seven reaction intermediates permits the calculation of specific rate constants and determination of the spectral properties of some of the intermediates. The interpretation of the results is quite different from that previously proposed on the basis of less extensive data (Czerlinski and Malkewitz, 1964). A detailed chemical mechanism can be postulated which is consistent with the available experimental data.

Experimental Section

The α subform of cytoplasmic aspartate aminotransferase (EC 2.6.1.1) was prepared from fresh pig hearts as previously described (Martinez-Carrion *et al.*, 1965, 1967). Enzyme concentrations were determined spectrophotometrically: a molar extinction coefficient of 8.2×10^3 M^{-1} cm^{-1} at 362 mμ and pH 8.5 was used (Martinez-Carrion *et al.*, 1965). Enzyme concentrations ranged from 0.2×10^{-4} to 1.0×10^{-4} M in coenzyme. DL-*erythro*-β-Hydroxyaspartic acid was obtained from Calbiochem (lot 511234) as was dihydroxymaleic acid (dihydroxyfumaric acid) (lot 104482). Thin-layer chromatography of *erythro*-β-hydroxyaspartic acid on silica gel in various ethanol–water mixtures gave a single ninhydrin spot under conditions where it is well resolved from aspartic acid. All other chemicals were standard reagent grade.

The temperature-jump apparatus used was a combined stopped flow temperature jump (Erman and Hammes, 1966; Faeder, 1969). This combination apparatus was employed so that the enzyme and substrate (~0.15 ml of each) could be mixed just prior to applying a temperature jump to the system. The stopped-flow apparatus used was a Durrum-Gibson stopped-flow spectrophotometer. A Varian Model G-14A-2 chart recorder was used to record the course of reaction for the process associated with τ_9. The input for the recorder was taken from a Tektronix Model 1A7 plug-in unit combined with a Model 549 oscilloscope. A Zeiss Model PMQII spectrometer and a Cary 14 recording spectrophotometer were used to determine the binding constants and the kinetics for the processes associated with τ_{10} and τ_{11}. The pH measurements were made using a Radiometer Model 26 pH meter. All experiments were carried out at 25° in 0.1 M sodium pyrophosphate titrated to pH 8.0 with HCl.

Results and Treatment of Data

Relaxation times were calculated from plots of the logarithm of the absorbancy *vs.* time. Usually the absorbancy changes were sufficiently small so that the change in transmission was directly proportional to the change in absorbancy; however in some stopped-flow experiments the absorbancy changes were sufficiently large so that the transmission changes had to be converted to absorbancy changes before plotting the data. In the neighborhood of equilibrium the change in absorbancy, Δa, is equal to $\sum_i C_i e^{-t/\tau_i}$ where the C_i are constants and the τ_i are relaxation times (*cf.* Amdur and Hammes, 1966). Ten relaxation times were found to be necessary to describe the data. In addition, a fast absorbancy change was observed ($\tau < 10$ μsec), which implied the occurrence of an eleventh relaxation process. In most cases, the relaxation times were sufficiently different that they could be easily separated simply by selecting the appropriate time range on the oscilloscope. In some cases, the relaxation times had to be resolved graphically (*cf.* Hammes and Hubbard, 1966), and in a few cases resolution of individual relaxation times was not possible, as will be discussed below. The changes in absorbancy were monitored at 310, 332, 362, 430, 460, and 492 mμ.

Table I lists the eleven relaxation times observed in mixtures of *erythro*-β-hydroxyaspartic acid and enzyme,

TABLE I: Summary of Relaxation Times.

τ_i	λ (mμ)	Δ^a	Method[b]	Range $1/\tau$ (sec^{-1})
1	430	D	TJ	$1.3–2 \times 10^4$
	362	I	TJ	
2	430	D	TJ	$5.5–6.5 \times 10^3$
	362	I	TJ	
3	492	D	TJ	$1–10 \times 10^2$
	430	D	TJ	
4	492	I	SF	$1–4.5 \times 10^2$
	430	I	SF	
	362	D	SF	
	362	I	TJ	
5	492	I	TJ	$1.8–3.1 \times 10^3$
	332	D	TJ	
6	492	I	TJ	$>1.5 \times 10^5$
	460	D	TJ	
7	492	D	SF	1–2.5
	430	D	SF	
	362	D	SF	
	332	I	SF	
8	332	I	TJ	$2–5.5 \times 10^2$
	310	D	TJ	
9	492	D	SF	$3–5.1 \times 10^{-2}$
	430	D	SF	
	362	D	SF	
	332	I	SF	
10	492	D	CZ	2.0×10^{-4}
	430	D	CZ	
	362	D	CZ	
	332	I	CZ	
11	492	D	CZ	$\sim 8 \times 10^{-6}$
	430	D	CZ	
	362	D	CZ	
	332	I	CZ	

[a] Change in absorbancy: I = increase, D = decrease. [b] TJ = temperature jump, SF = stopped flow, and CZ = Cary 14 or Zeiss Model PMQII.

the wavelengths at which they were observed, the method used, and the range in $1/\tau$ observed as the concentration of substrate was changed. Plots of eight of the relaxation times as a function of substrate concentration are shown in Figures 1–6. Two of the relaxation times, τ_{10} and τ_{11}, are concentration independent, and as previously mentioned one of the relaxation times, τ_6, was too short to measure. The concentration of the enzyme was always much lower than that of the substrate, so that the abscissas of Figures 1–6 show only the concentration of L-*erythro*-β-hydroxyaspartic acid. The D form of the substrate was assumed not to interact with the enzyme since D-amino acids have never been found to bind to the enzyme. The experimental uncertainties in $1/\tau$ are about $\pm 10\%$ except for $1/\tau_2$ and $1/\tau_5$ where they are about $\pm 20\%$. The results summarized in Table I and Figures 1–6 represent the analysis of more than 1200 kinetic traces.

The two longest relaxation times are clearly not involved in the enzymatic reaction since the turnover number for the enzyme-catalyzed transamination between ketoglutarate and *erythro*-β-hydroxyaspartic acid is known to be of the order of magnitude of 1 sec^{-1} (Jenkins, 1964). Moreover, τ_{10} and τ_{11} are independent of the enzyme and substrate concentrations. The product of the reaction, oxaloglycolate, is most stable in its enol form, dihydroxyfumarate, and dihydroxyfumarate is known to decarboxylate slowly (Hartree, 1953). Experiments with dihydroxyfumarate show a very slow decomposition reaction which can be observed at 290 mμ and has a relaxation time identical with τ_{11}. Although τ_{10} is not observed without enzyme, the enzyme could serve as an indicator for the formation of an intermediate in the product decomposition reaction. All other kinetic and equilibrium experiments were carried out in times short compared to τ_{10} and τ_{11} so that the relaxation processes associated with these times need not be considered further.

The concentration dependence of $1/\tau_1$ (Figure 1) is typical of that expected for a bimolecular reaction: $1/\tau_1$ increases continually with increasing concentration of substrate and the amplitude of the relaxation process decreases as the enzyme approaches saturation. Although the accessible concentration range is somewhat restricted, this relaxation time can be associated with the initial association–dissociation of enzyme and substrate. All other relaxation times approach a constant value at sufficiently high concentrations of *erythro*-β-hydroxyaspartate, which implies they are associated with intramolecular processes. The wavelength dependence of the amplitude of the relaxation process identified with τ_1 is the same as has been previously observed for the bimolecular step in the interaction of α-methylaspartic acid and aspartate aminotransferase (Hammes and Haslam, 1968). On the other hand no discernible concentration dependence is observed for $1/\tau_2$ (Figure 2). The amplitude of this relaxation process is small and can only be observed at 430 and 362 mμ.

Figure 3 shows the measured relaxation times from temperature-jump experiments at 492 and 430 mμ,

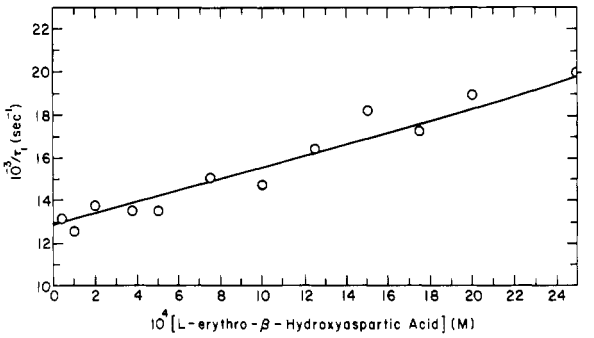

FIGURE 1: Variation of $1/\tau_1$ with L-*erythro*-β-hydroxyaspartic acid concentration. Circles are experimental values obtained at 430 and 362 mμ. The solid line is a theoretical line based on the mechanism of eq 1 and the rate constants in Table II.

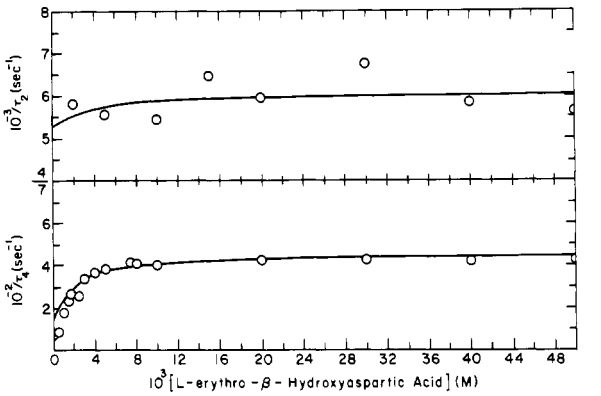

FIGURE 2: Variation of $1/\tau_2$ and $1/\tau_4$ with L-*erythro*-β-hydroxyaspartic acid concentration. Circles are experimental values obtained at 430 and 362 mμ for $1/\tau_2$ and 492, 430, and 362 mμ for $1/\tau_4$. The solid lines are theoretical lines based on the mechanism of eq 1 and the rate constants in Table II.

while the dashed line shows the relaxation times which are of a similar order of magnitude measured with the stopped-flow apparatus at 492, 430, and 362 mμ and with the temperature-jump apparatus at 362 mμ ($1/\tau_4$ of Figure 2). These measured relaxation times have been designated as τ_3 and τ_4; however it is evident that the actual relaxation times overlap so much at low substrate concentrations that a composite relaxation time is being measured, which is a weighted average of the two time constants. Only at concentrations of L-*erythro*-β-hydroxyaspartic acid greater than 10^{-2} M can the two relaxation times be clearly resolved. This overlapping of relaxation times also leads to an unusual concentration dependence of the amplitude of the relaxation process associated with "$1/\tau_3$": the amplitude is very large at low substrate concentrations and decreases at high concentrations. This is due to the close coupling of the relaxation times τ_3 and τ_4, and is also due to the fact that $1/\tau_5$ (Figure 4) has a relaxation time somewhat shorter than τ_3 with an associated absorption change at 492 mμ of opposite sign which increases in magnitude with increasing substrate concentration. Thus although τ_3, τ_4, and τ_5 are closely coupled at low substrate concentrations, the individual relaxation

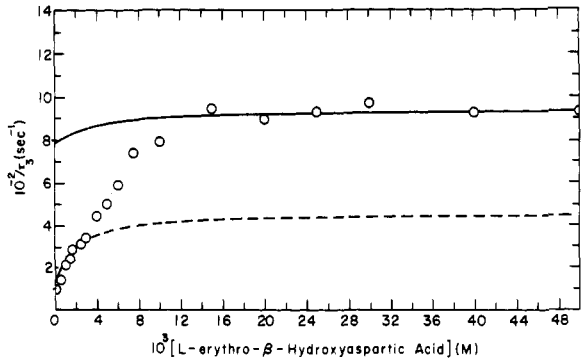

FIGURE 3: Variation of $1/\tau_3$ with L-*erythro*-β-hydroxyaspartic acid concentration. Circles are experimental values obtained at 492 and 430 mμ. The solid line is based on the mechanism of eq 1 and the rate constants in Table II. The dashed line is $1/\tau_4$ taken from Figure 2. See text for a discussion of this figure.

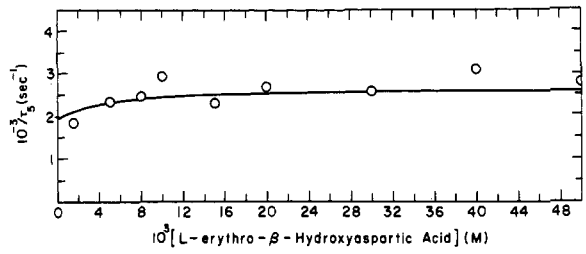

FIGURE 4: Variation of $1/\tau_5$ with L-*erythro*-β-hydroxyaspartic acid concentration. Circles are experimental points mainly at 492 mμ with a few also at 332 mμ. The solid line is a theoretical line based on the mechanism of eq 1 and the rate constants in Table II.

times can be clearly resolved at high concentrations ($>10^{-2}$ M). The relaxation time τ_5 was not observed at either 430 or 362 mμ, but a small effect was observed at 332 mμ in which the amplitude decreased. Unfortunately a slower relaxation process, associated with τ_7, has a much larger amplitude of opposite sign at 332 mμ making it very difficult to measure $1/\tau_5$ at this wavelength.

At substrate concentrations greater than 5×10^{-3} M a relaxation process was observed at 492 and 460 mμ which occurred in a time shorter than the apparatus resolution time. This process was observed only at these two wavelengths, and the amplitude was of opposite sign at 492 and 460 mμ.

Figure 5 shows the concentration dependence of τ_7 and τ_9. Both of these processes are observed by stopped-flow techniques; the absorbancy decreases at 492, 430, and 362 mμ and increases at 332 mμ. The amplitude of τ_7 was very large, while that of τ_9 was quite small. The amplitude of the relaxation process related to the relaxation time τ_8 (Figure 6) was observed to increase at 332 mμ and to decrease at wavelengths between 310 and 315 mμ; lower wavelengths are experimentally inaccessible because of the large absorption of the protein. This relaxation time could not be detected in a stopped-flow–temperature-jump experiment in which the temperature jump was applied 50 msec after mixing. This indicates that the relaxation process associated with $1/\tau_8$ must follow that associated with $1/\tau_7$.

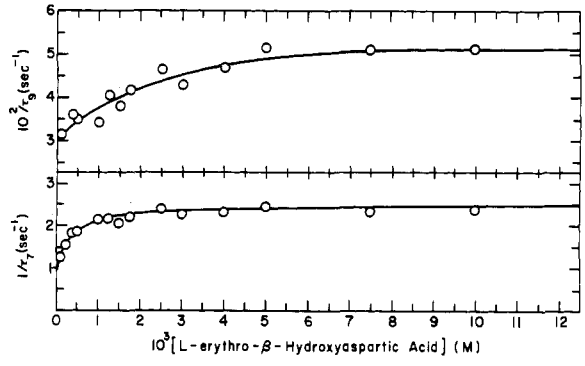

FIGURE 5: Variation of $1/\tau_7$ and $1/\tau_9$ with L-*erythro*-β-hydroxyaspartic acid concentration. Circles are experimental points at 492, 430, 362, and 332 mμ. The solid lines are smooth curves drawn through the points.

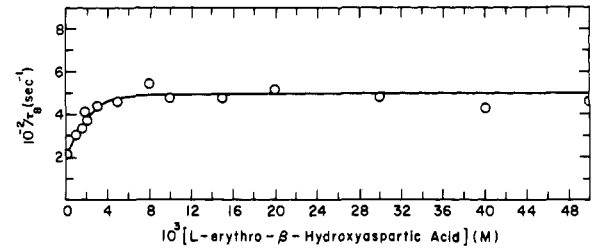

FIGURE 6: Variation of $1/\tau_8$ with L-*erythro*-β-hydroxyaspartic acid concentration. Circles are experimental points at 332 and 310–315 mμ. The solid line is a smooth curve drawn through the points.

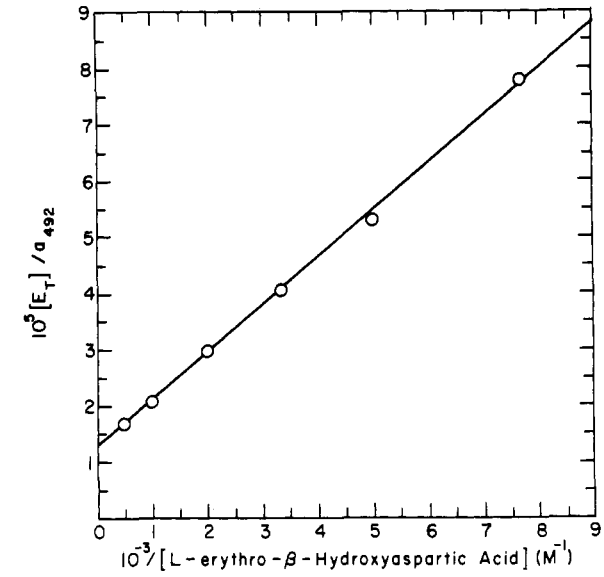

FIGURE 7: Plot of total enzyme concentration divided by the absorbance at 492 mμ in a 2-cm path-length cell *vs.* the reciprocal of the L-*erythro*-β-hydroxyaspartic acid concentration. The solid line is a least-squares fit of the data. See text for details.

Although a dependence of the enzymatic reaction upon the concentration of Mg^{2+} has been reported when the reaction progress was monitored at 290 mμ (Jenkins, 1964), the relaxation times τ_7 and τ_9–τ_{11} have been found to be independent of Mg^{2+} in solutions saturated with $MgCl_2$ (~0.03 M).

Two binding constants can be readily determined experimentally. One binding constant is defined as $K_B = \Sigma(X_i)/(E_L)(S)$ where $\Sigma(X_i)$ includes all reaction intermediates formed, E_L is the pyridoxal enzyme, and S is the substrate (the L form only). This constant was determined after the procedure of Jenkins (1964) and found to be 3.1×10^3 M^{-1}. This is somewhat smaller than the constant reported by Jenkins (1964), which was determined under different conditions. A second binding constant can be determined by measurement of the absorption at 492 mμ in the stopped-flow apparatus at times long relative to τ_5, but short relative to τ_7. This includes all of the reaction intermediates up to the relaxation process associated with τ_7. This binding constant can be determined by the procedure previously employed for the determination of the binding constant between aspartate aminotransferase and α-methylaspartate (Hammes and Haslam, 1968). A plot of $(E_T)/a_{492}$ *vs.* $1/(S)$ is shown in Figure 7. The intercept is equal to $1/\epsilon_{492}l$ and the slope is $K_A/\epsilon_{492}l$, where ϵ_{492} is the apparent extinction coefficient of the intermediates at 492 mμ, l is the path length, and K_A is the binding constant. The constant K_A has a value of 1.55×10^3 M^{-1} and ϵ_{492} is equal to 39,500 M^{-1} cm^{-1}.

Because of the many relaxation times observed, the mechanism of the enzymatic reaction must be complex. A simple mechanism consistent with the data is given in eq 1, where E_L and E_M are the pyridoxal and pyridoxamine forms of the enzyme, S and S′ are L-*erythro*-β-hydroxyaspartate and oxaloglycolate, and the X_i are enzyme–substrate complexes. The very rapid

$$E_L + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X_3 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \begin{matrix} X_4' \\ \uparrow\downarrow \\ X_4 \end{matrix} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} X_5 \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} X_6 \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} X_7 \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} E_M + S' \quad (1)$$

process (τ_6) between an intermediate absorbing at 492 and one at 460 mμ is assumed to be associated with the reaction $X_4 \rightleftharpoons X_4'$. The placement of this reaction as a shunt rather than on the main pathway is arbitrary, but in any event X_4 and X_4' must be directly coupled since all relaxation effects observed at 492 mμ are also observed at 460 mμ. The kinetic analysis is identical in both cases. The rate constants k_4, k_{-4}, k_5, and k_{-5} represent the rate constants for breakdown and formation of $(X_4 + X_4')$. More complex schemes are certainly possible, but this is the simplest one we have been able to find which is consistent with all of the data.

The first five relaxation times are sufficiently close together that the rate constants for the individual steps are a function of all five relaxation times. The rate constants and the relaxation times for these five steps are related by eq 2 (Castellan, 1963). (The assumption has been made that $(S) \gg (E_L)$.) This determinant is a polynomial in $1/\tau$ which can be represented as

$$\begin{vmatrix} k_1(S) + k_{-1} - 1/\tau & -k_{-1} & 0 & 0 & 0 \\ -k_2 & k_2 + k_{-2} - 1/\tau & -k_{-2} & 0 & 0 \\ 0 & -k_3 & k_3 + k_{-3} - 1/\tau & -k_{-3} & 0 \\ 0 & 0 & -k_4 & k_4 + k_{-4} - 1/\tau & -k_{-4} \\ 0 & 0 & 0 & -k_5 & k_5 + k_{-5} - 1/\tau \end{vmatrix} = 0 \quad (2)$$

$$-(1/\tau)^5 + A(1/\tau)^4 - B(1/\tau)^3 + C(1/\tau)^2 - D(1/\tau) + E = 0 \quad (3)$$

where A, B, C, D, and E are various expressions involving the rate constants and substrate concentration. The coefficients of eq 3 are related to the roots of the equation by eq 4–8. According to these equations, a

$$\sum_{i=1}^{5} 1/\tau_i = A \quad (4)$$

$$\sum_{i=1}^{4} \sum_{j=i+1}^{5} 1/\tau_i \cdot 1/\tau_j = B \quad (5)$$

$$\sum_{i=1}^{3} \sum_{j=i+1}^{4} \sum_{k=j+1}^{5} 1/\tau_i \cdot 1/\tau_j \cdot 1/\tau_k = C \quad (6)$$

$$\sum_{i=1}^{2} \sum_{j=i+1}^{3} \sum_{k=j+1}^{4} \sum_{l=k+1}^{5} 1/\tau_i \cdot 1/\tau_j \cdot 1/\tau_k \cdot 1/\tau_l = D \quad (7)$$

$$\prod_{i=1}^{5} 1/\tau_i = E \quad (8)$$

plot of A, B, C, D, and E against substrate concentration should be a straight line. From the values of the five slopes and five intercepts and their relation to the rate constants through A, B, C, D, and E, the ten rate constants can be evaluated. Unfortunately all of the relaxation times cannot be reliably determined over the entire concentration range. Therefore, values of A, B, C, D, and E were constructed from the data, and a computer was used to solve the polynomial equation (eq 3) at various substrate concentrations to give theoretical values of $1/\tau_i$. Variation of the coefficients A, B, C, D, and E were then made and the rate constants were calculated from each set of coefficients until reasonable agreement between calculated and experimental relaxation times was obtained. The independently measured binding constant K_A was used as an additional constraint on the system. The lines of Figures 1–4 are theoretical curves calculated in this manner. The ten rate constants obtained from this analysis are given in Table II. The fit of the theoretical curves is quite good. The only marked exception is in

TABLE II: Summary of Rate and Equilibrium Constants.

i	k_i (sec^{-1})	k_{-i} (sec^{-1})	K_i	X_i (%)
1	3.10×10^6 M^{-1}	1.09×10^4	284 M^{-1}	9.2
2	1.58×10^3	2.18×10^3	0.725	6.6
3	2.09×10^3	1.55×10^3	1.35	9.0
4	1.29×10^3	5.95×10^2	2.17	19.4
5	1.91×10^2	6.28×10^2	0.304	5.9
6	10.36	1.98	5.23	30.9
7	1.90×10^2	3.10×10^2	0.613	19.0
A			1.55×10^3 M^{-1}	50.0
B			3.10×10^3 M^{-1}	100.0

the case of τ_3. The measured reciprocal relaxation time, $1/\tau_3$, approaches the calculated value of $1/\tau_3$ at high substrate concentrations and the calculated value of $1/\tau_4$ at low concentrations, and has some sort of average value at intermediate concentrations. This can be seen in Figure 3 which includes the theoretical curves for both $1/\tau_3$ and $1/\tau_4$. As previously discussed, in this instance the true relaxation time cannot be determined experimentally over the entire concentration range so that this behavior is not surprising.

The rate constants for the next two steps in eq 1 were calculated from the data in Figures 5 and 6 and the equilibrium constants K_A and K_B. If the reaction $X_6 \rightleftharpoons X_7$ equilibriates slowly relative to the reaction $E + S' \rightleftharpoons X_7$ and rapidly relative to the reaction $X_5 \rightleftharpoons X_6$, then

$$1/\tau_8 = k_7 + \frac{k_{-7}}{1 + \dfrac{k_8/k_{-8}}{(E_M) + (S')}}$$

This equation is of the correct form to be consistent with the data, but unfortunately k_8/k_{-8} and $(E_M) + (S')$ cannot be determined so that a detailed analysis of the curve cannot be made. However, the intercept of the curve at zero substrate concentration is k_7, and the limiting value of $1/\tau_8$ at high substrate concentrations is $k_7 + k_{-7}$ so that k_7 and k_{-7} can be calculated from the data in Figure 6. The values of the rate constants obtained from this analysis are included in Table II.

The value of K_6 can now be calculated from the known equilibrium constants (see Table II). This gives $k_6/k_{-6} = 5.25$. The slowest relaxation time for the mechanism of eq 1 is $1/\tau_7$. When the enzyme is saturated with substrates, (*i.e.*, $1/\tau_7$ is equal to its limiting value at high substrate concentrations), the reciprocal relaxation time can be written as (*cf.* Hammes and Schimmel, 1966; Fasella and Hammes, 1967)

$$1/\tau_7 = \frac{k_6}{1 + \sum_{i=2}^{5} \prod_{j=i}^{5} (1/K_j)} + \frac{k_{-6}}{1 + K_7}$$

where the K_j's are given in Table II. Values of k_6 and k_{-6} can be calculated from these relationships and the data in Figure 5 and are given in Table II.

Attempts were made to incorporate the relaxation time τ_9 into both the catalytic path and as a dead end or shunt in equilibrium with X_5, X_6, or X_7. (Involvement in catalysis seems quite unlikely in view of the length of τ_9.) All these schemes gave values of the equilibrium constants such that nearly one-half of the concentration of intermediates was in the form X_9 with only a very small concentration in X_7. This is inconsistent with the observed amplitudes of the relaxation effects since the amplitude of the process associated with τ_7 is very large and that associated with τ_9 is small. A possibility is that τ_9 is involved with reactions occurring after the formation of the pyridoxamine enzyme. Jenkins (1964) has shown that the pyridoxamine form of the enzyme binds *erythro*-β-hydroxyaspartic acid quite strongly. The reactions following the rate-determining step are complicated by the conversion of the product of the reaction, oxaloglycolate, into dihydroxyfumarate and its slow decomposition (Hartree, 1953). We have not observed any relaxation process which can be associated with the formation of the pyridoxamine enzyme or its reactions with *erythro*-β-hydroxyaspartic acid. The relaxation times for these effects are either too fast to measure or are not detectable at the wavelengths investigated.

Discussion

A comparison of these results with those of Czerlinski and Malkewitz (1964) indicates that the actual values of the relaxation times reported by these investigators are quite similar to those observed with the temperature-jump apparatus at 490 mμ in this work. However, the considerably extended time and wavelength region investigated here has resulted in a quite different interpretation of the data. Eleven relaxation times have been found to be associated with the interaction between aspartate aminotransferase and *erythro*-β-hydroxyaspartic acid. Two of these, and probably a third, are not directly involved in the transamination reaction. Formulation of a unique mechanism is obviously not possible, but the mechanism of eq 1 appears to be the simplest consistent with the kinetic data. Within the framework of this mechanism the rate constants are uniquely defined in the sense that they do not depend on arbitrarily assigning relaxation times to particular steps.

However, because of the complexity of the mechanism, the precision in the rate constants is not great and probably ranges from 20 to 100% depending on the particular constant under consideration. Nevertheless, the number of significant figures given in Table II is necessary in order to reproduce the experimental relaxation times. This is because calculation of the relaxation times involves, among other things, taking the differences between rate constants. Extreme variations in the rate constants from the values given in Table II yield imaginary roots of eq 3 so that the order of magnitude of the rate constants is well defined.

The per cent of each intermediate present when the enzyme is saturated is given in Table II. All of the first three intermediates are present in relatively small amounts (<10%). The wavelength dependence of the amplitudes of the relaxation processes associated with τ_1 and τ_2 suggests the first two intermediates have spectral peaks at 430 and 362 mμ. The spectral properties of X_3 cannot be unambiguously derived from the data because of the close coupling between τ_3 and τ_4. However, the three intermediates formed when α-methylaspartic acid interacts with aspartate aminotransferase have spectral peaks at 430 and 362 mμ (Hammes and Haslam, 1968), and a reasonable assumption is that the first three intermediates of eq 1 represent the process of Schiff base formation. Thus according to the mechanism previously postulated (Hammes and Haslam, 1968) X_1 represents an initial complex, X_2 is an altered conformational state of X_1, and X_3 is a Schiff base. The rate constants, involved in Schiff base formation in the case of *erythro*-β-hydroxyaspartic acid are considerably larger than those found with α-methylaspartic acid.

The intermediates $X_4 + X_4'$ represent the most prevalent intermediates occurring before the rate-determining step. The amplitude of the relaxation process and the absorption spectra of equilibrium mixtures are consistent with $X_4 + X_4'$ having spectral peaks at 460 and 492 mμ. The extinction coefficient of these intermediates at 492 mμ can be calculated to be 118,000 M^{-1} cm^{-1}. The spectral peak at 492 mμ has been previously identified with a quinone-type structure (Jenkins, 1964), which is consistent with the extremely high extinction coefficient found. Both X_4 and X_4' are quinoid type intermediates. The interconversion between X_4 and X_4' is extremely rapid, and these two species may represent different conformational states or different ionization states. Evidence for a quinoid intermediate in the enzymatic reaction of the natural substrates has also been found (Jenkins and D'Ari, 1966; Jenkins and Taylor, 1965; Fasella and Hammes, 1967).

Assignment of specific structures to the remaining intermediates is not so simple. The intermediate X_5 apparently has a spectral maximum around 330 mμ and most logically represents the ketimine. The rate-determining step in the mechanism would then be hydrolysis of the ketimine or a conformational change between ketimine structures. The species X_6 and X_7 also have spectral peaks around 330 mμ. They may be ketimine structures, or may be keto acid–enzyme complexes not involved in a ketimine structure. The latter suggestion would be consistent with the pattern observed in many other enzymatic reactions, namely formation of a noncovalently linked enzyme–substrate complex followed by an isomerization or conformational change (Hammes, 1968a,b).

The rate constants observed in this system are considerably smaller than found with the natural substrates (Fasella and Hammes, 1967), but are still quite large. From these results the turnover number for the transamination reaction between *erythro*-β-aspartate and ketoglutarate can be calculated to be 1.2 sec^{-1} (*cf.* Fasella and Hammes, 1967). The corresponding reaction between oxaloglycolate and glutamate is predicted to have an identical turnover number. Although the exact turnover numbers are not known for the conditions used in this study, estimates made from literature data (Jenkins, 1964) are in qualitative agreement with these calculated values.

The results obtained in this study indicate the mechanism of enzymatic transamination is quite complex. The existence of three covalently different intermediates, an aldimine, a quinoid structure, and a ketimine, appears to be necessary. The rate-determining step can be tentatively assigned to the hydrolysis or a conformational change of the ketimine. In addition many different conformational states of the intermediates must exist. If the mechanism of eq 1 is correct, at least 16 different steps are involved in enzymatic transamination. The existence of a large number of intermediate states may be an important aspect of the unusual catalytic efficiency of enzymes (*cf.* Hammes, 1968a).

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The Spectroscopic Properties of Firefly Luciferin and Related Compounds. An Approach to Product Emission*

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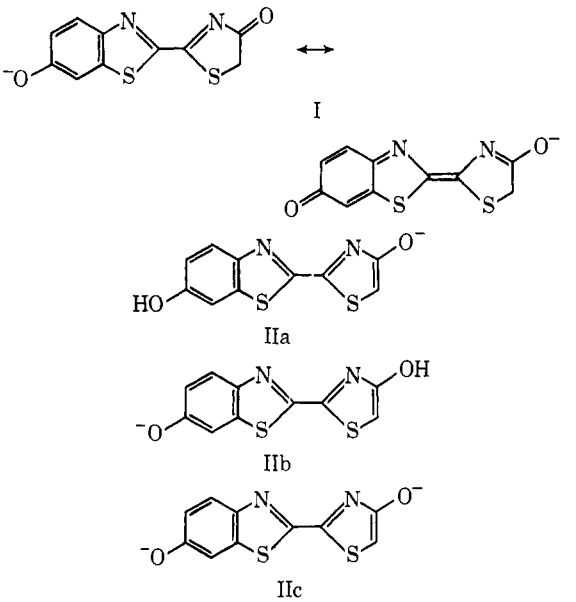
ABSTRACT: The spectroscopic properties of firefly luciferin, its adenylate, and several analogs were investigated in an effort to understand the excited state formed in firefly bioluminescence. It was found that in aqueous solutions, the 6′-hydroxyl group of firefly luciferin (phenol form) dissociates its proton in the excited state. Fluorescence is from the corresponding phenolate anion, the phenol form being relatively nonfluorescent in aqueous solutions. In addition, the fluorescence emission peak of the phenolate anion of luciferin is red-shifted by more than 3200 cm^{-1} in going from nonpolar solvents to water. In aqueous solutions, Zn^{2+} and Cd^{2+} ions produce a further red shift of the fluorescence emission peak. The spectra of the adenylates of luciferin and dehydroluciferin showed effects which could be interpreted in terms of an intramolecular interaction between the benzothiazole and adenine chromophores. All these perturbations of the luciferin chromophore result in significant shifts of the peaks but only small changes in the band width of luciferin emission. Finally, the relevance of these results to the bioluminescence is discussed.

The complete mechanism of firefly bioluminescence must ultimately include, in addition to the biochemical information normally sought for all enzymatic reactions, an understanding of the excited state which emits the visible light. Spectroscopy thus has a more central role than as a means for studying molecular structure. If the immediate oxidation product of the bioluminescent reaction could be extracted and identified, knowledge of its excited state(s) could be obtained by studying its spectroscopic properties. Recent identification of the product emittor in luciferyl adenylate red chemiluminescence as the monoanion of the decarboxylated, 4-keto derivative of luciferin (I) suggests that an anion of the enol form (IIa, b, or c) may be the bioluminescence emittor



I

IIa

IIb

IIc

(Hopkins *et al.*, 1967). These molecules, however, are not stable in aqueous solutions, and attempts to isolate the product of the enzymatic reaction have failed (Seliger and McElroy, 1966; Plant *et al.*, 1968). Neither have we been able to observe a molecular species in a

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