A Kinetic Investigation of the Interaction of α-Methylaspartic Acid with Aspartate Aminotransferase*

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ABSTRACT: Temperature-jump and flow techniques have been used to study the kinetics of the interaction of α-methylaspartic acid with the α subform of aspartate aminotransferase.

Three coupled relaxation processes are observed in the time range 1 msec–1 sec. The dependence of the relaxation times on concentration suggests that a bimolecular reaction followed by two unimolecular processes occurs. The six rate constants characterizing the mechanism have been obtained with a computer analysis of the data. All of the rate processes are considerably slower than for similar reactions involving the enzyme and natural substrates. The kinetic data at concentrations of substrate below 8×10^{-3} M are not entirely consistent with the simple mechanism mentioned above. If the mechanism is modified to include the possibility that two molecules of α-methylaspartic acid bind to 1 equiv of the enzyme, with one of them functioning as an activator, the kinetic data can be fit quantitatively. Some properties of the various forms of the complexes are derived, and a reaction mechanism is proposed.

The enzyme aspartate aminotransferase has been extensively studied with a variety of experimental techniques in a number of different laboratories (*cf.* Fasella, 1967). The general mechanism of action of the enzyme involves transfer of the amino group of the amino acid substrate to enzyme-bound pyridoxal phosphate, resulting in formation of a keto acid and enzyme-bound pyridoxamine. This is followed by transfer of the amino group of pyridoxamine to the keto acid substrate, yielding the pyridoxal enzyme and the new amino acid. However, the nature and number of reaction intermediates involved in this mechanism is still uncertain, although enzyme–substrate Schiff bases are known to be formed (Riva *et al.*, 1964; Malakhova and Torchinsky, 1965), and the role of the protein in the catalytic process remains to be elucidated. Previous temperature-jump kinetic studies with the natural substrates have established that a minimum of four enzyme–substrate complexes are involved in the over-all reaction and some of the pertinent kinetic parameters have been determined (Fasella and Hammes, 1967). The process of Schiff base formation between enzyme and substrates is very rapid and involves second-order rate constants greater than 10^7 M^{-1} sec^{-1} and first-order rate constants greater than 10^4 sec^{-1}. Because of the complexity and rapidity of the over-all reaction with natural substrates, it is of interest to examine the interaction between the enzyme and pseudo-substrates or inhibitors which are only able to undergo a portion of the over-all reaction. Such reactions are generally considerably slower than those involving the normal substrates and also permit detailed examination of a small portion of the over-all mechanism. An investigation of this sort with the substrate β-hydroxyaspartate has been reported (Czerlinski and Malkewitz, 1964), although the enzyme used was nonhomogeneous.

Equilibrium binding studies between α-methylaspartate and the α subform of the enzyme have been reported at various values of pH and temperature (Fasella *et al.*, 1966; Hammes and Tancredi, 1967). This amino acid is known to form a Schiff base with the enzyme-bound pyridoxal phosphate (Braunstein, 1964), but the presence of the methyl group in the α position prevents further reaction. The pH dependence of the binding constant suggests only the form of the enzyme with an absorption maximum at 362 mμ reacts with the enzyme. In this paper the results of a kinetic study of the interaction between α-methylaspartate and the α subform of aspartate aminotransferase with temperature-jump and stopped-flow techniques are reported. The process of Schiff base formation involves at least three discrete steps. The individual rate constants characterizing the three processes are reported, and a possible mechanism is presented.

Experimental Section

The α subform of cytoplasmic aspartate aminotransferase (EC 2.6.1.1) was prepared from pig hearts as previously described (Martinez-Carrion *et al.*, 1965, 1966). The ratio of the absorbance at 430 mμ to that at 340 mμ was 2.9–3.2 in 0.1 M sodium acetate buffer (pH 5.2). Enzyme concentrations were determined spectrophotometrically. A molar extinction coefficient (per mole of coenzyme) of 8.2×10^3 M^{-1} cm^{-1} at 362 mμ and pH 8.5 was used (Martinez-Carrion *et al.*, 1965). Enzyme solutions were $0.25–1.2 \times 10^{-4}$ M

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received November 20, 1967.* This work was supported by grants from the National Institutes of Health (GM 13292 and GM 12944).

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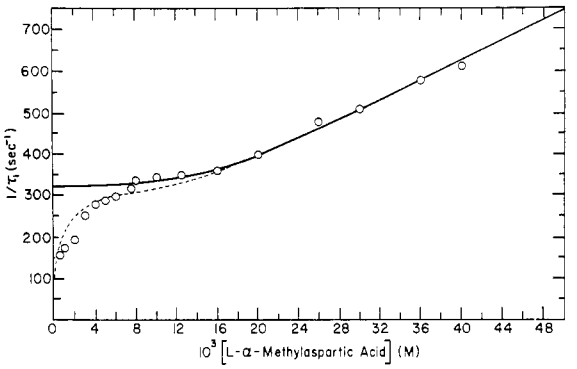

FIGURE 1: Variation of $1/\tau_1$ with L-α-methylaspartic acid concentration. Circles are experimental values obtained from temperature-jump data. The solid line is a theoretical line based on the mechanism of eq 1. The dashed line is a theoretical line based on the mechanism of eq 6.

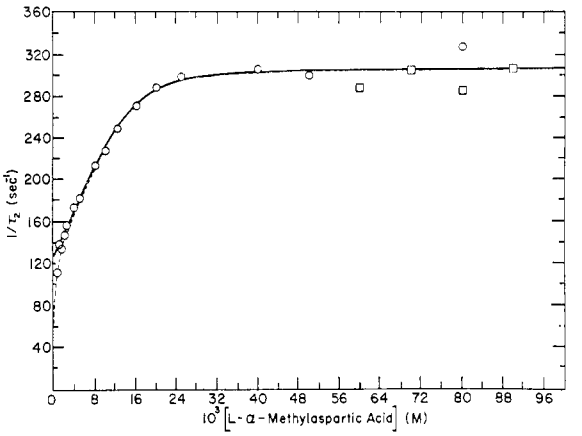

FIGURE 2: Variation of $1/\tau_2$ with L-α-methylaspartic acid concentration. Circles are experimental values obtained from stopped-flow data and squares are from temperature-jump data. The solid line is a theoretical line based on the mechanism of eq 1. The dashed line is a theoretical line based on the mechanism of eq 6.

in coenzyme. DL-α-Methylaspartic acid was obtained from Sigma Chemical Co. (lot 106 B-0050). All other chemicals were standard reagent grade.

The temperature-jump apparatus has been described previously (Hammes and Steinfeld, 1962) and the stopped-flow apparatus used was a Durrum–Gibson stopped-flow spectrophotometer. The dead time of the stopped-flow spectrophotometer was determined by studying the reaction of ferricyanide (10^{-3} M) and ascorbic acid (0.1–0.2 M) at pH 9.0. The half-times for this reaction can be easily varied by changing the ascorbic acid concentration. First-order plots of the data for reactions with varying half-times intersect at zero time. The difference between zero time and the time of the initial observation is the apparatus dead time. The average value of the dead time is 3.6 ± 0.8 msec. Relaxation times were calculated from plots of the logarithm of the amplitude *vs.* time in the usual manner. Spectral measurements for the determination of the equilibrium binding constant were made on a Zeiss Model PMQII spectrometer using the procedures previously described (Fasella *et al.*, 1966); pH measurements were made using a Radiometer TTT1 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

The binding constant for the reaction of L-α-methylaspartate with the α subform of aspartate aminotransferase in 0.1 M sodium pyrophosphate at 25° was determined as previously described (Fasella *et al.*, 1966; note that a minus sign was inadvertantly omitted after the equal sign in eq 5 of this reference). Spectrophotometric titration at 360 and 430 mμ gave an average value for the binding constant of 295 M^{-1}. (As in previous studies only the L isomer of α-methylaspartic acid is assumed to bind to the enzyme; in an independent experiment no evidence for the binding of D-aspartic acid to the enzyme was found.)

Two distinct relaxation processes were seen in experiments carried out with the temperature-jump method. At concentrations between approximately 10^{-3} and 4×10^{-2} M in L-α-methylaspartate a single relaxation process was seen in which the absorption at 430 mμ increased with a concomitant decrease at 362 mμ and an isobestic point at about 390 mμ. The relaxation times (τ_1) associated with this process are summarized in Figure 1. The estimated experimental error is ±10%. At substrate concentrations above 4×10^{-2} M, the amplitude of the first relaxation process became very small and another relaxation process with an absorption change of opposite sign to that associated with τ_1 occurred. The relaxation times associated with this process (τ_2) are shown as squares in Figure 2; the experimental error is estimated as ±15%. Stopped-flow experiments also revealed two relaxation processes. Relaxation times can be calculated from stopped-flow data exactly as with the temperature-jump data as long as measurements are made sufficiently close to equilibrium. The fastest relaxation process had a relaxation time which was identical with values of τ_2 obtained from temperature-jump experiments at comparable substrate concentrations, although this relaxation time could be measured over a much wider concentration range with the stopped-flow apparatus. The measured relaxation times (τ_2) are shown in Figure 2; the estimated error in the relaxation times is ±10%. In the stopped-flow experiments the change in absorbance associated with τ_2 was an increase in absorbance at 430 mμ and a decrease at 362 mμ. The relaxation process associated with τ_1 was not seen with the stopped-flow apparatus. At high concentrations of substrate, τ_1 is too short to measure with the flow apparatus; however it is surprising no indication of the relaxation process associated with τ_1 is observed with the flow apparatus at low concentrations of substrate. The third relaxation process was considerably slower than the others, had a very small amplitude (~0.01 absorbancy unit), and was only observable at relatively high amino acid concentrations. The absorption changes were the reverse of those associated with τ_2. Values of the longest relaxation times (τ_3) are presented in Figure 3; the ex-

perimental uncertainty in these time constants is about ±15%. No dependence of the relaxation times on enzyme concentration was observed in the necessarily limited concentration range investigated.

Since the relaxation times are independent of the method of perturbation of the system, the relaxation times obtained with the two experimental methods can be considered together. The amplitudes of the relaxation process, of course, are dependent on the perturbation technique used. The fact that one reciprocal relaxation time increases as the concentration of substrate is raised while the two others reach a constant value suggests that a bimolecular reaction and two intramolecular reactions occur.

The simplest mechanism consistent with the majority of the data is given in eq 1, where E represents

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} X_3 \qquad (1)$$

the enzyme; S, the L-α-methylaspartate; and the X_i, enzyme–substrate complexes. If the rate equations are linearized, it can be shown that the relaxation spectrum associated with the mechanism of eq 1 can be obtained by solving the determinant given in eq 2 (Eigen and de Maeyer, 1963; Castellan, 1963; Hammes and Schimmel, 1966). (In general, the substrate concentration should be replaced by the sum of the equilibrium concentrations of free substrate and free enzyme, but since the L-α-methylaspartate concentration is always considerably greater than the enzyme concentration the above formulation is sufficient.) However, the solution of the cubic equation which gives the three relaxation times is sufficiently intractable so as to make direct calculation of the rate constants from the individual relaxation times unfeasible. A cubic matrix can be shown to have three relationships (eq 3–5) between the elements and eigenvalues. (The general theorems from which these equations are derived are the sum of the eigenvalues is equal to the trace of the matrix, the sum of the product of all pairs of the eigenvalues is equal to the products of all pairs of the diagonal elements minus the products of the pairs of the off-diagonal elements of the type $a_{ij}a_{ji}$ ($j \neq i$), and the product of the three eigenvalues is equal to the determinant of the matrix.)

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

$$1/\tau_1 + 1/\tau_2 + 1/\tau_3 = k_1(S) + k_{-1} + k_2 + k_{-2} + k_3 + k_{-3} \qquad (3)$$

$$1/\tau_1\tau_2 + 1/\tau_1\tau_3 + 1/\tau_2\tau_3 = k_1(S)(k_2 + k_{-2} + k_3 + k_{-3}) + k_{-1}k_{-2} + k_{-2}k_{-3} + (k_{-1} + k_2)(k_3 + k_{-3}) \qquad (4)$$

$$1/\tau_1\tau_2\tau_3 = k_1(S)(k_2k_3 + k_2k_{-3} + k_{-2}k_{-3}) + k_{-1}k_{-2}k_{-3} \qquad (5)$$

The right-hand sides of eq 3–5 are all experimentally determined quantities; furthermore the right-hand side of each equation is a linear function of the concentration of L-α-methylaspartate. Thus from three appropriate plots, six independent combinations of the rate constants can be obtained (three slopes and three intercepts), and from these constants all six rate constants can be calculated. Note that the ratio of slope to intercept according to eq 5 is the equilibrium binding constant which has also been determined independently by spectrophotometric titration. Plots of the data were constructed according to eq 3–5 in the concentration region where all three relaxation times could be determined and are shown in Figures 4–6. All six rate constants were obtained, and these were then used to solve for the three individual relaxation times as a function of concentration by solution of eq 2 on a high-speed digital computer. A range of slopes and intercepts consistent with the data in Figures 4–6 was examined so as to obtain good agreement between the calculated and measured relaxation times. The solid lines in Figures 1–3 are the calculated curves obtained with the rate constants given in Table I. The estimated uncertainty in the rate constants is about ±20%. Mention should be made of the fact that in most cases where three relaxation processes are well separated on the time axis, the secular determinant (eq 2) can usually be factored to obtain rather simple expressions for the individual relaxation times. (*cf.* Hammes and Schimmel, 1966). At sufficiently high amino acid concentrations (>0.05 M) $1/\tau_1$ can be approximated simply as $k_1(S) + k_{-1}$; however the other two relaxation processes are always coupled because k_{-2} and k_3 are comparable in magnitude. Thus the complete cubic equation *must* be solved in general. At high concentrations of substrate where the two unimolecular reactions become uncoupled from the bimolecular step, the same secular equation is obtained for relaxation kinetics and for the solution of the general differential equations characterizing these former two reactions. It should be noted that under these conditions no simple way exists to obtain the four rate constants from the two eigenvalues.

The agreement between the calculated and experimentally determined relaxation times is very good above L-α-methylaspartate concentrations of 8×10^{-3} M. The deviations occurring at low concentrations are definitely real and can be explained by the following modification of the mechanism in eq 1.

$$\begin{array}{ccccccc} X_0 & + S \rightleftharpoons & X_1 & \rightleftharpoons & X_2 & \rightleftharpoons & X_3 \\ K_4 \uparrow\downarrow & & K_5 \uparrow\downarrow & & K_6 \uparrow\downarrow & & K_7 \uparrow\downarrow \\ E + S & & X_4 + S & & X_5 + S & & X_6 + S \end{array} \qquad (6)$$

This mechanism postulates substrate activation, *i.e.*, two molecules of L-α-methylaspartate bind to the en-

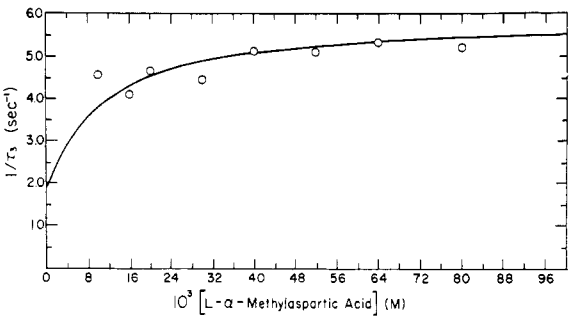

FIGURE 3: Variation of $1/\tau_3$ with L-α-methylaspartic acid concentration. Circles are experimental values obtained from stopped-flow data. The solid line is a theoretical line based on the mechanism of eq 1 and 6.

zyme per active site. If the equilibration between inactive and active forms, that is, between E and X_0, X_4 and X_1, X_5 and X_2, and X_6 and X_3, is rapid relative to the rates of the other reactions the secular determinant can be approximated by a determinant very similar to eq 2 except that each rate constant is divided by a factor $[1 + 1/K_i(S)]$, where the K_i's are association constants defined by eq 6; for k_1, $i = 4$; for k_{-1} and k_2, $i = 5$; for k_{-2} and k_3, $i = 6$; and for k_{-3}, $i = 7$. If K_4, K_5, and K_7 are much greater than K_6, and if K_6 is equal to 1.5×10^3 M^{-1}, the calculated dependence of the relaxation times on concentration is as shown by the dashed lines in Figures 1 and 2. The values of $1/\tau_3$ are virtually unchanged from those found for the simpler mechanism, and the calculated curves for the mechanism of eq 6 merge into those obtained for the mechanism of eq 1 at higher concentrations in the cases of $1/\tau_1$ and $1/\tau_2$. The more complex mechanism is also consistent with the spectrophotometric titration experiments if the active and inactive species are assumed to have the same spectra (see the discussion below). More complex activation mechanisms have been tried, but the one presented appears to be the simplest consistent with the data.

Discussion

We first consider the results in terms of the mechanism of eq 1. This is the simplest mechanism consistent with the number of relaxation times observed and the concentration dependence of the relaxation times (at concentrations of α-methylaspartate greater than 8×10^{-3} M). All other mechanisms would involve either *more* forms of the complex and/or a nonsequential pathway, *i.e.*, the formation of complexes not on the main reaction pathway. Thus the mechanism of eq 1 can be accepted with a reasonable amount of confidence. Given this mechanism, the assignment of the rate constants and relaxation times is unique. A chemical mechanism cannot be assigned with certainty, but existing evidence strongly suggests a mechanism of the type shown in Figure 7. The initial reaction is probably the reaction of the amino acid with the unprotonated internal Schiff base of the enzyme to form a complex

TABLE I: Summary of Rate and Equilibrium Constants.[a]

i	k_i	k_{-i} (sec^{-1})	K_i
1	1.21×10^4 M^{-1} sec^{-1}	133	90.5 M^{-1}
2	18.2 sec^{-1}	227	0.08
3	67.1 sec^{-1}	2.47	27.2

[a] Determined from plots of the data according to eq 3–5; the over-all binding constant is 295 M^{-1} (0.1 M pyrophosphate, pH 8.0, 25°).

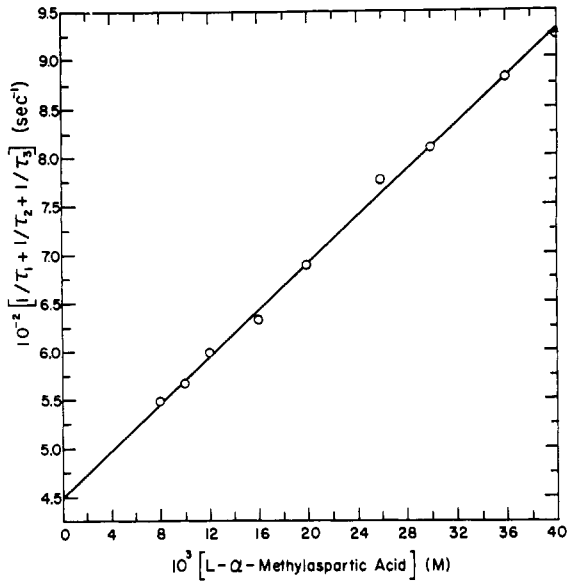

FIGURE 4: Plot of $1/\tau_1 + 1/\tau_2 + 1/\tau_3$ *vs.* L-α-methylaspartic acid concentration. The values of the slope and intercept are related to the various rate constants by eq 3.

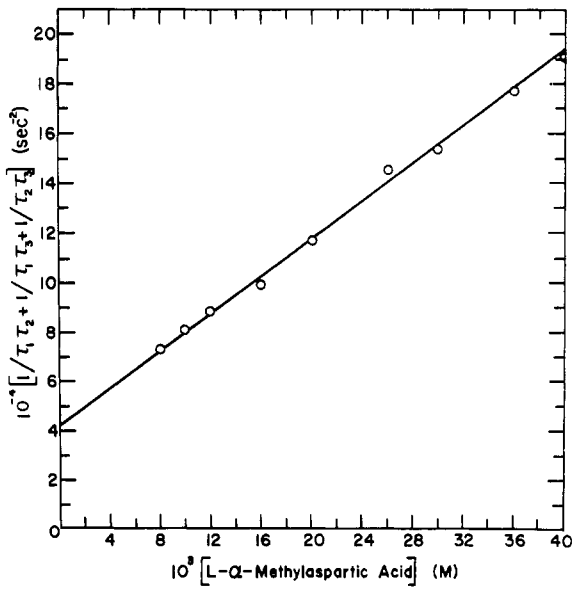

FIGURE 5: Plot of $1/\tau_1\tau_2 + 1/\tau_1\tau_3 + 1/\tau_2\tau_3$ *vs.* L-α-methylaspartic acid concentration. The values of the slope and intercept are related to the various rate constants by eq 4.

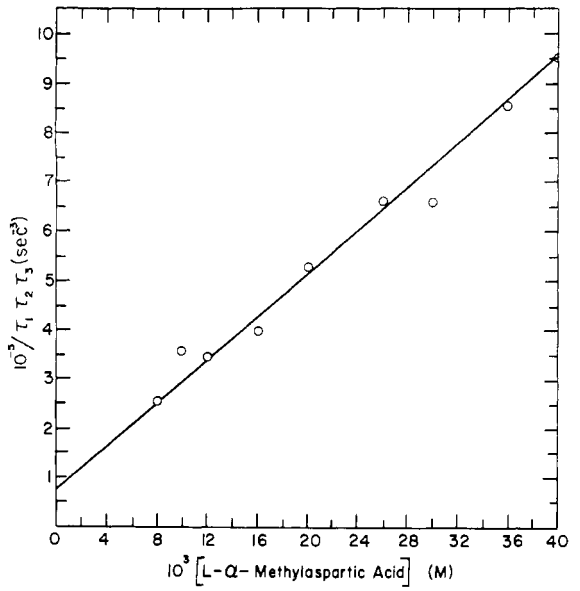

FIGURE 6: Plot of $1/\tau_1\tau_2\tau_3$ *vs.* L-α-methylaspartic acid concentration. The values of the slope and intercept are related to the various rate constants by eq 5.

not covalently linked. The involvement of the neutral internal aldimine is consistent with the pH dependence of the binding constant previously reported (Fasella *et al.*, 1966). However, it should be noted that this pH dependence was determined with different buffers at each pH, and the results were interpreted with the assumption that specific buffer effects are not of importance. This assumption, although consistent with the previous data, may not be correct since in the present study a binding constant of 295 M^{-1} was obtained at pH 8.0 in 0.1 M sodium pyrophosphate whereas a binding constant of 420 M^{-1} was obtained at pH 8.0 in 0.14 M Tris buffer. However, regardless of the quantitative aspects of this problem, it is quite certain the binding of L-α-methylaspartate is considerably weaker at low pH (~5) than at high pH (~8). Recent evidence suggests buffer ions play a mechanistic role in the binding of some dicarboxylic acids to aspartate aminotransferase (Jenkins and D'Ari, 1966). Since all the experiments in this work were done at constant buffer concentration, the role of the buffer ions in the mechanism (if any) cannot be ascertained. The final complex (X_3) in the mechanism is almost certainly a Schiff base (Braunstein, 1964). The central complex (X_2) must be an isomer or different conformational state of either X_1 or X_3. In Figure 7, X_2 is schematically shown as a conformational isomer of X_1 by changes in the dashed line designating the protein. The choice of this isomer was made because in several other enzyme systems in which covalent changes are not involved, isomerizations or conformational changes have been found to follow formation of the initial enzyme–substrate complex (Hammes, 1968).

Some information regarding the spectrum of the intermediates can be derived from the amplitudes of the relaxation effects. An exact analysis of this problem would involve obtaining the three eigenvectors of the

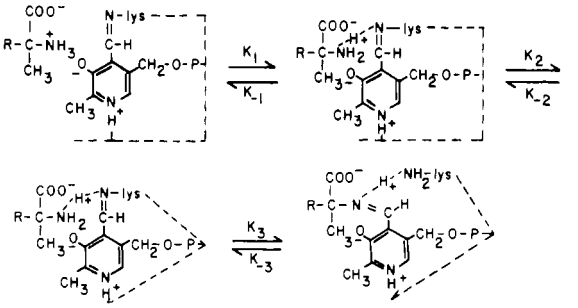

FIGURE 7: Possible reaction mechanism for the interaction of L-α-methylaspartic acid and aspartate aminotransferase. The dashed line schematically represents the protein.

secular determinant (eq 2) and relating these three normal concentration variables to the absorption changes (*cf.* Eigen and de Maeyer, 1963). Unfortunately, such an analysis would be of little practical use because of the complexity of the final expressions for the eigenvectors. However, at high concentrations of amino acid where the first relaxation time becomes very short, while the other two relaxation times reach constant limiting values, the spectral properties of the first intermediate (X_1) can be determined. As noted previously, under these conditions the bimolecular process is too rapid to be observed with the stopped-flow apparatus. Thus from experiments at various wavelengths about 430 and 362 mμ an extrapolation of the absorbancy change of the first process observed with the stopped-flow to zero time gives a value for the absorbance of the first intermediate equilibrated with the free enzyme. The extrapolation is possible since the dead time of the instrument has been determined and the process is known to be first order. Since the equilibrium constant for the first reaction is known as well as the extinction coefficients of the free enzyme, the extinction coefficients for X_1 can be calculated.

The absorbance at infinite substrate concentration is equal to $\epsilon_{X_1}(X_1) + \epsilon_{X_2}(X_2) + \epsilon_{X_3}(X_3)$; since $\epsilon_{X_1}(X_1)$ is known, $\epsilon_{X_2}(X_2) + \epsilon_{X_3}(X_3) = [(X_2) + (X_3)][\epsilon_{X_2}/(1 + K_3) + \epsilon_{X_3}/(1 + 1/K_3)]$ can be calculated. The constant K_3 is quite large (*i.e.*, the concentration of X_2 is quite small) so that the apparent extinction coefficient of $[(X_2) + (X_3)]$, $[\epsilon_{X_2}/(1 + K_3) + \epsilon_{X_3}/(1 + 1/K_3)]$, is essentially the extinction coefficient of X_3. The values of ϵ_E, ϵ_{X_1}, and $[\epsilon_{X_2}/(1 + K_3) + \epsilon_{X_3}/(1 + 1/K_3)]$ are given in Figure 8 over the wavelength region 300–500 mμ. The spectra should be considered quite approximate since data could only be obtained in the neighborhood of 430 and 360 mμ and these derived quantities depend on many variables which are not precisely known, *i.e.*, the dead time of the flow apparatus and the equilibrium constants of the individual steps in the reaction mechanism. However, it is apparent that X_1 has absorption peaks at 430 and 360 mμ, and X_3 has absorption peaks at 430 and 370 mμ; the small differences in the extinction coefficients between the two species is well within the experimental uncertainties. Although the extinction coefficient of X_2 cannot be obtained because it is present in relatively small concentrations at all times, the amplitude of the relaxation effects suggests it has a wave-

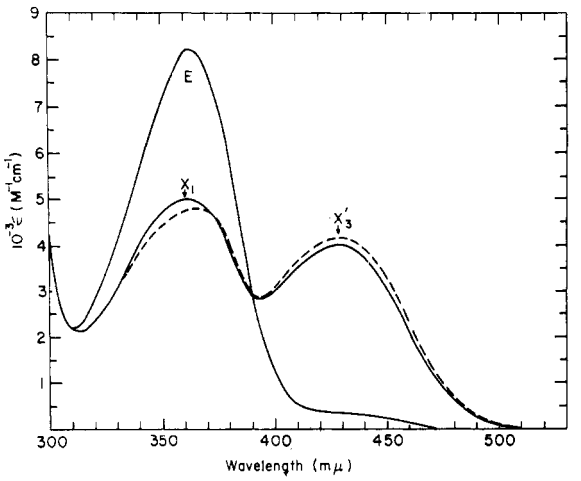

FIGURE 8: Extinction coefficients of the enzyme aspartate aminotransferase (E) and the various complexes formed with L-α-methylaspartic acid; X_3' refers to the apparent extinction coefficient, $[\epsilon_{X_3}/(1 + K_3) + \epsilon_{X_3}/(1 + 1/K_3)]$. See the text for further details.

length dependence very similar to the extinction coefficients of X_1 and X_3.

A possible interpretation of these results is that all three forms of the complex can exist either as a protonated aldimine with an absorption peak at 430 mμ or an unprotonated aldimine with an absorption peak at 360–370 mμ, analogous to the free enzyme. However, this is difficult to rationalize with the fact that the spectrum of the complex is virtually invariant with pH over the range 5–12 (Hammes and Tancredi, 1967). The only explanation of the spectral properties of the intermediates which is somewhat satisfactory is that each intermediate can exist as two rapidly equilibrating conformational species, possibly involving intramolecular proton transfer, one of which has an absorption maximum at 430 mμ, while the other has an absorption maximum at 360–370 mμ. Alternatively the two absorption peaks could be associated with a single species although this seems unlikely in view of the known spectral properties of the native enzyme.

The rate constants summarized in Table I are considerably smaller than those associated with the reaction between the enzyme and the natural substrates (Fasella and Hammes, 1967). The second-order rate constant is several orders of magnitude smaller than typical values associated with enzyme–substrate complex formation (Eigen and Hammes, 1964). However, these rate constants are quite similar in magnitude to those derived for the reaction between β-hydroxyaspartate and aspartate aminotransferase (Czerlinski and Malkewitz, 1964). Three forms of the complex were also postulated for this reaction, although one of these was proposed to form after Schiff base formation. However, since only light of a single wavelength was used to follow concentration changes (505 mμ), additional relaxation processes may be present which were not observed.

The possibility of amino acid activation occurring with this enzyme has not previously been suggested, but it appears to be the only explanation of the data at low amino acid concentrations. However, since only the concentration dependence of τ_1 at low concentrations deviates appreciably from the behavior predicted by the simple mechanism of eq 1 and since it has not been possible to obtain corroborative evidence for the more complex mechanism of eq 6, this latter mechanism must be regarded as quite tentative. This mechanism implies two molecules of amino acid bind per mole of active site. Because the binding constants between most substrates (and inhibitors) and enzyme are generally rather small ($\lesssim 10^3$ M^{-1}), the stoichiometry of these interactions has not been well characterized. If the activating molecule does not cause appreciable spectral changes when it binds to the enzyme and if it binds appreciably stronger than the second molecule, the activating process might easily go unnoticed in spectral titration experiments. Unfortunately amino acid activation can only be observed over a narrow concentration range in the present system. Since the possibility of amino acid activation may be of biological significance, detailed steady-state kinetics are now being carried out to see if this phenomenon exists for the over-all transamination reaction; both the monomer and dimer forms of the enzyme are being investigated.

Acknowledgment

The authors are indebted to Dr. David L. Miller who carried out preliminary experiments on this system and to Mrs. Y. C. Lee for her technical assistance.

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Reaction of Tetranitromethane with Protein Sulfhydryl Groups. Inactivation of Aldolase*

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ABSTRACT: The reactivity of protein tyrosyl and sulfhydryl groups toward tetranitromethane (TNM) was studied using aldolase of rabbit muscle. As in model compounds, oxidation of sulfhydryl groups in this protein occurs much faster than tyrosyl nitration. However, the rate of SH modification decreases with decreasing pH in contrast to previous findings with model compounds (Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582). Aldolase is rapidly and completely inactivated by a 23-fold M excess of TNM at pH 8.0. The loss in activity toward either fructose 1,6-diphosphate or fructose 1-phosphate correlates with modification of SH groups; eight to ten SH groups are oxidized in the completely inactivated enzyme while not more than 0.6 tyrosyl residue is nitrated. Other amino acid residues do not react with TNM under the mild conditions used. The same relationship between number of SH groups oxidized and degree of inactivation holds for all conditions of modification. The inactivation is only partially reversible upon incubation with mercaptoethanol suggesting only limited S–S bond formation. Other oxidized forms of cysteinyl residues occur during the reaction with TNM as evidenced by the detection of cysteic acid on amino acid analysis. Competitive inhibitors and fructose 1,6-diphosphate partially protect the enzyme against inactivation by TNM.

Nitration of tyrosine and related compounds with tetranitromethane (TNM)[1] proceeds readily under mild conditions (Riordan *et al.*, 1966). The reaction is well suited for modifying proteins (Sokolovsky *et al.*, 1966) and has proven useful for studying the tyrosyl residues of carboxypeptidase A (Riordan *et al.*, 1967). TNM has also been shown to react with sulfhydryl groups in model compounds, the product being a disulfide. Studies on sulfhydryl groups in proteins analogous to those on tyrosyl residues have not been carried out as yet and the reactivities of tyrosyl and cysteinyl residues toward TNM have not been compared in the same protein.

Aldolase seemed to be particularly suitable for such studies since it contains readily reactive tyrosyl and cysteinyl residues, both of which are thought to be involved in activity (Swenson and Boyer, 1957; Drechsler *et al.*, 1959; Kowal *et al.*, 1965; Schmid *et al.*, 1966; Kobashi and Horecker, 1967; Pugh and Horecker, 1967). However, it should be possible to examine the sulfhydryl groups independently of the tyrosyl residues. Studies with model compounds had shown that the pH dependencies of the reactions of these two residues with TNM are different (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966). Moreover, carboxypeptidase treatment removes the C-terminal tyrosyl residues of aldolase, reducing FDP activity to 5%. The residual activity of this derivative is not altered further by acetylation of tyrosine (Schmid *et al.*, 1966). Modification of sulfhydryl groups completely abolishes the activity of aldolase toward both fructose 1,6-diphosphate (FDP) and fructose 1-phosphate (F-1-P) (Kobashi and Horecker, 1967), while reactions involving the tyrosyl residues alter only activity toward FDP (Pugh and Horecker, 1967). Hence, activity can serve as a first approximation to the types of residues being modified.

Materials

Fructose 1,6-diphosphate aldolase from rabbit muscle was obtained from Worthington Biochemical Corp. as a crystal suspension in ammonium sulfate. Before use, the enzyme was dialyzed against the appropriate buffer. The specific activity at 25° was 11.3 IUB units/mg with fructose 1,6-diphosphate as substrate. Other materials and suppliers were: carboxypeptidase A, Worthington Biochemical Corp.; glycerol 1-phosphate dehydrogenase, triose phosphate isomerase, fructose 1,6-diphos-

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: TNM, tetranitromethane.