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EFFECT OF AGGREGATION ON THE KINETIC PROPERTIES OF ASPARTATE AMINOTRANSFERASE *

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Summary

In this investigation the steady-state kinetic parameters of the α subform of aspartate aminotransferase (EC 2.6.1.1) were determined in 0.2 M Tris · HCl, pH 8.0, at 25°C. The kinetic parameters for both the forward and reverse reactions were determined under conditions where the enzyme is monomeric, while only the steady-state parameters associated with the forward reaction could be determined under conditions where the enzyme is dimeric. The catalytic center activity of the forward reaction for dimeric enzyme decreased relative to that of monomeric enzyme, 245 versus 360 s^{-1}, while the K_m for aspartate increased, 3.3 versus 2.6 mM. No significant change in the Michaelis constant for ketoglutarate was observed. The steady-state parameters of dimeric enzyme are slightly altered in 0.1 M $Na_4P_2O_7$, pH 8.0, the catalytic center activity and Michaelis constant for ketoglutarate being slightly larger. From the dependence of the initial velocity on enzyme concentration the dissociation constant for the monomer-dimer equilibrium is estimated to be $2 \cdot 10^{-8}$ M. A similar value of the dissociation constant was estimated from Sephadex gel filtration experiments.

Introduction

The enzyme aspartate aminotransferase (EC 2.6.1.1) has been extensively studied by many investigators and with many different experimental techniques [1]. A general mechanism for the physiological reaction catalyzed by the enzyme is

$$\begin{aligned} &E_L + Asp \rightleftharpoons X_1 \rightleftharpoons \cdots X_n \rightleftharpoons E_M + Oa \\ &E_M + Kg \rightleftharpoons Y_1 \rightleftharpoons \cdots Y_n \rightleftharpoons E_L + Gm \end{aligned} \qquad (1)$$

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where Asp is aspartate, Oa is oxaloacetate, Kg is ketoglutarate, Gm is glutamate, E_L is the pyridoxal form of the enzyme, E_M is the pyridoxamine form of the enzyme, and the X_1 and Y_1 are enzyme-substrate complexes. The results of both steady-state and relaxation kinetic studies of this enzyme are consistent with the above mechanism [2—6]. Moreover, the results of temperature jump experiments indicate a large number of reaction intermediates occur in the overall reaction [2—4].

Unfortunately much of the early work done on aspartate aminotransferase utilized an enzyme which was a mixture of several subforms, with the α subform being the most active and most prevalent [7]. This is particularly true of the steady-state kinetic studies. In addition this enzyme exists as a dimer at concentrations where most of the equilibrium and temperature jump studies have been carried out, while the enzyme is monomeric at the concentrations used in steady-state kinetic studies [8,9]. Thus a comparison of experimental results obtained at different concentrations is difficult. Experimental results have been reported which suggest a monomer-dimer equilibrium is not of importance at the concentrations in question [10]. The purpose of this investigation is 3-fold: first to determine the steady-state kinetic properties of the α subform of the enzyme, second to estimate the monomer-dimer equilibrium constant by Sephadex gel filtration, and third to determine the steady-state parameters under conditions where the enzyme is either essentially monomeric or dimeric so that the kinetic properties of these two aggregation states can be compared.

Experimental Procedure

The α subform of supernatant aspartate aminotransferase was prepared from pig heart as previously described [7]. The specific activity measured under standard conditions [7] was 230 mol $\cdot$ min^{-1} $\cdot$ mg^{-1}. Oxaloacetic, aspartic, glutamic and α-ketoglutaric acids were purchased from Calbiochem. Tris, three times crystallized, was obtained from Nutritional Biochemical Corp. Bovine serum albumin and β-lactoglobulin were obtained from Pentex Inc. and Sephadex G-100 was obtained from Pharmacia Chemicals Inc. All other chemicals were reagent grade.

Enzyme concentrations were determined spectrophotometrically using either a molar extinction coefficient per active site of $8.05 \cdot 10^3$ M^{-1} $\cdot$ cm^{-1} at 362 nm at high enzyme concentrations or a molar extinction coefficient per active site of $7.16 \cdot 10^4$ M^{-1} $\cdot$ cm^{-1} at 280 nm at low enzyme concentration [11]. Enzyme molecular weight was assumed 47 000 per monomer [7]. The measurements of both wavelengths gave identical results when a comparison was possible.

Sephadex G-100 was allowed to swell 5 days in the buffer used in the experiments and packed at 25°C. The column was 9 mm internal diameter. Column effluent (flow rate, 4 ml/h) was collected in 0.5-ml fractions. The exact volume of each fraction was determined by weighing. The marker proteins were at a concentration of 1—3 mg/ml and were detected using optical absorption at 280 nm. The aspartate aminotransferase was detected with spectrophotometric assay using 0.2 M Tris $\cdot$ HCl, pH 8.00, $3 \cdot 10^{-3}$ M α-ketoglu-

tarate and $1.5 \cdot 10^{-2}$ M aspartate The reaction was monitored at 280 nm.

Steady-state kinetic studies at approx. 10^{-9} M enzyme were done in a manner similar to that described by Velick and Vavra [5]. Results of the Sephadex gel filtration experiments and the results of other workers indicate that the enzyme is essentially monomeric at this concentration [8,9]. The substrates were incubated in 0.2 M Tris · HCl, pH 8.0, and upon addition of 25 μl of enzyme (to a total volume of 10 ml) the change in absorption at 280 nm was followed on a Cary 15 recording spectrophotometer using a 0—0.1 slide-wire and a 50 mm path length cell. The initial velocity was obtained from the slope of the absorbance versus time plot. No appreciable lag period was observed. A molar extinction coefficient for oxaloacetate of 570 $M^{-1} \cdot cm^{-1}$ at 280 nm was used to measure changes in concentration. In the assays involving oxaloacetate as substrate, the solution was made immediately prior to the kinetic experiments; in no case were the oxaloacetate solutions used later than 1 h after they had been prepared.

The steady-state study of the concentrated enzyme necessitated special procedures. The reactants were incubated in 0.2 M Tris · HCl, pH 8.0, or in 0.1 M $Na_4P_2O_7$, pH 8.0. An enzyme concentration of approx. 10^{-6} M was chosen for two reasons: at any lower concentration an appreciable fraction of the total enzyme would be in the monomeric form; on the other hand at higher concentrations the steady-state approximation would no longer be valid because the total enzyme concentration would not be much less than total substrate concentration over the range of substrate concentration of interest. At such high enzyme concentrations, in the range of substrate concentrations of interest, the enolization of oxaloacetate is slow compared to rate of enzyme reaction so that a direct spectrophotometric assay cannot be used. Accordingly a coupled assay utilizing malate dehydrogenase and NADH was used. The aspartate aminotransferase was incubated with malate dehydrogenase (20 μg/ml) and NADH ($2 \cdot 10^{-4}$ M). The enzyme and reactants were mixed in a Durrum-Gibson stopped-flow spectrophotometer and the ensuing change in transmission at 340 nm due to the decrease in concentration of NADH was recorded on an oscilloscope. The logarithm of the ratio of the signal amplitudes in the absence and presence of the reaction mixture, i.e. the absorbance, was plotted against time to yield the initial velocity. A molar extinction coefficient for NADH of $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 340 nm was used to determine the rate of NADH disappearance [12].

Experiments to measure the equilibrium constant for the dimerization of aspartate aminotransferase were carried out in 0.2 M Tris · HCl at pH 8.0. The initial velocity of transamination was measured as a function of enzyme concentration at fixed substrate concentrations. Generally an assay solution containing 9.8 mM α-ketoglutarate, 0.5—1.0 mM aspartate, malate dehydrogenase (20 μg/ml) and NADH ($2 \cdot 10^{-4}$ M) was used. The Michaelis constant for α-ketoglutarate is the same for the monomer and dimer while that for aspartate varied 2-fold between the two forms (see results section). Therefore, the concentrations of substrate were chosen so as to saturate the enzyme with α-ketoglutarate while only partially saturating with aspartate. Under these conditions the initial velocity is a sensitive function of the aggregation state of the enzyme. The enzyme concentration was varied by diluting a solution of known

concentration 2-fold several times and then adding 1 ml of the enzyme solution to 2 ml of assay solution and measuring the initial velocity.

All experiments were done at 25 ± 0.2° C.

Results and Treatment of Data

Sephadex gel filtration

The effect of dilution of the enzyme on the elution volume from Sephadex G-100 is shown in Fig. 1. In order to analyze the results quantitatively in terms of the monomer-dimer equilibrium, enzyme, in a total volume of 8—10 ml, was added to the column (the total column volume was 28.2 ml) and eluted with 0.2 M Tris · HCl, pH 8.0, and buffer containing additionally 1.5 mM aspartate and 0.3 mM α-ketoglutarate. A plateau region occurs in which the concentration of the enzyme is the same as the initial concentration. The elution volume is taken as the centroid of the leading edge of the protein peak [13—15]. A plot of elution volume versus the enzyme concentration is shown in Fig. 1. The estimated error in the elution volume is 0.3 ml. The elution volume and the concentration in the plateau region can be used to determine a dissociation constant. Winzor and Scheraga [13,14] and Ackers and Thompson [15] have shown that in dimerizing systems

$$V_E = \alpha V_M + (1 - \alpha) V_D \tag{2}$$

$$K = \alpha^2 [E_0]/(1 - \alpha) = [E_1]^2/[E_2] \tag{3}$$

where α is the fraction of enzyme in monomeric form, V_E, V_M, and V_D are the elution volumes of the sample, monomer and dimer, respectively, and $[E_0]$ is the protein concentration at the plateau. The dimer dissociation constant, K, is defined by Eqn. 3. The lines in Fig. 1 are calculated using V_D = 13.6 ml, V_M = 15.8 ml and $K = 1 \cdot 10^{-8}$ M (A) $2 \cdot 10^{-8}$ M (B) and $5 \cdot 10^{-8}$ M (C). A dissocia-

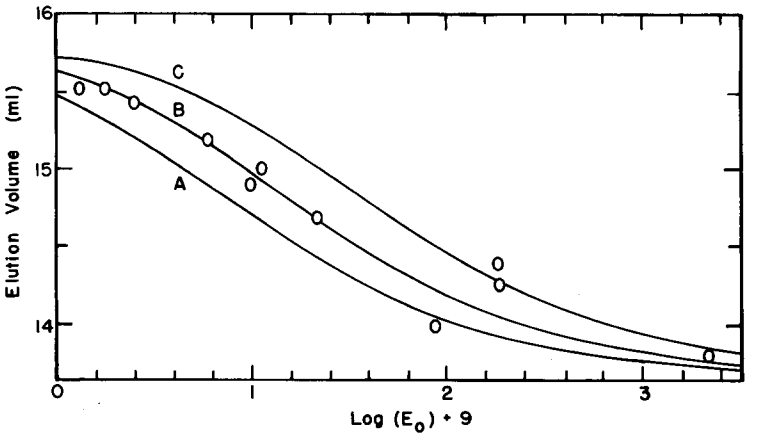


Fig. 1. Plot of elution volume of aspartate aminotransferase as a function of logarithm of initial concentration. The lines were calculated according to Eqns 2 and 3 using a dimerization dissociation constant of (A) 10 nM, (B) 20 nM, (C) 50 nM. The column volume is 20.2 ml.

tion constant of about $2 \cdot 10^{-8}$ M fits the data adequately. The values for the elution volumes of the dimer and monomer were obtained by interpolation on an Andrews' plot [16]. Standards used and their molecular weights are β-lactoglobulin (36 000) [17], ovalbumin (45 000) [18] and bovine serum albumin (68 900) [19]. Essentially all the applied activity was recovered.

Steady-state kinetics

The initial steady-state velocity, v, for the mechanism of Eqn 1 can be written as

$$v = \frac{k[E_0]}{1 + \frac{K_{AA}}{[AA]} + \frac{K_{KA}}{[KA]}} \tag{4}$$

where k is the catalytic center activity, K_{AA} is the Michaelis constant of the amino acid AA, K_{KA} is the Michaelis constant of the keto acid KA, and $[E_0]$ is the total enzyme concentration. This equation can be rewritten as

$$[E_0]\,[AA]/v = K_{AA}/k + [AA]\,(1 + K_{KA}/[KA])/k \tag{5}$$

so that a plot of $[E_0]\,[AA]/v$ versus $[AA]$ at constant $[KA]$ should be a straight line with an intercept equal to K_{AA}/k and a slope, S, equal to (1 +

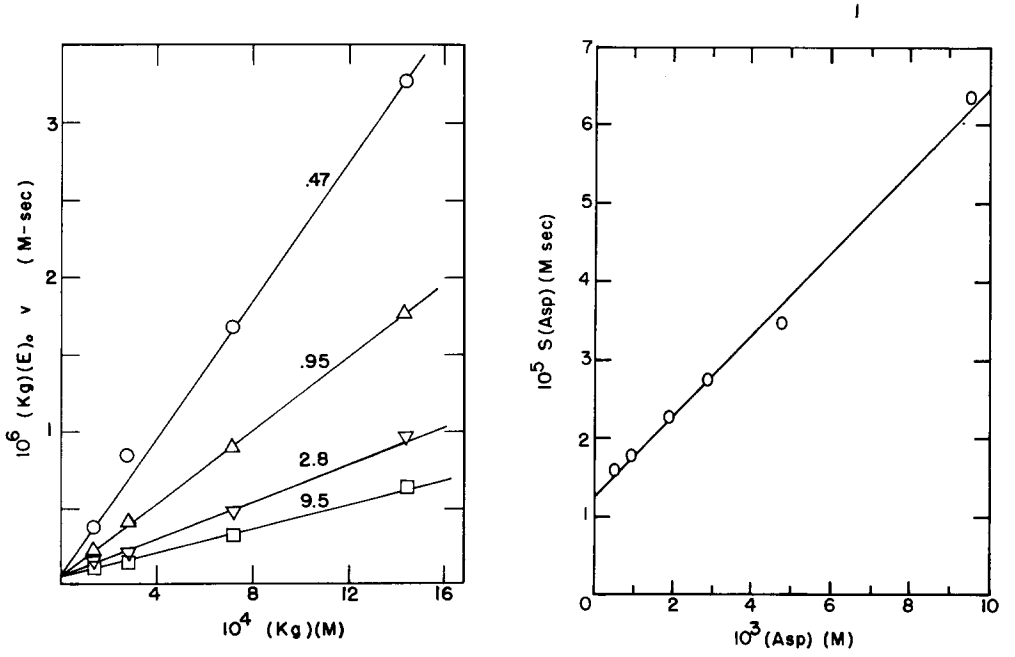


Fig. 2. Plot of $[Kg]\,[E_0]/v$ versus $[Kg]$ according to Eqn 5. The lines were calculated by least squares analysis. The assays were done in 0.2 M Tris · HCl, pH 8.0, 25°C, $[E_0] = 2.2 \cdot 10^{-9}$ M. The concentration of aspartate (mM) is given by each line.

Fig. 3. Replot of data in Fig. 2 (two additional points) in the form $S[Asp]$ against $[Asp]$ as discussed in the text. The line was calculated by a least squares analysis.

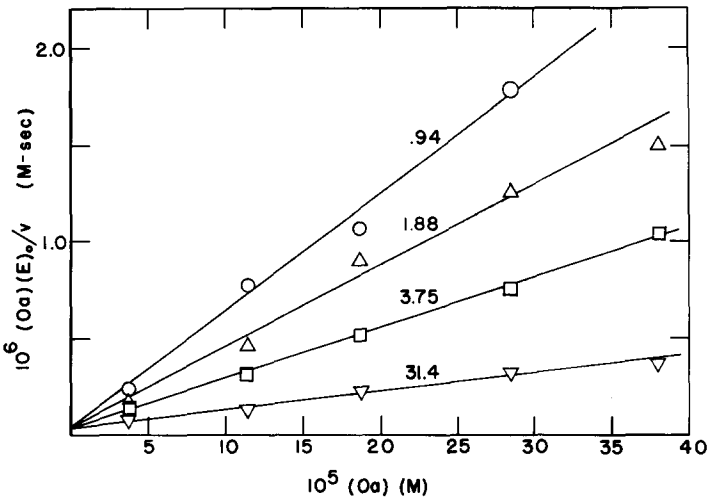


Fig. 4. Plot of [Oa] [E_0] /v against [Oa]. The conditions are the same as in Fig. 2. The line was calculated by a least squares analysis. The concentration of glutamate (mM) is given by each line.

K_{KA}/[KA])/k. A secondary plot can then be constructed of S[KA] versus [KA] which has a slope of $1/k$ and an intercept of K_{KA}/k. Obviously the keto acid and amino acid concentrations can be interchanged in these calculations since they appear symmetrically in the rate law (Eqn 4). Thus all three kinetic parameters can be obtained. Some typical primary plots of the data using α-ketoglutarate and oxaloacetate as variable are shown in Figs 2 and 4. The corresponding secondary plots are shown in Figs 3 and 5. All of these results were obtained with an enzyme concentration of approx. 10^{-9} M. Figs 6 and 7 show some representative data obtained with an enzyme concentration of approx. 10^{-6} M. A least squares analysis of the data was used in all cases. All of the results are consistent with the rate law given in Eqn 4.

The steady-state kinetic parameters obtained are summarized in Tables I

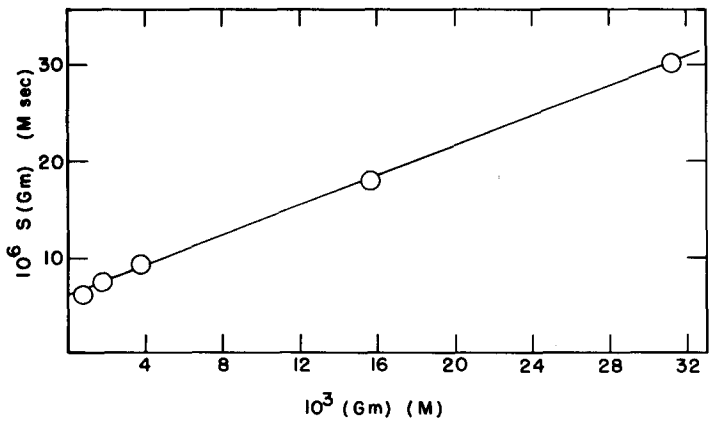
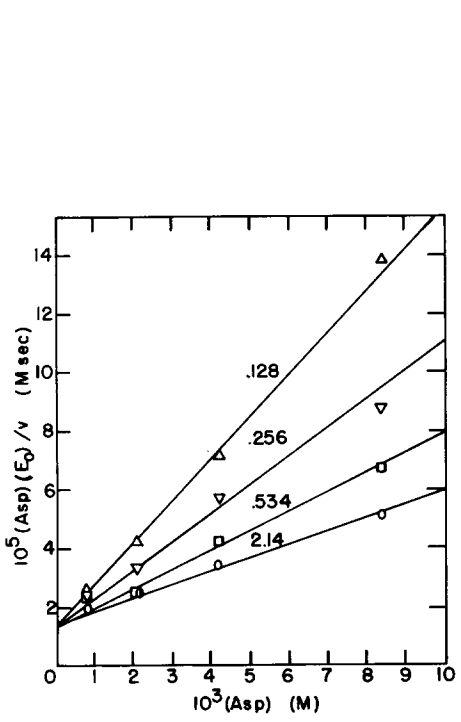


Fig. 5. Replot of the data in Fig. 4 (plus one additional point) in the form S[Gm] against [Gm] as discussed in the text. The line was calculated by a least squares analysis.



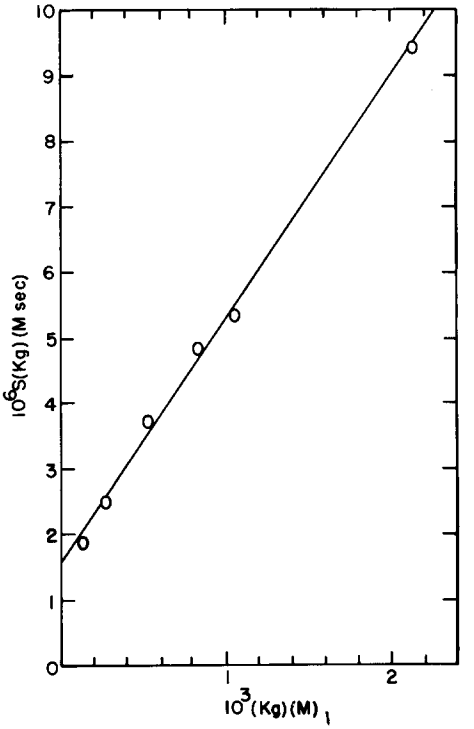


Fig. 6. Plot of [Asp] $[E_0]/v$ against [Asp]. The lines were calculated by least squares analysis. The assays were done in 0.1 M pyrophosphate, pH 8.0, 25°C, $[E_0] = 8.1 \cdot 10^{-7}$ M. The concentration of ketoglutarate (mM) is given by each line.

Fig. 7. Replot of the data (plus two additional points) in Fig. 6 in the form S[Kg] against [Kg] as discussed in the text. The line was calculated from a least squares analysis.

and II. Each initial velocity used in the kinetic plots with 10^{-9} M enzyme was the mean of three determinations, while those at 10^{-6} M enzyme were the mean of 5—8 determinations. The error associated with the parameters in Tables I and II is estimated to be about ±15%. A typical experiment involved the variation of at least five concentrations of each substrate. The kinetic parameters given are the average of a number of determinations; the number of determinations is included in the tables.

The effect on the initial velocity of varying the enzyme concentration at

TABLE I

KINETIC PARAMETERS OF MONOMERIC ASPARTATE AMINOTRANSFERASE IN 0.2 M TRIS · HCl, pH 8.0, 25°C

Substrate	K_m (mM)	k (s^{-1})	Number of experiments
Aspartate	1.8	326	5
α-Ketoglutarate	0.20		
Glutamate	10.5	1240	3
Oxaloacetate	0.052		

TABLE II

KINETIC PARAMETERS OF DIMERIC ASPARTATE AMINOTRANSFERASE AT 25°C

K_α and K_A are the Michaelis constants for α-keto-glutarate and aspartate, respectively, and k_f is the catalytic center activity for the forward reaction of Eqn 1.

	Buffer	
	0.2 M Tris · HCl, pH 8.0	0.1 M $Na_4P_2O_7$, pH 8.0
K_α (mM)	0.18	0.51
K_A (mM)	3.2	3.4
K_f (s^{-1})	224	304
Number of experiments	2	2

constant substrate concentrations is shown in Fig. 8. The experiment was done in 0.2 M Tris · HCl at pH 8.0 with $5.0 \cdot 10^{-4}$ M aspartate and $9.8 \cdot 10^{-3}$ M α-ketoglutarate as substrates. The decrease of $v/[E_0]$ as the enzyme concentrations is raised is consistent with the constants given in Tables I and II since the dimer has a slightly lower catalytic center activity and a slightly larger Michaelis constant for aspartate. The results shown in Fig. 8 can be quantitatively analyzed if the observed behavior is attributed to a monomer-dimer equilibrium. Since the kinetic properties of the monomer and dimer are not markedly different, the amount of monomer relative to dimer will be approximately constant in the absence and presence of substrates (this will be discussed further in Discussion). In this case the initial velocity can be written as

$$v = \gamma_1[E_1] + \gamma_2[E_2] \tag{6}$$

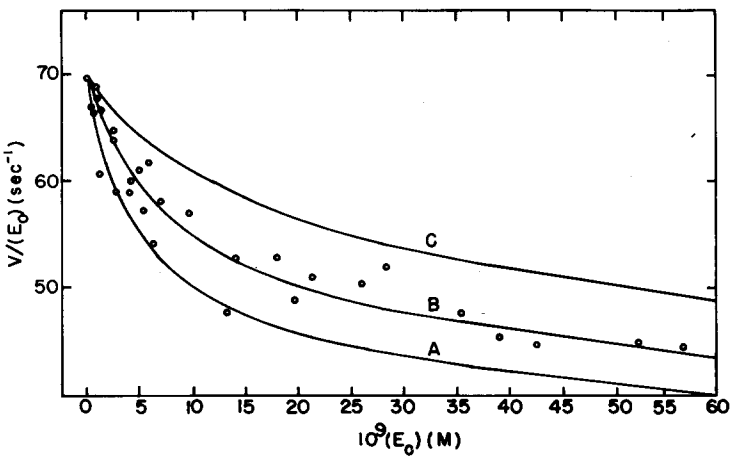


Fig. 8. Plot of initial specific activity as a function of enzyme concentration. The reaction mixture included $9.8 \cdot 10^{-3}$ M ketoglutarate and $5.0 \cdot 10^{-4}$ M aspartate in 0.2 M Tris · HCl, pH 8.0. The lines were calculated according to Eqn 9 using the constants in Tables I and II and constants of (A) 10 nM, (B) 20 nM, (C) 50 nM. The symbols differentiate three separate experiments.

where $[E_1]$ represents the total concentration of monomer, $[E_2]$ the total concentration of dimer and

$$\gamma_i = i\, k_{f(i)} \Big/ \left(1 + \frac{K_{AA(i)}}{[AA]} + \frac{K_{KA(i)}}{[KA]}\right) \tag{7}$$

In addition Eqn 3 and the following relation hold:

$$[E_0] = [E_1] + 2[E_2] \tag{8}$$

Combinations of Eqns 3, 6 and 8 give

$$v/[E_0] = (\gamma_1/4 - \gamma_2/8)(\sqrt{1 + 8[E_0]/K} - 1)(K/[E_0])) + \gamma_2/2 \tag{9}$$

Since the only unknown in Eqn 9 is K, a trial and error procedure was used to obtain a value of K consistent with this equation and the data. The solid lines in Fig. 8 are calculated according to Eqn 9, the data in Tables I and II and values of K of $1 \cdot 10^{-8}$, $2 \cdot 10^{-8}$, and $5 \cdot 10^{-8}$ M A dissociation constant of approx. $2 \cdot 10^{-8}$ M appears to fit the data best.

Discussion

The steady-state kinetic parameters for the monomeric α subform of aspartate aminotransferase are summarized in Table I. The equilibrium constant for the overall reaction, K_{eq}, can be written in terms of the steady-state parameters [5] as

$$K_{eq} = \left(\frac{k_r}{k_f}\right)^2 \frac{K_A K_\alpha}{K_G K_O} \tag{10}$$

where k_f and k_r are the catalytic center activities for the forward and reverse reactions of Eqn 1, and K_A, K_α, K_G, and K_O are the Michaelis constants for aspartate, α-ketoglutarate, glutamate, and oxaloacetate, respectively. Substitution of the data of Table 1 in Eqn 10 gives an overall equilibrium constant of 9.9. This agrees reasonably well with those directly determined, 7.8 [20], 6.7 [21] and 6.5 [22], and those determined from other steady-state investigations, 6.2 [5], and 4.6 [6].

An important consideration in this investigation is the method of assaying the enzymatic activity. By far the most convenient assay is the spectrophotometric measurement of the increase or decrease in oxaloacetate concentration at 280 or 260 nm. However, since the absorption band of oxaloacetate at these wavelengths is due to the enol form of the acid, while the keto acid is utilized by the enzyme, this method requires that the keto-enol equilibrium be adjusted rapidly relative to the rate of the enzymatic reaction. The keto-enol reaction is subject to general base catalysis, and one of the most efficient bases is Tris. This was the primary reason for choosing Tris buffer in this investigation. However, even with this buffer, the rate of the enzymic reaction exceeds the

rate of equilibration of the keto and enol forms at some of the enzyme and substrate concentrations of interest here.

A useful assay for measurement of the production of oxaloacetate, which is not dependent on the rates of the keto-enol reaction, is a coupled system employing malate dehydrogenase. This enzyme utilizes the keto form of oxaloacetate and reduces it to malate with a corresponding redox reaction involving NADH-NAD. The progress of this reaction can be easily monitored by spectrophotometric measurement of the decrease in the concentration of NADH. This assay is applicable as long as the rate of the reduction of oxaloacetate is rapid relative to transamination; this can usually be arranged by appropriate adjustment of the concentration of malate dehydrogenase.

Unfortunately, we have not been able to devise an assay which can be conveniently utilized to measure the rate of the transamination reaction between oxaloacetate and glutamate. Therefore, extensive kinetic studies on the dimer form of the enzyme have only been possible with aspartate and α-ketoglutarate as substrates. The direct assay has been used to study the kinetic properties of the monomer form of the enzyme for both the forward and reverse reactions.

In Table III a summary is given of the kinetic properties of the monomeric enzyme recorded in the literature. Unfortunately virtually every investigator has used a different buffer system so that quantitative comparison of the results is not meaningful. The steady-state kinetic parameters have been shown to be markedly dependent on the ionic composition of the medium [21,22]. However, the Michaelis constants and turnover numbers in all cases are not markedly different from those found for the α subform in 0.2 M Tris · HCl, pH 8.0, at 25° C. This is undoubtedly due to the fact that the α subform is the most active and prevalent form of the enzyme so that the results of previous investigations with less purified enzyme are largely dominated by the properties of the α subform.

A comparison of the parameters in Tables I and II shows that the kinetic properties of the monomer and dimer forms of the enzyme are not markedly different in 0.2 M Tris · HCl: the Michaelis constant for ketoglutarate is essentially identical, the Michaelis constant for aspartate of the dimer is less than twice that of the monomer, and the catalytic center activity of the dimer is slightly less than that of the monomer. This is only in semiquantitative agreement with the results of Polyanovskii and Ivanov [8,9] who reported a 4-fold increase in both the Michaelis constants for the dimer relative to the monomer, and an identical catalytic center activity for both aggregation states of the enzyme. However, their studies were carried out in 0.05 M Tris/sodium acetate, pH 8.5, at 15° C. Unfortunately, the steady-state parameters associated with the dimer could only be obtained for the forward reaction because of the difficulty in assaying the enzyme as discussed in detail earlier. This precludes a check of the kinetic data by use of Eqn 10. The dimerization dissociation constant found in this work, $2 \cdot 10^{-8}$ M, disagrees with that reported by Polyanovskii and Ivanov, $1.6 \cdot 10^{-7}$ M [9]. The method used to obtain the constant in this work is based upon the premise that either the aggregation process is slow relative to the overall enzymatic reaction or the aggregation of all enzymic species, i.e. free enzyme and enzyme-substrate complexes, is characterized by

TABLE III

SUMMARY OF THE KINETIC PARAMETERS OF ASPARTATE AMINOTRANSFERASE

K_A, K_α, K_O and K_G are the Michaelis constants for aspartate, α-ketoglutarate, oxaloacetate and glutamate, respectively, and k_f and k_r are the catalytic center activities for the forward and reverse reactions, respectively, of Eqn 1.

K_A (mM)	K_α (mM)	K_O (mM)	K_G (mM)	k_f (s^{-1})	k_r (s^{-1})	Conditions	Reference
1.8	0.20	0.052	10.5	326	1260	0.2 M Tris, pH 8.0, $\approx 10^{-9}$ M aspartate aminotransferase, 25°C	this work
3.2	0.18	—	—	224	—	0.2 M Tris, pH 8.0, $\approx 10^{-6}$ M aspartate aminotransferase, 25°C	this work
3.4	0.51	—	—	304	—	0.1 M Pyrophosphate, pH 8.0, $\approx 10^{-6}$ M aspartate aminotransferase, 25°C	this work
4.2	0.7	0.105	35	370	920	0.2 M Pyrophosphate, pH 8.0, $\approx 10^{-4}$ M aspartate aminotransferase, 25°C	2*
1.0	0.09	0.04	4	310	1000**	0.04 M Sodium arsenate, pH 8.0, 26°C, dilute enzyme	5
4.4	0.38	0.095	9.6	—	—	0.025 M arsenate, pH 7.4, 37°C, dilute enzyme	6
2.4	0.14	—	—	—	—	0.1 M Tris · HCl, pH 8.0, 25°C, dilute enzyme	7
3.9	0.57,	—	—	—	—	67 mM Phosphate, pH 7.4, 37°C, dilute enzyme	25
4.9	0.61						
5.9	12	0.17	5.4	—	—	16 mM Pyrophosphate, pH 7.4, 25°C, dilute enzyme	24
2.78	0.13	0.013	4.05	—	—	Imidazole I = 0.05, pH 6.9, 25°C, dilute enzyme	26

* The kinetic parameters were calculated from microscopic kinetic parameters determined by relaxation techniques with the assumption that the Michaelis constants are equilibrium dissociation constants.

** Estimated from the ratio of V_f/V_r given.

the same dimerization dissociation constant. At present no definitive evidence exists with regard to the first premise, although it is certain dimerization is not extremely slow. The second premise cannot be exactly correct since the principle of detailed balance would then require that the binding constants of the substrates to enzyme would be identical for both monomer and dimer. This is seen in the analogous equation for binding in a polymerizing system (Eqn 10 of ref. 27) in which the saturation curve of the polymer exactly duplicates that of the monomer if no binding sites and the intrinsic binding constants are unchanged. Since the change in the Michaelis constant is small, the dissociation constant should be a reliable estimate of the apparent dissociation constant and can be used to verify the assumption that the protein is essentially monomeric at 10^{-6} M.

These results are not in accord with those of Banks et al. [10] who suggest that dissociation of the enzyme, if it occurs at all, is incomplete at enzyme concentrations of $3 \cdot 10^{-9}$ M. At that concentration two activity peaks were found in the Sephadex elution pattern, a result not expected for a rapidly equilibrating dissociating system. Their experiments were done under quite different conditions (pH, buffer and temperature) with an enzyme which was probably a mixture of subforms. However, this seems insufficient to explain the qualitative differences found. It should be noted that gel filtration experiments on dissociating systems must be quantitatively interpreted by use of the frontal analyses used here or analysis of receding boundaries. The experimental conditions necessary for this analysis are not the same as those used for preparative gel filtration. Banks et al. [10], also found no variation in specific activity with enzyme concentration. However, these assays were done in the presence of inhibitors so that the effect of enzyme dissociation may have been obscured.

The results shown in Fig. 1 indicate that in our experiments aspartate aminotransferase is retarded in Sephadex gel filtration on dilution. The data are consistent with the occurrence of a reversible dissociation. Moreover, the value of the dimer dissociation constant inferred from both Sephadex gel filtration and steady-state kinetics is identical, namely $2 \cdot 10^{-8}$ M.

The kinetic properties of the dimer in 0.1 M pyrophosphate differ somewhat from those found in 0.2 M Tris · HCl, although this difference is not great. The purpose of obtaining the steady-state kinetic parameters in pyrophosphate was so that a meaningful comparison could be made between the steady-state kinetic studies and the kinetic studies of the enzyme carried out in pyrophosphate buffer with the temperature jump method. The temperature jump results predicted a steady-state catalytic center activity of 270 s^{-1} and dissociation constants for aspartate and ketoglutarate of $4.2 \cdot 10^{-3}$ and $7.0 \cdot 10^{-4}$ M, respectively [2], which are in reasonable accord with the data in Table II. One of the original purposes of this investigation was to see if substrate activation occurred with aspartate at low amino acid concentrations. The possibility of such a phenomenon was suggested by the concentration dependence of one of the relaxation times associated with the interaction of α-methyl aspartate and aspartate aminotransferase at low concentrations of α-methyl aspartate [3]. No appreciable deviations from the behavior predicted by Eqn 4 were observed, so that substrate activation seems quite improbable in the case

of aspartate. The concentration dependence of the relaxation time at very low α-methyl aspartate concentration probably requires an alternative explanation.

In summary, the steady-state kinetic properties of monomeric and dimeric α-aspartate aminotransferase have been determined and the dimerization constant has been estimated to be about $2 \cdot 10^{-8}$ M. The results obtained are consistent with the mechanism of Eqn 1 and indicate the aggregation state of the enzyme does not have a marked influence on its kinetic properties [21].

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