Glutamic-Aspartic Transaminase

VIII. EQUILIBRIUM KINETICS WITH ASPARTATE

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Fig heart "soluble" glutamic-aspartic transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) will catalyze the exchange of an amino group between glutamate and ketoglutarate without the participation of any other amino acid or keto acid (1). This "exchange transamination" also occurs between aspartate and oxaloacetate. The rates of these reactions are comparable to the physiological reversible transfer from aspartate to ketoglutarate under identical conditions. Because the concentrations of substrates do not change, such "exchange transaminations" provide ideal steady state systems for the spectroscopic analysis of enzyme substrate complexes when the enzyme concentration is greatly increased.

Two forms of the enzyme, (E₁) and (E₂), have been prepared (2-5) which contain pyridoxal phosphate and pyridoxamine phosphate, respectively. Since these two forms of the enzyme are instantaneously, and specifically, interconverted by the addition of amino acid and keto acid substrates, they must be considered to be among the components of the exchange transamination equilibria (Fig. 1). Although the pyridoxal and pyridoxamine forms of the enzyme have been well characterised spectroscopically, rigorous characterization of the other complexes in the equilibria has not yet been accomplished, owing to the fact that a wide variety of such complexes exists and their spectra closely resemble those of the pyridoxal and pyridoxamine forms of the enzyme (1, 2, 6). Until now this has prevented the determination of the relative stabilities of the enzyme substrate complexes and hence the elucidation of the detailed mechanism of the reaction.

Although exchange transamination at a constant pH between amino acid (A) and keto acid (O) involves many forms of the enzyme, these are equilibrium mixtures of relatively few possible species as shown in Fig. 1. Three possible types of binary complexes are shown in Fig. 1. The complex (O·E₁) between keto acid (O) and the pyridoxal form of the enzyme (E₁) together with that (A·E₂) between amino acid (A) and the pyridoxamine form of the enzyme (E₂) we term "abortive complexes." This term has been used to describe ternary complexes with lactate dehydrogenase which contain either lactate and reduced diphosphopyridine nucleotide or pyruvate and diphosphopyridine nucleotide. We refer to (EX) as the intermediary binary complex because it appears to be an obligatory intermediate on the route from the pyridoxal form of the enzyme (E₁) to the pyridoxamine form (E₂).

No convincing evidence for the participation of the ternary complexes (O·EX) and (A·EX) as even minor components of such exchange transamination equilibria has yet been found. Since the data are adequately and consistently described by assuming the formation of only the binary complexes, (OE₁), (EX), and (A·E₂), simplified equations, without consideration of the ternary complexes, will be presented in this paper. This is equivalent to an assumption that the dissociation constants K₁ and K₂ are much greater than the concentrations of amino acid and keto acid employed. For the experiments in this paper, these concentrations were even insufficient to form appreciable concentrations of the "abortive" complexes O·E₁ and A·E₂.

These experiments are concerned solely with the substrate pair aspartate and oxaloacetate. The interactions of aspartate and oxaloacetate provide a favorable system to introduce and compare several different methods we have recently developed for the determination of substrate dissociation constants, because there is an enzyme substrate complex in the equilibrium mixture (EX) which absorbs at 430 mµ. The protonated pyridoxal form of the enzyme (E₁) and its complex with oxaloacetate also absorb at 430 mµ (7), but experimental conditions were selected so that neither was present in appreciable amounts. A further advantage of the aspartate-oxaloacetate system is the high affinity of the pyridoxal form (E₁) for oxaloacetate, which permits the intermediary complex mixture absorbing at 430 mµ to be formed in substantial amounts upon the addition of only the amino acid to the pyridoxal form of the enzyme. A similar situation has been investigated with the substrate analogue β-hydroxyaspartate (8).

EXPERIMENTAL PROCEDURE AND RESULTS

Materials

Malic dehydrogenase was obtained from Worthington.

L-Aspartic acid and oxaloacetate were obtained from Calbiochem. The oxaloacetate was found to be better than 95% pure by enzymatic assay with malic dehydrogenase.

Radioactive aspartic acid was also obtained commercially. Although it appeared to contain no radiochemical impurities other than aspartic acid, 6% of the radioactivity was inactive as a transaminase substrate. Appropriate corrections were made for this contaminant, which presumably was n-aspartate.

Glutamic-aspartic transaminase was prepared with glutarate...
A + OEX $\xrightarrow{K_2} A$

A + E $\xrightarrow{K_1} EX \xrightarrow{K_2} E + O$

FIG. 1. The possible binary and ternary complexes involved in exchange transamination.

$D_1$ and $D_2$ are the 430 nm absorbances at concentrations $S_1$ and $S_2$, respectively. The lines illustrate the effects of choosing different values ($S_1$) for the reference absorbance $D_1$. The dissociation constant (0.112 M) was determined by the method of least squares from the data for ($S_1$) = 0, $D_1$ = 0.032, because these points have the least variances. The other lines are visual estimates based upon this same dissociation constant. All solutions contained 0.1 M pyrophosphate buffer, pH 8.0, and 1.44 x 10^-4 M bound pyridoxal phosphate. The numbers are the reference millimolar concentrations of oxaloacetate.

buffer instead of maleate buffer (9) by the procedure described earlier (7). Elution from a column of carboxymethyl Sephadex with 0.18 M acetate buffer (pH 5.25) and precipitation with 50% acetone (10) yielded additional purification. It was noted that the ratio of absorbances, $A_{430}:A_{468}$, varied through the elution peak from the carboxymethyl Sephadex column from 1 to 3.1. Therefore, only the later fractions were pooled. After acetone fractionation the enzyme which was used in these experiments had absorbance ratios $A_{430}:A_{468} = 2.8$ (0.1 M acetate, pH 5.2) and $A_{427}:A_{468} = 11.4$ (0.1 M pyrophosphate, pH 8.0). This enzyme has been crystallized, but even the crystals yielded three anionic bands upon starch gel electrophoresis (cf. Fig. 3 of Reference 11). No apparent effects of this heterogeneity have ever been observed in the titrations of the enzyme with substrate or inhibitors, but the heterogeneity may obviously affect the reproducibility of the results with different enzyme preparations.

The pyrophosphate buffer was made by titrating tetrasodium pyrophosphate with hydrochloric acid.

**Determination of Radioactive Exchange Rates**

Reactions were carried out in 1-ml volumes. To separate the aspartate from oxaloacetate 15-μliter aliquots were removed and spotted on paper strips which had been previously moistened with 97% formic acid to stop the reaction. The strips were then dried and subjected to electrophoresis in a Spinco Durrum apparatus for 3 hours at 400 volts with a buffer containing 31.2 ml of 97% formic acid and 98.2 ml of glacial acetic acid per liter.

The strips were again dried, and the radioactive zones were located with a Vanguard Autoscanne (model 880). Each zone was cut into two strips (2 x 7 cm) which were fitted around the bottom of a polystyrene vial for scintillation counting in a Packard Tri-Carb scintillation spectrometer (model 314 EX-2). A scintillation fluid (10 ml) containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2'-(5'-diphenyloxazolyl)benzene in toluene yielded a counting efficiency of 60% by this procedure.

**Spectroscopic Determination of Dissociation Constants in Simple Systems**

The simplest type of interaction to analyze is that in which one colored component (E) reacts with a colorless component (S) to yield a colored complex (ES).

$$E + S \rightleftharpoons ES$$

It is assumed that the concentration of the free (S) is known, either because it is supplied by some "buffer" system or, alternatively, because the amount added originally is much greater than the total concentration of enzyme (E0). It can be shown that the absorbance observed (D) is related to the extinction coefficients of E ($e_1$) and of ES ($e_2$) by the expression

$$D = e_1D_1 + e_2E_0$$

where

$$E_0 = (E) + (ES)$$

and

$$\frac{(E)(S)}{(ES)} = K$$

It follows that

$$\frac{(S_2 - S_1)}{(D_2 - D_1)} = \frac{(K + S_1)(K + S_2)}{K(e_1 - e_2)}$$

where $(D_1 - D_2)$ is the change in absorbance at any wave length upon increasing the substrate concentration from $S_1$ to $S_2$. Fig. 2 shows the application of this equation to the determination of the dissociation constant of the interaction of oxaloacetate with the pyridoxal form of the transaminase at pH 8, where the interaction causes an increase in the absorbance at 430 nm. The figure illustrates the chief drawback of the equation. Since it involves the reciprocal of a difference, the scatter of the experimental points increases markedly for the smaller differences in

1 M. Martinez-Carrion and W. T. Jenkins, unpublished experiments.
It can be seen that the dissociation constant for oxaloacetate at pH 8 (0.112 M) is very much greater than the concentrations of enzyme required to measure the absorbances. When the ratio $S_1:S_2$ is small, a more suitable form of the equation for plotting is

\[
\frac{(1 - S_1/S_2)}{(D_1 - D_2)} = \frac{(1 + K/S_2)(1 + S_1/K)}{(e_1 - e_2)(E_0)}
\]

(2)

It has been customary to make the ratio $S_1:S_2 = 0$ by measuring absorbances relative either to that observed when $S_1 = 0$ or, alternatively, to that observed when $S_2$ was infinitely large. Since both of these are impracticable in many spectroscopic analyses of the enzyme-substrate interactions, the unmodified Equation 2 is used.

Fig. 3 shows the application of Equation 2 to the protolytic dissociation of the pyridoxal form of the enzyme. The combination of the prosthetic group with a proton causes a decrease in absorbance at 362 μm and an increase in absorbance at 430 μm (7). The proton concentration is assumed to be proportional to the ratio of the concentrations of the acidic and basic species of the phosphate buffer (12). The figure shows that the same dissociation constant is obtained when the measurements are made at any wave length and plotted, in this case, relative to the absorbance observed with a ratio of monobasic phosphate to dibasic phosphate of 50.

**Spectroscopic Determination of Dissociation Constant upon Addition of Both Amino Acid and Keto Acid**

When no ternary complexes are formed, the scheme shown in Fig. 1 can be considerably simplified by measuring the variation of the total concentration of all the intermediary binary complexes (EX) with the amino acid (A), keeping the ratio of amino acid to keto acid constant at a known value, R. It can be easily shown (13) that one obtains an apparent dissociation constant for (A) of $K'$ where

\[
K' = \frac{(K_1 + R \cdot K_2)}{(1 + K_1/R \cdot K_2)} + \frac{[R \cdot K_2]/K_2}{1 + K_1/R \cdot K_2}
\]

It has been suggested that the rate of exchange transamination or the absorbance at about 500 μm could be used as appropriate measures of (EX) (13). A more detailed subsequent analysis based upon Equation 2 showed that in fact the absorbance could be measured at any wave length, the only restriction being that the concentrations of substrates were not appreciably altered by the amounts bound to the enzyme or formed in the reaction ("Appendix"). This approach had been investigated earlier (2) but was then abandoned. It was thought to be impracticable to measure a reference absorbance at either zero or infinite substrate concentrations or when no unique absorption maximum could be ascribed to the (EX) mixture.

The linear form of the equation enables an accurate prediction of the absorbance for zero substrate concentration. This is extremely valuable because the resulting apparent extinction coefficient ($e'$) provides an independent measure of the ratio of the two dissociation constants ($K_1/K_2$) from the equation ($e' = e_1/R = (e_1 - e_2)/K_1$, since the extinction coefficients of the pyridoxal form ($e_1$) or pyridoxamine form ($e_2$), or both, are known independently.

Fig. 4 shows the application of this equation to the aspartate-oxaloacetate interaction for amino acid to keto acid ratios (R) of 1 and 10. It was found that the apparent dissociation constant was almost independent of the ratio and that the apparent zero concentration extinction coefficient was very close to that of the pyridoxal form of the enzyme.

Analysis of the data in Fig. 4 showed that the value of $K_1$ was 3.2 mM, and that the ratio of the amino acid dissociation constant to that of the keto acid dissociation constant ($K_1/K_2$) was of the order of 100. $K_4$ could not be accurately determined but was greater than 10 mM.
Some of the mixture of intermediary binary complexes \((EX)\) which absorbs at 430 m\(\mu\) at pH 8 is formed when only aspartate is added to the pyridoxal form of the enzyme (Fig. 5). It was possible to compare these results, therefore, with those obtained by an independent method described previously (8).

This earlier analysis was based upon the fact that at an appropriate wave length at which an absorbance change \((D = \epsilon \cdot (EX))\) is an accurate measure of the change only in the intermediary binary complex \((EX)\), there is a simple relationship between this change and the total enzyme concentration employed \((E_0)\).

\[
\frac{(E_0)}{D} = \frac{1 + K_1/(A)}{\epsilon} + \sqrt{\frac{K_2 [1 + (A)/K_1]}{\epsilon \cdot D}}
\]

Thus, when the reciprocal of the experimental apparent extinction coefficient \((E_0)/D\) is plotted, at a series of amino acid concentrations \((A)\), against the reciprocal of the square root of the observed absorbance \((D)\), one obtains straight lines. The ordinate intercepts of these lines \([1 + K_1/(A)]/\epsilon\) may be replotted to yield both the amino acid dissociation constant \(K_1\) and the maximum extinction coefficient \(\epsilon\). The slopes may be replotted to yield the keto acid dissociation constant \((K_2)\) and the amino acid abortive complex dissociation constant \((K_3)\). A constant slope implies that \(K_3\) is much larger than the amino acid concentration \((A)\).

Fig. 6 shows the results which were obtained upon the titration of a variety of enzyme concentrations with small aliquots of aspartate. The values obtained for the dissociation constants (3.3 mM and 16 \(\mu\)M) agree very well with those found by the method described above. Moreover, the fact that the slopes of the lines did not vary confirms the previous observation that did not dissociate into keto acid and the pyridoxamine form of the enzyme. The slope is the square root of the ratio of the dissociation constant for the keto acid to the molar absorbance of the intermediary binary complex at this wave length. This latter may be determined from the secondary plot in B, which is a secondary plot based on the ordinate intercepts of A. The dissociation constant for aspartate can be shown to be equivalent to the negative intercept on the abscissa of such a plot. The slope is the reciprocal of the molar absorbance of the intermediary binary complex.
the amino acid does not appreciably combine with the pyridoxamine form of the enzyme (i.e. $K_a$ is very large). These results are in marked contrast with the interaction which was observed with the substrate analogue $\text{c} \text{yste} \text{n-oxy-3-hydroxy-}$

\text{aspartate} (8).

**Determination of Dissociation Constants from Exchange Reaction Kinetics**

To measure the rate of the exchange reaction it is necessary to dilute the enzyme considerably (1000-fold). Since the enzyme is a dimer (7), this might cause a dissociation into subunits and hence exert a drastic effect upon the substrate dissociation constants.

It is assumed that the rate of exchange transamination is a measure of the relative amount of ($EX$). If the ratio of amino acid to keto acid is kept constant ($R$), this rate ($v$) is given by the simple equation

$$
V/v = 1 + K'/v'
$$

where $K'$, as before, is

$$
(K_1 + R \cdot K_2)/(1 + K_2/(R \cdot K_2) + [R \cdot K_2]/K_2)
$$

and $V_e$ is the maximum rate of exchange for a particular concentration ratio of amino acid to keto acid.

The rate of radioactive exchange is given by the equation

$$
\frac{-\ln(C_0 - C_t)/(C_0 - C_\infty)}{t} = (1 + R) \cdot \frac{t}{v'}
$$

where $t$ is the time and $C$ is the observed count, at times indicated by the subscripts, measured under standard conditions. Data from the course of a reaction are shown for aspartate in Fig. 7 to indicate the excellent reliability we obtained from the experimental points. Erratic results were obtained for oxaloacetate owing to its decomposition.

To determine the apparent dissociation constant $K'$, a relatively small value of the ratio of amino acid to keto acid concentrations ($R$) was chosen and the reactions were allowed to proceed until exchange reached about 50% completion. The data were then plotted in accordance with the combined equation

$$
\frac{(A)/v}{v} = (1 + R) \cdot \frac{t}{\ln[(C_0 - C_t)/(C_0 - C_\infty)]} = [(A)/v] + \frac{K'}{v'}
$$

where $A$ is the absorbance increment (A) relative to those at 362 nm (0.490) and 430 nm (0.420) with an amino acid concentration of 0.04 M. Solutions contained 0.1 M pyrophosphate buffer, pH 8.

The values of $K'$ found in three experiments were 2.2, 2.3, and 2.8 mM (Fig. 8). The corresponding values of $V_e$ were 17,000, 16,200, and 19,000 per molecule of bound pyridoxal phosphate. From these figures we calculated a value of $K_1 = 3.1$ mM, assuming a value of 0.112 M for $K_s$ and a value for the ratio $K_1:K_2$ of more than 50.

The value of $K'$ determined spectrophotometrically under comparable reaction conditions was found to be 4.7 mM (Fig. 9).

It was of interest to compare the value found for $K_1$ for aspartate with its Michaelis constant ($K_m$) derived from the reaction with ketoglutarate. $K_m$ should, of course, be greater than $K_1$ because of the Briggs-Haldane steady state correction, and because $K_1$ is relative to all of the possible intermediary binary complexes, whereas $K_m$ is relative only to those on the aspartate side of the rate-limiting step in the reaction. Fig. 10 shows that concentrations of 2 and 4 mM ketoglutarate (O) were almost
assay mixtures initially contained $10^{-4}$ M DPNH, an excess of the concentration of substrates (A) and (O), $V_f/v = 1 + K_A/(A) + K_O/(O)$, values of 2.9 mM and 36,400 per mole of bound pyridoxal phosphate per min were derived for the $K_m$ for aspartate ($K_a$) and the maximum forward velocity ($V_f$), respectively. Inhibition by ketoglutarate was not considered since at 25°, but otherwise, under comparable conditions, the dissociation constant for ketoglutarate from its complex with the pyridoxal form of the enzyme was found to be 84 mM by the spectroscopic procedure of Fig. 3. This is much higher than the concentration of ketoglutarate employed.

FIG. 10. Determination of the $K_m$ for aspartate at 37°. All assay mixtures initially contained $10^{-4}$ M DPNH, an excess of malic dehydrogenase, 0.1 M pyrophosphate buffer (pH 8), and enzyme containing $4.2 \times 10^{-9}$ m bound pyridoxal phosphate. The data illustrate the velocity dependence upon aspartate concentration with two series of keto acid concentrations.

DISCUSSION

The different methods for the determination of enzyme-substrate dissociation constants gave values which were in surprisingly good agreement over a considerable concentration range. The average aspartate dissociation constant at 37° determined kinetically with $6.3 \times 10^{-9}$ m bound pyridoxal phosphate was 2.4 mM, whereas determined spectroscopically with $1.56 \times 10^{-9}$ m bound pyridoxal phosphate it was 4.7 mM. We thought that the discrepancy was not significant in view of the errors involved and the fact that for the latter experiment a different enzyme preparation had to be used. A recent paper by Polyanovskii and Ivanov (14) shows, however, that upon dilution the enzyme appears to dissociate, as judged by the changes in the polarization of fluorescence. They associated this dissociation with a small decrease in the $K_m$ for aspartate from 5.1 mM to 1.2 mM (Tris-acetate buffer, pH 8.5) as the concentration of bound pyridoxal phosphate was decreased from $4 \times 10^{-6}$ M to $5 \times 10^{-6}$ M. The small decrease we found for the dissociation constant with dilution would thus be consistent with their findings since comparable concentration ranges were studied in both cases. Since the $K_m$ derived from the data in Fig. 10 was obtained with an enzyme concentration 15-fold lower than the dissociation constant derived from the data in Fig. 8, they are not strictly comparable, the $K_m$ figure being possibly slightly higher at the lesser dilution. The good agreement, which was observed, would otherwise indicate that the Michaelis constant is a true dissociation constant and that the equilibria in the steady state exchange system are such that no appreciable amount of enzyme exists as a binary complex to the pyridoxamine side of the reaction, which is rate-limiting in the transamination with ketoglutarate.

The ratio of amino acid dissociation constant to keto acid dissociation constants ($K_1:K_2$) is an important ratio closely related to the relative binding constants for pyridoxal phosphate and pyridoxamine phosphate (13). This ratio may be obtained (by the methods described in this paper) either directly or, less accurately, by the separate determination of $K_1$ and $K_2$. It has been suggested that this ratio may be derived from the amount of amino acid required to cause a half-maximal spectral change of the pyridoxal form of the enzyme (5). Such a procedure is clearly invalid if appreciable amounts of binary complexes are formed, as is the case for the reaction with aspartate.

Hammes and Fasella (15) have calculated values for the individual rate constants for the aspartate-oxaloacetic reaction from rapid changes in absorbance at 430 m$m$ after an even more rapid “temperature jump.” The value for $K_1 = (E)/(A)/(EX)$ derived from these rate constants is an order of magnitude less than that reported here. Their treatment appears to have been based on an invalid assumption that their unknown indicator, absorbing at 430 m$m$, provided a satisfactory measure of two slow reactions separated by a fast reaction.

The spectra with a high concentration of both enzyme and aspartate showing maxima at 330 m$m$ and 430 m$m$ and an inflection at about 490 m$m$ (Fig. 5) were found to be identical at pH values 8 and 10. Therefore, it appears that the observable equilibria between the aspartate binary complexes do not involve protolytic dissociations, a fact which had been deduced from the lack of variation of $V_{max}$ with pH (5). They are comparable in this respect to what has been observed previously with the pig heart glutamic-alanine transaminase (13) and the glutamic-aspartic transaminase reaction with the substrate analogue β-hydroxyaspartate (8).

The reaction with glutamate and ketoglutarate together with some other methods for the determination of enzyme-substrate dissociation constants will be described in a later paper.

It is hoped that the methods presented in this paper will be generally useful in the study of other enzymatic steady state spectral changes and exchange reactions. They should permit more rigorous, detailed analyses of the reaction mechanism by the methods of “temperature jump” rapid kinetics (16) and circular dichroism (17).

SUMMARY

1. The pig heart soluble glutamic-aspartic transaminase was shown to react with mixtures of aspartate and oxaloacetate to form a characteristic binary complex absorbing at 430 m$m$.
2. A method is presented for the spectroscope determination of dissociation constants when only a restricted range of reagent concentrations may be employed.
3. It is shown how such a method may be extended to analyze the enzyme-substrate dissociation constants in transaminase “exchange transamination” equilibria.
4. The dissociation constants obtained by this method, which is generally applicable, were shown to be identical with those obtained by an independent, less versatile, spectroscope procedure described previously.
5. A kinetic analysis of the transamination reaction from labeled aspartate to oxaloacetate with a much lower enzyme concentration yielded a comparable dissociation constant for
aspartate and proceeded at a maximum rate about half that of
the reaction of aspartate with ketoglutarate under comparable
conditions.

6. The $K_m$ constant for aspartate reacting with an excess of ketoglutarate was found to be virtually identical with the disso-
ciation constants derived from the rate of the exchange trans-
amination reaction and with the dissociation constant derived
spectroscopically at much higher enzyme concentrations.

APPENDIX

If the ratio of amino acid to keto acid ($R$) is kept constant,
it can easily be shown that the reaction becomes simply the con-
version of an equilibrium mixture of forms of the free enzyme
$[(E_1) + (E_2)]$ with apparent extinction coefficient $e_1'$ into an
equilibrium mixture of binary complexes $[(O \cdot E_1) + (EX) +
(A \cdot E_2)]$ with an apparent extinction coefficient $e_2'$, where

$$
\frac{(A)[(E_1) + (E_2)]}{[(O \cdot E_1) + (EX) + (A \cdot E_2)]} = K' = \frac{(K_1 + RK_2)}{[1 + K_1/(RK_2) + (RK_2)/K_1]}
$$

$e_1'$ is related to the extinction coefficients for $(E_1)$ and $(E_2)$, $e_1$ and
$e_2$, respectively, by the relationship

$$
e_1' = \frac{e_1 + e_2(RK_2)/K_1}{1 + (RK_2)/K_1}
$$

On the other hand, $e_2'$ is related to those of the binary com-
plexes $(O \cdot E_1)$, $(EX)$, and $(A \cdot E_2)$. If these are $e_0$, $e_4$, and $e_5$, re-
spectively, $e_2'$ is given by the expression

$$
e_2' = \frac{e_0K_1 + e_5RK_2}{1 + K_1/(RK_2) + (RK_2)/K_1}
$$

The value of $K'$ can be determined from Equation 2, in which
the apparent dissociation constant and extinction coefficients
replace the true values of the simpler systems.

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