Kinetic Studies of Rat Liver Glutamic-Alanine Transaminase*

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With the discovery that pyridoxal phosphate and pyridoxamine phosphate were cofactors for enzymatic transamination, it was proposed that the reaction was biphasic, involving first an amino group transfer from the amino acid substrate to enzyme-bound pyridoxal phosphate, followed by a transfer of the amino group to the keto acid substrate (for review, see (1)). With the availability of a highly purified transaminase, Jenkins and Sizer (2) were able to study individual half-reactions and obtained results consistent with the proposed role of pyridoxal as an amino group carrier.

These experiments, however, were unable to distinguish between the alternatives of a single enzymatic site for all substrates versus two sites of attachment, each specific for a keto acid-amino acid pair. More recently, the extensive kinetic studies of Velick and Vavra (5) with glutamic-aspartic transaminase of pig heart have ruled out certain types of two-site mechanisms for this enzyme and have led to the proposal of a single site of attachment of all substrates as the most attractive mechanism.

Similar kinetic studies have been performed with a highly purified glutamic-alanine transaminase of rat liver (6) and are reported in the present communication.

EXPERIMENTAL PROCEDURE

Preparation of the enzyme and the method of assay of the reaction in the direction of pyruvate and glutamate formation have been described (6). For assay in the direction of α-ketoglutarate and alanine formation, α-ketoglutarate production was coupled to DPNH oxidation via the glutamic dehydrogenase system. Pyruvate, which is a substrate for the transamination reaction in this direction, is slowly reduced by DPNH in the presence of glutamic dehydrogenase (7). Therefore, an amount of glutamic dehydrogenase was used that allowed only a minimal rate of pyruvate reduction but still was not rate-limiting in measurements of α-ketoglutarate production. In each assay, the rate of DPNH oxidation by pyruvate before the addition of the transaminase was measured and subtracted from the rate of DPNH oxidation after addition of the transaminase. The other substrate for the transamination reaction, glutamate, inhibits the glutamic dehydrogenase-catalyzed reduction of α-ketoglutarate, and therefore the maximal amount of glutamate that could be added to the assay system was limited.

Aminooxyacetate was a gift from Dr. P. W. O'Connell of the Upjohn Company.

RESULTS

Michaelis Constants—The determination of apparent Michaelis constants of all four substrates at several levels of their respective cosubstrate is shown in Figs. 1 to 4, together with the extrapolations to absolute values of the Michaelis constants and maximal velocity at infinite concentration of cosubstrate, on the basis of Equation 1. It was noted that alanine was inhibitory at high concentrations, when the α-ketoglutarate concentration was low.

The values of absolute Michaelis constants and absolute maximal velocities from these experiments are summarized in Table I.

Inhibition by Aminooxyacetate—Aminooxyacetate, which has been reported by Wallach (8) to be a potent inhibitor of glutamic-γ-aminobutyrate transaminase of brain and Escherichia coli, also strongly inhibited liver glutamic-alanine transaminase. This inhibition was highly dependent upon pH in the range from 7 to 8 (Fig. 5). Since the pK values of the dissociable groups of aminooxyacetate are approximately 3.5 and 5.3, the pH effect must depend upon a dissociable group of the enzyme (see "Discussion").

The inhibition by aminooxyacetate was competitive with the amino acid substrates and of a type that is formally identical with uncompetitive kinetics (9) with respect to the keto acid substrates (Fig. 6). From Equations 3 and 4, values of the inhibitor-binding constant at pH 7.3 of 3.4, 8.3, 9.7, and 4.0 × 10⁻⁸ M (average, 6.4 × 10⁻⁸ M) can be calculated from the data with alanine, glutamate, pyruvate, and α-ketoglutarate as the variable substrate, respectively (Fig. 6).

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1 A third type of mechanism involving a site specific for keto acids and one for amino acids has been proposed (2), but Velick and Vavra (4) have presented a cogent theoretical objection to this proposal.
2 C. Frieden, personal communication.
3 The terms, "apparent Michaelis constant" and "apparent maximal velocity," refer to values that are dependent upon the concentration of the cosubstrate. By "absolute Michaelis constant" is meant the concentration of substrate that gives half-maximal velocity at infinite cosubstrate concentration (extrapolated). By "absolute maximal velocity" is meant the velocity at infinite concentration of both substrates (extrapolated).
4 For method of calculation of these parameters, see the Appendix.
5 P. W. O'Connell, personal communication. We have also confirmed the location of these pK values.
Fig. 1. Kinetics with alanine as the variable substrate. The medium contained, in a final volume of 3.1 ml, 300 μmoles of sodium pyrophosphate, pH 7.8, 400 μg of DPNH, and approximately 4 units (micromoles per minute) of crystalline lactic dehydrogenase. Three different levels of cosubstrate were used; their final concentrations are shown on the curves. \( v_0 \) is expressed in micromoles formed per minute. \( v_0/(\text{ALA} \times 10^3) \) was calculated from the millimolar concentration of L-alanine. The reaction was started by the addition of 0.29 μg of enzyme. The reaction was measured in a Cary model 11 spectrophotometer water-jacketed at 37°C. Curves B and C show the extrapolations of the apparent maxima velocities and Michaelis constants (in millimolar units), respectively, to infinite α-ketoglutarate (α-KG) concentration.

Fig. 2. Kinetics with α-ketoglutarate as the variable substrate. Conditions and symbols are as described in Fig. 1.

extrapolations involved in the calculations is considered, these values are in good agreement.

Inhibition by Maleate and Acrylate—Maleate inhibits heart glutamic-aspartic transaminase competitively with all substrates (5) and protects that enzyme against heat denaturation (10). Acrylate, the analogous α,β-unaturated derivative of the 3-carbon substrate in the glutamic-alanine system, protects this enzyme against heat denaturation (8). Therefore, it was of interest to test these compounds as inhibitors of the glutamic-alanine enzyme (Figs. 7 and 8). With both maleate and acrylate, essentially the same value of the affinity constant (0.035 to 0.055 μ) was obtained whether binding to the 3-carbon site or the 5-carbon site, or to the pyridoxal or pyridoxamine form of the enzyme, was measured, as was the case with maleate inhibition of glutamic-aspartic transaminase (5).

Exchange Reactions—The rates of transamination from C14-labeled alanine to pyruvate and from C14-labeled glutamate to α-ketoglutarate are shown in Fig. 9. The initial rates were 0.042 μmole exchanged per minute in both systems, which may be compared with a rate of 0.037 μmole of pyruvate formed per minute from alanine plus α-ketoglutarate and a rate of approximately 0.05 μmole of α-ketoglutarate formed per minute (calculated) from glutamate plus pyruvate under the same conditions.

DISCUSSION

A reaction pathway that is in harmony with the kinetic results reported here is the following,

\[
E + A \xrightarrow{k_1} X \xrightarrow{k_2} \frac{E'}{k_3} + D \\
E' + B \xrightarrow{k_5} Y \xrightarrow{k_3} E + C
\]

where \( E' \) and \( E'' \) are distinct forms of the enzyme (in this case...
TABLE I

Kinetic parameters of glutamic-alanine transaminase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Absolute Michaelis constant</th>
<th>Absolute maximal velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>34 mM</td>
<td>145 μmoles min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>1.1</td>
<td>121 μmoles min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>15</td>
<td>125 μmoles min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.9</td>
<td>139 μmoles min⁻¹ mg⁻¹</td>
</tr>
</tbody>
</table>

* Average values.

pyridoxal and pyridoxamine forms), A and B are reactants, C and D are products, and X and Y are enzyme-substrate complexes.⁶

The rate equation for this sequence has been presented by Alberty (11) and may be written for the forward reaction in the form,

\[ v_f = \frac{k_3[E_o]}{1 + \frac{k_2}{k_1} \left( \frac{k_B}{(B_o)} \right)} \]

where \( v_f \) is the initial velocity of the forward reaction, \((A_o)\), \((B_o)\), and \((E_o)\) are initial concentrations of reactants and enzyme. \( K_A = (k_2 + k_4)/k_1 \) and \( K_B = (k_6 + k_7)/k_5 \).

At any concentration of \( B \), the value of the apparent maximal velocity at infinite \((A_o)\), \( V'_A \), and of the apparent Michaelis constant of \( A, K'_{m-A} \), are

\[ V'_A = \frac{k_3(E_o)}{1 + \frac{k_2}{k_1} \left( \frac{k_B}{(B_o)} \right)} \]

and

\[ K'_{m-A} = \frac{K_A}{1 + \frac{k_2}{k_1} \left( \frac{k_B}{(B_o)} \right)} \]

The absolute maximal velocity of the forward reaction and the absolute Michaelis constant of \( A \) at infinite \((B_o)\) are

\[ v_f = \frac{k_3(E_o)}{1 + \frac{k_2}{k_1}} \]

and

\[ K_{m-A} = \frac{K_A}{1 + \frac{k_2}{k_1}} \]

⁶ This mechanism can be expanded to include any number of unimolecular rearrangements of the enzyme-substrate complex. However, these do not alter the form of the rate equation, but only the meaning of the rate constants. Velick and Vavra (4) have discussed the interpretation of the rate constants in these cases.

![Figure 5](http://www.jbc.org/)

**Fig. 5.** pH dependence of aminooxyacetate inhibition. Conditions were as described in Fig. 1, except that the pH of the pyrophosphate buffer was varied and the L-alanine and α-ketoglutarate concentrations were 17 mM and 2.0 mM, respectively. A final concentration of 10⁻¹ m aminooxyacetate was used. The upper curve in A is in the absence of inhibitor, and the lower curve in A is in the presence of inhibitor. The curve in B is calculated from the data in A.

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Kinetics of aminooxyacetate inhibition. Conditions and symbols are as described in Figs. 1 and 3, except that the pH of the buffer was 7.3 throughout. The variable substrate is indicated in each figure. The concentrations of cosubstrates were: α-ketoglutarate, 2.0 mM; L-alanine, 25 mM; pyruvate, 1.0 mM; L-glutamate, 12 mM. The upper curve in each figure is in the absence of inhibitor, and the lower curve, in the presence of 10⁻¹ m aminooxyacetate.

Both \( V'_A \) and \( K'_{m-A} \) increase with increasing \((B_o)\), and by the same proportion. Therefore, the ratio, \( V'_A/K'_{m-A} \), is a constant independent of \((B_o)\) (equal to \( V_f/K_{m-A} \) and \( k_3(E_o)/K_A \)). Entirely analogous rate equations arise for \( v_f \) as a function of \((B_o)\).
FIG. 7. Kinetics of maleate inhibition. Conditions and symbols are as described in Fig. 6, except that KPO₄ buffer was used and the cosubstrate concentrations were 4.0 mM for the keto acids, 150 mM for L-alanine, and 17 mM for L-glutamate. The inhibitor concentration was 0.05 m.

FIG. 8. Kinetics of acrylate inhibition. Conditions and symbols are as described in Fig. 7.

at constant (A₀) and for vᵢ as a function of (C₀) and (D₀).⁷ These properties of the rate equations are consistent with the data in Figs. 1 to 4.

From the relationship for this mechanism between the equilibrium constant and the kinetic parameters (10),

$$K_{eq} = \left[ \frac{v_{eq}}{v_{eq}} \right] = \frac{K_{m-A}K_{m-B}}{K_{m-C}K_{m-D}}$$

a value of 2.6 is calculated for K_{eq} for the reaction as written:

L-Glutamate + pyruvate → α-ketoglutarate + L-alanine

⁷To consider vᵢ as a function of (B₀) at constant (A₀), Equation 1 can be written in a suitable form by interchanging (A₀) and (B₀), K₄ and K₅, and k₆ and k₇. Equations of identical form can also be written for vᵢ, (initial velocity of the reverse reaction) as a function of (C₀) or (D₀) by employing the appropriate rate constants.

Their inclusion in the rate equation leads to

$$vᵢ = \frac{k₁(E₀)}{1 + k₅ \left[ 1 + \frac{K₄}{(B₀) \left( 1 + \frac{(I₀)}{K₅'} \right)} \right] + (A₀)}$$

If I can combine with both E and E', i.e. K₁ and K₁' both are finite, V'A decreases with increasing (I₀), whereas K₄m₋ₐ may increase, remain constant, or decrease, but to a lesser extent than V'A, with increasing (I₀), depending upon the relative values of K₄ and K₅', and the concentration of the cosubstrate.⁸

⁸If the ratio, K₁/K₁', equals [1 + (B₀)/K₅₋ₐ], the apparent Michaelis constant of A will be unaffected by (I₀). If the ratio is larger or smaller than this term, the apparent Michaelis constant of A will decrease or increase with increasing (I₀), respectively. If K₁ < or = K₁', the ratio, K₁/K₁', is always less than [1 + (B₀)/K₅₋ₐ], and K₅₋ₐ therefore always increases with (I₀).
The inhibitions by maleate and acrylate fit this mechanism, with $K_I = K'I_1$, as has also been reported by Velick and Vavra for the maleate inhibition of glutamic-aspartic transaminase (5).

The data presented in Fig. 6, on the other hand, for aminooxyacetate inhibition of glutamic-alanine transaminase fit a mechanism in which the inhibitor combines only with one form of the enzyme. If the inhibitor combines only with the form $E'$, i.e. the same form with which $A$ combines, then $K'I = \infty$. Therefore, Equation 2 becomes

$$v_i = \frac{k_i(B)}{K_A' \left[ 1 + \frac{K_B}{(B_o)} \right] + (A)} + (A)$$

Equation 3 predicts that $V'_A$ is independent of $(I_o)$ and that $K'_m-A$ increases with $(I_o)$, i.e. that $I$ is competitive with $A$. This is the relationship between aminooxyacetate and the amino acid substrates, as can be seen from Fig. 6.

If, on the other hand, the inhibitor combines only with $E'$, the same form with which the substrate, $A$, does not combine, then $K_I = \infty$, and Equation 2 becomes

$$v_i = \frac{k_i(E_o)}{1 + \frac{k_i}{k_l} \left[ 1 + \frac{K_B}{(B_o)} \right] + (A)}$$

Equation 4 predicts that both $V'_A$ and $K'_m-A$ decrease with increasing $(I_o)$, and in the same proportion. This behavior is formally identical with uncompetitive kinetics (9). This is the relationship between aminooxyacetate and the keto acid substrates, as can be seen from Fig. 6.

Thus the kinetic results are in complete accord with a mechanism in which the inhibitor, aminooxyacetate, combines only with that form of the enzyme with which the amino acid substrates combine. This mechanism would also explain the observations made by Leibman (3) with serine-alanine transaminase, that isoserine inhibited competitively with the amino acid substrates but not with the keto acid substrates.1

The pH dependence of aminooxyacetate inhibition requires explanation. The curve of percentage inhibition versus pH

If $K_I > K'I_1$, the effect of $(I_o)$ on $K'_m-A$ will depend upon $(B_o)$. In general, if $K_I = K'I_1$, the apparent Michaelis constants of all four substrates will increase with $(I_o)$, whereas if $K_I \neq K'I_1$, the apparent Michaelis constants of one type of substrate, e.g. the keto acids, will increase with $(I_o)$, and those of the other type, e.g. the amino acids, will decrease, remain constant, or increase with increasing $(I_o)$ as the concentration of cosubstrate is increased. Even though these kinetics are not those usually associated with competitive inhibition, Velick and Vavra (5) refer to this type of inhibition as competitive, since it results from a reaction mechanism in which the inhibitor competes with the substrates for a site or sites on the enzyme.

1 It is permissible to interchange the meanings of $A$ and $B$, $E$ and $E'$, and $K_I$ and $K'I_1$ to make this analysis, since the form of the rate equation is identical for all four substrates (see footnote 7).
substrates for exchange would have to compete for a single site, whereas those for net reaction would not.

On the basis of the single site postulation and the known specificity of the enzyme (6), it appears to be necessary to visualize a binding site with one point of attachment for the α-amino or keto group, another for the α-carboxyl group, and either a third, which can accommodate specifically the methyl group of alanine or pyruvate and the carboxyethylene group of glutamate or α-ketoglutarate, or two separate points of attachment for these groups, sufficiently close to fall within the limits of distortion of the bond angles of the α-carbon.

SUMMARY

The kinetics of glutamic-alanine transaminase have been studied and found to fit a reaction sequence in which the enzyme shuttles between two distinct forms. Michaelis constants have been determined for the four substrates of the forward and reverse reactions. Aminooxyacetate strongly inhibits the reaction by a mechanism involving combination with the pyridoxal form of the enzyme only. Maleate and acrylate are much weaker inhibitors and combine with both forms of the enzyme with the same affinity. The exchange reactions between the 3-carbon substrates and the 5-carbon substrates have been measured, and the rates have been found to be as rapid as the net reactions. On the basis of the data, it is proposed that there is a single site of attachment for all four substrates.

Acknowledgment—We are indebted to Drs. Velick and Vavra for the opportunity of seeing the results of their experiments before their submission for publication and before the completion of our own kinetic studies.

APPENDIX

Calculation of Absolute Maximal Velocities and Absolute Michaelis Constants—The apparent Michaelis constant is

\[ K_{m,A}^{'} = \frac{K_A}{1 + \frac{k_2}{k_1}(B_0)} \]

and the absolute Michaelis constant, at infinite \(B_0\), is

\[ K_{m,A} = \frac{K_A}{1 + \frac{k_2}{k_1}} \]

From Equation 5 above,

\[ \frac{1}{K_{m,A}^{'}} = \frac{1}{K_{m,A}} + \frac{k_2}{k_1} \frac{1}{K_A (B_0)} \]

Therefore, the ordinate intercept of a plot of \(1/K_{m,A}^{'}\) versus \(1/(B_0)\) equals \(1/K_{m,A}\). Absolute maximal velocities are obtained in an analogous manner.

10 The carboxyl group is apparently essential, since the analogue of pyruvate with a fully reduced carboxyl group (acetone) and the decarboxylated analogue (acetaldehyde) are inactive as substrates.

11 The slope of this line is \((k_2/K_A)/(k_2/K_A)\), which can be shown to be equal to \((K_{m,B})/(K_{m,A})\). Comparison of appropriate ratios of the absolute Michaelis constants in Table I with these slopes fits this requirement closely. Values for \(k_2\), \(k_2\), \(K_A\), \(K_B\), and the analogous terms in the reverse reaction cannot be evaluated individually from steady state kinetic data from the uninhibited reaction, but it is possible to do so from data from product inhibition (5).

Calculation of Inhibitor-binding Constants—From Equation 3, for the case in which the inhibitor (aminooxyacetate) combines only with the same form of the enzyme as \(A\) (the variable substrate), the apparent Michaelis constant of the inhibited reaction, \(K_{m,A-I}^{'}\), is

\[ K_{m,A-I}^{'} = \frac{K_A}{1 + \frac{(I_0)}{K_I}} = \frac{K_{m,A}}{1 + \frac{(I_0)}{K_I}} \]  

\(K_{m,A-I}^{'}\) and \(K_{m,A}^{'}\) are obtained from the slopes of the plots of the data for the inhibited and uninhibited reactions, respectively, in Fig. 6, where the amino acids are the variable substrates. \((I_0)\) is known, and therefore \(K_I\) can be calculated from Equation 6.

From Equation 4, for the case in which the inhibitor combines only with the form of the enzyme with which the variable substrate does not combine, the apparent maximal velocity of the inhibited reaction, \(V_{m-I}^{'}\), is

\[ V_{m-I}^{'} = \frac{k_2}{k_1} \frac{1 + \frac{K_B}{(B_0)} \frac{1 + \frac{(I_0)}{K_I}}}{1 + \frac{(I_0)}{K_I}} \]

Therefore

\[ \frac{1}{V_{m-A}^{'} - \frac{1}{V_{m-A}^{}} = \frac{K_B}{(B_0)} \frac{1}{K_I} \]

\(V_{m-I}^{'}\) and \(V_{m-A}^{'}\) are obtained from the ordinate intercepts of the plots of the data for the inhibited and uninhibited reactions, respectively, in Fig. 6, where the keto acids are the variable substrates. \(K_B/k_2(E_0)\) equals \(K_{m-B}/V_B\) (see "Discussion"), both of which are available from Table I. \((I_0)\) and \(B_0\) are known, and therefore \(K_I\) can be calculated from Equation 7.

From Equation 3, for the case in which the inhibitor (maleate and acrylate) combines with both forms of the enzyme, the inhibitor-binding constant, \(K_I\), can also be calculated with the use of Equation 7 from the apparent maximal velocity of the inhibited and uninhibited reactions (Figs. 7 and 8). Here the ratio, \(K_B/k_2(E_0)\), was calculated from the apparent Michaelis constants and apparent maximal velocities, since \(K_B/k_2(E_0)\) also equals \(K_{m-B}/V_B\) (see "Discussion"). The other inhibitor-binding constant, \(K_{I-I}\), can be obtained from the apparent Michaelis constants with the same set of data, once \(K_I^{'}\) has been obtained. The apparent Michaelis constant for the inhibited reaction, \(K_{m,A-I}^{'}\), is, from Equation 2,

\[ K_{m,A-I}^{'} = \frac{K_A}{1 + \frac{(I_0)}{K_I}} \]

Therefore

\[ \frac{1 + \frac{(I_0)}{K_I}}{K_{m,A-I}^{'}} = \frac{1 + \frac{k_2K_B}{(B_0)}}{k_2K_A} \cdot \frac{1}{(I_0)} \cdot \frac{1}{K_I} \]
Since \( k_3K_B/k_2K_A = K_{m-B}/K_{m-A} \) (see footnote 11), this becomes

\[
\frac{1}{K_{m-A}} + \frac{(I_0)}{K_I} = \frac{1}{K_{m-A}'} + \frac{K_{m-B}}{K_{m-A}'} \left( \frac{1}{K_I} \right)
\]

\((I_0), (B_0), \text{and} K_I\) are known. \(K_{m-A}'-1\) and \(K_{m-A}'\) are obtained from the slop of the plots of the data for the inhibited and uninhibited reactions, respectively (Figs. 7 and 8), and \(K_{m-B}\) and \(K_{m-A}\) are available from Table I.

Addendum—Since the original submission of this manuscript, the English translation of a paper by Braunstein, Azarkh, and Tin-Sen' (12) has become available to us. In studies with L-cycloserine inhibition of rat liver glutamic-asparagine and pig heart glutamic-alanine transaminases, these workers have found that the inhibition is competitive with the amino acid substrates and apparently uncompetitive with the keto acids. The results were not as straightforward as those reported here with amino-oxyacetate, however, because of an irreversible, two-stage reaction of the inhibitor with the enzyme. However, evidence is cited suggesting that the cycloserine ring ruptures upon reaction with the enzyme giving rise to an aminoxy derivative analogous to the amino-oxyacetate utilized in these experiments. Decyclized derivatives of cycloserine containing a free aminoxy group have been reported to be strong inhibitors of transaminases (13).

REFERENCES

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