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 bihapic, 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 cosubstrates 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). When the number of

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‡ A third type of mechanism involving a site specific for keto acids and one for amino acids has been proposed (5), but Velick and Vavra (4) have presented a cogent theoretical objection to this proposal.
Fig. 1. Kinetics with alanine as the variable substrate. The medium contained, in a final volume of 3.1 ml, 300 pmoles 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°. Curves B and C show the extrapolations of the apparent maxima velocities and Michaelis constants (in millimolar units), respectively, to infinite \( \alpha \)-ketoglutarate (\( \alpha \)-KG) concentration.

Fig. 2. Kinetics with \( \alpha \)-ketoglutarate as the variable substrate. Conditions and symbols are as described in Fig. 1.

Fig. 3. Kinetics with glutamate as the variable substrate. Conditions and symbols are as described in Fig. 1, except approximately 1 unit of crystalline glutamic dehydrogenase and 100 µmoles of neutral ammonium sulfate replaced lactic dehydrogenase. GLU and PYR refer to L-glutamate and pyruvate, respectively.

Fig. 4. Kinetics with pyruvate as the variable substrate. Conditions and symbols are as described in Fig. 3.

The enzyme, was measured, as was the case with maleate inhibition of glutamic-aspartic transaminase (5).

Exchange Reactions—The rates of transamination from \( ^{14} \)C-labeled alanine to pyruvate and from \( ^{14} \)C-labeled glutamate to \( \alpha \)-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 \( \alpha \)-ketoglutarate and a rate of approximately 0.05 µmole of \( \alpha \)-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:

\[
E + A \xrightarrow{k_1} X \xrightarrow{k_2} E' + D
\]

\[
E' + B \xrightarrow{k_5} Y \xrightarrow{k_6} E + C
\]

where \( E' \) and \( E'' \) are distinct forms of the enzyme (in this case...
pyridoxal and pyridoxamine forms), $A$ and $B$ are reactants, $C$ and $D$ are products, and $X$ and $Y$ are enzyme-substrate complexes.¹

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

$$v_i = \frac{\frac{k_3(E_0)}{k_2} + \frac{(A_0)}{K_A}}{1 + \frac{k_2}{k_7} \left[ 1 + \frac{K_B}{(B_0)} \right] + \frac{(A_0)}{K_A}}$$

where $v_i$ is the initial velocity of the forward reaction, $(A_0)$, $(B_0)$, and $(E_0)$ are initial concentrations of reactants and enzyme. $K_A = (k_6 + k_9)/k_1$, and $K_B = (k_6 + k_9)/k_8$.

At any concentration of $B$, the value of the apparent maximal velocity at infinite $(A_0)$, $V'_A$, and of the apparent Michaelis constant of $A$, $K'_{m-A}$, are

$$V'_A = \frac{k_3(E_0)}{1 + \frac{k_2}{k_7} \left[ 1 + \frac{K_B}{(B_0)} \right]}$$

and

$$K'_{m-A} = \frac{K_A}{1 + \frac{k_2}{k_7} \left[ 1 + \frac{K_B}{(B_0)} \right]}$$

The absolute maximal velocity of the forward reaction and the absolute Michaelis constant of $A$ at infinite $(B_0)$ are

$$V_f = \frac{k_3(E_0)}{1 + \frac{k_2}{k_7}}$$

and

$$K_{m-A} = \frac{K_A}{1 + \frac{k_2}{k_7}}$$

¹ 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.

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**TABLE I**

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

* Average values.
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 (Aₒ) and for vₛ as a function of (Cₒ) and (Dₒ). 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),

\[ K_{eq} = \frac{v_f}{v_0} \frac{V_{cat}}{K_{cat} V_{mBR}} \]

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

L-Glutamate + pyruvate \rightleftharpoons \alpha-ketoglutarate + L-alanine

7 To consider vᵣ as a function of (Bₒ) at constant (Aₒ), Equation 1 can be written in a suitable form by interchanging (Aₒ) and (Bₒ), Kₐ and K₋, and k₂ and k₁. Equations of identical form can also be written for vᵣ (initial velocity of the reverse reaction) as a function of (Cₒ) or (Dₒ) by employing the appropriate rate constants.

This may be compared with the value of 1.6 determined directly (6).

For consideration of the effect of inhibitors, two additional reactions are required.

\[ E + I \rightleftharpoons EI \]

\[ K_I = \frac{(E)(I)}{(EI)} \]

and

\[ E' + I \rightleftharpoons EI' \]

\[ K_{I'} = \frac{(E')(I)}{(EI')} \]

Their inclusion in the rate equation leads to

\[ v_f = \frac{k_1(E₀)}{1 + \frac{k_2}{k_3} \left[ \frac{1 + \frac{K_p}{(B_o)}}{1 + \frac{(I_o)}{K_I}} \right] (A_o) + \frac{k_1}{k_3} \left[ \frac{1 + \frac{K_p}{(B_o)}}{1 + \frac{(I_o)}{K_I}} \right] (A_o) + (A_o)}{K_A \left[ 1 + \frac{(I_o)}{K_I} \right] + (A_o)} \]

If I can combine with both E and E', i.e. K_I and K_{I'} both are finite, V′ₐ decreases with increasing (Iₒ), whereas K_{m₋A} may increase, remain constant, or decrease, but to a lesser extent than Vₐ, with increasing (Iₒ), depending upon the relative values of K_I and K_{I'}. If K_I < or = K_{I'}, the ratio, K_I/K_{I'}, is always less than [1 + (Bₒ)/K₋ₐ], and K_{m₋} therefore always increases with (Iₒ).

8 If the ratio, K_I/K_{I'}, equals [1 + (Bₒ)/K₋ₐ], the apparent Michaelis constant of A will be unaffected by (Iₒ). If the ratio is larger or smaller than this term, the apparent Michaelis constant of A will decrease or increase with increasing (Iₒ), respectively. If K_I < or = K_{I'}, the ratio, K_I/K_{I'}, is always less than [1 + (Bₒ)/K₋ₐ], and K_{m₋} therefore always increases with (Iₒ).
The inhibitions by maleate and acrylate fit this mechanism, with \( K_I = K_I' \), 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

\[
v_f = \frac{k_0(E_0)}{1 + \frac{k_2}{k_1} \left[ \frac{1 + K_B}{K_B} \frac{1 + (I_0)}{K_I} \right] + (A_0)}
\]

Equation 3 predicts that \( V'_A \) is independent of \( (I_0) \) and that \( K'm-A \) increases with \( (I_0) \), i.e. that \( I \) is competitive with \( A \). This is the relationship between aminooxyacetate and the keto acid substrates, as can be seen from Fig. 6.

If, on the other hand, the inhibitor combines only with \( E' \), i.e. the form of the enzyme with which the substrate, \( A \), does not combine, then \( K_I = \infty \), and Equation 2 becomes

\[
v_f = \frac{k_0(E_0)}{1 + \frac{k_2}{k_1} \left[ \frac{1 + K_B}{K_B} \frac{1 + (I_0)}{K_I'} \right] + (A_0)}
\]

Equation 4 predicts that both \( V'_A \) and \( K'm-A \) decrease with increasing \( (I_0) \), and in the same proportion. This behavior is formally identical with uncompetitive kinetics (9). This is the relationship between aminooxyacetate and the amino 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 strongly suggests the titration of a group with a pK of about 7.5, which must be in the acid form for inhibitor binding. Since the pK values of the titratable groups of aminooxyacetate, itself, are approximately 3.5 and 5, the group in question must be on the enzyme and must be in the more positively charged form (or less negatively charged form) for inhibitor binding, consistent with the existence of aminooxyacetate as an anion at these pH values. However, since there is no indication of a marked difference in the \( K_m \) values between pH 7.3 and 7.8, it appears that the dissociable group involved in the binding of the inhibitor plays no role in binding of the substrates. Since it is only binding of the inhibitor to active forms of the enzyme that produces a measured inhibition, one possibility is that the status of the group involved in inhibitor binding is not a factor in the activity of the enzyme. Another possibility is that binding of the inhibitor to the enzyme produces a shift to a higher value in the pK of the group whose dissociation is responsible for the acid limb of the pH-activity curve.

It should be noted that the reaction sequence from which the rate equation (Equation 1) was derived is noncomittal with respect to the question of a single site for all reactants versus separate sites for the 3-carbon and 5-carbon substrates, although it implies that only one substrate at a time is present on the enzyme. Therefore, conformity with Equation 1 cannot serve to distinguish these alternatives. Furthermore, a reaction sequence that involves separate sites for the 3-carbon and 5-carbon compounds, both of which may be occupied at the same time, e.g. \( E + B = EB, EB + A = XB, \) etc., also leads to the same rate equation, provided that the presence of one substrate on the enzyme does not affect the binding of the other. Therefore, Equation 1 is applicable to this mechanism as well. Thus, from the kinetics of the uninhibited reaction, at least three possibilities exist: (a) there is a single site of attachment for all four substrates; (b) there are separate sites for the 3-carbon and 5-carbon substrates, but only one site is occupied at a time; and (c) there are separate sites, both of which may be occupied at the same time, but the presence of one substrate on the enzyme does not affect the attachment of the other.

However, the nature of the inhibition by aminooxyacetate and other considerations provide further information which favors the single site mechanism. Firstly, the similarity of the values of the inhibitor-binding constants for the 3-carbon site and the 5-carbon site with all three inhibitors tested means either that the binding of the inhibitor to the same site is being measured each time or that there is a fortuitous equality in the binding constants of two separate sites. Secondly, the mechanism of separate sites, both of which may be occupied at the same time, seems unlikely, since it requires that the amino acid substrate be able to occupy its site even when the enzyme is in the pyridoxamine form, whereas aminooxyacetate cannot be bound to either site when the enzyme is in this form. Thirdly, the mechanism of separate sites with only one substrate bound at a time also has contradictory aspects. It requires that, in the pyridoxal form of the enzyme, for example, both the 5-carbon and the 3-carbon sites be available to the respective amino acids and completely unavailable to the respective keto acids. Finally, the fact that the rates of the exchange reactions were as rapid as those of the net reactions argues in favor of the single site mechanism. If separate sites for the 3-carbon and 5-carbon substrates existed, the rates of the exchange reactions would be expected to be less than those of the net reactions, since the
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 \( \alpha \)-amino or keto group, another for the \( \alpha \)-carboxyl group, and either a third, which can accommodate specifically the methyl group of alanine or pyruvate and the carboxyethylene group of glutamate or \( \alpha \)-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 \( \alpha \)-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

\[
K'_{m,A} = \frac{K_A}{1 + \frac{k_3}{k_7} \left( 1 + \frac{K_A}{(B_0)} \right)}
\]

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

\[
K_{m,A} = \frac{K_A}{1 + \frac{k_3}{k_7}}
\]

From Equation 5 above,

\[
\frac{1}{K'_{m,A}} = \frac{1}{K_{m,A}} + \frac{k_3}{k_7} \frac{1}{K_A (B_0)}
\]

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.

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.

\(10\) is known, and therefore \(K_{r} \) can be calculated from Equation 7.

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, \(V'_{m-A}\), is

\[
V'_{m-A} = \frac{k_3}{k_7} \left[ 1 + \frac{K_A}{(B_0)} \left( 1 + \frac{(I_0)}{K_I} \right) \right]
\]

Therefore

\[
\frac{1}{V'_{m-A}} = \frac{1}{k_3} \left[ \frac{1 + K_A}{(B_0)} \left( 1 + \frac{(I_0)}{K_I} \right) \right]
\]

\(V'_{m-A}\) 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 amino acids are the variable substrates. \((I_0)\) is known, and therefore \(K_I\) can be calculated from Equation 6.

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, \(K_A/k_3(E_0)\), was calculated from the apparent Michaelis constants and apparent maximal velocities, since \(K_A/k_3(E_0)\) also equals \(K'_{m-B}/V'_{B}\) (see "Discussion"). The other inhibitor-binding constant, \(K_I\), 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,

\[
K'_{m,A-I} = \frac{K_A \left[ 1 + \frac{(I_0)}{K_I} \right]}{1 + \frac{k_3}{k_7} \left[ 1 + \frac{K_A}{(B_0)} \left( 1 + \frac{(I_0)}{K_I} \right) \right]}
\]

Therefore

\[
\frac{1}{K'_{m,A-I}} = \frac{1}{K'_{m,A}} + \frac{k_3K_A}{K_I (B_0)} \cdot \frac{1}{K_I} \cdot \frac{(I_0)}{K_I}
\]
Since \( \frac{k_{30} K_B}{k_{20} K_A} = \frac{K_{m-B}}{K_{m-A}} \) (see footnote 11), this becomes

\[
\frac{1 + \frac{(I_0)}{K_I}}{K_m^* - A} = \frac{1}{K_{m-A}} + \frac{1}{K_{m-B}} \frac{(I_0)}{K'' \cdot R_0}
\]

\( (I_0), (B_0), \) and \( K_I^* \) are known. \( K_{m-A}^* \) and \( K_{m-A} \) are obtained from the slopes 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 aminoxyacetate 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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