Glutamic Aspartic Transaminase

V. THE REACTION WITH L-ALANINE*

W. Terry Jenkins

From the Department of Biochemistry, University of California, Berkeley, California

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Aspartate and glutamate react instantaneously with the pyridoxal form of the pig heart glutamic aspartic transaminase (1) to yield the corresponding keto acid, converting the enzyme-bound pyridoxal phosphate to bound pyridoxamine phosphate (2). Other amino acids such as methionine sulfone, methionine sulfoxide, and alanine react much more slowly with the enzyme, but the reaction itself appears to be essentially the same.

This paper describes experiments performed with L-alanine to study the mechanism of transamination.

EXPERIMENTAL PROCEDURE

Materials—The pyridoxal and pyridoxamine forms of the pig heart glutamic aspartic transaminase were prepared as previously described (1, 2). L-Alanine, D-alanine, and ketoglutarate were obtained from the California Corporation for Biochemical Research and sodium pyruvate from Nutritional Biochemicals Corporation.

Methods—The reaction of the enzyme with L-alanine may be followed readily as a function of time. Aliquots of an incubation mixture of the pyridoxal form of the enzyme in 0.1 M Tris-HCl buffer, pH 8.3, 0.025 M with respect to L-alanine, were removed at the times indicated in Fig. 1 and assayed for both pyruvate and enzyme-bound pyridoxal phosphate by a modification of the method previously described (3).

The 1-ml aliquots were allowed to react for 10 minutes with 2 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl and then extracted with 6.5 ml of ethyl acetate. Pyridoxal phosphate hydrazone was determined spectrophotometrically at 415 nm in the aqueous phase after 1.5 ml were extracted with a further 2 ml of ethyl acetate to remove residual 2,4-dinitrophenylhydrazine and pyruvate hydrazone. To determine the pyruvate as its hydrazone, 5 ml of the ethyl acetate were extracted with 2 ml of 2 N HCl, and the pyruvate hydrazone was then extracted from 4 ml of the washed ethyl acetate with 0.5 ml of 10% sodium carbonate. A 0.3-ml aliquot of the sodium carbonate was then added to 0.5 ml of 2 N NaOH to develop the characteristic red color. The absorbancy at 452 nm is proportional to the pyruvate concentration originally present. Fig. 1 shows that the reaction is slow, even with a relatively high concentration of alanine, and that the loss of enzyme-bound pyridoxal phosphate is accompanied by the formation of pyruvate.

The reaction between alanine and the pyridoxal form of the enzyme may be more readily followed at pH 8.3 as a decrease in absorbance resulting from the latter at 360 nm (Fig. 2). Reactions were followed in a Cary recording spectrophotometer at constant temperature.

To show that the reaction did not depend upon catalytic amounts of ketoglutarate, low levels were included in the reaction mixtures. It can be seen from Fig. 2 that ketoglutarate affects the reaction two ways. (a) It causes a virtually complete inhibition of the decrease in the pyridoxal enzyme. If the time taken for the reaction to reach its maximal rate (absorbancy about 0.5) is considered, it can be seen that this inhibition persists only for a length of time which is proportional to the amount of ketoglutarate initially present. The proportionality constant may be considered as an apparent rate of inactivation of ketoglutarate. (b) It affects the final equilibrium, as expected, to favor the pyridoxal form of the enzyme at the higher levels of the keto acid.

Ketoglutarate has little effect upon the rate of the reaction once the initial inhibition is overcome. The initial rate of transamination with the enzyme in the absence of ketoglutarate was the same as the apparent rate of inactivation observed for ketoglutarate. Since, as it is known, low levels of ketoglutarate will instantly and almost quantitatively convert the pyridoxamine form of the enzyme to the pyridoxal form (2), ketoglutarate will prevent the accumulation of pyridoxamine enzyme. Ultimately all the ketoglutarate is converted to glutamate. These results are consistent only with the following scheme:

- Alanine → Pyridoxal enzyme → Glutamate
- Pyruvate → Pyridoxamine enzyme → Ketoglutarate

A large excess of alanine causes a decrease of about 77% in the absorbancy at 360 nm. This decrease in absorbancy is associated with the conversion of virtually all the enzyme to the pyridoxamine form (Fig. 2). The difference between the absorbancy at any time and that at infinite time in the presence of an excess of alanine is thus proportional to the concentration of the pyridoxal form at that time. With a large excess of alanine (>0.1 M) the rate of the reverse reaction between the pyruvate and pyridoxamine enzyme, formed in the reaction, is comparable to the forward reaction only when most of the pyridoxal enzyme has reacted. More than 90% of the reaction under these conditions is observed to be first order (Fig. 3). With lower concentrations of alanine, the reverse reaction be-

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comes appreciable, and an equilibrium is ultimately reached between the two forms of the enzyme, the alanine added and the pyruvate formed. With 0.005 M alanine and 0.00006 M bound vitamin B₆, approximately equal concentrations of the two forms of the enzyme are present in the final equilibrium mixture. Because the rate of the reaction decreases rapidly with time, it was found that plotting the reaction as if it were always of first order in pyridoxal enzyme, even with the lower concentrations of alanine, enabled a more practicable linear representation of the data obtained. Initial rates are, therefore, given in the form of first order rate constants. It can be seen from Fig. 3 that this method is applicable over a wide range of alanine concentrations, and that the rate constant so obtained is proportional to the alanine concentration. It should be emphasized that this is a technical device to measure the initial velocity accurately and does not mean that the reaction can be described by either a reversible or irreversible first order equation at low alanine concentrations. Fig. 4 shows that the initial velocity (which is proportional to the reaction constant k) is increased in the presence of low concentrations of formate. The observed rates may be described in terms of the molarity of

FIG. 1. Reaction of L-alanine (0.025 M) with the pyridoxal form of the glutamic aspartic transaminase in 0.01 M Tris-HCl buffer, pH 8.3, 25°, as a function of time. The analytical procedure for the simultaneous determination of pyruvate and bound pyridoxal phosphate is described in the text.

FIG. 2. The effect of low concentrations of ketoglutarate upon the loss of the pyridoxal form of the transaminase in 0.1 M Tris-HCl buffer, pH 8.3, 30°. All solutions were 0.2 M with respect to L-alanine. The numerals on the graph are 10,000 times the initial concentration of ketoglutarate.

formate (A) by the empirical equation:

\[
k = 0.031 \left[ \frac{1 + \frac{7.0}{1 + \frac{0.05}{(A)}}} \right]
\]

(1)

Effect of pH—The glutamic aspartic transaminase is stable at room temperature only in the pH range from 4.5 to 11. It possesses measurable catalytic activity in the range from about 5 to 10.5, but two pH ranges are of particular interest. In the range from 5 to 7.5 the enzyme acts as a pH indicator, but the substrate degree of ionization does not change appreciably. In the range from 8 to 10.5 alanine dissociates, and it might be expected that only one ionic form would be a substrate for the enzyme. In this pH range, the spectra of the enzyme do not change.
Figure 5. A. The initial rate of the reaction of L-alanine (0.1 M) with the pyridoxal form of the transaminase plotted as a function of pH at 30°C. (I) Pyrophosphate buffers 0.04 M; (II) phosphate buffers 0.1 M. The initial rate is expressed as a rate constant k. B. The rate constants in 0.1 M potassium phosphate buffers of Fig. 5A plotted as a function of the absorbancies at 360 nm and 426 nm corrected for dilution by the addition of the substrate. Rate constants were determined as previously described in the range pH 5 to 7.5 in both pyrophosphate (I) and phosphate (II) buffers by measuring the reaction at the isosbestic point for the pyridoxal form of the enzyme (389 nm). The pH values in Fig. 5A were determined on simulated reaction mixtures without enzyme. Two different curves were obtained for the two buffers. The rate constant in the presence of a mixture of phosphate and pyrophosphate buffers at pH 7 was the same as that with pyrophosphate alone. This suggests that the higher rate in the presence of pyrophosphate is due to activation by pyrophosphate.

To correlate the activities with the ionic forms of the enzyme, spectra were recorded just before the start of the reaction by the addition of alanine. The decrease in absorbancy at 426 nm observed as the pH increases is proportional to the concomitant increase in absorbancy at 360 nm (1). For both series of buffers, the reactivity in this pH range is proportional to the concentration of the form with absorption maximum at 360 nm (Fig. 5B). The corollary is that the increase in activity is also proportional to the loss of the acidic form (λ_max 426 nm). This form appears to be inactive.

In the pH range 8 to 10.5, it was noted that the rate constant decreased only slightly, certainly not as much as would be expected if only one ionic form of the substrate were active. Such an effect may be explained by a compensating ionization of either the enzyme (Scheme I) or the enzyme-substrate complex (Scheme II). The reverse reactions have been omitted from the schemes, since only the initial velocity is considered and no products are then present.

\[
\begin{align*}
H^+ + S + EH & \rightarrow SH + E \rightleftharpoons ESH \xrightarrow{k_2} \text{Products} \\
\text{Scheme I} & \\
S + E & \rightarrow \text{S} + E \rightleftharpoons ES + H^+ \xrightarrow{k_1} \text{Products} \\
\text{Scheme II}
\end{align*}
\]

Scheme II yields equations which describe the experimental data best. On the basis of this scheme it can be shown that if the substrate anion (S) is kept constant, the initial rate of reaction (R) is described by the expression

\[
R = \left( K_1 + \frac{K_2}{K_1} (S) \right) \frac{K_2}{K_1} [E] [S] = \frac{k_2 [E] [S]}{K_1 + \frac{K_2}{K_1} (S)}
\]

where [E] is the total pyridoxal enzyme concentration present initially and K_1 and K_2 are defined by the following steady state concentrations of substrate (S, SH), free enzyme (E), and enzyme-substrate complex (ES, ESH):

\[
K_1 = \frac{(S)(H^+)}{(SH)} \quad K_2 = \frac{(ES)(H^+)}{(ESH)} \quad K_3 = \frac{(E)(S)}{(ES)} \quad K_4 = \frac{(SH)(E)}{(ESH)}
\]

with high concentrations of alanine, the reaction is a pseudo first order reaction in pyridoxal enzyme. K_4, therefore, has to be much greater than (SH) or K_2/K_1 (S), and the terms in (S) and (SH) drop out of the left-hand side of the expression. The formulation predicts:

1. That at a constant anion concentration, the rate constant (R) will be linearly related to the concentration of the zwitter ion (SH).
2. The slope of this line should be almost independent of the concentration of anion.
3. The negative X axis intercept for a plot of the rate constant against zwitter ion concentration at different anion concentrations should be proportional to the anion concentration with a proportionality constant equal to (k_1/k_2)(K_2/K_1).

Fig. 6 shows that all these theoretical predictions are supported by the experimental data; the value derived for (k_1/k_2) (K_2/K_1) was 0.8.

Pyruvate reacts more rapidly with the pyridoxamine form of the enzyme than does alanine with the pyridoxal form. To measure the rates accurately, low concentrations of pyruvate must be used. This requirement makes an accurate determination of the initial velocity by the present methods impracticable; therefore, the reaction with pyruvate was not studied in any great detail.

It has previously been shown that it is possible to calculate the enzyme-substrate dissociation constants spectrophotometrically (2), since the loss of the pyridoxal form of the enzyme as the concentration of alanine varied may be measured as a
FIG. 6. The effect of the addition of the zwitter ionic form of alanine upon the initial rate of reaction (given as a pseudo first order reaction constant) measured at 30° in the presence of (I) 0.1 M sodium alaninate and (II) 0.05 M sodium alaninate.

decrease in absorbancy at 360 mμ if fixed levels of pyruvate are used. For the system

\[ E_1 + S_1 \xrightarrow{K_{S_1}} ES_1 \xrightarrow{K} ES_2 \xrightarrow{K_{S_2}} E_2 + S_2 \]

it was shown that

\[ \frac{S_1}{\Delta E_1} = \frac{1}{E_0} \left[ S_1 + \frac{KK_{S_1}}{(1 + K + K_{S_2})S_2} \right] \]  

where \( E_1, E_2 \) are forms of the enzyme, \( ES_1, ES_2 \) are postulated enzyme substrate complexes, \( E_0 \) the total enzyme concentration, and \( \Delta E_1 \) the loss of pyridoxal enzyme (\( E_1 \)), upon the addition of alanine (\( S_1 \)), in the presence of a constant level of pyruvate (\( S_2 \)). The equilibrium concentrations of the components define the constants

\[ K_{S_1} = \frac{(E_1)(S_1)}{(ES_1)}, \quad K = \frac{(ES_2)}{(ES_2)}, \quad K_{S_2} = \frac{(E_2)(S_2)}{(ES_2)} \]  

With constant high concentrations of pyruvate, \( ES_1, ES_2 \), and \( E_2 \) are in equilibrium, if it is assumed that at a fixed pH the equilibrium mixture and \( E_1 \) both follow Beer’s law at 360 mμ. The loss in absorbancy at 360 mμ (\( \Delta A_s, 360 \mu \)) which is observed upon the addition of alanine, is proportional to the associated decrease in the concentration of pyridoxal enzyme (\( \Delta E_1 \)).

A plot of \( S_1/\Delta A_s, 360 \mu \) against alanine, therefore, has an intercept on the X axis (see Fig. 7A and Reference (2)) equal to

\[ \frac{K \cdot K_{S_1}}{1 + K + K_{S_2}} \]

The reciprocal of this value, when plotted against the reciprocal of the different concentrations of pyruvate (\( S_2 \)), should yield values for \( K \cdot K_{S_1}/(1 + K) \) and \( K_{S_2}/(1 + K) \) (Fig. 7B).

Experimentally these are both much greater than the concentrations of alanine and pyruvate used. The concentration of the pyridoxal form of the enzyme is equal to that of the pyridoxamine form when the ratio of alanine to pyruvate is \( K \cdot K_{S_1}/K_{S_2} = 160 \). Reaction mixtures which contain equimolar amounts of alanine and pyruvate under these conditions of pH and temperature, consequently have a corresponding ratio of pyridoxal form to pyridoxamine form.

**DISCUSSION**

Braunstein and Kritzman (3) originally studied the reversible transamination of alanine-pyruvate with other amino acids and found that catalytic amounts of ketoglutarate were required. It was subsequently shown (4, 5) that the reversible transamination from aspartate to pyruvate occurred most readily with a combination of two enzymes and catalytic amounts of ketoglutarate-glutamate. The direct reaction of alanine with the glutamic-aspartic transaminase is several orders of magnitude less in rate per mole of enzyme than the coupled enzymatic reactions described above. This low reactivity makes the reaction potentially valuable for the study of the mechanism of transamination.

We are indebted to Dr. S. F. Velick for pointing out an error in our previous derivation.
In the pH range 5 to 7.5, two demonstrable forms of the pyridoxal enzyme occur which differ in state of ionization and have absorption maxima at 360 mμ and 426 mμ. In the same pH range, the reactivity with alanine shows a marked increase. Over a wide range of pH, the increment in activity is proportional to the observed increment in the more basic form. Furthermore, when the enzyme is wholly converted into its acidic form, as judged spectrophotometrically, it is inactive, whereas when converted into its basic form, by the same criteria, it possesses maximal activity. In studies in which the rate of transamination from glutamate to ketoglutarate was measured as a function of pH and the reactivity correlated with spectral changes observed under comparable conditions, results indicated indirectly that only the basic form was active in transamination (6). The two findings therefore support the hypothesis that the mechanisms of both reactions are essentially similar.

It has been shown that the active site of the enzyme is pyridoxal phosphate and that this is converted into bound pyridoxamine phosphate in the transamination reaction (2). Fischer and Krebs (7) have found that the pyridoxal phosphate aldehyde group of this enzyme is bound in Schiff base linkage with a protein lysine ε-amino group. It was proposed, therefore, that such a group must be liberated during the formation of the enzyme substrate complex (8). Because of the ionization of this amino group when liberated, the equilibrium between enzyme and substrate may be written between enzyme and either anion or zwitter ion forms of the substrate (Scheme II). It is not unreasonable to expect that the rate constants for the reaction of acidic (k1) and basic (k2) forms of the enzyme substrate complex are of comparable magnitude, so that the pK of this complex must be close to that of the amino group of alanine (i.e. K1 = K2). This is consistent with the suggestion that the group responsible is the ε-amino group of a protein-bound lysine molecule (9) and hence supports the postulated reaction mechanism.

SUMMARY

1. Alanine reacts very slowly with the pig heart glutamic aspartic transaminase. Pyruvate formation in this reaction is equivalent to the loss of bound pyridoxal phosphate.

2. Ketoglutarate is an effective inhibitor of the reaction because it reconverts the pyridoxamine form of the enzyme back to the pyridoxal form.

3. Only one of the two ionic forms of the pyridoxal enzyme known to be present in the pH range from 5 to 7.5 is reactive. The effect of pH on the rate of the reaction is due to the interconversion of the two ionic forms.

4. The effect of pH in the range of pH from 8 to 10.5 was studied in the absence of buffer salts other than the substrate. The fact that the rate could not be correlated with the concentration of either alanine or ketoglutarate supports the hypothesis that the ionization of the substrate was compensated for by another ionization of a different residue, or of the enzyme substrate complex. The experimental evidence supports an earlier hypothesis that the ε-amino group of a protein lysine molecule is liberated upon formation of the enzyme substrate complex.

5. The enzyme was shown to have a very low affinity for the substrates alanine and pyruvate. This low affinity was also indicated by the fact that the initial velocity of the reaction was proportional to the concentration of alanine even above 0.1 M. The enzyme substrate equilibria are such as to favor greatly the pyridoxal form of the enzyme in the presence of equimolar amounts of amino and keto acid substrates.

6. The reaction is specific for L-alanine. The terminal carboxyl group of the physiological substrates aspartate and glutamate, therefore, does not determine the stereospecificity of the reaction.

Acknowledgment—The original observation that alanine is capable of reacting with the glutamic aspartic transaminase was made in collaboration with Dr. I. W. Sizer and was described previously. (Dissertation, Massachusetts Institute of Technology, Boston, Massachusetts, 1957.)

REFERENCES

Glutamic Aspartic Transaminase: V. THE REACTION WITH L-ALANINE
W. Terry Jenkins


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