Kinetic Properties of Native and Carboxypeptidase-altered Rabbit Muscle Aldolase*

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In the accompanying publication (2), it is shown that the interaction of rabbit muscle aldolase and dihydroxyacetone phosphate yielded a product that could be visualized spectrophotometrically with properties consistent with an enzyme-substrate complex. Similar affinities were found for dihydroxyacetone phosphate with both native and carboxypeptidase-treated aldolase. It was of interest, therefore, to compare the apparent affinities of the two preparations for this substrate in the aldol condensation. A coupled reaction with \( \alpha \)-glycerophosphate dehydrogenase was devised to permit measurement of the kinetic constants of the condensation reaction of aldolase.

The effect of carboxypeptidase treatment was previously noted by Drechsler, Boyer, and Kowalsky (3) as decreasing the rate of cleavage of fructose diphosphate by approximately one order of magnitude, although leaving the rate of cleavage of fructose 1-phosphate and the Michaelis constants of these two substrates essentially unchanged. The results to be presented show that the altered enzyme reacts more efficiently with aldhydes than does the native enzyme. This finding and others, involving the effect of \( \phi \) on the rate of reaction and the \( K_m \) values of the hexose phosphates and the limitation of the rate of cleavage by aldhydes, define properties of aldolase that must be considered in analyzing its mechanism of action.

EXPERIMENTAL PROCEDURE

Rabbit muscle aldolase was prepared by the method of Taylor, Green, and Cori (4) or was purchased from Boehringer und Sohne. The suspensions of crystals in ammonium sulfate contained small but significant amounts of triose phosphate isomerase. Overnight dialysis against flowing cold distilled \( \mathrm{H}_2\mathrm{O} \) usually resulted in practical elimination of this contaminant, but complete elimination was assured by passage through DEAE-cellulose; the aldolase is eluted before the isomerase by dilute salts (5). \( \alpha \)-Glycerophosphate dehydrogenase was purchased from Boehringer und Sohne.

Dihydroxyacetone phosphate, \( \alpha \)-glyceraldehyde 3-phosphate, and \( \alpha \)-glyceraldehyde were purchased commercially. \( \beta \)-Glyceraldehyde 3-phosphate was kindly given by Dr. C. E. Ballou.

Spectrophotometric assays were performed in Cary model 11 and 14 recording spectrophotometers.

* Part of the material in this paper has been presented in preliminary form (1).

RESULTS

The assay of the condensation reaction of aldolase involved coupling with \( \alpha \)-glycerophosphate dehydrogenase. This combination had previously been used by others (6) to measure the cleavage reactions of aldolase, since the dihydroxyacetone phosphate formed from all substrates can be measured quantitatively in the reaction with DPNH catalyzed by the dehydrogenase. However, if relatively large concentrations of L-\( \alpha \)-glycerophosphate and DPN are incubated with the dehydrogenase at somewhat alkaline \( \phi \) values, an equilibrium is established in which measurable concentrations of DPNH, and presumably equivalent amounts of dihydroxyacetone phosphate, accumulate. The assay for aldolase depends upon the formation of DPNH as the dehydrogenase produces more DPNH and dihydroxyacetone phosphate in response to the removal of dihydroxyacetone phosphate by aldolase. Fig. 1 shows the linear recordings obtained during the early portion of the reaction from which rates are measured and the proportionality of these reactions to aldolase concentration.

The dihydroxyacetone phosphate concentration can be poised at any desired value within wide limits by adjusting the concentrations of glycerophosphate, DPN, and hydrogen ion. Since the equilibrium constant of \( 5 \times 10^{-12} \) makes it difficult to raise the dihydroxyacetone phosphate concentration much beyond \( 10^{-4} \) at \( \phi 9 \) by increasing the concentrations of substrates for the dehydrogenase, higher concentrations of dihydroxyacetone phosphate were obtained by adding this substrate to systems containing the dehydrogenase equilibrium mixture. This resulted in the formation of a new equilibrium mixture, in which dihydroxyacetone phosphate and DPNH were no longer equivalent. The equilibrium concentration of dihydroxyacetone phosphate was determined by measurement of the change in DPNH or by calculation from the equilibrium constant of the dehydrogenase. The response of the dehydrogenase system to aldolase continued to be linear and proportional to aldolase in the presence of added dihydroxyacetone phosphate, and remained unchanged by increasing amounts of dehydrogenase. The rate of the aldolase reaction as a function of dihydroxyacetone phosphate concentration is shown in Fig. 2. It can be seen that the reaction is inhibited by this substrate at concentrations below saturation. No appreciable alterations in shape or position of these curves were found when various concentra-

1 Unpublished experiments. This value is very similar to that reported by Baranowski (7).
The curves represent the optical densities recorded by the Cary model 11 spectrophotometer during the first minute after the contents of the cuvettes were mixed. In each vessel was added 0.2 ml of 0.5 M glycine buffer, pH 10.0, 3 pmoles of DPN, 15 pmoles of α-glycerophosphate, 0.1 mg of glycerophosphate dehydrogenase, and water to give a final volume of 1.0 ml. Aldolase in the volumes indicated (in microliters; denoted by λ in the figure) was added to start the reaction, the cuvettes were mixed by inversion with a Parafilm cover, and optical density at 340 mμ was recorded from approximately 10 seconds after the addition of the aldolase.

Fig. 1. Spectrophotometric assay of condensation reaction of aldolase. The curves represent the optical densities recorded by the Cary model 11 spectrophotometer during the first minute after the contents of the cuvettes were mixed. In each vessel was added 0.2 ml of 0.5 M glycine buffer, pH 10.0, 3 pmoles of DPN, 15 pmoles of α-glycerophosphate, 0.1 mg of glycerophosphate dehydrogenase, and water to give a final volume of 1.0 ml. Aldolase in the volumes indicated (in microliters; denoted by λ in the figure) was added to start the reaction, the cuvettes were mixed by inversion with a Parafilm cover, and optical density at 340 mμ was recorded from approximately 10 seconds after the addition of the aldolase.

Fig. 2. Condensation reaction of native (left) and carboxypeptidase-altered (right) aldolase with varying concentrations of dihydroxyacetone phosphate. To each cuvette were added 40 μmoles of glycine buffer, pH 10.2, 0.3 μmole of DPN, 10 μmoles of α,α'-glyceraldehyde, 10 μg of α-glycerophosphate dehydrogenase, dL-α-glycerophosphate in varying concentrations, and water to give a final volume of 1.0 ml; at zero time, 91 μg of aldolase or 180 μg of carboxypeptidase-altered aldolase were added. The final pH was 9.3. Rates were determined as initial slopes of the curves recorded by the Cary spectrophotometer at 340 mμ.

The rates of the condensation reaction as functions of aldehyde concentration are shown in Figs. 3 and 4 for glyceraldehyde and its phosphate ester, respectively. The importance of the phosphate group both in increasing the affinity of the aldehyde as a substrate and in causing it to inhibit in higher concentrations is apparent on inspection of these figures. A less expected result was the greatly increased affinity for alddehydes shown by carboxypeptidase-treated aldolase. The $K_m$ values for glyceraldehyde with native and carboxypeptidase-altered enzyme were calculated from Lineweaver-Burk plots (8) as 0.028 μM and 0.0018 μM, respectively, and the corresponding values for glyceraldehyde 3-phosphate were found to be $1.9 \times 10^{-4}$ μM and $2.2 \times 10^{-4}$ μM. Thus, the apparent affinity of the altered enzyme for alddehydes is approximately one order of magnitude greater than that of native aldolase. The increased affinity is also characteristic of the inhibition by excess substrate. The maximal velocity of the condensation reaction calculated from extrapolated reciprocal rate versus substrate plots is approximately the same for both alddehydes in the presence of optimal levels of dihydroxyacetone phosphate, but is faster with native than with carboxypeptidase-treated aldolase. All of the experiments reported in this paper were carried out with dL-glyceraldehydes, but essentially identical reaction rates were observed with equimolar concentrations of a commercial syrupy preparation of dL-glyceraldehyde and with a sample of synthetic dL-glyceraldehyde 3-phosphate instead of the racemic mixture.

Glyceraldehyde 3-phosphate is also a potent inhibitor of the cleavage reactions of aldolase. Two mechanisms may be considered as likely possibilities for the inhibition of the cleavage reaction: a reversal of the reaction by condensation of enzyme-bound dihydroxyacetone phosphate with the free aldehyde and competitive inhibition of the binding of fructose diphosphate by the triose phosphate. Reciprocal plots of the rates of cleavage of fructose diphosphate versus substrate concentration in the presence and absence of the inhibitor gave inconsistent results, in some experiments suggesting competitive, and in others uncompetitive, inhibition. The probability that both types of inhibition are significant contributors to the overall effect of glyceraldehyde 3-phosphate is indicated by the following experiments. Glyceraldehyde at high concentrations can participate in the condensation reaction at rates comparable to those of its phosphate ester. When the free aldehyde was used as an in-
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**Fig. 3.** Condensation reaction of native (left) and carboxypeptidase-altered (right) aldolase with varying concentrations of D,L-glyceraldehyde. Conditions were those given in the legend to Fig. 2, with α-glycerophosphate at $2.5 \times 10^{-3}$ M and $1 \times 10^{-3}$ M for native and degraded aldolase, respectively, to produce optimal concentrations of dihydroxyacetone phosphate.

**Fig. 4.** Condensation reaction of native (left) and carboxypeptidase-altered (right) aldolase with varying concentrations of D,L-glyceraldehyde-3-phosphate. Conditions were those given in the legend to Fig. 3.

**Fig. 5.** The cleavage of fructose diphosphate was carried out with 250 μg of carboxypeptidase-treated aldolase in 0.05 M phosphate buffer, pH 7.5, with no aldehyde (●), $3.5 \times 10^{-4}$ M glyceraldehyde-3-phosphate (○), or 0.02 M glyceraldehyde (□).

Inhibitor of the cleavage reaction, reciprocal plots were obtained that clearly show uncompetitive inhibition, as would be expected if the only effect of the inhibitor were to divert an enzyme-substrate intermediate from its normal pathway. This result demonstrates the possibility of an aldehyde inhibiting by acting as a substrate for the reverse reaction, and suggests that this should be a property of all aldehydes that are substrates. To demonstrate the competitive part of the inhibition, the reaction was studied with relatively low degrees of saturation of both substrate and inhibitor. This cannot be done easily under optimal conditions of activity because concentrations below the $K_m$ are too small to allow accurate rate measurements in conventional 1-cm cuvettes. Therefore, the reactions were carried out in the presence of high concentrations of phosphate buffer, which is a competitive inhibitor of the substrates and produces large increases in the apparent $K_m$ values. Under these conditions, in contrast to reactions carried out in media of low ionic strength, the inhibition by glyceraldehyde-3-phosphate appears to be completely competitive, whereas the inhibition by non-esterified glyceraldehyde remains clearly uncompetitive (Fig. 5).

The role of the phosphate in determining the affinity of the aldehyde led to an investigation of the role of the ionic groups in other reactions of aldolase. Several previous investigations (reviewed in (9)), using different assay methods, resulted in disagreement about the effect of pH on the activity of aldolase, with optima of 7.5 and 9 being reported. With the glycero-phosphate dehydrogenase assay, the maximal velocity of rabbit...
muscle aldolase is essentially independent of pH from pH 5 to 10. The decreased activity of the dehydrogenase below pH 5 makes it difficult to extend the curve to lower values. The irreversible inactivation of aldolase during manipulations at pH 4 or lower also discourages further attempts to extend this curve. Similar results were obtained by Stumpf with aldolase of peas (10).

In Fig. 6 are shown the effects of pH on the $K_m$ values of fructose 1- and 1,6-diphosphates. At neutral pH values, there is little effect of pH, but toward both extremes the apparent affinity for substrate decreases sharply. Since both substrates are essentially completely ionized above pH 7, changes in $K_m$ at higher pH values must be attributed to alteration of the enzyme. At lower pH values, however, the phosphate groups become partially un-ionized, and the binding of protons to the substrates may alter their availability to the enzyme. That the effect of low pH actually is the result of alteration of the substrate, not the enzyme, is indicated by the difference in the positions of the curves for the two substrates; that of fructose diphosphate rises more at higher pH values than that of fructose 1-phosphate, and this difference is correlated with the difference in the acidic dissociations of the substrates. The second pK of fructose 1-phosphate was measured at 5.8. The two secondary groups of fructose diphosphate are titrated together over a wider pH range than a single group, so that a definitive pK cannot be given for the weaker acid. However, the addition of sufficient acid to protonate one-half of one group of fructose diphosphate lowers the pH only to 6.5.

In studies with various substrates and analogues as protectors of the enzyme against destruction by proteolytic enzymes, it was found that inorganic potassium phosphate has appreciable protective action. This finding led to an examination of this protective action. This finding led to an examination of this protective action. This finding led to an examination of this protective action. In this way, studies with glyceraldehyde 3-phosphate phosphate buffer, 0.05 M; MgCl₂, 0.05 M; phorate buffer, 0.05 M; MgCl₂, 0.05 M; and MgSO₄, 0.03 M. $K_t$ values were calculated from the slopes of plots of $1/V$ vs $1/S$, by using the relationship,

$$K_t = \frac{[I]}{(slope_{inhb}/slope_{non-inh}) - 1}$$

Table 1

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<thead>
<tr>
<th>Salt</th>
<th>$K_t$</th>
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<tr>
<td>NaCl</td>
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<tr>
<td>CH₃COOK</td>
<td>0.25</td>
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<tr>
<td>KH₂PO₄-K₂HPO₄ pH 7.0</td>
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<td>MgCl₂</td>
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<td>MgSO₄</td>
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**Discussion**

The use of an enzyme to generate a substrate for a second enzyme reaction is very familiar. There are certain advantages, however, in using a generating system at equilibrium to measure the rate of a second enzyme. This is the reverse of the more familiar use of a "coupled" reaction to remove a product quantitatively; i.e. to "pull" the reaction being measured, as glycero-

The reactions were carried out in 0.02 M imidazole buffer, pH 7.0, with limiting concentrations of fructose diphosphate, 0.1 

$\mu$ mole of DPNH, 50 

 phosphorylated trioses, it is apparent from the rates of reaction at low substrate concentra-

Table 1

**Inhibition of aldolase by salts**

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**Fig. 6.** $K_m$ values for the cleavage of fructose 1-phosphate ($F-1-P$) and fructose diphosphate (HDP) obtained from Lineweaver-Burk plots at the pH values indicated in acetate, imida-

tion that these compounds react with aldolase much more efficiently than does the unphosphorylated aldehyde, and that carboxypeptidase-treated aldolase reacts more effectively with glyceraldehyde 3-phosphate than does native aldolase.

The increase in $K_m$ values for the hexose phosphates near the pK values is most obviously interpreted as indicating that the form in which the substrate reacts with aldolase is the fully ionized anion. The importance of the phosphate groups had already been established by the finding that the $K_m$ of fructose diphosphate is two orders of magnitude less than that of the corresponding monophosphate (3). It seems probable, then, that a major force in binding the substrate to the enzyme is electrostatic attraction. This conclusion is reinforced by the effect of ionic strength on the binding; the competitive nature of the inhibition of all of the salts tested suggests that the attraction of oppositely charged ions by both enzyme and substrate results in a weakened interaction between them.

Experiments in which the rates of exchange of the two triose phosphates and fructose diphosphate were measured at conditions near equilibrium have shown that the aldehyde exchanges more rapidly than dihydroxyacetone phosphate (12). This result suggests that there may be a compulsory order of reaction, in which the aldehyde must dissociate before the dihydroxyacetone phosphate can leave the enzyme. In this case, the rate of dissociation of the tightly bound glyceraldehyde 3-phosphate could limit the rate of cleavage of fructose diphosphate. When aldolase is treated with carboxypeptidase, the decrease in the rate of fructose diphosphate cleavage could be correlated with the increased affinity for the aldehyde produced. Since fructose 1-phosphate does not yield a tightly bound aldehyde, the dissociation step could not limit the cleavage of this substrate, and consequently the rate would be unchanged by carboxypeptidase treatment.

Although this scheme is consistent with the kinetic results, results of experiments showing that the rate of exchange of glyceraldehyde 3-phosphate into fructose diphosphate is essentially unchanged by carboxypeptidase treatment are difficult to reconcile. It may be, therefore, that the increase in affinity for aldehydes occurs coincidentally with other alterations in the rates of other steps in the overall reaction.

SUMMARY

A spectrophotometric assay for the condensation reaction of aldolase has been devised, involving coupling with α-glycerophosphate dehydrogenase. With this assay, the kinetics of the condensation reaction have been examined. The effects of pH on the kinetic constants of the cleavage reaction indicate that only fully ionized phosphate esters serve as substrates, and that weak basic groups in the enzyme participate in the binding. The $K_m$ values of the various substrates with native and carboxypeptidase-treated aldolase are similar, except for the greatly decreased values for aldehydes with the altered enzyme.

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