Mechanism of the Aldolase Reaction*

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Upon treatment with carboxypeptidase, muscle aldolase (ketose-1-phosphate aldehyde-lyase, EC 4.1.2.7) loses 3 COOH-terminal tyrosine residues (3, 4) and exhibits a 20-fold fall in the rate of fructose diphosphate cleavage (3) and a 500-fold fall in the rate of exchange detrinitation of dihydroxyacetone phosphate (3). The cleavage of fructose-1-P is relatively unchanged (3). The present investigation shows that it is not necessary to invoke an unusual explanation (6) for these differential effects but that they follow from the fact that different steps are rate-determining in the three processes examined and hence these would not be expected to change concomitantly when only a single reaction step has been modified by the carboxypeptidase treatment, as will be shown is the case.

By use of isotope exchange at equilibrium, comparing native with carboxypeptidase-modified enzyme, information is obtained about the order of substrate reaction with the enzyme in the condensation reaction. Further exchange experiments are done in an attempt to observe isomerization steps in the mechanism.

EXPERIMENTAL PROCEDURE

Rabbit muscle aldolase was obtained commercially. The preparation of Sigma Chemical Company (specific activity, 21 units per mg) was used generally except in those experiments done with preparations of Boehringer und Sohne: Table II, 10 units per mg; and Table III, 3 units per mg. All preparations contained only insignificant amounts of triosephosphate isomerase. Yeast aldolase was prepared by the method of Richards and Rutter (7). Carboxypeptidase-modified muscle aldolase was prepared by mixing 2 mg of aldolase and 0.25 mg of carboxypeptidase-DFP (Worthington Biochemical Corporation). The aldolase activity fell rapidly to about 4% of the rate before treatment and remained at that level. No attempt was made to reisolate the aldolase, but the solution was used as such for over a year without apparent change.

Other enzymes were obtained as follows. a-Glycerophosphate dehydrogenase, triosephosphate isomerase, phosphoglycerate kinase, and phosphoglucone isomerase were from Boehringer; hexokinase was from Sigma; phosphofructokinase was prepared from rabbit muscle (8).

Fructose 1,6-diphosphate and dihydroxyacetone phosphate (as the ketal) were preparations of Calbiochem; p-glyceraldehyde-3-phosphate was prepared by the controlled periodate oxidation of FDP in good yield according to Sauveur (9). These compounds were assayed spectrophotometrically as disappearance of DPNH with a-glycerophosphate dehydrogenase alone, with aldolase, or with triosephosphate isomerase.

Dihydroxyacetone phosphate containing tritium was prepared as before (10). A similar procedure with D2O was used to make the deuterated species. Fructose diphosphate-6-32P was prepared from glucose, ATPS3P, and hexokinase, with subsequent conversion of the glucose 6-phosphate to fructose 6-phosphate and then to FDP by the combined action of phosphoglucone isomerase and phosphofructokinase and unlabeled ATP. FDP-1-32P was prepared similarly but with the ATP3P used in the last step only. The FDP was isolated on Dowex 1-CF3(−) column elution with 0.1 N HCl in the cold and immediately brought to pH 6 before concentrating.

For determination of the loss of tritium from DHAP in the exchange and condensation studies, samples were run into AgNO3 (5 mM final), which inactivates aldolase, or added to a solution of DPNH and a-glyceraldehyde dehydrogenase followed, after 30 seconds, by AgNO3 (5 mM final). The latter method was especially useful in eliminating any subsequent labilization of tritium. The samples were put into small distilling tubes, where they were sublimed in high vacuum to recovery of all the water. Control samples were always taken to allow the determination of the zero time volatile counts and those labilized after complete aldolase reaction with additional enzyme. For determination of the rates of condensation at various times, samples were either placed in 0.1 N HCl to inactivate aldolase and the remaining DHAP determined enzymatically (done for Table II) or otherwise placed in 1 N NaOH to destroy DHAP and the FDP formed was determined.

Exchange of 32P from FDP to triose phosphate was initiated by addition of enzyme to the equilibrium mixture containing FDP32P. Samples were placed in NaOH (0.5 N final) and after 15 minutes at room temperature the 32P associated with triose phosphate was extracted by isobutanol as an inorganic phosphate complex (11). A part of the isobutanol phase was counted directly by liquid scintillation radiometry. Samples were taken at zero time and after sufficient time for complete equilibration.

The abbreviations used are: FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; H* and D* refer to DHAP and its carbonion; G refers to an aldehyde, and DG to a condensation product such as FDP. H*, D*, and G* refer to the equilibrium rates of exchange of the activated tritium of DHAP3H with protons of the medium, of C-1,2,3 of DG with DHAP, and of C-4,5,6 of DG with glyceraldehyde-3-P, respectively.

For an individual step, n, of a reaction sequence, the rate constant in the forward direction is kₙ, for the backward direction is k₋ₙ, and the value Kₙ is k₋ₙ/kₙ.
of isotope. The latter values showed that the 32P distributed itself in accordance with the initial composition of the mixture, indicating that the equilibrium ratios were valid.

All exchange rates were determined after making the first order correction. Thus, for tritium exchange

$$v = \frac{-2.3 \times (\text{DHAP})}{\text{time}} \cdot \log (1 - f)$$

and for triose-P exchange

$$v = \frac{-23 \times (\text{FDP}) \times (\text{triose-P})}{\text{time} \times [\text{FDP}] + [\text{triose-P}]} \cdot \log (1 - f)$$
in which \(f\) is the fraction of complete isotopic equilibration that has been achieved. Exchange rates given are the average of two or three independent values, which agreed to within 10% of the mean value.

All rates are related to the units of aldolase activity as determined in the standard assay at 35°C and pH 7.5 (tris(hydroxymethyl)amino methane) with FDP (1 mM), serum albumin (0.1%), EDTA (1 mM), DPNH (0.1 mM), and with α-glycerophosphate dehydrogenase, and triosephosphate isomerase in excess. When the yeast enzyme was assayed, the mixture contained potassium acetate (0.1 mM), Zn(SO₄)₂ (1 mM), EDTA (0.3 mM), and mercaptoethanol (1.6 mM). All assays were at 35°C unless otherwise specified. A unit of aldolase activity refers to the micromoles of FDP consumed per minute in the standard assay.

The equilibrium constants for FDP = DHAP + glyceraldehyde-3-P were determined at 35°C for the pH range studied, with the following results: \(K_m\) was 0.042 (pH 5), 0.062 (pH 6), 0.085 (pH 7), 0.124 (pH 8.1), and 0.13 (pH 9). The derivation of the exchange rate equations was by the method of Boyer (12, 13).

### RESULTS AND DISCUSSION

The effect of carboxypeptidase treatment on the equilibrium exchange rates of the FDP = DHAP + glyceraldehyde-3-P equilibrium was examined in the hope of localizing the step(s) affected by the partial digestion. The exchange rates at 3 pH values of DHAP with C-1, 2, 3 of FDP (D*) and of glyceraldehyde-3-P with C-4, 5, 6 (G*) were determined and are expressed as rate (micromoles exchanged per minute) per unit of aldolase activity for the native enzyme or for the carboxypeptidase-treated enzyme prior to treatment (Table I). Whereas carboxypeptidase treatment caused the 14- to 100-fold decrease in DHAP:C-1,2,3 exchange, there was no effect on the glyceraldehyde-3-P:C-4,5,6 exchange rate.

This result has important consequences in establishing the sequence of the reaction of the two triose phosphates with the enzyme. Consider the alternate routes in Scheme 1.

![Scheme 1](image)

The steps concerned with \(G^*\) are Steps 3, 7, and 8 for the mechanism in which DHAP reacts first and Steps 4, 5, 6, 7, and 8 for the glyceraldehyde-3-P-on-first mechanism. According to the upper route (see Equation 3 below), one could even eliminate Step 1, hence prevent \(D^*\) (which depends on Steps 1, 2, 3, 7, and 8) without affecting \(G^*\) (which depends on the rate constants \(k\) values of Steps 3, 7, and 8 and the concentration of \(ED\), which is determined by the equilibrium constants, \(K_N\), \(K_S\), and \(K_E\)). On the other hand, a large decrease in \(D^*\) in the lower mechanism implies an effect of carboxypeptidase treatment on Steps 5, 6, 7, or 8, all of which are also involved in determining \(G^*\) by this path and would be expected to have an effect on \(G^*\). Thus, to the extent to which carboxypeptidase treatment alters \(D^*\) without altering \(G^*\) (a factor as large as 100-fold), so the upper path must dominate in determining \(G^*\).

That an obligatory sequence with DHAP reacting first is shown by a further use of isotope exchange at equilibrium. The equations for exchange of tritium between DHAP and water under conditions of the whole reaction equilibrium are shown in Equations 1 and 2 for the corresponding alternate paths.

$$H^* = \frac{k_{c-2} e^{K_{C} K_{S} K_{E}}} {k_1 + k_2 K_{C} K_{S} K_{E} + K_{C} (D) (1 + K_{S}) + (D) (G) (1 + K_{E})}$$

$$H^* = \frac{k_{c-4} e^{K_{C} K_{S} K_{E}}} {k_1 + k_2 K_{C} K_{S} K_{E} + K_{C} (D) (1 + K_{S}) + (D) (G) (1 + K_{E})}$$

The two equations are affected differently by the glyceraldehyde-3-P concentration. In the upper path, \(H^*\) goes from a finite value when \(G = 0\) to 0 as \(G\) becomes very large. It is already known that DHAP undergoes hydrogen exchange catalyzed by aldolase in the absence of aldehyde (10, 14, 15) but the relevance of this phenomenon has been questioned (6) because the exchange rate was greatly depressed by carboxypeptidase treatment of the enzyme. In the lower path, \(H^*\) should increase with increasing glyceraldehyde-3-P until a “saturating” region is reached.

Because of the form of the equilibrium expression

$$K_{eq} = \frac{\text{DHAP} \cdot \kappa \text{glyceraldehyde-3-P}}{\text{FDP}}$$

it is possible to maintain a constant DHAP concentration if the ratio (glyceraldehyde-3-P):(FDP) is held constant, and the
absolute concentration of glyceraldehyde-3-P may be varied if the FDP is varied in the same manner. When such an experiment is done (Fig. 1), it is observed that the rate of tritium exchange falls strongly with increasing glyceraldehyde-3-P. The plot of 1/\(H^*\) versus glyceraldehyde-3-P makes it clear that the value obtained with glyceraldehyde-3-P = 0 corresponds to the extrapolated value indicating that the exchange in the absence of aldehyde is not unrelated to the condensation reaction. A similar result was obtained with the carboxypeptidase-treated muscle enzyme (Fig. 2). The question of whether the alternate path with glyceraldehyde-3-P reacting first occurs to any extent depends on whether a limiting value of \(H^*\) is reached at high glyceraldehyde-3-P. The results of Figs. 1 and 2 indicate that at the highest concentration of glyceraldehyde-3-P tested the values of \(H^*\) are still falling (1/\(H^*\) has not become independent of glyceraldehyde-3-P concentration). Hence, it can be said that the participation of the glyceraldehyde-3-P-on-first path for the native muscle aldolase is less than 2%, for the carboxypeptidase-modified enzyme less than 15%, and for the yeast enzyme less than 8%, since these are the values to which \(H^*\) has been decreased by the highest concentration of glyceraldehyde-3-P used.

One may attempt to define more closely the step in the reaction that is altered by the carboxypeptidase treatment. That some step in handling the DHAP has been affected is shown by the large fall in the exchange rate between DHAP and C-1,2,3 of FDP and also by the large fall in the exchange of tritium between DHAP and water in the absence of aldehyde that was reported to result from carboxypeptidase treatment (5). The fact that the exchange of glyceraldehyde-3-P with C-4,5,6 of FDP is unaffected suggests that the process of Schiff's base formation between FDP and enzyme (16) is not altered by carboxypeptidase treatment. Hence, it is likely that the reasonably analogous process between DHAP and enzyme would also be unaffected. This suggests that the step involved in activating the proton of DHAP might be the one altered. If indeed this step has become rate-determining, it would be expected to show an isotope effect. A comparison of the rates of condensation of \(\Delta\)-glyceraldehyde with DHAP substituted with either \(H\) or \(D\) using the native and modified aldolase is given in Table II. 

Hence, it seems clear that no more than a slight fall in the condensation reaction occurs with carboxypeptidase treatment (only 2.2-fold) has been the result of an at least 200-fold fall in the rate of condensation of \(\Delta\)-glyceraldehyde (16). The effect of carboxypeptidase treatment in causing approximately a 200-fold decrease in the rate of tritium exchange is observed during the condensation reaction as well as in the isolated exchange reaction. Hence, it is likely that the effect of carboxypeptidase treatment is to cause a substantially greater isotope effect in the cleavage reaction than in the condensation reaction.

That \(C-H\) bond making has become rate-limiting for the cleavage of FDP by carboxypeptidase-treated aldolase is shown by comparing the rates in \(H_2O\) and \(D_2O\) (Table III). The question of whether with native enzyme the apparent isotope effect is related to the protonation to form DHAP or is an unrelated medium effect will be discussed further in a later section. In any case, the effect of carboxypeptidase treatment is to cause a substantially greater isotope effect in the cleavage reaction as well as in the condensation direction.

If it is true that the predominant sequence of reactions is that in which glyceraldehyde-3-P reacts with enzyme-DHAP intermediate, it is important to ask whether a prerequisite to this interaction is that the intermediate has lost the proton that is to
The isotope effect in condensation reaction

The aldol cleavage of FDP and the exchange deetrification of DHAP-H were assayed under standard conditions with either native (N) or carboxypeptidase-treated aldolase (CP) in which the same amount of protein is compared. Incubations of 0.1 M glyceraldehyde with 1 mM DHAP (Line 3), DHAP-H (Line 4), or DHAP containing H (Line 5) with either native or modified enzymes were in the presence of bovine serum albumin (0.2%), EDTA (1 mM), and triethanolamine (0.1 M, pH 7.3). The disappearance of DHAP was determined in Lines 3 and 4 and the appearance of volatile counts in Line 5. All rates are expressed as the percentage of that of the native enzyme in the standard assay of the enzyme.

<table>
<thead>
<tr>
<th>Rate measured</th>
<th>Relative rate with</th>
<th>Ratio of rates (N:CP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-aldolase</td>
<td>CP-aldolase</td>
<td></td>
</tr>
<tr>
<td>1. FDP → DHAP + glyceraldehyde-3-P</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>2. DHAP-H exchange</td>
<td>98</td>
<td>0.5</td>
</tr>
<tr>
<td>Condensation of glyceraldehyde with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. DHAP</td>
<td>61</td>
<td>24</td>
</tr>
<tr>
<td>4. DHAP-H</td>
<td>60</td>
<td>3.5</td>
</tr>
<tr>
<td>5. DHAP containing H (measure appearance of volatile counts)</td>
<td>240</td>
<td>1.3</td>
</tr>
</tbody>
</table>

be replaced by the aldehyde or whether the aldehyde can react with the enzyme bearing the protonated form of the DHAP. The problem is one of distinguishing between the alternate paths in Scheme 2.

The participation of the alternative paths can be evaluated by considering the relative effects of carboxypeptidase treatment on glyceraldehyde-3-P (= FDP) in the concentrations noted, 100 µmoles of sodium cacodylate buffer (pH 6), 0.8 mg of bovine serum albumin, 0.2 µmole of EDTA, 0.8 µmole of ZnSO₄, 2 µmoles of mercaptoethanol, 100 µmoles of potassium acetate, and 0.02 to 0.08 unit of yeast aldolase.
Effect of D₂O on rate of FDP cleavage

The components were those of the standard assay (triethanolamine (pH 7.5), EDTA, bovine serum albumin, and FDP), except that DPNH and enzymes were mixed, dried in a vacuum, and dissolved in H₂O or D₂O (99.5%). The subsequent additions of aldolase, α-glycerophosphate dehydrogenase, and triosephosphate isomerase reduced the D₂O content to 96%. Rates were measured at 25°C. The FDP concentration was 1 mM; the same rates were observed with 10 mM FDP.

<table>
<thead>
<tr>
<th>Aldolase</th>
<th>FDP cleavage</th>
<th>In H₂O</th>
<th>In D₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umoles/min/mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native muscle</td>
<td>3.0</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Carboxypeptidase muscle</td>
<td>0.31</td>
<td>0.049</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Test for isomerization step (muscle aldolase)

Native muscle aldolase was used under the conditions of equilibrium (FDP = 0.1 mM, and triose phosphate = 0.066, 0.092, or 0.114 mM each at pH 5, 7, and 9, respectively) with DHAP-3H, FDP-1,3P or FDP-6-2P at pH 5 (cacodylate, 0.2 M), or at pH 7 and 9 (triethanolamine, 0.2 M). The exchange rates were measured at 35°C and expressed as micromoles of isotope exchanged per minute per unit of enzyme assayed under standard conditions for cleavage of FDP. The ratio of condensation to detritiation was determined with DHAP-3H and glyceraldehyde-3-P with each triosephosphate 0.5 mM under comparable conditions. The FDP and volatile counts formed were determined at times before equilibrium amounts of FDP had been formed.

<table>
<thead>
<tr>
<th>pH</th>
<th>Exchange rate</th>
<th>H⁺ G⁻</th>
<th>D⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of condensation</td>
<td>Rate of detritiation</td>
<td>Corrected¹</td>
</tr>
<tr>
<td>5</td>
<td>0.35</td>
<td>0.26</td>
<td>0.21</td>
</tr>
<tr>
<td>7</td>
<td>0.61</td>
<td>0.21</td>
<td>0.34</td>
</tr>
<tr>
<td>9</td>
<td>0.92</td>
<td>0.11</td>
<td>0.32</td>
</tr>
</tbody>
</table>

¹ The values of H⁺ used in the corrected expression are the experimental values multiplied by the apparent isotope effect.

The effect of carboxypeptidase treatment is to alter H⁺ by very large factors under conditions which do not alter G⁻. Thus, any interaction of free glyceraldehyde-3-P with the enzyme-DHAP complex can be productive of condensation product only when the DHAP has lost its proton prior to such interaction.

The results obtained support an ordered sequence of reactions: DHAP reaction with the enzyme (to form a Schiff's base), the dissociation of the proton, and then reaction with the aldehyde resulting in condensation and dissociation of the condensation product. Speculation about a mechanism that would permit a change in the rate of reaction of a proton with C-3 of DHAP without an apparent effect on the corresponding reaction of the carbonyl group of an aldehyde led to consideration of an isomerization step between the steps involving C—H and C—C bond formation, as indicated in Scheme 3.

\[
E + DH \xrightarrow{a} E - DH \xrightarrow{b} E - D^- \xrightarrow{c} E' + DG \xrightarrow{d} \frac{D^-}{G} \xrightarrow{e} E' - DG \xrightarrow{f} E + DG \xrightarrow{g} \frac{E + DG}{G}
\]

Scheme 3

This might be a steric rearrangement or a redistribution of charge that would require that the species of \(E - D^-\) to be protonated or to react with aldehyde not be the same. One may examine this possibility by comparing the rates of the three equilibrium exchange reactions, \(H^+\), \(D^+\), and \(G^+\), measured under identical conditions. For this mechanism, the three exchange rates have a necessary relation only when \(E - D^-\) is identical with \(E' - D^-\), or when Step i is very rapid. In these cases, it can be shown that

\[
D^+ = \frac{H^+ G^+}{H^+ + G^+}
\]

When Step i is not fast compared to the other steps, so that \(E - D^-\) and \(E' - D^-\) are kinetically distinguishable, then

\[
D^+ < \frac{H^+ G^+}{H^+ + G^+}
\]

Thus, it is clear that if Step i were made very slow it would have no effect on \(H^+\) or \(G^+\), but would decrease \(D^+\) below the predicted value.

Studies with the enzymes from muscle and from yeast are reported in Tables V and VI. In no case is the determined value of \(D^+\) significantly below the value predicted for no isomerization, and in some cases the agreement is quite good. In others, the value obtained for \(D^+\) is higher than the calculated value, suggesting either a correction required in the mechanism or an error in the analysis.

This relation for the case where there is no isomerization can be obtained directly. Consider

\[
DH + E \xrightarrow{a} E - DH \xrightarrow{b} E - D^- \xrightarrow{c} E' + DG
\]

Then \(H^+ = V_a\), \(G^+ = V_b\), and \(D^+ = V_a\) times the fraction of \(E - D^-\) that is derived directly from \(DH\), not \(DG\), thus

\[
D^+ = V_a \left( \frac{V_a}{V_a + V_b} \right) = \frac{H^+ G^+}{H^+ + G^+}
\]

This same equation is obtained from the expressions for \(D^+\), \(H^+\), and \(G^+\) obtained for the mechanism containing Steps 1, 2, 3, 7, and 8, and is an excellent verification of these expressions.
isotope effect in the tritium exchange measurements leading to an underestimation of the value of proton exchange, $H^*$. That the latter is, in part, the explanation is suggested by the observation that $H^*$ is sometimes less than $D^*$, a situation not allowed by the basic mechanism, and was shown by a direct determination of the discrimination against tritium in the condensation of DHAP (containing tritium) with glyceraldehyde-3-P. Unlike the condensation with $D^*$-glyceraldehyde (Table II) in which the detrition rate greatly exceeds condensation, with glyceraldehyde-3-P the condensation with the prototized form of DHAP occurs preferentially, i.e. $\log$ (condensation)/$\log$ (detrition) > 1. Thus, it may be that a part of the effect of DzO on the rate of FDP cleavage with the native muscle enzyme (Table III) may have been due to a kinetic isotope effect and not simply a "medium effect." Apparently, glyceraldehyde-3-P is much more effective than glyceraldehyde in the capture of DzO so that no excess detrition is observed. Hence, in the case with glyceraldehyde-3-P, the rate-determining step precedes condensation. Certainly, the C-H bond cleavage step cannot be considered rate-determining for the net reaction since the isotope effect observed with the muscle enzyme is much smaller than the 20-fold that would be expected from the studies with the carboxypeptidase-treated enzyme.3 When the value of $H^*$ is corrected by the determined isotope effect, the calculated value ($H^* G^*/(H^* + G^*)$ comes close to the measured value of $D^*$ and does not exceed it significantly. Ideally, it would be expected to result in equivalence at least. The conclusion seems justified that for both the muscle and yeast enzyme an isomerization step at this point cannot be of importance in determining the rate of the over-all reaction, as represented by $D^*$.

According to present evidence the chief and perhaps only kinetic effect of removing the 3 carboxyl-terminal tyrosine residues by carboxypeptidase treatment of muscle aldolase is to decrease the rate of the step in which the C-H bond to DHAP is made and broken, thus making the liberation of DHAP the slow step in the cleavage reaction. This consequence is in agreement with certain observations on the kinetic behavior of the modified enzyme. We have observed that in the assay of the carboxypeptidase-treated enzyme by FDP cleavage coupled with DPNH oxidation by $\alpha$-glycerophosphate dehydrogenase, the rate of DHAP production fell greatly as the reaction proceeded. This was not the case when native enzyme was used. This effect was overcome by the addition of triosephosphate isomerase. The inhibitory effect of accumulating glyceraldehyde-3-P has been reported to affect native liver aldolase activity.

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The assumption is made that the intrinsic mechanism of the $-\text{C}--\text{H}$ cleavage step is not altered by carboxypeptidase treatment. The magnitude of the isotope effect that is found in a step is determined by the extent to which the bond to hydrogen is weakened in the transition state, being largest when there is the least amount of bonding to both the donating and receiving atoms (17). The magnitude of the isotope effect seen for a multi-step reaction depends not only on the isotope effect of the particular step but on the extent to which the rate of over-all reaction would reflect a change in rate of that one step, i.e. rate limitation. Hence, the inhibition of DHAP with glyceraldehyde-3-P at pH 8 with yeast aldolase, the titrated species reacts at only one-ninth the rate of protonated DHAP (Table VI). At pH 6 and 7, the two rates are more nearly equal. Although it is possible to imagine a change in the nature of the catalysis due to an ionization in the active site, it is more reasonable that the change is a rate effect altering the degree to which the proton-dissociating step determines the net rate. Likewise for the change upon carboxypeptidase treatment.

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### Table VI

<table>
<thead>
<tr>
<th>pH</th>
<th>Exchange rate</th>
<th>$H^* G^*$</th>
<th>$H^* + G^*$</th>
<th>$D^*$</th>
<th>$D^* - G^*$ corrected$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.07</td>
<td>0.6</td>
<td>0.057</td>
<td>0.063</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td>0.05</td>
<td>0.06</td>
<td>0.028</td>
<td>0.027</td>
<td>1.9</td>
</tr>
<tr>
<td>8</td>
<td>0.01</td>
<td>0.004</td>
<td>0.0047</td>
<td>0.003</td>
<td>7</td>
</tr>
</tbody>
</table>

$^a$ As in Table V.

### Table VII

Effect of acetaldehyde on "aldolase assay"

Cuvettes contained in 1 ml of triethanolamine buffer (0.1 M, pH 8), serum albumin (1%), EDTA (1 mM), FDP (1 mM), DPNH (0.15 mM), and $\alpha$-glycerophosphate dehydrogenase (0.03 mg) at 25°C. Additions of acetaldehyde (25 $\mu$moles), triose-P isomerase (0.01 mg) and either native (0.007 unit) or carboxypeptidase-treated (corresponding to 0.02 unit before treatment) muscle aldolase were present as indicated. The initial rate of absorbance change at 340 nm was measured immediately after the addition of the aldolase and may be used to calculate the rate of FDP cleavage.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Isomerase</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaldehyde</td>
<td>Native</td>
<td>Carboxypeptidase-treated</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

in the same way (18), and it has been found in this laboratory that carboxypeptidase treatment of liver aldolase intensifies this effect, which is also eliminated by addition of isomerase. It has been suggested previously that aldehyde can inhibit aldol cleavage by condensing with enzyme-bound DHAP (19). The great change in sensitivity of the assay to glyceraldehyde-3-P when aldolase is treated with carboxypeptidase is clearly caused by the decreased rate of protonation of the aldolase-DHAP-anion, which makes the condensation reaction with glyceraldehyde-3-P relatively more effective.

Spolter, Adelman, and Weinhouse (20) have recently reported that several aldehydes greatly increased the rate of carboxypeptidase-modified aldolase acting on FDP in the assay with $\alpha$-glycerophosphate dehydrogenase and triose-P isomerase. In this case, the DPNH oxidized is a measure of combined DHAP and glyceraldehyde-3-P. No increase was seen with the native enzyme and FDP nor when fructose-1-P was the substrate with the carboxypeptidase-modified enzyme. An explanation for the stimulation by aldehyde can be given based on the conclusion that carboxypeptidase enzyme is very ineffective in releasing DHAP from the enzyme-DHAP-anion intermediate and that the
latter can react just as well as native enzyme with aldehyde. In this view, the effect of aldehyde is to accelerate the liberation of glyceraldehyde-3-P and free enzyme by an alternate route. The accelerated DPNH oxidation observed in the experiments of Spolter et al. could then be entirely due to reduction of DHAP formed from glyceraldehyde-3-P, which should be equivalent to the aldehyde which condenses. That this is indeed the case is shown by the fact that in the absence of triosephosphate isomerase there is no effect of the addition of acetaldehyde on the rate of DHAP oxidation, indicating that the effect is caused by glyceraldehyde-3-P production. This is shown in Table VII. In the presence of aldehyde the rate of FDP cleavage by the carboxypeptidase-treated enzyme is returned to the value obtained before the native enzyme lost 95% of its ability to form DHAP from FDP. Thus, the 20-fold decrease in rate of FDP cleavage when acetaldehyde is omitted compares well with the 23-fold loss in rate of FDP cleavage that resulted from carboxypeptidase treatment. The presence of 5-deoxypentulose-1-P in the expected amount was shown by the DPNH oxidation resulting from the addition of native aldolase and alcohol dehydrogenase after free acetaldehyde had been removed by aeration.

The formation of a condensation product of acetaldehyde under the conditions described above is a transaldolase reaction, in which the DHAP does not dissociate from the enzyme. It has been reported (21) that fructose-6-P transaldolase catalyzes a slow exchange between dihydroxyacetone and fructose-6-P. The simplest mechanism for a transaldolase reaction is one in which the aldehyde reacts with the enzyme-C-1,2,3 intermediate and not with the free enzyme. The present results with yeast and muscle aldolase indicate that in cleavage and condensation, the aldehyde is the first to leave and the last to combine and does not interact fruitfully with the free enzyme. It seems apparent, also, that the protonation step, necessary for net aldol cleavage, is due to an activating group of the enzyme that does not function in the transaldolase phenomenon.

Although the present results imply only a small role (if any) for a pathway in which aldehyde reacts with free enzyme first, glyceraldehyde-3-P (18) and other aldehydes (20) have been reported to be competitive inhibitors of aldol cleavage under some conditions. The implication of these results is that aldehyde reacts with free enzyme. It may be said, however, that such data provide no indication of the quantitative importance of this mechanism and further it may be that there is no relation between the inhibitor and substrate activities of the aldohydases.

Among various interpretations of the nonuniform effect of carboxypeptidase treatment of aldolase on the several activities of the muscle enzyme, it has been suggested that the tyrosines removed by the treatment have a more critical role in the cleavage of FDP than of fructose-1-P, possibly by binding to the phosphate in position 6 of FDP (3). The present results indicate that loss of the tyrosines does not affect the cleavage of the carbon chain of FDP. The effect is in the proton addition step necessary for the formation of DHAP, a step that should be identical in the cleavage of both FDP and fructose-1-P. Since the rate of DHAP formation from fructose-1-P is normally only 2% of that from FDP, it is clear that the difference in rate must be the result of a much slower rate-limiting step in the fructose-1-P reaction that occurs prior to DHAP liberation. Hence, decreasing the rate of this latter by carboxypeptidase treatment will have a more marked effect with FDP than with fructose-1-P. The fact that both cleavage reactions proceed at the same rate after carboxypeptidase treatment may indicate that both reactions are now limited by the —C—H bond-forming step.

**Summary**

1. Both the yeast and muscle aldolase reactions are shown to proceed almost exclusively by an ordered sequence.

   Enzyme + fructose diphosphate ⇌ glyceraldehyde 3-phosphate + enzyme-dihydroxyacetone phosphate (lacking a proton on the α-carbon) ⇌ enzyme + dihydroxyacetone phosphate

2. The proton exchanges known to occur between water and dihydroxyacetone phosphate in the absence of aldolase are shown to occur at rates predicted from the observed dependence of proton exchange on glyceraldehyde 3-phosphate concentration under equilibrium conditions for both the yeast and muscle enzymes.

3. Carboxypeptidase treatment of muscle aldolase is shown to affect the exchange of dihydroxyacetone phosphate with fructose diphosphate and not that of glyceraldehyde 3-phosphate with fructose diphosphate. The particular step that is altered is the step concerned with formation of the —C—H bond of dihydroxyacetone phosphate. This step occurs at only 0.8% the rate found with native aldolase when measured as release of tritium from tritiated dihydroxyacetone phosphate and is shown to be rate-limiting in both aldol synthesis and cleavage reactions. The differential large decrease in tritium exchange reactions is the result of a very large isotope effect that becomes evident when the —C—H bond-forming step becomes rate-limiting following carboxypeptidase treatment.

4. Isotope exchange studies at equilibrium have been used to investigate the occurrence of a reactant-independent step, isomerization, in the sequence of steps. No evidence for such a step was detected with either the yeast or muscle enzymes over a range of pH values.

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**References**

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