Studies on the Interaction of Aldolase with Substrate Analogues*

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SUMMARY

Interactions of specific and nonspecific agents at the dihydroxyacetone-P and glyceraldehyde-3-P sites of rabbit muscle aldolase are shown by several methods: quenching of protein fluorescence, protection against the destructive action of trypsin, the inhibition of initial rates and of equilibrium exchange rates, and the inactivation caused by NaBH₄. It is concluded from inactivation and labeling studies that glycolaldehyde-P can form a Schiff’s base at the dihydroxyacetone-P site. D-Glyceraldehyde-3-P and erythrose-4-P, like dihydroxyacetone-P, cause fluorescence quenching at low concentration, whereas L-glyceraldehyde-3-P, which is a good acceptor for dihydroxyacetone-P, does not. The competitive inhibition shown by small anions such as Cl⁻, P₄, and ethyl-P is first order for these anions with fructose-1-P as the substrate but approximately second order with fructose-1,6-di-P as substrate. This suggests that binding of fructose-1-P to the enzyme is prevented by an anion bound to the dihydroxyacetone-P site, but not by one at the aldehyde-P site, whereas fructose-di-P binding is prevented by anion interaction with either site.

O

Acetol-P (CH₃—C—CH₂OPO₃⁻) is a substrate for the proton exchange reaction of aldolase. It has a much higher Kₘ than dihydroxyacetone-P, the maximum velocity of exchange is about 17% as great, and its rate of condensation with glyceraldehyde-3-P only about 1% that found with dihydroxyacetone-P.

L-Glyceraldehyde-3-P is a competitive inhibitor of the proton exchange reaction of acetol-P but does not react with the dihydroxyacetone-P site as shown by lack of fluorescence quenching. This suggests that, although the enzyme can bind aldehyde, the complex is inappropriate for productive reaction with dihydroxyacetone-P. This is consistent with earlier studies requiring that the condensation is an ordered reaction with dihydroxyacetone-P reacting before aldehyde.

The inactivation of aldolase as a result of reaction with L-

glyceraldehyde-3-P is like that produced by carboxypeptidase in that it seems to inhibit the rate of —C—H bond cleavage and not —C—C bond cleavage of fructose-di-P. This is concluded since that transaldolase action of the treated enzyme with acetaldehyde as an acceptor is not much slower than that of untreated enzyme. The rate of inactivation with L-glyceraldehyde-3-P shows “saturation” kinetics with a Kᵢ that agrees with its Kᵢ as a competitive inhibitor of fructose-1-P cleavage. However, in view of the finding that the transaldolase activity of the treated enzyme is unimpaired, the locus of this L-glyceraldehyde-3-P action cannot be that of the fructose-di-P binding.

Those compounds that quench protein fluorescence also protect aldolase from inactivation by trypsin, whereas aldehydes such as DL-glyceraldehyde and L-glyceraldehyde-3-P form complexes that are more susceptible to trypsin action.

Carboxypeptidase treatment of aldolase is observed to produce a large decrease in Kᵢ as a competitive inhibitor of fructose-di-P. This is contrary to the findings of others, but is required by theory if the ordered reaction mechanism is correct and if the effect of carboxypeptidase action is restricted to the step for protonation of the enzyme-dihydroxyacetone-P eneamine, as is believed.

Kinetic studies on the mechanism of muscle fructose-di-P aldolase have indicated that the liberation of D-glyceraldehyde-P precedes that of dihydroxyacetone-P (1).

E + FDP \[\xrightarrow{\text{glyceraldehyde-3-P}}\] E-FDP + DHAP + H⁺

The main evidence for this ordered sequence is that carboxypeptidase treatment of aldolase resulting in a 20-fold decrease in Vₘₐₓ of FDP cleavage is without effect on the glyceraldehyde-

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1 The abbreviations used are: FDP, fructose-1,6-di-P; DHAP, dihydroxyacetone-P; Kᵢ, the concentration of a compound that gives half-maximal quenching of aldolase fluorescence.
EDTA (1 mM) aldolase was made available by Dr. P. Meloche. 2-Keto-3-deoxygluconate-6-P was not destroyed by alkali. It was possible to obtain the complex that does not react with DHAP. In addition, studies comparing FDP cleavage kinetics of native and carboxypeptidase treated aldolase have been reported (3) in which no change in $K_m$ of FDP is observed. This result proves inconsistent with the ordered mechanism of Scheme 1 in the case that a large decrease in $V_{max}$ as previously proposed (1).

The present paper is an attempt to deal with these contradictions. In addition, several criteria are found which distinguish between action of compounds at the DHAP and aldehyde sites.

**MATERIALS AND METHODS**

Four aldolase preparations were used in these studies. Preparation I, from Sigma, was freed of traces of triose-P isomerase on DEAE cellulose (4) in 1964. At that time it had a specific activity of 21 units per mg. Preparation II, from Boehringer, was found to have 13.2 units per mg and was freed of isomerase. Preparation III was freshly prepared from rabbit muscle (5) and was used without crystallization with a specific activity of 15.8 units per mg. Preparation IV was obtained from Sigma and, after chromatography to remove isomerase, had a specific activity of 9 units per mg. Preparation II, from Boehringer, was found to have 13.2 units per mg and was freed of isomerase.

**RESULTS**

In the addition of acetaldehyde (1, 2).

The DHAP + glyceraldehyde-3-P, 1,4-diacylglyceraldehyde-3-P, FDP, and fructose 1-P were purchased from Calbiochem. D-Glyceraldehyde-3-P was prepared by HIO oxidation of FDP according to Szewczuk et al. (6). L-Glyceraldehyde-3-P was prepared from D-glyceraldehyde-3-P by removal of the a isomer by reaction first with 2-keto-3-deoxygluconate-6-P aldolase in the presence of excess pyruvate followed by barium precipitation of the condensation product at pH 3.5 (7). Residual D-glyceraldehyde-3-P was removed with triose-P isomerase and a-glycerol-P dehydrogenase. The L-glyceraldehyde-3-P was obtained by Dowex 1-Cl- chromatography with 0.02 N HCl. 1,4-Butanediol-di-P was prepared according to Hartman and Barker (8).

DHAP-1,3-14C was prepared from 1,3-14C glycerol by enzymatic phosphorylation and oxidation in the presence of hydrazine under similar conditions to those used for the assay of glycerol (9). The hydrazine was removed by extraction with benzaldehyde (10) and the DHAP was purified on Dowex 1-Cl- with elution by 0.025 N HCl. D-Glyceraldehyde-3-P, 3-14C was prepared from glucose-6-14C by successive reaction with the pure glycolytic enzymes to FDP, which was isolated on Dowex 1-Cl-, 0.1 N HCl elution. This was reacted with aldolase and glyceraldehyde-3-P dehydrogenase to produce a mixture of a-glyceraldehyde-P and D-glyceraldehyde-3-P, 3-14C which was purified on Dowex 1-Cl-, 0.025 N HCl elution. Glyceraldehyde-2-P, 2-14C was prepared from glyceraldehyde-3-P, 3-14C by reaction with HIO until glyceraldehyde-3-P was completely destroyed as shown by enzymatic assay. It was then isolated on Dowex 1-Cl-, eluted by 0.025 N HCl.

Assays for DHAP, FDP, and D-glyceraldehyde-3-P were done in the spectrophotometer by coupling to DPNH oxidation with glyceraldehyde-3-P dehydrogenase alone or with the addition of aldolase and triose-P isomerase. L-Glyceraldehyde-3-P was assayed as P, after treatment for 10 min at 25°C with 0.1 N NaOH. Acetol-P could be assayed with glyceraldehyde-3-P dehydrogenase. Glycolaldehyde-2-P was determined as P after acid degradation. The standard assay for aldolase was at 35°C with triethanolamine buffer (0.1 M, pH 7.5), FDP (1 mM), serum albumin (0.1%), EDTA (1 mM), DPNH (0.1 mM), and a-glyceraldehyde-3-P dehydrogenase and triose-P isomerase in excess.

Detritiation of acetol-P by aldolase was measured by stopping the reaction with an equal volume of ethanol and determining the radioactivity that passed through a column, 1 x 3 cm, of Dowex 1-acetate. Exchange rates were calculated as before (1).

Fluorescence quenching studies were done with the Amino-Bowman spectrophotofluorimeter. Fluorescence at 355 mμ was measured upon activation at 280 mμ. Bovine serum albumin (50 μg per ml) was used as a standard. Aldolase solutions at this concentration were stable during the measurements in which small volumes of test compounds were added successively, followed by the measurement of fluorescence. After correcting for volume change, the fraction of the total fluorescence lost as a result of quenching gave a normal hyperbolic relation with respect to concentration of added compound (Fig. 1). By extrapolation of a linear double reciprocal plot one could obtain the maximum quenching fraction and the affinity constant, $K_{d/2}$. The neutralized solution was placed on Dowex 1-Cl- and the labeled product was eluted with 0.025 N HCl. Radioactivity was randomly distributed among the five "stable" positions as judged by the finding that prolonged incubation with aldolase exchanged 60% of the tritium with water.
but at 10³ higher concentration, consistent with its higher Ki for interact with aldolase, various inhibitors of FDP cleavage were used and reflecting the general difference between diphosphates and monophosphates (8). On the other hand, the monophosphates—DHAP, n-glyceraldehyde-3-P, glycolaldehyde-P, and erythrose-4-P—had high affinities in causing quenching, suggesting that a carbonyl group might be required to produce high affinity if a second phosphate group is not present. Although the last three compounds are aldehydes which are known to accept DHAP in condensation, n-n-glyceraldehyde and l-glyceraldehyde-3-P, both of which are acceptors of DHAP and inhibitors of fructose-1-P cleavage, did not result in detectable quenching. As independently observed by Lai et al. (11) and shown below, l-glyceraldehyde-3-P causes inactivation of aldolase with a half-time of 7 to 10 min at saturation. It was therefore important in testing for quenching by l-glyceraldehyde-3-P to look for effects prior to the occurrence of significant inactivation, i.e., within 30 sec. No quenching could be observed at any time. Consideration was therefore given to the possibility that the common feature of the quenching compounds was reaction at the DHAP sites. Consistent with this idea was the observation that aldolase that had been inactivated by BH₄⁻ treatment in the presence of DHAP was unreactive with either DHAP or n-glyceraldehyde-3-P in the quenching of fluorescence. It has not been determined whether the fluorescence yield of this protein differs from that of native enzyme. The differences in the maximum extent of quenching by the active compounds (Table I) need not imply that different sites of action are involved. The protein conformation of the various complexes may differ as a result of detailed differences in steric, electrostatic, and chemical interactions with the particular test compound.

Inactivation with NaBH₄—Reduction by BH₄⁻ of an imine formed between a carbonyl compound such as DHAP and an amino group on the protein may provide a specific criterion for reaction of a test compound at the DHAP site. The condensation of DHAP with formaldehyde in H₂¹⁸O leads to incorporation of much less than 1 atom of ¹⁸O into a stable carbinal position, suggesting that imine formation is not a feature of the aldehyde site in the reaction mechanism. Therefore, inactivation of aldolase with BH₄⁻ was determined in the presence of some of the compounds studied by fluorescence quenching. In these experiments 2 mg of aldolase, in 1 ml of 0.15 M sodium succinate (pH 6.0), and the test compounds at 3 mM were treated at 4°C with eight 0.02-ml additions of 1 M NaBH₄ over a 30-min period. Nonyl alcohol, 0.05 ml, was added to minimize foaming and HCl vapor was added to maintain the pH. Under these conditions 10 to 22% of the activity was lost with no addition, whereas the addition of those compounds that caused quenching resulted in greater loss of activity, as follows: DHAP (96%), glycolaldehyde-P (84%), erythrose-4-P (50%), and n-glyceraldehyde-3-P (36%). On the other hand, l-glyceraldehyde-3-P results in rapid inactivation in the absence of BH₄⁻ (11), making it difficult to establish a BH₄⁻ effect. The ¹⁴C-labeled DHAP, glycolaldehyde-P, and D-glyceraldehyde-3-P were prepared to test the covalent labeling of the enzyme dependent on BH₄⁻ treatment. All three resulted in labeling the protein; however, some labeling by D-glyceraldehyde-3-P occurred in the absence of BH₄⁻. More than 10 eq were bound in the absence of BH₄⁻ when enzyme and D-glyceraldehyde-3-P were incubated under alkaline conditions, pH > 8. Because of the difficulty in preventing local alkalinity in the hydrolysis of NaBH₄, the possibility of non-

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximum extent of quenching (%)</th>
<th>K₉/2 (μM)</th>
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</thead>
<tbody>
<tr>
<td>DHAP</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>Acetol-P</td>
<td>27</td>
<td>6700</td>
</tr>
<tr>
<td>Glycolaldehyde-P</td>
<td>6</td>
<td>2.5</td>
</tr>
<tr>
<td>n-Glyceraldehyde-3-P</td>
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<td>12.5</td>
</tr>
<tr>
<td>L-Glyceraldehyde-3-P</td>
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<td>0</td>
</tr>
<tr>
<td>l-Glyceraldehyde</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Erythrose-4-P</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>F₃</td>
<td>8</td>
<td>1300</td>
</tr>
<tr>
<td>1,4-Butanediol-di-P</td>
<td>7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*The highest concentration used was 200 μM.

*The highest concentration used was 10 mM.

from the concentration that gave half-maximal quenching. Since very little of the total substrate could be present in a complex, the K₉/2 would correspond closely to the dissociation constant of the complex between enzyme and test compound.

### Results

**Structural Requirements for Fluorescence Quenching of Aldolase**

In an attempt to characterize the manner in which aldehydes interact with aldolase, various inhibitors of FDP cleavage were studied for their effect on the protein's fluorescence (Table I). A carbonyl group is not required to produce quenching, since 1,4-butanediol-di-P, an excellent competitive inhibitor (8), quenches at very low concentrations. F₃ had a similar effect, but at 10⁹ higher concentration, consistent with its higher Kᵢ contributing to the general difference between diphosphates and monophosphates (8). On the other hand, the monophosphates—DHAP, n-glyceraldehyde-3-P, glycolaldehyde-P, and erythrose-4-P—had high affinities in causing quenching, suggesting that a carbonyl group might be required to produce high affinity if a second phosphate group is not present. Although the last three compounds are aldehydes which are known to accept DHAP in condensation, n-n-glyceraldehyde and l-glyceraldehyde-3-P, both of which are acceptors of DHAP and inhibitors of fructose-1-P cleavage, did not result in detectable quenching. As independently observed by Lai et al. (11) and shown below, l-glyceraldehyde-3-P causes inactivation of aldolase with a half-time of 7 to 10 min at saturation. It was therefore important in testing for quenching by l-glyceraldehyde-3-P to look for effects prior to the occurrence of significant inactivation, i.e., within 30 sec. No quenching could be observed at any time. Consideration was therefore given to the possibility that the common feature of the quenching compounds was reaction at the DHAP sites. Consistent with this idea was the observation that aldolase that had been inactivated by BH₄⁻ treatment in the presence of DHAP was unreactive with either DHAP or n-glyceraldehyde-3-P in the quenching of fluorescence. It has not been determined whether the fluorescence yield of this protein differs from that of native enzyme. The differences in the maximum extent of quenching by the active compounds (Table I) need not imply that different sites of action are involved. The protein conformation of the various complexes may differ as a result of detailed differences in steric, electrostatic, and chemical interactions with the particular test compound.

Inactivation with NaBH₄—Reduction by BH₄⁻ of an imine formed between a carbonyl compound such as DHAP and an amino group on the protein may provide a specific criterion for reaction of a test compound at the DHAP site. The condensation of DHAP with formaldehyde in H₂¹⁸O leads to incorporation of much less than 1 atom of ¹⁸O into a stable carbinal position, suggesting that imine formation is not a feature of the aldehyde site in the reaction mechanism. Therefore, inactivation of aldolase with BH₄⁻ was determined in the presence of some of the compounds studied by fluorescence quenching. In these experiments 2 mg of aldolase, in 1 ml of 0.15 M sodium succinate (pH 6.0), and the test compounds at 3 mM were treated at 4°C with eight 0.02-ml additions of 1 M NaBH₄ over a 30-min period. Nonyl alcohol, 0.05 ml, was added to minimize foaming and HCl vapor was added to maintain the pH. Under these conditions 10 to 22% of the activity was lost with no addition, whereas the addition of those compounds that caused quenching resulted in greater loss of activity, as follows: DHAP (96%), glycolaldehyde-P (84%), erythrose-4-P (50%), and n-glyceraldehyde-3-P (36%). On the other hand, l-glyceraldehyde-3-P results in rapid inactivation in the absence of BH₄⁻ (11), making it difficult to establish a BH₄⁻ effect. The ¹⁴C-labeled DHAP, glycolaldehyde-P, and D-glyceraldehyde-3-P were prepared to test the covalent labeling of the enzyme dependent on BH₄⁻ treatment. All three resulted in labeling the protein; however, some labeling by D-glyceraldehyde-3-P occurred in the absence of BH₄⁻. More than 10 eq were bound in the absence of BH₄⁻ when enzyme and D-glyceraldehyde-3-P were incubated under alkaline conditions, pH > 8. Because of the difficulty in preventing local alkalinity in the hydrolysis of NaBH₄, the possibility of non-

[Unpublished results.]
specific labeling must be considered. In an experiment with the
14C-substrates used as before the expected inactivation was ob-
tained (96%, 84%, and 36%) and the nondialyzable and tri-
chloroacetic acid-insoluble radioactivity found corresponded to
1.7, 0.68, and 1.6 eq of DHAP, glycolaldehyde-P, and δ-glyceral-
dehyde-3-P, respectively, per eq of 150,000 molecular weight.
An electrophoretic separation of a trypsin-nagarse digest of the
three labeled proteins was performed to determine whether sim-
ilar labeled peptides were present. It is clear from Fig. 2 that
DHAP and glycolaldehyde-P label the enzyme identically. Much
less radioactivity from δ-glyceraldehyde-3-P remained
with these peptides through the digestions, and that which did so
did not follow the same pattern, indicating that labeling at several
sites may have occurred with the δ-glyceraldehyde-3-P.
A second characteristic of the interaction between aldolase and
DHAP is the absorbance increase at 240 μm observed by Mehler
sites may have occurred with the δ-glyceraldehyde-3-P.

The fluorescence quenching measurement provides a simple
direct method for determining the binding constants of DHAP and
butanediol-di-P as a function of pH. As seen in Fig. 3, the
KQiz of DHAP was the same for the native and carboxypeptidase
enzymes, and showed a sharp decrease in affinity at pH > 8.3.
The degree of quenching by DHAP was not greatly altered by pH
or carboxypeptidase treatment, being 16 to 20%. The KQiz
values for butanediol-di-P also show a sharp increase at alkaline
pH—about 1 pH unit higher than for DHAP. A similar effect
is seen in the KQiz values for FDP and fructose 1 P (13).

Acetol-P—This compound, which differs from DHAP in having
a —CH2 group in place of a —CH2OH group, has a poor affinity
as judged by fluorescence quenching in marked contrast to glyco-
aldehyde-P. Although this result provides no indication for
specific binding, the possibility that acetol-P might function as an
analogue of DHAP was tested by studying the detritiation of the
pentatriitated molecule. When tested at the millimolar concen-
tration an active exchange rate could be observed with native
aldolase that was reduced to <0.2% by carboxypeptidase treat-
ment of the aldolase. A KQiz for detritiation of 6.7 mM compared
well with KQiz (Table I), but was much greater than the KQiz
for detritiation of DHAP, 0.07 mM (14). The Vmax for exchange
was about 17% of that of DHAP with the native muscle enzyme.
Richards and Rutter (4) reported that acetol-P exchanged hy-
drogen and condensed with aldehydes at rates about 104 slower
than DHAP. In retrospect it seems that the high KQiz caused
this difference. Testing the 4H compound at 4 mM the rate of
condensation with 0.1 mM δ-glyceraldehyde-3-P was determined
by determining the radioactivity that was displaced to the FDP
intermediate, and perhaps by inactivation with BH4—. Loss of FDP
cleavage activity as a result of trypsin treatment of aldolase has

Fig. 2. High voltage electrophoresis of 14C-peptides from
aldolase treated with BH4— in the presence of (A) 14C-DHAP
(×), (B) 14C-δ-glyceraldehyde-3-P (△), or (C) 14C-glycolalde-
hyde-P (○). The trichloroacetic acid-precipitated aldolase was
dissolved in alkali at 80°C for 5 min and treated with trypsin +
calcium and with nagarse. The three preparations were run at
2000 volts, pH 6.5, pyridine-acetic acid- H2O (10:0.4:90), on What-
man No. 3MM for 2 hours. Identically placed spots could be
seen in the ultraviolet in the three strips and are indicated by the
shaded areas. Segments of 1-cm width were cut and counted and
their distribution is shown as a function of the distance of migra-
tion toward the anode.

Fig. 3. The effect of pH on KQiz caused by DHAP and 1,4-bu-
tanediol-di-P. KQiz was determined for DHAP as in Fig. 1 with K-
cacodylate buffer (0.02 M, pH 6 to 0.5) and triethanolamine-HCl
buffer (0.02 M, pH 6.9 to 9.5) with native aldolase, Preparation 1
(●), or carboxypeptidase-treated enzyme (○) and for butanediol-
di-P with native enzyme (△).
Fig. 4. Effect of DHAP, D-glyceraldehyde-3-P (D-G3P), and butanediol-di-P (Bu-diol-di-P) on trypsin inactivation of aldol cleavage. Aldolase (0.15 mg per ml of Preparation II) was incubated at 25°C with 0.1 M triethanolamine-HCl buffer (pH 7.4), EDTA (1 mM), bovine serum albumin (1 mg per ml), and either trypsin (0.02 mg per ml) alone (X) or with 0.2 mM of one of the following: DHAP (O), D-glyceraldehyde-3-P (A), or 1,4-butane-

previously been reported to be prevented by FDP (10). The data of Fig. 4 indicate that those compounds that quench fluorescence also protect aldolase against inactivation by trypsin. On the other hand, D-glyceraldehyde (50 mM) and D-glyceraldehyde-3-P (40 mM) formed complexes that were not resistant to trypsin action but rather somewhat more sensitive to trypsin (Fig. 5, A and B).

Action of L-Glyceraldehyde-3-P—As noted in Fig. 6, L-glyceraldehyde-3-P is a competitive inhibitor of the ³H exchange of acetal-P. The Kᵦ is the same as found for inhibition of fructose-1-P cleavage (Fig. 7). A replot of the Kᵦ of acetal-P against L-glyceraldehyde-3-P concentration (inset, Fig. 6) suggests that acetal-P has affinity for the enzyme-L-glyceraldehyde-3-P complex the resulting ternary complex is unreactive. This indicates that E-aldehyde is a dead end complex and that the competitive character of the inhibition of fructose-1-P cleavage cannot be taken as support for a random order mechanism.

As indicated earlier (11) and shown in Fig. 5C, L-glyceraldehyde-3-P inactivates aldolase. The affinity of L-glyceraldehyde-3-P as determined from the concentration dependence of this inactivation is 10 μM (Fig. 8), and compares well with the competitive Kᵦ when used to inhibit fructose-1-P cleavage or acetal-P exchange. The enzyme that has been treated with L-glyceraldehyde-3-P at 0.1 mM for 3 hours still retains about 3% of its activity for the cleavage of FDP (Table II). It is much less altered in the cleavage of fructose-1-P, however. In this respect, and in the large decrease in tritium exchange rates, it is similar to carboxypeptidase-treated enzyme (16, 17). In further analogy (1, 2) the L-glyceraldehyde-3-P-inactivated enzyme was stimulated 18-fold by addition of acetaldehyde to the assay of FDP cleavage. Since the enzyme action before exposure to L-glyceraldehyde-3-P was not stimulated by acetaldehyde, this result indicates that the L-glyceraldehyde-3-P-enzyme is only slightly decreased in the rate of transaldolase reaction: FDP + acetaldehyde → D-glyceraldehyde-3-P + 5-deoxyxylulose-1-P.

Site of Interaction with Nonspecific Anions—It is known that muscle aldolase reacts nonspecifically with inorganic anions (13), which are strictly competitive inhibitors of both FDP and fructose-1-P cleavage. An interesting difference between the two substrates is shown in Fig. 9 and Table III. The increase in Kᵦ of FDP becomes second order with either P₀ or Cl⁻, whereas the Kᵦ of fructose-1-P is always linearly increased. This result indicates that there is an anion-specific site on aldolase which when occupied stands in the way of FDP binding, but not fructose-1-P binding. It is reasonable to suggest that this is the site which normally binds the C₅-phosphate of FDP. It was of interest to determine whether fructose-1-P would overlap an aliphatic phosphate ester bond to that site. As seen in Fig. 9, ethyl-P produced the same effects as P₀.

Effect of Carboxypeptidase Treatment of Aldolase on Kᵦ of FDP
Fig. 6. Effect of L-glyceraldehyde-3-P (L-GSP) on the initial rate of detritiation of acetol-P. Each milliliter of incubation at 25°C contained 3.2 units of aldolase, Preparation II, triethanolamine-HCl (pH 7.5, 0.1 M), bovine serum albumin (1 mg per ml), EDTA (1 mM), uniformly labeled acetol-P-3H (4 to 24 mM with 7600 cpm per pmole of C-H position), and L-glyceraldehyde-3-P at 0 μM (X), 32 μM (□), and 64 μM (○). The maximal rate of 3H exchange was 0.52 pmole of hydrogen per min per ml. The $K_i$ of acetol-P was 2.9 mM. The $K_i$ of L-glyceraldehyde-3-P was 14 μM.

D-GSP, d-glyceraldehyde-3-P.

Fig. 7. Effect of L-glyceraldehyde-3-P (L-GSP) on the initial rate of fructose-1-P cleavage. Each cuvette contained per ml: 0.30 unit of aldolase, Preparation II, imidazole-HCl buffer (20 mM, pH 7.5), EDTA (1 mM), bovine serum albumin (1 mg), DPNH, fructose-1-P (4 to 32 mM), α-glycerol-P dehydrogenase (0.3 unit), and L-glyceraldehyde-3-P at 0 μM (X), 20 μM (□), or 30 μM (○). The $K_m$ of fructose-1-P was 7.5 mM. The $K_i$ of L-glyceraldehyde-3-P was 19 μM. $F1P^\prime$, fructose-1-P$^\prime$.

Fig. 8. Concentration dependence of aldolase inactivation by L-glyceraldehyde-3-P (L-G-3-P). Aldolase (0.64 mg per ml of Preparation IV) was incubated at 25°C with triethanolamine-HCl (pH 7.4, 0.1 M), EDTA (2 mM), bovine serum albumin (0.8 mg per ml), and L-glyceraldehyde-3-P (0, 5, 10, 15, or 25 μM). Samples were diluted at 15 and 30 min 10-fold in buffer-EDTA-bovine serum albumin medium containing FDP (50 μM) and an aliquot was assayed for FDP cleavage activity. The ordinate is the reciprocal of the rate of inactivation as measured from a semilog plot of percentage activity against time. The maximum rate of inactivation is 5% per min; $K_i$ = 10 μM.

Table II

Comparison of reaction rates of native and L-glyceraldehyde-3-P-treated aldolase

<table>
<thead>
<tr>
<th>Reaction measured</th>
<th>Native</th>
<th>Treated</th>
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<tbody>
<tr>
<td>1. FDP → DHAP + glyceraldehyde-3-P</td>
<td>13.3</td>
<td>0.38</td>
</tr>
<tr>
<td>2. FDP + CH$_2$CHO → 5-deoxyxylulose-1-P + glyceraldehyde-3-P</td>
<td>13.3</td>
<td>6.8</td>
</tr>
<tr>
<td>3. Fructose-1-P → DHAP + glyceraldehyde-3-P</td>
<td>0.45</td>
<td>0.11</td>
</tr>
<tr>
<td>4. Acetol-P-3H → H$^\prime$</td>
<td>1.1</td>
<td>0.03</td>
</tr>
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</table>

The initial rate equation for the cleavage of FDP and the ordered release of product as shown in Scheme 1 is

$$v = \frac{k_2 \cdot k_5 \cdot E_P}{k_1 + k_1} \cdot \frac{E_P}{k_2 + k_3 \cdot k_5} \cdot \frac{E_P}{k_2}$$
parallel is borne out by the data of Spolter, Adelman, and Weinhouse (a), but not by that of Mehler and Cusic (3) who report that usually much larger than the decrease in carboxypeptidase treatment on aldolase indicate that a large decrease in the rate of release of tissue action of multiple alleles may be considered (18). It may be of significance in this respect that the $V_{\text{max}}$ values after carboxypeptidase treatment of the three enzymes were much closer to each other than with the "native" enzymes. On the other hand, it is possible that rate constants for Steps 1 and 2 may be slightly altered by carboxypeptidase treatment in a way that affects $K_m$ more than $V_{\text{max}}$. It is of interest to note that for $k_3 < k_2$, as observed for the carboxypeptidase enzyme, one could find values of $K_m$ much less than the dissociation constant of the enzyme-FDP, $k_{-1}/k_1$.

**DISCUSSION**

It is interesting that all the agents that act as inhibitors of the aldol cleavage reaction fall into two classes which are distinguished by their effect on protein fluorescence and on the rate of trypsin-catalyzed destruction of activity. Those agents that inhibit trypsin action also quench fluorescence. Those that stimulate trypsin action do not quench fluorescence. These latter are L-glyceraldehyde-3-P and dl-glyceraldehyde. It may be not safe to conclude that these compounds react with the free enzyme at the site with which aldehyde reacts in the enzyme-DHAP anion complex. For one thing such a site need not exist. The possibility of heterogeneity of aldolase as a result of tissue action of multiple alleles may be considered. It may be of significance in this respect that the $V_{\text{max}}$ values after carboxypeptidase treatment of the three enzymes were much closer to each other than with the "native" enzymes. On the other hand, it is possible that rate constants for Steps 1 and 2 may be slightly altered by carboxypeptidase treatment in a way that affects $K_m$ more than $V_{\text{max}}$. It is of interest to note that for $k_3 < k_2$, as observed for the carboxypeptidase enzyme, one could find values of $K_m$ much less than the dissociation constant of the enzyme-FDP, $k_{-1}/k_1$.

**Table IV**

Comparison of $K_m$ and $V_{\text{max}}$ of FDP cleavage for native and carboxypeptidase aldolases

<table>
<thead>
<tr>
<th>Added Pi</th>
<th>Native Aldolase</th>
<th>Carboxypeptidase Aldolase</th>
<th>$V_{\text{max}}/K_m$ ratio</th>
<th>$K_m/K_{mP}$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mM)</td>
<td>$V_{\text{max}}$ (units/mg)</td>
<td>$K_m$ (mM)</td>
<td>$V_{\text{max}}$ (units/mg)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>I 10</td>
<td>10.8</td>
<td>87</td>
<td>0.62</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>620</td>
<td>0.62</td>
<td>17.5</td>
</tr>
<tr>
<td>II 50</td>
<td>6.0</td>
<td>294</td>
<td>0.83</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>670</td>
<td>0.83</td>
<td>31.0</td>
</tr>
<tr>
<td>III 0</td>
<td>17.0</td>
<td>1.8</td>
<td>0.50</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>15.5</td>
<td>685.0</td>
<td>0.45</td>
</tr>
</tbody>
</table>

$^a$ CP, carboxypeptidase.

Measurements were made on a Cary model 15 spectrophotometer, 0 to 0.1 scale expansion with cells of 5-cm path length.
by n-n-glyceraldehyde with the liver enzyme, with linear replots. It is of interest that the "inactivation" by L-glyceraldehyde-3-P of the FDP aldolase action is also allosteric as shown by its failure to prevent FDP-acetaldehyde-transaldolase action. Since L-glyceraldehyde-3-P seems only to show simple competitive inhibition over the concentration range studied (Figs. 6 and 7), one might suppose that L-glyceraldehyde-3-P first binds to the anion-binding site that is specific for the G-phosphate group of FDP and is then able to reach, with its carboxyl group, the "allosteric" aldehyde site with which it reacts covalently in some unexplained manner. Once this has happened the enzyme is still able to bind FDP since the phosphoryl group of the original L-glyceraldehyde-3-P, although present in a high local concentration, would be subject to competition by FDP for interaction with the substrate-binding site. In keeping with this picture it is found that the \( K_m \) of FDP in transaldolase reaction with acetaldehyde is increased about 5-fold by the L-glyceraldehyde-3-P treatment. If the \( K_m \) of fructose-1-P were similarly increased from the 6 mM value observed with native enzyme (Fig. 6), the \( V_{max} \) of fructose-1-P cleavage with the L-glyceraldehyde-3-P-treated enzyme would be about 2.5 times greater than shown in Table II. This would make the effect of L-glyceraldehyde-3-P treatment on fructose-1-P cleavage and the transaldolase rate about the same as for the native enzyme. The L-glyceraldehyde-3-P-treated enzyme recovers activity over a period of weeks in the cold. The use of acetaldehyde in these tests for transaldolase activity did not result in any increase in FDP aldolase activity during the period of measurement in the spectrophotometer, however.

It is not clear that quenching and protection from trypsin action are necessarily characteristics of binding to the DHAP site, although this is the simplest conclusion. This becomes ambiguous in the cases of D-glyceraldehyde-3-P and D-erythrose-3-P which both quench and protect, but are obviously capable of acting at the aldehyde site (sites). A more specific characteristic of the DHAP site is the absorbance increase in the far ultraviolet region that is seen when DHAP is mixed with aldolase but is not observed with glyceraldehyde-3-P (12) or glycolaldehyde-P and so may be characteristic of an intermediate subsequent to the imine. Since the step that brings about proton abstraction from DHAP becomes rate-limiting in the cleavage of FDP with the carboxypeptidase-treated enzyme, the absorbance increase, if it were due to the enamine, could not proceed more rapidly than the cleavage rate. Since the FDP cleavage rate with carboxypeptidase enzyme is about 1 sec\(^{-1}\) (1) it was feasible to ask whether the absorbance increase at 240 nm that follows the mixing of DHAP with carboxypeptidase aldolase could be observed with the stopped flow apparatus. Dr. Julian Sturtevant performed such experiments with the carboxypeptidase enzyme. The rate of absorbance increase was too rapid to follow even at 4°, suggesting that it precedes the rate-limiting proton release. It cannot be decided from present information whether proton abstraction from DHAP or release of the proton from the enzyme is limiting. Only in the former case would it be possible to say that enamine formation was not responsible for the absorbance change.

Studies with aldolase show several examples of the large contribution that multiple binding makes to the affinity of a reagent. This was shown by Hartman and Barker (8), who observed that a second ester phosphate group suitably spaced with respect to the first in a compound caused about 100-fold decrease in the \( K_m \) value of an inhibitor. In the present work, the \( K_v \) of L-glyceraldehyde-3-P (14 \( \mu \)M), when compared with that of glyceraldehyde (4 to 10 \( \mu \)M, and \( P_i > 1 \) \( \mu \)M) (13), indicates that a \( 10^4 \) to \( 10^9 \) increase in affinity results from prior binding at one end of the L-glyceraldehyde-3-P. Likewise, binding to the DHAP site is apparently helped by a carboxyl group as well as a phosphate, leading to a lower quenching constant for glycolaldehyde-P than for \( P_i \). Thus, it is of interest to find that acetol-P, which certainly can undergo reactions subsequent to binding through its phosphate group, shows about the same poor affinity as \( P_i \) when measured by quenching and as suggested by its high \( K_m \) for proton exchange when compared with glycolaldehyde-P and DHAP. Purified acetol-P has an absorbance peak characteristic of the carbonyl group (\( E_{265}^{\text{max}} = 18 \) which compares well with the value for acetone (\( E_{265}^{\text{max}} = 17.5 \)) (19). Thus, extensive hydration should not be a problem. The importance of electron-withdrawing groups a to the carbonyl in determining the equilibria for carbinolamine formation (20, 21) and semicarbazone formation (19) may relate to the decreased effectiveness of \(-\text{CH}_2\text{OH}\) compared with \(-\text{H}\) and \(-\text{CH}_3\text{OH}\). The fact that the affinity is increased by acid around pH 8.5 where the binding of butanedial-di-P is not affected is suggestive of the titration of a group other than one responsible for binding the phosphate dianion. This could be the carbinolamine and imine nitrogens in the complex.

The very low reactivity of acetol-P in the condensation reaction at a concentration of substrate that is near to saturation for the proton exchange reaction may be considered from several viewpoints. The presence of \(-\text{OH}\) group may be of particular importance for the protein conformation involved in condensation or may contribute through hydrogen bonding to the activation and orientation of the attacking carbonyl group. It may have some bearing on the question of the role of \(-\text{OH}\) on the orientation of the incoming carbonyl that 4-hydroxy-2-keto glutarate aldolase, for which pyruvate is the ketone product, is known to be nonspecific with respect to the configuration of the \( C_4 \) position (22, 23).

With the finding that compounds other than DHAP are reactive in imine and enamine formation with muscle aldolase the effects of systematic structural alterations on the binding and reaction rates become a feasible subject for future study. Experiments with yeast aldolase show that both the proton exchange and aldolase condensation reactions occur at moderate rates with acetol-P, making such a study of wider applicability. Recent experiments of Kobes and Dekker indicate that formation of imines as indicated by inactivation with BH\(_4\) may not be highly specific in the 4-hydroxy-2-ketogluturate aldolase reaction (24).

According to the ordered sequence of product release shown in Scheme I, with the trapping of products with triose-P isomerase and glycerol-P dehydrogenase, the inhibition of aldol cleavage by added aldehyde should be uncompetitive. This has never been reported to be the case for any of the known acceptors of DHAP (2) acting as inhibitors with muscle or liver aldolases in the native or carboxypeptidase form. All of the inhibition studies tend to favor a random order which is contra-
indicated by the isotope exchange studies (1). The crux of the argument depends on whether the enzyme-aldehyde complex, indicated to exist by inhibition studies, reacts productively with DHAP. A critical test would seem to be the ability of DHAP proton exchange to occur with the aldehyde present on the enzyme. In a previous study (1), it was shown that n-glyceraldehyde-3-P suppressed the exchange rate to <2% with no evidence that the ternary complex enzyme-glyceraldehyde-3-P-DHAP could undergo any proton exchange. In further support of this it is now shown (Fig. 8) that n-glyceraldehyde-3-P acts as a competitive inhibitor of the proton exchange of acetol-P.

The effect of carboxypeptidase treatment on the $K_m$ of FDP (Table IV) confirms the results of Spoiler et al. (2), who also observed that the $K_m$ of FDP returned to the value of the native enzyme when acetaldehyde was present in the assay. It was subsequently established (1) that the effect of acetaldehyde was to circumvent Step 3 of Scheme 1 by a transaldolase reaction between the enzyme-DHAP anion and acetaldehyde. This observation has been confirmed in unpublished work of this laboratory where acetaldehyde was shown to raise $V_{\text{max}}$ and $K_m$ of FDP exactly in parallel. A related phenomenon may be the kinetic dependence on FDP for native and carboxypeptidase enzyme in the production of nitroform from tetraniromethane as reported by Christen and Riorden (25).

It is of interest that the effects of carboxypeptidase treatment, namely, a large decrease in rate with FDP but not with fructose-1-P (16), a normal transaldolase rate with FDP (1, 2), and a low rate of proton exchange with DHAP can be produced in other ways—treatment with acetyl-imidazole (26) and with n-glyceraldehyde-3-P. Although it was initially postulated (15) and subsequently reiterated (25) that the primary effect of this modification is in a deletion of the binding of the C-P phosphate of FDP, it follows from the isotope exchange studies (1) that no change in this property occurs. If, indeed, the primary effect is to decrease by >100-fold the rate of proton abstraction from DHAP and the protonation of the DHAP eneamine (1), then it would seem that this function exists in a distinct and labile segment of the protein structure. It has been proposed (4) that these unrelated events of inactivation, in fact, may destroy completely the enzyme catalysis of this step and that the residual rates of cleavage and condensation are due to the instability of the DHAP-enzyme Schiff's base toward reaction in aqueous medium. The observation that the modified enzyme catalyzes 3H-exchange of DHAP with the same stereospecificity as the native form does not necessarily rule this out.

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