ON THE NATURE OF THE TRANSALDOLASE-DIHYDROXYACETONE COMPLEX

BY B. L. HORECKER, S. PONTREMOLI, * C. RICCI, † AND T. CHENG

DEPARTMENT OF MICROBIOLOGY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

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During recent years there has been considerable progress in the elucidation of the mechanism of action of the aldolases, particularly with respect to the activation of the substrate. (For a review of this subject, see ref. 1.) However, little information has been gained relating to the nature of the active site on the enzyme, apart from the work of Swenson and Boyer2 with sulphydryl reagents.

The enzyme transaldolase is distinguished from other aldolases by the fact that it catalyzes not merely the cleavage of an aldol linkage to form a pair of products, but rather a transfer of a dihydroxyacetone group from one molecule to another: dihydroxyacetone itself is not a substrate for the enzyme. It has been proposed3-4 that a stable intermediate is formed with the dihydroxyacetone group firmly bound to the enzyme, from which it is removed only when a suitable acceptor is present or when the enzyme is denatured. This enzyme-dihydroxyacetone complex has recently been described by Venkataraman and Racker.5

A study of the formation and properties of the transaldolase-dihydroxyacetone intermediate has been in progress in our laboratory. We have identified the bound group as dihydroxyacetone; when the enzyme complex is treated with borohydride
the dihydroxyacetone group is reduced and becomes firmly and irreversibly fixed to the enzyme protein. The complex can then be hydrolyzed with acid to yield a small molecule containing the bound group. In this way it has been established that the dihydroxyacetone group must be linked through the β-carbon atom. Some of the properties of the reduced fragment are described in this report.

**Experimental Procedures.—Materials:** Most of the experiments reported were carried out with a preparation of transaldolase⁴ which is about 30–60 per cent pure, compared with the crystalline preparation.⁴

D-Erythrose 4-phosphate was kindly provided by Drs. Sprinson and Srinivasan of the College of Physicians and Surgeons of Columbia University. Commercial dihydroxyacetone was recrystallized from hot ethanol. This was essential to remove glyceraldehyde which was present in significant amounts. Other enzymes and substrates were as previously reported.⁴

**Methods:** Transaldolase was measured spectrophotometrically as described in the preceding paper.⁴ Other procedures were as previously described.⁵⁶

**Results.—Formation and stability of the enzyme-dihydroxyacetone complex:** The conditions for formation and precipitation of the radioactive complex are described in the preceding report.⁴ With the noncrystalline enzyme preparations similar conditions were employed, except that hexose phosphate isomerase was present as a contaminant and addition of this enzyme was unnecessary. With the less pure enzyme preparations the extent of labeling was less than has been observed with the crystalline enzyme and with prolonged incubation the amount of complex decreased (Table 1).

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONDITIONS FOR FORMATION OF THE TRANSALDOLASE-DIHYDROXYACETONE COMPLEX</strong></td>
</tr>
<tr>
<td>Time of incubation, minutes</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

The incubation mixture (0.075 ml) contained 0.6 mg of transaldolase (specific activity = 16.1), 0.1 M triethanolamine buffer, pH 7.4, and 10⁻³ M glucose 6-phosphate-1-C¹⁴ (1 × 10⁶ cpm per μmole). After incubation at 34° for the period indicated, 0.15-ml samples were mixed at 0° with 1 ml of 85 per cent saturated ammonium sulfate solution, centrifuged, the precipitate resuspended in 0.2 ml of 85 per cent saturated ammonium sulfate, centrifuged, and the residue dissolved in 0.2 ml of triethanolamine buffer. An aliquot was plated for counting and another taken for the determination of protein.

The enzyme complex is stable to repeated precipitation with ammonium sulfate (Table 2). The first precipitate is contaminated with substrate from the incubation mixture, but following the second precipitation at 0° there is no further loss in activity. With each successive precipitation at 37° about 5–10 per cent of the label is lost. At 80° the complex is completely dissociated and the precipitated protein shows only traces of radioactivity.

**Characterization of the enzyme-dihydroxyacetone complex:** When glucose 6-phosphate-1-C¹⁴ is replaced by glucose 6-phosphate-P³², there is little labeling of the protein (Table 3). Therefore it is not the intact substrate molecule which is involved in complex formation. When the labeled enzyme is incubated with a suitable acceptor, such as erythrose 4-phosphate or glyceraldehyde, all of the radioactivity is removed from the protein (Table 3).
The product formed from the labeled protein and D-erythrose 4-phosphate was identified as sedoheptulose 7-phosphate by co-chromatography with an authentic sample (Fig. 1). The autoradiogram obtained on exposure of the paper to x-ray film showed a perfect fingerprint reproduction of the spot developed by spraying with aniline phthalate reagent. The enzyme complex therefore has the properties of a transaldolase-dihydroxyacetone complex.

This was confirmed by identification of dihydroxyacetone itself following decomposition of the complex at 80°. The isolated product, co-chromatographed with dihydroxyacetone in two different solvents, showed identical spots on autoradiograms and the paper developed with aniline phthalate reagent. One of these chromatograms is shown in Figure 2.

**Fixation of the labeled group by reduction with borohydride:** While the enzyme-dihydroxyacetone complex is relatively stable under ordinary conditions, it would not survive hydrolysis of the protein, which is essential to a determination of the nature of the linkage. It was found, however, that the labeled group could be fixed to the protein by reduction with borohydride, and indeed without changing the solubility properties of the enzyme. Reduction was found to be most effective at pH 6.0. Following borohydride treatment at this pH, none of the counts were removed from the protein by incubation with D-glyceraldehyde. At pH 7.5, reduction was far less effective, and almost three-fourths of the counts remained transferable to D-glyceraldehyde. Without borohydride treatment, even at pH

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**TABLE 2**

**STABILITY OF TRANSALDOLASE-DIHYDROXYACETONE COMPLEX**

<table>
<thead>
<tr>
<th>Precipitation</th>
<th>Total counts, cpm</th>
<th>Specific activity, cpm/mg protein</th>
<th>Precipitate dissolved as</th>
<th>Total counts, cpm</th>
<th>Specific activity, cpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>63,300</td>
<td>9,500</td>
<td>60,800</td>
<td>9,100</td>
<td></td>
</tr>
<tr>
<td>Second</td>
<td>38,700</td>
<td>7,400</td>
<td>36,500</td>
<td>6,500</td>
<td></td>
</tr>
<tr>
<td>Third</td>
<td>38,900</td>
<td>7,600</td>
<td>32,800</td>
<td>6,200</td>
<td></td>
</tr>
<tr>
<td>Fourth</td>
<td>36,500</td>
<td>7,900</td>
<td>25,200</td>
<td>5,500</td>
<td></td>
</tr>
<tr>
<td>Fifth (80°)</td>
<td>3,500</td>
<td>100</td>
<td>7,700</td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>

Each incubation mixture (0.25 ml) contained 0.01 M triethanolamine buffer, pH 7.4, 0.25 X 10^{-2} M glucose 6-phosphate-1-C^{14} (3 X 10^{6} cpm/amole) and 1.8 mg of transaldolase (specific activity = 16.4). After 3 min at 34° the solutions were precipitated with 10 volumes of 85 per cent saturated ammonium sulfate solution, centrifuged, and the precipitate dissolved in 0.2 ml of triethanolamine buffer. One solution was kept at 0° for 3 min and the other at 37°. Each was then precipitated as before and the process repeated. Aliquots of each solution were taken for determination of radioactivity and protein content. The last solutions, following the fourth precipitation, were kept for 3 min at 80° and then treated with ammonium sulfate. The overall recovery of protein was about 60 per cent.

**TABLE 3**

**DISSOCIATION OF THE COMPLEX BY TRANSALDOLASE ACCEPTORS**

<table>
<thead>
<tr>
<th>Glucose 6-phosphate-1-C^{14}</th>
<th>Glucose 6-phosphate-P^{32}</th>
<th>First incubation addition</th>
<th>Total counts, cpm</th>
<th>Specific activity, cpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>Second incubation addition</td>
<td>3,200</td>
<td>0.68</td>
</tr>
<tr>
<td>D-erythrose 4-phosphate</td>
<td>D-glyceraldehyde</td>
<td></td>
<td>20</td>
<td>0.004</td>
</tr>
<tr>
<td>D-glyceraldehyde</td>
<td></td>
<td></td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>0.05</td>
</tr>
</tbody>
</table>

The first incubation mixtures contained 1.2 mg of transaldolase (specific activity = 16.2 units/mg), 0.01 M triethanolamine buffer, pH 7.6, and 0.11 amole of glucose 6-phosphate-1-C^{14} (1 X 10^{6} cpm/amole) or 0.11 amole of glucose 6-phosphate-P^{32} (6.4 X 10^{6} cpm/amole). After incubation for 3 min at 34° the protein was precipitated with 5 ml of 85 per cent saturated ammonium sulfate, centrifuged, and washed with 1 ml of 85 per cent saturated ammonium sulfate. The washed precipitate was dissolved in 0.1 ml of 0.04 M triethanolamine buffer, pH 7.6. Aliquots were removed for counting and for protein determination. The remainder was incubated for 3 min at 34° with no other addition or with 0.12 amole of D-erythrose 4-phosphate or 0.36 amole of D-glyceraldehyde. The enzyme was then precipitated as before, and the precipitate washed and dissolved in 0.1 ml of triethanolamine buffer and counted.
In this experiment 4.5 mg of transaldolase (specific activity = 14.5) was incubated with 1.3 μmoles of glucose 6-phosphate-1-C14 (3.0 × 10^6 cpm per μmole) in 0.07 ml of 0.01 M triethanolamine buffer, pH 7.6. After 3 min at 34° the reaction mixture was precipitated with 4.0 ml of saturated ammonium sulfate solution, centrifuged, and the precipitate dissolved in 0.5 ml of 0.04 M triethanolamine buffer, pH 7.6. The protein was again precipitated as before and dissolved in 0.2 ml of 0.04 M triethanolamine buffer and treated with 0.05 ml of p-erythrose 4-phosphate solution containing 0.35 μmole. After 3 min at 34°, 3 μmoles of carrier sedoheptulose 7-phosphate was added and the solution deproteinized with 0.02 ml of 100 per cent trichloroacetic acid solution. The supernatant solution contained 80 per cent of the counts in the original enzyme complex (1.11 × 10^6 cpm, total). The trichloroacetic acid solution was thoroughly extracted with ether and the phosphate ester precipitated with barium acetate and 4 volumes of ethanol. The precipitate was dissolved in 0.5 ml of 0.01 M acetic acid. Barium ion was removed with a slight excess of sodium sulfate and the supernatant solution concentrated to a small volume, placed on Whatman No. 1 paper, and chromatographed with butanol:pyridine:water (6:4:3).

Fig. 2.—(a) Chromatogram developed with aniline phthalate spray and (b) autoradiogram of dihydroxyacetone released by warming the transaldolase complex to 80°. The formation of the labeled enzyme complex was as in Fig. 1, with 4.0 mg of transaldolase (specific activity = 24.6). The protein was precipitated twice with ammonium sulfate, dissolved in 0.5 ml of 0.1 N acetic acid to maintain an acidic pH, and warmed to 80° for 10 min. The solution was cooled and 0.04 ml of 0.1 M dihydroxyacetone was added as carrier. The solution was then deproteinized with 2.5 ml ethanol and 0.002 ml of mercaptoethanol added to prevent oxidation of dihydroxyacetone. Ethanol was removed by evaporation under a stream of N2, and the residue dissolved in water containing 10^-3 M mercaptoethanol. The solution was deionized by passage through a 1-ml bed of Amberlite MB-3 resin. The effluent was concentrated to a small volume and placed on Whatman No. 1 paper for ascending chromatography in water-saturated phenol. The over-all yield of counts from the labeled enzyme was 35 per cent. A similar chromatogram was obtained by chromatography in 80 per cent acetone-water solvent.

In Fig. 2(a) the left-hand spot is a dihydroxyacetone standard.
Two possibilities in complex, complex formation.

One-half carried out to of conditions slowly similar which corresponds.

The following activity corresponds a nonradioactive completely.

The control experiments were identical except that borohydride was not added.

6.0, the enzyme-complex still retained full activity, and the dihydroxyacetone group was quantitatively transferred to n-glyceraldehyde, leaving the protein completely nonradioactive (Table 4).

Reduction of the enzyme-dihydroxyacetone complex with borohydride permitted a determination of the extent of formation of the complex. The loss of enzymatic activity corresponds to the degree of blocking of the active site by the reduced group. The reduced complex, as has been shown above, is without enzymatic activity. Following addition of the dihydroxyacetone group and reduction of the complex with borohydride, 48 per cent of the enzyme activity was lost (Table 5),

<table>
<thead>
<tr>
<th>Reduced at pH</th>
<th>Total counts</th>
<th>Fixed counts*</th>
<th>Fixed, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>3,600</td>
<td>960</td>
<td>27</td>
</tr>
<tr>
<td>6.5</td>
<td>6,200</td>
<td>3,200</td>
<td>52</td>
</tr>
<tr>
<td>6.0</td>
<td>3,300</td>
<td>3,500</td>
<td>100</td>
</tr>
</tbody>
</table>

Control at pH

6.5

6.0

3,200

5,000

390

0

12

0

* Remaining after incubation with n-glyceraldehyde. The enzyme-transaldolase complex was prepared as in Fig. 1 with 2.7 mg of transaldolase (specific activity = 9.7) and 0.05 amole of glucose 6-phosphate-1-C14. The labeled enzyme was twice precipitated with ammonium sulfate, and finally dissolved in 0.5 ml of phosphate buffer (0.05 M) at the pH indicated. Reduction with borohydride was carried out at 0° by the addition of 0.01 ml quantities of fresh borohydride solution containing 40 mg of sodium borohydride per ml. Ten such additions were made over a period of 30 min; the pH was maintained at the initial level by the addition of 0.01-ml portions of 1 N acetic acid. Following the last addition of borohydride, the mixture was kept at 0° for 30 min, treated with 4.0 ml of saturated ammonium sulfate solution and centrifuged. To test for completeness of reduction, the precipitate was dissolved in 0.4 ml of 0.02 M triethanolamine buffer, pH 7.4, and treated with 0.005 ml of 1.8 M n-glyceraldehyde. This solution was warmed to 34° for 3 min and the protein precipitated with 2.5 ml of saturated ammonium sulfate solution. The last precipitate was dissolved in 0.2 ml of 0.04 M triethanolamine buffer, pH 7.4, and an aliquot counted.

which corresponds almost exactly to the per cent of enzyme protein labeled in a similar experiment with fructose 6-phosphate 1-C14.

Stability to dialysis: The C14-labeled transaldolase-dihydroxyacetone complex was slowly dissociated during dialysis against distilled water; in 16 hr approximately one-half of the radioactivity of the C14-labeled complex was lost. Under the same conditions of dialysis the reduced complex lost no radioactivity.

Hydrolysis and periodate oxidation: Some preliminary experiments have been carried out to determine which carbon atom of dihydroxyacetone is involved in complex formation. For this purpose we have used the borohydride-reduced complex, in which the three-carbon group is fixed by a stable covalent linkage. Two possibilities must be considered. The complex may be either a derivative
involving the carbonyl group at position 2 of the original fructose 6-phosphate molecule or a derivative formed through carbon atom 3. Following reduction with borohydride the former would yield a \( \beta \)-glycerol derivative, while the latter would be converted to an \( \alpha \)-glycerol derivative. These can be distinguished by their susceptibility to oxidation with periodate since the \( \alpha \)-glycerol derivative would yield formaldehyde derived from the C-1 of fructose 6-phosphate, while the \( \beta \)-glycerol derivative would not be attacked. For this purpose, the borohydride reduced complex, derived from glucose 6-phosphate 1-\( ^{14} \mathrm{C} \), was dialyzed overnight against water and hydrolyzed in a sealed tube with 6 \( \text{N} \) \( \text{HCl} \) at 110° for 22 hr. \( \text{HCl} \) was removed by repeated evaporation in vacuo over KOH and \( \text{CaCl}_2 \) and an aliquot of the hydrolysate, containing 1,500 cpm, was treated with excess periodate at pH 4.5. After 1 hr at room temperature glycerol was added to remove excess periodate and the formaldehyde formed collected as the dimedon derivative. This was found to be completely nonradioactive and all the counts were recovered in the supernatant solution. A similar experiment was carried out with the reduced enzyme complex, without acid hydrolysis, and again no activity was obtained in the dimedon derivative. These results suggest that dihydroxyacetone is linked to the enzyme through carbon atom 2, rather than through the hydroxymethylene group at carbon 3.

Properties of the active site: With respect to the possible nature of the amino acid involved in formation of the complex some information is available. Following hydrolysis in 6 \( \text{N} \) \( \text{HCl} \) all of the radioactivity is absorbed by Dowex 50-H\(^+\) resin, but not by Dowex 1-formate or Dowex 1-OH\(^-\). The reduced complex is therefore not a strong anionic compound. On the other hand, following exposure to nitrous acid all of the counts passed through a Dowex 50-H\(^+\) column, which indicates that the reduced complex possesses a free amino group.

Treatment with HBr or HI failed to yield glycerol, suggesting that the reduced 3-carbon residue is not attached by an 0-ether linkage to serine or threonine. It is pertinent to note that transaldolase is not inhibited by diisopropylfluorophosphate (DFP).

The possibility remains that dihydroxyacetone is attached to a protein SH-group as a thioacetal derivative. However, transaldolase is not significantly inhibited by iodoacetic acid nor by incubation with oxidized glutathione.

In two-dimensional paper chromatography, with methanol, pyridine, water (60:20:20) as the first solvent and t-butanol, water, diethylamine (85:15:4) as the second solvent, the radioactive fragment formed after borohydride reduction and acid hydrolysis is found to be located between aspartic acid and serine. It has thus been converted to a molecule of low molecular weight, not identical with glycerol.

Discussion.—We have described a stable \( ^{14} \text{C} \)-labeled transaldolase complex formed when the enzyme is incubated with fructose 6-phosphate-1-\( ^{14} \text{C} \). The labeled group can be transferred to erythrose 4-phosphate to form sedoheptulose 7-phosphate, or liberated as free dihydroxyacetone when the enzyme is warmed to 80°. This confirms the work of Venekataraman and Racker\(^4\) and proves that the intermediate formed in the enzymic action of transaldolase is, as originally postulated,\(^3\) an enzyme-dihydroxyacetone complex.

Some of the properties of this complex have been determined. It can be re-
duced with sodium borohydride to form a stable derivative, presumably a glycerol derivative. This derivative does not yield labeled formaldehyde when treated with periodate, and we have therefore concluded that the dihydroxyacetone group is attached through the 2-position, possibly as a ketal derivative, rather than through the terminal hydroxymethylene group.

The borohydride reduced protein complex has been hydrolyzed with 6N HCl at 110°. This procedure yields a small molecule which is strongly adsorbed by cationic but not by anionic resins. The cationic group is probably a free amino group, since the molecule is no longer adsorbed following treatment with nitrous acid.

It is unlikely that either serine or threonine is involved in formation of the complex since the reduced product is not susceptible to cleavage by hydrobromic or hydroiodic acids, as would be expected for an O-ether linkage.

Swenson and Boyer have obtained evidence which indicates that the active group in aldolase may be a sulfhydryl group. This does not appear to be the case for transaldolase. However, in view of the similarity in the action of this enzyme and transaldolase it is reasonable to expect that they will possess similar groups at the active site. In the case of transaldolase a definitive answer to this question may now be possible since we can obtain the active group firmly linked as a C14-labeled derivative, presumably a derivative of glycerol. The availability of large quantities of crystalline enzyme should facilitate an approach to this problem.

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* Present address: Institute of Biological Chemistry of the University of Genoa, Genoa, Italy.
† Fulbright Exchange Fellow. Present address: Institute of Biological Chemistry of the University of Siena, Siena, Italy.

1 Horecker, B. L., J. Cell and Comp. Physiol., 54, Sup. 1, 89 (1959).