A New Intermediate of the Aldolase Reaction, the Pyruvaldehyde–Aldolase–Orthophosphate Complex

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(Received 9 December 1977)

Fructose 1,6-bisphosphate aldolase from rabbit muscle forms by reaction with dihydroxyacetone phosphate a pyruvaldehyde–aldolase–orthophosphate complex that is in equilibrium with the enamine intermediate. The new intermediate accumulates in two phases. The first one is practically complete in 40 ms, and the second occurs with an apparent first-order rate constant of $4.6 \pm 0.5 \text{ s}^{-1}$. The new intermediate breaks down slowly with the release into the medium of pyruvaldehyde and P_i . The rate of the spontaneous release is higher at acidic than at neutral pH.

We describe here the chemical identification of a new intermediate of the aldolase reaction, the pyruvaldehyde–aldolase–orthophosphate complex. This new intermediate is in equilibrium with the aldolase–dihydroxyacetone phosphate complex and breaks down very slowly to give rise to free pyruvaldehyde and free P_i , the rate of the release being higher at acidic than at neutral pH.

The formation of the new intermediate is subsequent to the formation of the Schiff base between aldolase and dihydroxyacetone phosphate (Grazi *et al.*, 1962) and of the enamine intermediate (Rose & Rose, 1969).

The aldolase-catalysed release of P_i from fructose bisphosphate (the prevailing substrate in the muscle) is even slower than the release from dihydroxyacetone phosphate. The reaction thus appears to be of limited physiological significance.

Materials

Fructose bisphosphate aldolase (EC 4.1.2.13) was prepared from rabbit muscle by the procedure of Taylor *et al.* (1948) and was recrystallized five times. The specific activity of the preparation used in these experiments was 16 units/mg of protein, where 1 unit is defined as the amount that catalyses the cleavage of 1 μmol of fructose 1,6-bisphosphate/min under standard assay conditions. Dihydroxy[^{14}C]acetone phosphate was prepared from commercial [^{14}C]fructose 1,6-bisphosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) by the procedure of Ginsburg & Mehler (1966). Pyruvaldehyde was prepared from the dimethylacetal (Schuchardt, Hohenbrunn, Germany), by hydrolysis for 24 h at 37°C in 1M-HCl. Dihydroxyacetone phosphate was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Hexitol 1,6-bisphosphate was prepared from fructose bisphosphate by the procedure of Ginsburg

& Mehler (1966). The calcium salt of D-glyceraldehyde 3-phosphate was prepared by the procedure of Szwczuk *et al.* (1961), and converted into the sodium salt by treatment with Dowex 50 (H^+ form) followed by neutralization with NaOH.

Glycerophosphate dehydrogenase (EC 1.1.1.8) and glyoxalase I (EC 4.4.1.5) were purchased from Boehringer, Mannheim, W. Germany. Carboxypeptidase A (EC 3.4.12.2) (treated with di-isopropyl phosphorofluoridate) was purchased from Sigma.

Methods

Fructose bisphosphate aldolase activity was measured in the test system described by Racker (1947).

Dihydroxyacetone phosphate was determined by following the oxidation of NADH in the presence of glycerophosphate dehydrogenase. Pyruvaldehyde was assayed with glyoxalase I and glutathione as described by Racker (1950), the ϵ_{240} ($\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) for lactoylglutathione being taken as 3370. P_i was determined by the procedure of Tashima & Yoshimura (1975). The protein concentration was measured from the A_{280} , assuming that the absorbance of 1 mg of pure aldolase/ml (light-path 1 cm) is 0.91 (Baranowski & Niederland, 1949).

The rapid-quenching experiments were performed with the Durum multi-mixer apparatus. The effectiveness of the quenching of the reaction by 0.15M-trichloroacetic acid was checked in control experiments where the 'enzyme syringe' contained buffered aldolase solution, the 'substrate syringe' contained buffer alone, and the third syringe contained trichloroacetic acid plus the substrate. Under these conditions no P_i production was detected.

Radioactive determinations were made in a Packard Tri-Carb liquid-scintillation counter in 10 ml of Bray's (1960) solution. Digestion of aldolase

with carboxypeptidase A was performed as described by Rutter *et al.* (1961), except that the aldolase/carboxypeptidase ratio was 70. The specific activity of carboxypeptidase-treated aldolase was 0.22 units/mg of protein.

Results

Aldolase catalyses the cleavage of dihydroxyacetone phosphate to pyruvaldehyde and P_i

When aldolase and dihydroxyacetone phosphate are mixed, a slow release of P_i occurs, accompanied by the production of pyruvaldehyde. The latter

Fig. 1. Chromatographic identification of the pyruvaldehyde formed by reaction of aldolase with dihydroxyacetone phosphate

The incubation mixtures (0.5 ml) contained 46 μ M-aldolase subunit, 130 μ M-[U-¹⁴C]dihydroxyacetone phosphate (specific radioactivity 740 c.p.m./nmol) and 20 mM-acetate buffer, pH 5.5. After 60 min of incubation at 20°C, the reaction was stopped by the addition of 0.5 ml of acetone. A control sample, where acetone was added before aldolase, was also prepared. The protein precipitate was removed by centrifugation and 0.1 ml of the supernatant (to which 3 μ mol each of dihydroxyacetone and of pyruvaldehyde had been added) was streaked in a 4 cm band on silica-gel plates. The chromatogram was developed for 4 h with the solvent system butan-1-ol-ethanol-water (52:32:16, by vol.). To locate the areas of radioactivity, silica gel was removed, at regular intervals, from the central part (3 cm wide) of the lane, washed with 0.5 ml of water and the radioactive content of water was determined. To locate the internal standards (A, dihydroxyacetone phosphate; B, dihydroxyacetone; C, pyruvaldehyde) the edges of the lane (0.5 cm from each side) were sprayed with alkaline potassium permanganate (aq. 1% KMnO₄ containing 2% Na₂CO₃). . . ., Control sample; —, incubated sample.

compound was identified by its reactivity with glutathione in the presence of glyoxalase I as well as by t.l.c. with the solvent system 1 [butan-1-ol/ethanol/water (52:32:16, by vol.)] (Fig. 1).

The stoichiometry of the reaction was also verified and it was found that, out of 52 nmol of dihydroxyacetone phosphate consumed, 39 nmol of pyruvaldehyde and 52 nmol of P_i were recovered. (Table 1). The difference in the recovery between the two compounds is due to the modality of the assay procedures. With pyruvaldehyde only the amount free in the medium was determined; with P_i both the amount free in the medium and that released from the enzyme by HClO₄ were measured (see the next section). In the course of the incubation the catalytic activity of aldolase, as assayed by the conventional method, was completely retained.

Aldolase does not catalyse the cleavage of α -gly-

Table 1. Stoichiometry of the cleavage of dihydroxyacetone phosphate

The incubation mixtures (1 ml) contained 0.11 mM-dihydroxyacetone phosphate, 1.4 mM-glutathione, 10 μ g of glyoxalase I and 20 mM-acetate buffer, pH 6.0. The reaction was started by the addition of 35.0 μ M-aldolase subunit. The production of pyruvaldehyde was determined from the changes in the A₂₄₀ as described in the Methods section. After 2 h of incubation at 25°C the reaction was stopped by the addition of 0.03 ml of 70% (w/w) HClO₄. The protein precipitate was separated by centrifugation. Dihydroxyacetone phosphate and P_i were determined as described in the Methods section.

	Dihydroxyacetone phosphate (nmol)	P _i (nmol)	Pyruvaldehyde (nmol)
Aldolase omitted	110	3	3
Dihydroxyacetone phosphate omitted	—	5	2
Complete system	58	56	42

Table 2. Reaction of cleavage of dihydroxyacetone phosphate by aldolase

The incubation mixture (1 ml) contained 40 μ M-aldolase subunit, 20 mM-acetate buffer, pH 6.0, and the compound indicated, at 0.5 mM concentration. At timed intervals the reaction was stopped by the addition of 2% (w/w) HClO₄ and P_i was determined as described in the Methods section.

Addition	P _i formed (nmol/min)
Dihydroxyacetone phosphate	0.43
Fructose biphosphate	0.10
D-Glyceraldehyde 3-phosphate	0.02
α -Glycerolphosphate	0.00
Hexitol 1,6-bisphosphate	0.00

Fig. 2. Effect of pH on the rate of cleavage of dihydroxyacetone phosphate by aldolase

The incubation mixture (1 ml) contained dihydroxyacetone phosphate (0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 mM), 42 μM-aldolase subunit and either 20 mM-acetate (pH 4.5–5.8) or 20 mM-Tris/HCl buffer (pH 6.0–9.0). Ionic strength was 0.02. Temperature was 22°C. At timed intervals the reaction was stopped by the addition of 2% HClO₄ and P_i was assayed as described in the Methods section. The rates of the reaction were calculated after subtraction of the initial (10 s) P_i liberation. The value of V_{max} at each pH was obtained by extrapolation of Lineweaver-Burk plots. V_{max} was expressed as nmol of P_i produced/min per nmol of aldolase subunit.

Fig. 3. Initial burst of P_i after reaction of aldolase with excess of dihydroxyacetone phosphate

A sample (96 μM) of aldolase subunit (●) or carboxypeptidase-treated aldolase subunit (○), dissolved in 20 mM-Tris/HCl buffer, pH 7.5, was mixed with an equal volume of a solution containing 0.22 mM-dihydroxyacetone phosphate (●, ○) or 0.22 mM-fructose biphosphate (▲) and 20 mM-Tris/HCl buffer, pH 7.5. Temperature was 22°C. At various timed intervals the reaction was stopped by trichloroacetic acid (final concentration 0.15 M). P_i was determined as described in the Methods section.

cerophosphate and of hexitol 1,6-bisphosphate. Dephosphorylation of D-glyceraldehyde 3-phosphate is barely detectable. The dephosphorylation of fructose 1,6-bisphosphate is probably subsequent to its conversion into dihydroxyacetone phosphate and is insensitive to the addition of 1 mM-EDTA; this excludes the possible involvement of fructose 1,6-bisphosphatase (Table 2).

The rate of P_i liberation increases sharply from

Fig. 4. Initial burst of P_i after reaction of dihydroxyacetone phosphate with an excess of aldolase

The incubation mixtures (0.2 ml) contained dihydroxyacetone phosphate (11 nmol), aldolase subunit as indicated and 20 mM-Tris/HCl buffer, pH 7.5. After 1 s of incubation at 22°C the reaction was stopped by the addition of 0.2 ml of 0.3 M-trichloroacetic acid, and P_i was assayed as described in the Methods section.

Table 3. Time course of pyruvaldehyde formation

The incubation mixture (1 ml) contained 49 μM-aldolase subunit, 1.1 mM-dihydroxyacetone phosphate, 1.4 mM-glutathione, 10 μg of glyoxalase I and either 20 mM-acetate buffer, pH 6.0, or 20 mM-Tris/HCl buffer, pH 7.5. Temperature was 22°C. The reaction was started by the addition of dihydroxyacetone phosphate. The formation of pyruvaldehyde was followed from the changes in A₂₄₀ as described in the Methods section.

	Pyruvaldehyde formed (nmol)	
Time	pH 6.0	pH 7.5
15 s	<1.0	<1.0
30 s	<1.0	<1.0
5 min	3.5	<1.0
10 min	8.0	1.0
20 min	16.6	1.8
30 min	22.7	3.1

glyoxalase I system (a typical experiment is shown in Table 3); (2) the aldolase-bound P_i was again converted into organic phosphate when the equilibrium of the reaction was displaced in favour of free dihydroxyacetone phosphate by the addition of 1M-NaCl, which increased the dissociation constant of the aldolase-dihydroxyacetone phosphate complex from $4\mu\text{M}$ to about 1 mM (Grazi & Trombetta, 1974) (Table 4).

It is noteworthy that the formation of the new intermediate from dihydroxyacetone phosphate was slower with carboxypeptidase-treated aldolase (Fig. 3). This suggests that the new intermediate is formed after the enamine [compound (b), Scheme 1], since enamine formation is the rate-limiting step in the carboxypeptidase-treated aldolase (Rose *et al.*, 1965).

The formation of the new intermediate was not detected when native aldolase reacted with fructose 1,6-bisphosphate as the substrate (Fig. 3). This was expected since the steady-state concentration of the enamine intermediate is less than 6% of the total enzyme-substrate intermediates when fructose 1,6-bisphosphate is the substrate (Grazi, 1975).

The proposed mechanism for the formation of the pyruvaldehyde-aldolase-orthophosphate complex is presented in Scheme 1.

We thank Dr. F. Dallochio for stimulating discussions. This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche.

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