INTERACTION BETWEEN D-FRUCTOSE-1,6-BISPHOSPHATE ALDOLASE AND TRIOSEPHOSPHATE ISOMERASE

Mutual protection against perchloric acid denaturation

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1. Introduction

D-Fructose-1,6-bisphosphate aldolase may participate in the formation of heterologous complexes with glycolytic and gluconeogenetic enzymes: Myogen, a sarcoplasmic preparation rich in glycolytic enzymes, forms a single slow-moving peak in boundary electrophoresis [1]; one of its fractions (myogen A) which contains aldolase and glycerophosphate dehydrogenase crystallizes readily and seems to be homogeneous in sedimentation equilibrium analysis [2]. Physicochemical evidence has been provided for interaction between rabbit muscle aldolase and D-glyceraldehyde-3-phosphate dehydrogenase [3], rabbit muscle aldolase and glycerophosphate dehydrogenase [4], rabbit liver aldolase and fructose-1,6-bisphosphatase [5,6], and insect muscle aldolase and triosephosphate isomerase [7].

A direct transfer of the intermediary product, glyceraldehyde-3-phosphate, between the complexed aldolase and D-glyceraldehyde-3-phosphate dehydrogenase has been suggested by analyzing the kinetics of the consecutive reactions catalyzed by the coupled enzymes [8]. This hypothesis was not substantiated in further experiments [9]: the addition of triosephosphate isomerase decreased the rate of formation of 3-phosphoglycerol-enzyme intermediates both in the reaction system where aldolase and D-glyceraldehyde-3-phosphate dehydrogenase have been freshly mixed and in the system where the 2 enzymes have been preincubated to form the heterologous complex.

We have decided to reinvestigate these problems. To study the effect of triosephosphate isomerase on the reaction catalyzed by aldolase, perchloric acid was added at different periods of time to solutions containing fructose bisphosphate, aldolase, and isomerase and the concentration of triosephosphates was measured in the neutralized perchloric extracts according to [9,10]. Since we found an unexpectedly high concentration of triosephosphates in the extracts, we investigated its cause.

Here, we present evidence that:

(i) Rabbit muscle aldolase and isomerase form a heterologous enzyme complex at pH 7.5;
(ii) Both aldolase and isomerase can be completely and irreversibly denatured by perchloric acid in the absence of the other enzyme;
(iii) If aldolase and isomerase are present together in the same solution, then the 2 enzymes became resistant to acid denaturation.

2. Materials and methods

Ammonium sulfate suspension of rabbit muscle D-fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) and triosephosphate isomerase (EC 5.3.1.1) were purchased from Sigma and Boehringer, respectively. The enzymes were also purified from rabbit muscle in our laboratory as in [11,12]. Crystalline rabbit muscle L-gycerol-3-phosphate dehydrogenase (EC 1.1.1.8) was purified according to [13]. Bovine serum albumin, bovine γ-globulin, and equine myoglobin were obtained from Serva.

D-Glyceraldehyde-3-phosphate was prepared as in [14] or was a Boehringer product. All other reagents were high purity commercial preparations from Merck, Aldrich and Pharmacia.

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Before use, the enzyme solutions were chromatographed on a Sephadex G-50 column equilibrated with 50 mM Tris–HCl, 1 mM EDTA, 1 mM mercaptoethanol (pH 7.5) to remove ammonium sulfate. Unless otherwise mentioned, this buffer was used in all further experiments.

The concentration of aldolase (M_r 160 000) and of isomerase (M_r 55 000) was determined spectrophotometrically at 280 nm [10,11]. Aldolase and isomerase activities were assayed at 25°C in the presence of 0.1 mM NADH and 1.5 U/ml L-glycerol-3-phosphate dehydrogenase. 2 mM D-fructose-1,6-bisphosphate and 0.3 mM D-glyceraldehyde-3-phosphate were used as substrates for aldolase and isomerase, respectively. The specific activity of aldolase was 65 U/mg and that of isomerase was 5000 U/mg.

The isomerase (10 μM) was labelled with fluorescein isothiocyanate (60–100 μM) in 50 mM Tris–HCl, 1 mM EDTA (pH 7.5). The mixture was incubated for 30–60 min in the dark at 4°C. Free fluorescent dye was removed from the protein by gel-filtration through a Sephadex G-50 column (1.5 × 30 cm) equilibrated with the same buffer. The latter procedure removed all traces of free dye, as demonstrated by a second chromatography through the same column in which the protein peak was coincident with the fluorescent peak. The degree of labelling was 0.1–1 mol dye/mol isomerase. The labelled enzyme retained, within experimental error, its original activity. The polarization and anisotropy of labelled isomerase were determined at 20°C as in [3].

3. Results and discussion

Perchloric acid (0.15 ml 60%) was added to 3 ml ice-cold solutions containing D-fructose-1,6-bisphosphate aldolase (0.02 μM) and triosephosphate isomerase (0.03–0.45 μM). The solutions remained clear and no precipitation occurred up to 10 min after acidification. The ice-cold acidified protein solutions were neutralized (pH 7) by addition of 0.45 ml of a mixture of 2 M K_2CO_3 and 0.5 M triethanolamine. The potassium perchlorate precipitate was removed by centrifugation and the clear supernatants were used for determination of aldolase activity.

The more isomerase was added to the aldolase solution before acidification, the more aldolase activity was measured after neutralization (table 1). In the presence of excess of isomerase, 30% of the original aldolase activity was recovered.

Table 1
Denaturation in perchloric acid

Before acidification		Recovered activity of aldolase (%)
Isomerase (μM)	Aldolase (μM)	
–	0.022	0
0.030	0.022	2
0.150	0.022	15
0.300	0.022	30
0.450	0.022	30

Error of determination of recovered activity is ± 1% (SD)

No appreciable aldolase activity was detectable if: (i) isomerase was not added to the solution prior to acidification; or (ii) perchloric acid was separately added to the enzyme solutions (0.04 μM aldolase and 0.9 μM isomerase) and these solutions were mixed and then neutralized.

The results obtained with commercial enzyme preparations were in good agreement with those obtained with enzyme purified in our laboratory and, within experimental error, independent of the preincubation time of the 2 enzymes in buffer and of the incubation time (1–10 min) of the enzymes in ice-cold perchloric acid.

Bovine serum albumin, bovine γ-globulin and equine myoglobin (≤0.5 mg/ml) did not protect aldolase against perchloric acid denaturation.

In the absence of aldolase, isomerase was irreversibly denatured by perchloric acid. Nevertheless, if the strong acid was added to solutions containing 0.03 μM isomerase and excess of aldolase (1 μM), 40% of isomerase activity was recovered after neutralization.

The mutual protection against perchloric acid could be interpreted assuming that aldolase binds isomerase, the heterologous complex being resistant to acid denaturation. Indeed physicochemical evidence has been reported for the interaction between aldolase and isomerase from flight insect *Ceratitis capitata* [7]. Fluorescence polarization experiments suggest that also the enzymes purified from rabbit muscle interact at pH 7.5, forming a heterologous complex. The anisotropy of fluorescein-labelled isomerase (0.2 μM) increased with increasing aldolase concentration (from 0–12 μM) and approached a limiting value with excess of aldolase (fig.1). The quantum yield [15] of the protein-conjugated dye was, within the experimental error, independent of aldolase concentration. Moreover the degree of labelling of isomerase

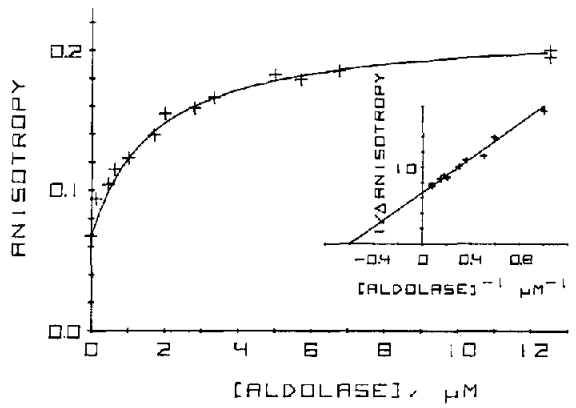

Fig.1. Anisotropy of fluorescein-labelled triosephosphate isomerase (0.2 μM), measured after 10 h incubation at 0°C, as a function of D-fructose-1,6-bisphosphate aldolase concentration. The experimental conditions are described in section 2. Inset: double-reciprocal plot of the change in anisotropy against aldolase concentration.

with the fluorescent dye, within ~10-fold range, did not appreciably affect the titration curve (not shown). These findings appear to exclude the possibility that aldolase binding was due to the fluorescent moiety. The apparent dissociation constant (K_d 1.7 μM) of the heterologous complex was calculated from a double reciprocal plot of anisotropy variation *vs* aldolase concentration [7], using only the values from those experiments where the concentration of total aldolase was much larger than the concentration of fluorescein-labelled isomerase. The apparent dissociation constant of the rabbit muscle heterologous complex was similar to that determined with enzymes purified from flight insect.

Assuming that aldolase and isomerase form heterologous macromolecular aggregates in perchloric acid, these complexes are probably quite different from those formed at pH around neutrality. The recovery of aldolase activity in the neutralized perchloric extract is ~2-times larger than that expected from the association constant of the 2 native interacting enzymes. Moreover the protein aggregation in perchloric acid cannot be a reversible process since the uncomplexed enzymes are completely and irreversibly denatured by the strong acid.

The study of the physicochemical properties of mixtures of aldolase and isomerase in perchloric acid is difficult because only very dilute enzymes are soluble under these conditions. It could be hypothesized that aldolase and isomerase interact in an intermediate denatured state and that the aggregation slows down the irreversible denaturation process. Another explanation is that unknown compounds, present in the enzyme preparations, could protect aldolase and isomerase against denaturation.

Whatever, the mechanism of the observed phenomenon, the discovery of resistance of mixtures of aldolase and isomerase against perchloric acid denaturation has practical importance. Perchloric acid has been widely employed in quantitative determination of intermediate metabolites [9,10,16–19]. A critical re-examination of the experimental results obtained in this way would be necessary.

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