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SUBSTRATE INACTIVATION OF FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE FROM *BACILLUS STEAROTHERMOPHILUS*

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SUMMARY

The fructose diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) from *Bacillus stearothermophilus* is inactivated at 60° by fructose 1,6-diphosphate (Fru-1,6-P_2), dihydroxyacetone phosphate or glyceraldehyde 3-phosphate. Inactivation occurs in phosphate, triethanolamine or *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer but does not occur in Tris or glycylglycine buffers. Inactivation is also prevented by addition of EDTA, dithiothreitol or manganous ion to phosphate, triethanolamine, or TES. An increase in the molar ellipticity at 220 nm accompanies inactivation of the thermophilic aldolase by Fru-1,6-P_2.

The inactivation of rabbit muscle Fru-1,6-P_2 aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13), a Type I aldolase, by high levels of Fru-1,6-P_2 or by stoichiometric concentrations of glyceraldehyde 3-phosphate or erythrose 4-phosphate has previously been shown[1,2]. This report deals with the inactivation by Fru-1,6-P_2, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate of a Type II aldolase isolated from *Bacillus stearothermophilus*.

Fru-1,6-P_2 aldolase was prepared from *B. stearothermophilus* essentially as described by Quinn[3]. Aldolase activity was assayed as described by Christian[4] at 43° in the presence of 0.17 mM $MnCl_2$. Protein concentration was estimated from the absorbance at 280 nm using $E^{1\%}_{280\ nm} = 5.9$ (R. P. Quinn, R. L. Howard and R. R. Becker, unpublished results). Yeast Fru-1,6-P_2 aldolase was prepared as described by Kobes *et al.*[5]. The preparation used was that obtained after the second crystallization. Yeast aldolase was assayed as described by Rutter and Hunsley[6].

Thompson and Thompson[7] reported that the Fru-1,6-P_2 aldolase from *B. stearothermophilus* (NCA 2184) in water was stable for 60 min at 70°. More recently, Sugimoto and Nosoh[8] have reported a 48% loss in activity when the aldolase was incubated for 30 min at 70°. Preliminary experiments with the enzyme prepared here

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

indicated a similar loss of activity (50% in 30 min) when the aldolase was incubated at 70° in either 0.05 M potassium phosphate or 0.1 M Tris–HCl at pH 7.5. To test for substrate stabilization, Fru-1,6-P_2(1 mg/ml) was added to the incubation mixture. In phosphate buffer, complete loss of aldolase activity occurred within 10 min.

The results of additional experiments, carried out at 60°, are shown in Fig. 1. In 0.05 M potassium phosphate, pH 7.5, the aldolase is rapidly inactivated by a 5–10 molar excess of Fru-1,6-P_2, dihydroxyacetone phosphate or glyceraldehyde 3-phosphate. When 0.1 M Tris–HCl, pH 7.5, was used as the buffer, little or no inactivation occurred (*cf.* Sugimoto and Nosoh[8]).

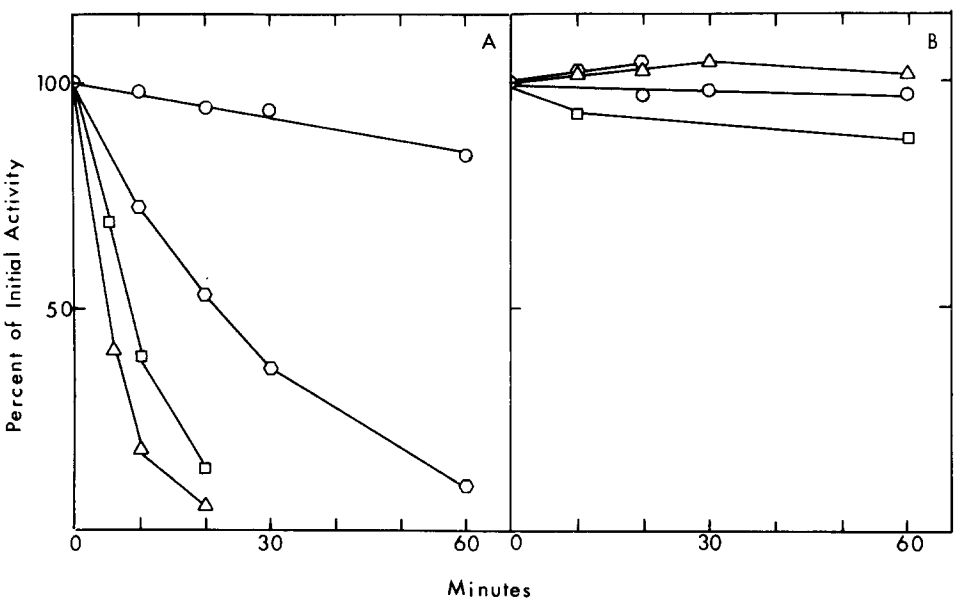

Fig. 1. Inactivation of thermophilic aldolase in presence of substrate. (A) Thermophilic aldolase ($2 \cdot 10^{-6}$ M) was incubated at 61° in (○) 0.05 M potassium phosphate, pH 7.5 containing (△) $2.6 \cdot 10^{-5}$ M Fru-1,6-P_2, (□) $1.2 \cdot 10^{-5}$ M dihydroxyacetane phosphate or (◇) $1.0 \cdot 10^{-5}$ M glyceraldehyde 3-phosphate. At the times indicated, suitable aliquots were with drawn and assayed for aldolase activity at 43° as described in the text. (B) Same as A except 0.1 M Tris–HCl, pH 7.5 (at 61°) was used in place of potassium phosphate. The preparation used had a spec. act. of 143 at 43° in the standard assay.

Other buffers and reagents have also been tested. The inactivation (by Fru-1,6-P_2, glyceraldehyde 3-phosphate, or dihydroxyacetone phosphate) occurred in *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), a secondary amine, or triethanolamine, a tertiary amine, as well as in phosphate buffer. On the other hand, both primary amine buffers tested, Tris and glycylglycine, prevented the inactivation. Substrate inactivation was also prevented when 1 mM EDTA, 1 mM dithiothreitol or 0.1 mM Mn^{2+} were present in the phosphate, triethanolamine or TES incubation mixtures. Attempts to reverse the substrate inactivation by excess β-mercaptoethanol, dithiothreitol or Tris have been unsuccessful.

Since it appeared that inactivation would occur at fairly low substrate concentrations a series of Fru-1,6-P_2 levels was tested (Fig. 2). Some inactivation occurs at

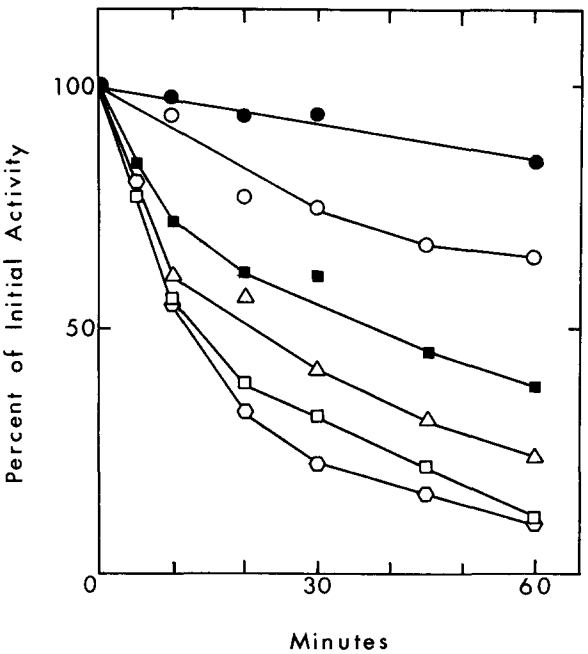

Fig. 2. Inactivation of thermophilic aldolase by low levels of Fru-1,6-P_2 and dihydroxyacetone phosphate. Thermophilic aldolase ($2 \cdot 10^{-6}$ M) was incubated at 61° in (●) 0.05 M potassium phosphate, pH 7.5 containing (○) 0.45 μM, (△) 0.9 μM, (□) 1.35 μM, (◇) 1.8 μM Fru-1,6-P_2 or (■) 0.5 μM dihydroxyacetone phosphate. Suitable aliquots were removed at the times indicated and assayed for aldolase activity in the standard assay. The preparation used had a spec. act. of 143 at 43°.

substrate levels less than one-quarter of the enzyme concentration. Dihydroxyacetone phosphate is even more effective than Fru-1,6-P_2 at these low concentrations.

The circular dichroic spectra of thermophilic aldolase was obtained at 55° under conditions similar to those used for inactivation. The results of these experiments (Table I) indicate that substrate in phosphate buffer produces an increase in the molar ellipticity at 220 nm that does not occur in Tris buffer.

As is true of many properties of Type I and II aldolases[9] the substrate inactivation of the aldolase from *B. stearothermophilus* differs in several respects from that observed with the rabbit muscle enzyme[2]. Dihydroxyacetone phosphate protects

TABLE I

MOLAR ELLIPTICITY OF THERMOPHILIC ALDOLASE

Thermophilic aldolase ($2.8 \cdot 10^{-7}$ M) was incubated at 55° for 60 min in phosphate or Tris buffer with or without Fru-1,6-P_2, $5 \cdot 10^{-5}$ M.

Buffer	$[\theta]_{220\ nm}$ *(degrees·cm² per dmole)*	
	−Fru-1,6-P_2	*+Fru-1,6-P_2*
0.05 M phosphate, pH 7.5	$-0.82 \cdot 10^4$	$-0.66 \cdot 10^4$
0.01 M Tris–HCl, pH 7.5 (at 55°)	$-0.79 \cdot 10^4$	$-0.77 \cdot 10^4$

against glyceraldehyde 3-phosphate inactivation of the rabbit muscle enzyme but is a potent inactivator of the thermophilic aldolase. Substrate inactivation of the rabbit muscle enzyme proceeds in both Tris and phosphate buffers in contrast to the results reported here.

Substrate inactivation of yeast aldolase, the prototype of Type II aldolases is also observed (R. D. Kobes and Y. M. Lin, personal communication). Inactivation occurred in imidazole or phosphate buffers at pH 6.5 and 25° with 1 mM dihydroxyacetone phosphate, Fru-1,6-P_2 or glyceraldehyde 3-phosphate. Preliminary experiments in our laboratory, at 30° or 36°, with a partially purified Fru-1,6-P_2 aldolase from yeast (spec. act. 68 at 30°) confirmed their result in imidazole buffer. However, our preparation was not inactivated when either phosphate or Tris buffers were employed. The reason for this discrepancy is not clear but may have to do with the difference in incubation temperature or with the purity of our preparation.

It is not known whether dihydroxyacetone phosphate, Fru-1,6-P_2, glyceraldehyde 3-phosphate or all three are responsible for the observed inactivation of the thermophilic aldolase. The results of Fig. 2 indicate that dihydroxyacetone phosphate is more effective than Fru-1,6-P_2 in inactivating the enzyme at similar concentrations. Although our experiments indicate glyceraldehyde 3-phosphate also inactivates the aldolase, even a trace of triose phosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) could convert sufficient glyceraldehyde 3-phosphate to dihydroxyacetone phosphate to cause the inactivation observed. Since the triose phosphate isomerase activity of the preparation used was about 0.15% of the aldolase activity, a role for this substrate in inactivation is questionable.

Initial circular dichroism experiments indicate a significant increase in the molar ellipticity at 220 nm when the thermophilic aldolase is incubated at 55° in 100-fold molar excess Fru-1,6-P_2 in phosphate buffer. No such change occurs when the enzyme is incubated in phosphate alone or in Tris with or without substrate. Although this change has been studied in the presence of relatively high levels of substrate, the fact that it is also prevented by Tris would suggest a relationship between loss of catalytic activity and the presumed conformational change indicated by changes in the circular dichroism. Additional experiments are needed to identify the groups involved in loss of enzymic activity and to define the role of the metal ion and the nature of the increase in molar ellipticity at 220 nm.

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