BBA 66243

PROPERTIES OF FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE FROM TWO THERMOPHILIC AND A MESOPHILIC CLOSTRIDIA

ELIZABETH M. BARNES, JAMES M. AKAGI AND RICHARD H. HIMES

Departments of Biochemistry and Microbiology, University of Kansas, Lawrence, Kan. 66044 (U.S.A.)

(Received July 17th, 1970)

SUMMARY

Fructose-1,6-diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) from the two thermophiles *Clostridium tartarivorum* and *Clostridium thermosaccharolyticum* and from the mesophile *Clostridium pasteurianum* has been purified about 55-fold. The partially purified proteins exhibited properties characteristic of Class II aldolases, *i.e.* they showed a requirement for a divalent cation best satisfied by Co^{2+}, were stimulated by K^+ or NH_4^+, exhibited a sharp pH optimum of 7.3–7.6, were not inactivated by carboxypeptidase or treatment with $NaBH_4$ in the presence of dihydroxyacetone phosphate and had a molecular weight of about 68000. The Michaelis constants for the cofactors and the substrate fructose 1,6-diphosphate were found to be similar for all three clostridial enzymes. The presence of cysteine or dithiothreitol in the absence of Co^{2+} caused a 2-fold stimulation in activity. However, in the presence of Co^{2+} the thiol compounds caused inhibition. Reaction with the sulfhydryl reagents *p*-chloromercuribenzoate and 5,5'-dithiobis-(2-nitrobenzoic acid) caused complete inactivation of the enzyme which could be reversed by the addition of 2-mercaptoethanol. Co^{2+} but not fructose 1,6-diphosphate protected the enzyme from inactivation by the sulfhydryl reagents. *C. pasteurianum* aldolase exhibited maximum activity when assayed at 42° whereas the thermophile enzymes showed a maximum at 70–72°. The mesophile enzyme was completely inactivated within 5 min when incubated at 57°; the thermophiles aldolases remained stable for at least 4 h. Both the mesophile and thermophile enzymes were found to be equally susceptible to the denaturants urea and guanidine·HCl.

The subject of thermophily in bacteria has received renewed attention in recent years. One reason for the interest is that proteins isolated from such organisms usually show a high degree of resistance to heat denaturation. By studying thermophile proteins it is anticipated that more information concerning the relationship between primary and higher orders of protein structure can be obtained. We have been in-

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

vestigating thermophily in the genus Clostridia and have reported on the properties of ferredoxin from thermophilic Clostridia[1]. We have also shown[2] that in crude extracts most of the glycolytic enzymes from the thermophiles *Clostridium tartarivorum* and *Clostridium thermosaccharolyticum* are much more stable to heat inactivation than the corresponding enzymes from the mesophile *Clostridium pasteurianum*. Fructose-1,6-diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) is one enzyme that we have chosen for further study and this report presents some properties of the partially purified protein from the three clostridial species.

Fructose-1,6-diphosphate aldolase was purified from the three organisms using the following steps: (1) ferredoxin was removed from the autolysate by passing it through a DEAE-cellulose column[1], (2) the enzyme was precipitated with $(NH_4)_2SO_4$ (50–80%), (3) the precipitate was suspended in buffer, dialyzed and adjusted to pH 4.0 and centrifuged, (4) the pH 4.0 supernate was fractionated with $(NH_4)_2SO_4$ (67–77%), (5) the enzyme was further purified on DEAE-cellulose using a linear KCl gradient from 0 to 0.3 M in 0.05 M potassium phosphate buffer, pH 7.3. This procedure resulted in about a 50-fold purification with about a 60% recovery in activity units. The final specific activity was 48 which is about 50% the maximum specific activity found for yeast aldolase[3].

The three enzymes exhibited the characteristics expected for Class II aldolases[4,5]. The molecular weight of the enzyme determined by Sephadex G-200 chromatography was 68000. The enzymes were not inactivated by carboxypeptidase or by treatment with $NaBH_4$ in the presence of dihydroxyacetone phosphate. They showed a sharp pH optimum between 7.3 and 7.6 when assayed using the spectrophotometric assay[6]. The activity of the enzymes using the colorimetric assay[7], which does not involve the use of coupling enzymes, was stimulated 9–10-fold by KCl and NH_4Cl and 2-fold by NaCl. A divalent cation was also required for maximal activity. This requirement was best satisfied by Co^{2+} which produced a 10-fold stimulation. The maximum stimulation by Fe^{2+} was 4.3-fold. Other divalent cations (Mg^{2+}, Mn^{2+} and Zn^{2+}) were essentially ineffective. When Co^{2+} and NH_4^+ were included together a synergistic increase in activity was observed. The K_m values for the substrate and cofactors determined with the enzyme from *C. thermosaccharolyticum* at 55° using cation-free reagents were: fructose 1,6-diphosphate, $1.4 \cdot 10^{-3}$ M; Co^{2+}, $1.8 \cdot 10^{-6}$ M; NH_4^+, $2.3 \cdot 10^{-3}$ M. Similar values were obtained with the enzyme from *C. tartarivorum* at 55° and *C. pasteurianum* at 37°.

The enzymes were found to be inhibited by the sulfhydryl reagents *p*-chloromercuribenzoate (PCMB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Of particular interest is that the inhibition is completely reversed by 2-mercaptoethanol and prevented by the inclusion of Co^{2+} (Table I). These results suggest that the divalent cation may bind to a sulfhydryl group in the enzyme. When added to the assay mixture, cysteine caused a slight stimulation of activity in the absence of Co^{2+}, but in the presence of the divalent cation an inhibition was observed. This presumably is due to removal of Co^{2+} by complex formation.

Although the afore-mentioned properties were similar for the mesophile and thermophile enzymes the response of these proteins to temperature was quite different. Shown in Fig. 1 is the temperature optima of the enzymes using the colorimetric assay. The temperature optimum for *C. pasteurianum* was found to be about

TABLE I

EFFECT OF PCMB AND DTNB ON *C. tartarivorum* ALDOLASE ACTIVITY

PCMB or DTNB was incubated with 0.5 mg of the protein in 1 ml of 0.05 M imidazole·HCl, pH 7.4, at room temperature, in the absence and presence of 0.4 mM $CoCl_2$. Samples were withdrawn, diluted and assayed using the spectrophotometric assay. At the time shown 2-mercaptoethanol was added to make the final concentration of 0.1 M.

Reagent	*Concn. (mM)*	*Incubation time (h)*	*Percent of initial activity*	
			$-CoCl_2$	$+CoCl_2$
PCMB	0.16	0	100	100
		0.5	75	100
		3.0	40	101
		3.0 then 2-mercaptoethanol	94	98
	0.50	0	100	100
		0.5	50	100
		1.0	0	98
		1.0 then 2-mercaptoethanol	97	99
DTNB	0.09	0	100	100
		0.25	80	100
		1.0	56	98
		1.0 then 2-mercaptoethanol	99	99
	0.19	0	100	—
		0.5	54	—
		2.5	0	—
		2.5 then 2-mercaptoethanol	93	—

42° whereas the temperature optimum for the thermophile enzymes was about 70–72°. A difference was also apparent in the thermostability of the enzymes. Fig. 2 shows the results of thermostability experiments with purified preparations of the clostridial aldolases as well as with aldolase from rabbit muscle. The most striking feature of

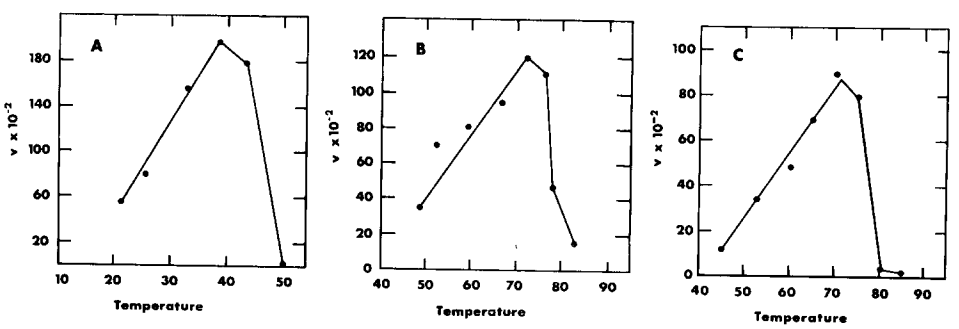

Fig. 1. Temperature optimum of Clostridial aldolase. The colorimetric assay was used to determine enzyme activity. A. *C. pasteurianum* aldolase. B. *C. tartarivorum* aldolase. C. *C. thermosaccharolyticum* aldolase. The velocity is expressed as μmoles alkali-labile phosphorus formed per min.

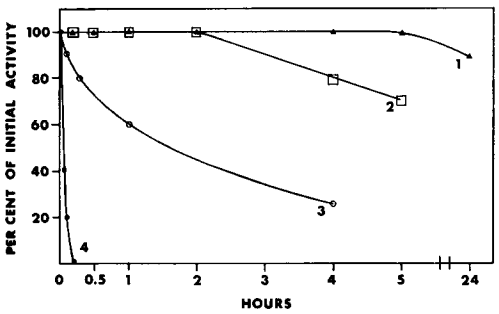

Fig. 2. Thermostability of fructose-1,6-diphosphate aldolase. The enzyme solutions (0.05 mg protein in 1 ml of 0.02 M potassium phosphate buffer, pH 7.4) were incubated at 57°. Samples were removed and assayed spectrophotometrically at 30°; Co^{2+} and NH_4^+ were not added for the assay of muscle aldolase activity. 1, *C. tartarivorum* aldolase; 2, *C. thermosaccharolyticum* aldolase; 3, muscle aldolase; 4, *C. pasteurianum* aldolase.

these results is that *C. pasteurianum* aldolase is rapidly inactivated (100% within 5 min) at 57° whereas the thermophile enzymes remain stable for hours. Muscle aldolase appears to be relatively stable for one hour but looses activity after that time. In these experiments, noticeable precipitation of protein was evident with muscle and *C. pasteurianum* aldolase. No clouding could be observed with the thermophile enzymes until inactivation began.

Although there was a large difference in the thermostability of the mesophile and thermophile enzymes the rates of inactivation by urea and guanidine·HCl were quite similar. Shown in Table II are the results of incubation of both enzymes with

TABLE II

EFFECT OF UREA AND GUANIDINE·HCl ON *C. pasteurianum* AND *C. thermosaccharolyticum* ALDOLASE ACTIVITY

Partially purified aldolase was incubated at 25° at a concentration of 0.2 mg/ml in 0.05 M potassium phosphate, pH 7.4, containing 6 M urea or 2 M guanidine·HCl. Samples were removed and assayed at 30° by the spectrophotometric assay. In the absence of denaturant no loss in activity occurred.

Denaturant	*Time (min)*	*Percent of initial activity*	
		C. pasteurianum	*C. thermosaccharolyticum*
6 M urea	0	100	100
	5	70	70
	30	50	—
	60	—	50
	90	29	40
	150	15	31
2 M guanidine·HCl	0	100	100
	5	80	—
	7	63	79
	20	55	71
	90	40	55

6 M urea and 2 M guanidine·HCl. Apparently the structure of both enzymes are affected in a similar manner by these denaturants. However, it must be pointed out that after incubation with the denaturants the enzyme was diluted for assay. Thus, reversible changes in structure would not be seen.

It is apparent from these studies that fructose-1,6-diphosphate aldolase from the three Clostridia used is a Class II aldolase. In practically all respects the mesophile and thermophile enzymes are identical. The main difference between them is their response to temperature. The thermophile enzymes have a temperature optimum about 30° higher than the mesophile enzyme and also show marked thermostability at 57° compared to the rapid inactivation of the mesophile protein. The greater heat stability of the thermophile proteins is probably not due to a greater degree of hydrogen bonding since mesophile and thermophile aldolases were similarly affected by the denaturants urea and guanidine·HCl.

ACKNOWLEDGMENTS

This work was supported by Grant NSG-17-002-042 from the National Aeronautics and Space Administration and was taken from a thesis submitted by E. M. Barnes to the Graduate School of the University of Kansas in partial fulfillment of the requirements for the Ph. D. degree.

J.M.A. and R.H.H. are U.S. Public Health Service Career Development Awardees.

REFERENCES

1 T. DEVANATHAN, J. M. AKAGI, R. T. HERSH AND R. H. HIMES, *J. Biol. Chem.*, 244 (1969) 2846.
2 N. HOWELL, J. M. AKAGI AND R. H. HIMES, *Can. J. Microbiol.*, 15 (1969) 461.
3 O. C. RICHARDS AND W. J. RUTTER, *J. Biol. Chem.*, 236 (1961) 3177.
4 W. J. RUTTER, J. R. HUNSLEY, W. E. GROVES, J. CALDER, T. V. RAJKUMAR AND B. M. WOODFIN, *Methods Enzymol.*, 9 (1965) 479.
5 W. J. RUTTER, *Federation Proc.*, 23 (1964) 1248.
6 R. WU AND E. RACKER, *J. Biol. Chem.*, 234 (1959) 1029.
7 J. A. SIBLEY AND A. L. LEHNINGER, *J. Biol. Chem.*, 177 (1949) 859.