THE THERMOSTABLE ALLOSTERIC NATURE OF FRUCTOSE 1,6-DIPHOSPHATASE FROM AN EXTREME THERMOPHILE.

Masasuke Yoshida and Tairo Oshima

Department of Agricultural Chemistry, Faculty of Agriculture,
the University of Tokyo, Bunkyo-ku, Tokyo, Japan

Received August 18, 1971

Summary: The thermostable fructose 1,6-diphosphatase from F. thermophilum HB8 was partially purified and separated from nonspecific phosphatases. The enzyme activity was inhibited by AMP and activated by phosphoenolpyruvate with allosteric manner even at 70°. Catalytic activity and susceptibility to allosteric effectors remained unchanged after heat treatment at 70° for 1 hour. The other properties of the enzyme are similar to those reported for fructose 1,6-diphosphatases from other mesophilic sources: a low value of Km for fructose 1,6-diphosphate, requirement for Mg^{++} or Mn^{++} ion, an alkaline pH optimum, and inhibition by excess substrate.

Introduction: An extreme thermophilic bacterium, Flavobacterium thermophilum HB8 (sp.n.) was isolated from a hot spring at Mine, Shizuoka, Japan. The maximum temperature for the growth of this bacterium was observed to be 85° (1), and some properties of cell constituents have being studied (2,3). In order to elucidate regulatory mechanism of glycolytic pathway of this organism, phosphofructokinase, which is supposed to be one of the key enzymes in the path (4), has been studied (5). Phosphofructokinase (PFK) from this thermophile was inhibited by phosphoenolpyruvate (PEP) and this inhibition was relieved by the addition of ADP. To examine the possibility that the concentrations of PEP and ADP in the cellular environment are among essential parameters in the regulation, the study of fructose 1,6-diphosphatase (FDPase, D-fructose 1,6-diphosphate 1-phosphohydrolase, E C 3.1.3.11) of this thermophile has been carried out. FDPase is considered to play an essential role in the regulation of gluconeogenesis and is sensitive to allosteric inhibition by AMP for various organisms (6-16). In this communication, thermostability of the enzyme and allosteric effects of PEP and AMP are presented.

Experimental: The cells of F. thermophilum aerobically grown at 75° in a medium consisting of 8g of polypeptone, 4g of yeast extract and 2g of

NaCl per liter, were harvested at the later stage of exponential growth, and were stored in frozen state at -20° until use. The frozen cells were thawed and resuspended in 50mM Tris-HCl buffer, pH 7.5, and sonicated for 10 min. All the following operations were performed at 4° or below. The sonic extract obtained by centrifugation, was further subjected to ultracentrifugal fractionation at 75,000 x g for 4 hours. To the supernatant fraction, solid ammonium sulfate was slowly added with stirring. The precipitate formed between 0.3 and 0.5 saturation was collected by centrifugation, dissolved in 50 mM Tris-HCl buffer, pH 7.5. After gel filtration on Sephadex G-25 column, the enzyme fraction was applied with a column of DEAE-cellulose equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The enzyme fraction was eluted with a linear gradient system of 0-0.5M NaCl in 50 mM Tris-HCl buffer, pH 7.5. Two discrete fractions of activity hydrolyzing FDP were eluted at 0.06M and 0.23M NaCl concentrations. The former activity showed a larger Km value (1 X 10^{-4}M) for FDP than the latter (3 X 10^{-6}M), was unaffected by the addition of AMP and PEP, and hydrolyzed p-nitrophenylphosphate (at about 1/2 v_{FDP}) whereas the latter showed no hydrolytic activity whatsoever. The former activity seemed to be non-specific phosphatase, and the latter, specific FDPase. The latter fraction was concentrated by ammonium sulfate precipitation, dissolved in 50 mM Tris-HCl buffer, pH 7.5, and stored at 4° . At this stage the yield was 20% and the specific activity was raised about 10 times that of the sonic extract. The activity assay was performed by measuring enzymatically the amount of F-6-P formed, in the presence of NADP, glucose 6-phosphate dehydrogenase and hexose 6-phosphate isomerase. The reduction of NADP was followed by its absorbance at 340 nm using a recording spectrophotometer (Gilford Model 240). The reaction was usually initiated by the addition of enzyme.

Results and discussion: The activity of F. thermophilum FDPase was dependent on the presence of Mg^{++} or Mn^{++}. The optimal concentrations were 5 mM for Mg^{++} and 0.5 mM for Mn^{++}. Mn^{++} was more effective at low concentrations but a small excess of Mn^{++} ion (>1mM) had an inhibitory effect. EDTA was only slightly stimulatory in the presence of excess amount of Mg^{++} ion.

The heat inactivation of the enzyme solution was tested at various temperatures and results are shown in Fig. 1. The enzyme was stable or slowly inactivated below at 80° , whereas the activity was lost rapidly at 90° . In this experiment no desensitizing phenomenon was observed. The effect of temperature on the enzyme activity is presented by an Arrhenius plot

(Fig. 2). The maximum activity under the conditions described in legend was observed around 80° (-PEP) and at 65° (+PEP). The stimulating effect of PEP was observed below at 80° .

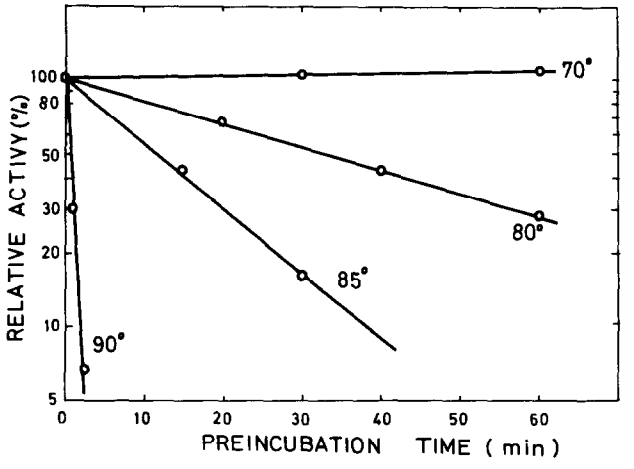

Fig. 1. Heat inactivation of F. thermophilum FDPase.
Enzyme solutions, containing 1.7 mg/ml protein, in 150 mM Glycine -NaOH buffer, pH 8.5 (at 25°), were incubated at each temperature. At appropriate intervals, an aliquot of the enzyme solution was taken and the activity was measured at 30° .

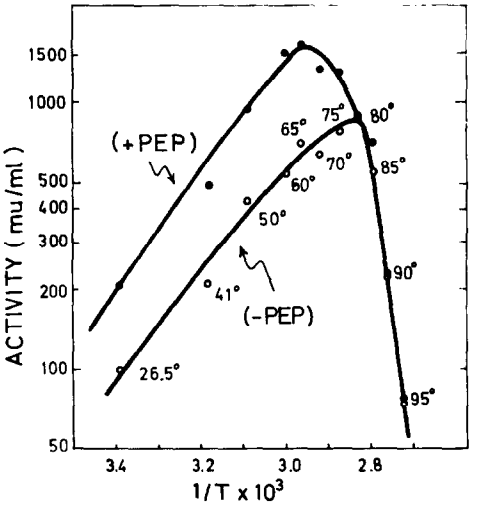

Fig. 2. Effect of temperature on the activity of F. thermophilum FDPase.
Reaction mixture contained 125 mM Glycine-NaOH buffer, pH 8.2 at each temperature; 5 mM $MgCl_2$; 0.5 mM FDP; and 1 mM PEP (—●—) or without PEP(—○—). After 5 min the reaction was stopped by the addition of sodium cyclohexane diamine tetraacetate (chelating reagent) to final concentration 60 mM. Fructose 6-phosphate formed was measured by corresponding NADP reduction at room temperature.

The enzyme activity was inhibited by the presence of excess substrate, FDP, and strongly inhibited by AMP (apparent Ki=1.2 X 10^{-6}M). ADP was observed to be inhibitory (apparent Ki=1.2 X 10^{-5}M) for the present enzymatic activity. It remains to be solved in a future study whether the inhibition was due to the true action of ADP, the presence of AMP in the ADP preparation used, or the conversion of ADP to AMP during the enzyme assay. It was found that PEP stimulated the activity several fold (Fig. 3). The concentration required for 50% activation was 2 X 10^{-4}M and the maximum activation was obtained at a value above 5 X 10^{-4}M of PEP.

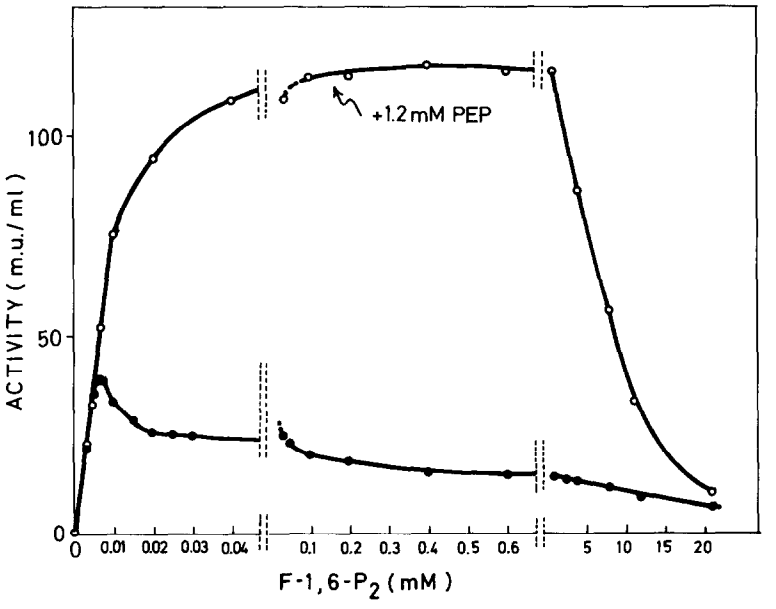

Fig. 3. The effect of PEP on kinetics of F. thermophilum FDPase. Reaction mixture contained 50 mM Glycine-NaOH buffer, pH 8.9; 10 mM $MgCl_2$; 1 mM EDTA; 0.2 mM NADP; excess amount of auxiliary enzymes; and 1.2 mM PEP (-o-) or without PEP (-●-). The assay was performed at 30°.

The maximum activity of the enzyme obtained at pH 8.1 in the absence of PEP and in the presence of PEP at 8.8 (Fig. 4). The ratio of the activation by PEP varied with pH and the maximum activation was observed around pH 9.1.

The present investigation reveals that FDPase from F. thermophilum demonstrates a thermostable allosteric nature. The results of these studies of PEP activation and AMP inhibition, taken together with our previous reports (4) indicating that PFK of F. thermophilum was inhibited by PEP and ADP relieved this inhibition, suggest that the concentrations of PEP,

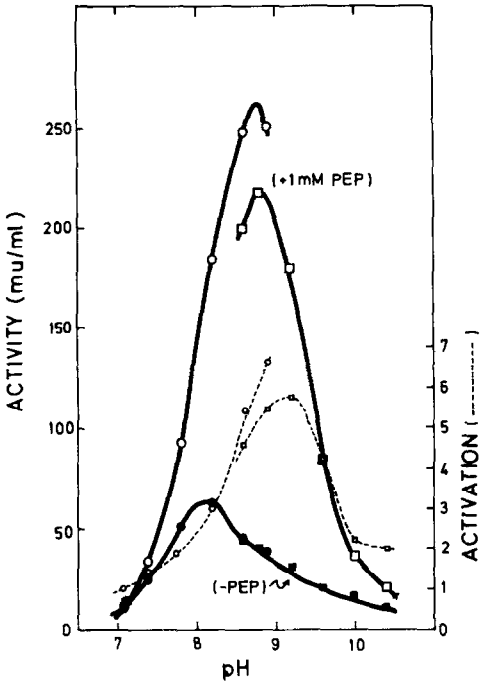

Fig. 4. The effect of pH on the enzyme activity and the activation by PEP. Reaction mixture contained (●,○) 100 mM Tris-HCl buffer or (■,□) 100 mM Glycine-NaOH buffer, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM FDP, 0.2 mM NADP and excess auxiliary enzymes. The assay was performed at 30°.

AMP and ADP may serve to control the direction of the Embden-Meyerhof pathway in F. thermophilum.

Acknowledgment: The authors thank Prof. K. Imahori for his encouragement and useful discussions. The study was in part aided by a grant from Ministry of Education of Japan.

References

1. Oshima, T., J. Gen. Appl. Microbiol., in press.
2. Yoshizaki, F., Oshima, T., and Imahori, K., J. Biochem., 69, 1083 (1971).
3. Oshima, T., and Imahori, K., The abstract of papers, the 24th Annual Meeting of the Chemical Society of Japan, Osaka, April 4, Vol. III, p. 1843 (1971) (in Japanese).
4. Stadtman, E. R., Advan. Enzymol., 28, 41 (1967).
5. Yoshida, M., Oshima, T., and Imahori, K., Biochem. Biophys. Res. Commun., 43, 36 (1971).
6. Fraenkel, D., and Horecker, B. L., J. Bacteriol., 90, 837 (1965).
7. Taketa, K., and Pogell, B. M., Biochem. Biophys. Res. Commun., 12, 229 (1963).

8. Newsholme, E. A., Biochem. J., 89, 389 (1963).
9. Mendicino, J., and Vasarhely, F., J. Biol. Chem., 238, 3528 (1963).
10. Krebs, H. A., and Woodford, M., Biochem. J., 94, 436 (1965).
11. Rosen, O. M., Rosen, S. M., and Horecker, B. L., Arch. Biochem. Biophys., 112, 411 (1965).
12. Gancedo, C., Salas, M. L., Giner, A., and Sols, A., Biochem. Biophys. Res. Commun., 20, 15 (1965).
13. Fraenkel, D. G., Pontremoli, S., and Horecker, B. L., Arch. Biochem. Biophys., 114, 4 (1966).
14. Opie, L. H., and Newsholme, E. A., Biochem. J., 104, 353 (1967).
15. Scala, J., Ketner, G., and Jyung, W. H., Arch. Biochem. Biophys., 131, 111 (1969).
16. Behrisch, H. W., Biochem. J., 121, 399 (1971).