THE THERMOSTABLE ALLOSTERIC ENZYME: PHOSPHOFRUCTOKINASE FROM AN EXTREME THERMOPHILE.

M. Yoshida, T. Oshima and K. Imahori
Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Received February 10, 1971

Summary: Phosphofructokinase from an extreme thermophilic bacterium was partially purified and it showed a thermostable allosteric nature. No detectable loss of activity was observed after incubation of the enzyme solution at 80° for 1 hour. The enzyme activity followed a simple Michaelis-Menten kinetics with respect to each substrate, fructose 6-phosphate and ATP. Phosphoenolpyruvate was a strong inhibitor and by its addition a normal saturation kinetic curve for fructose 6-phosphate was turned into a sigmoidal one, independent of ATP concentration. ADP relieved this inhibition.

An extreme thermophile, tentatively named Flavobacterium thermophilum sp.n.(to be described elsewhere (1)), was isolated from a hot spring at Mine, Shizuoka-ken, Japan. The maximum temperature for the growth of this bacterium (strain HB8) was observed to be 85° (1). In order to elucidate the mechanisms which enable the bacterium to grow at such high temperature, the investigation of the structures of its macromolecular constituents and regulation mechanisms were undertaken. So far it has been found that several enzymes included in the glycolytic pathway, ribosome and tRNA are extremely thermostable (2). In this context, phosphofructokinase (PFK) (ATP:D-fructose 6-phosphate 1-phosphotransferase, E.C.2.7.1.11) was extracted from this bacterium and its properties have been studied. In various organisms, PFK is supposed to play an essential role in the regulation of glycolytic metabolism, and it exhibits allosteric natures (3). In this communication, thermostability of the enzyme and allosteric effects of phosphoenolpyruvate and ADP will be presented.

Experimental. The cells of F. thermophilum HB8 aerobically grown at 75° in a medium consisting 10g 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, containing 0.1mM dithiothreitol and 0.02mM EDTA (Buffer A), and sonicated for 10min. All the following

operations were performed at 6° or below. The disrupted cells were centrifuged and solid ammonium sulfate was slowly added with stirring to the supernatant fraction. The precipitate formed between 200 and 300 g/l ammonium sulfate was collected by centrifugation, and dissolved in Buffer A. This fraction was absorbed on DEAE-cellulose equilibrated with the same buffer. To the fraction that eluted between 0.25 and 0.35M NaCl concentration, solid ammonium sulfate was slowly added with stirring. The precipitate that formed between 210 and 270 g/l was collected by centrifugation, dissolved in Buffer A, and stored at 4° . At this stage, the yield was 28% and the specific activity was raised to 33 times of the crude extract. This preparation did not contain any detectable activity of pyruvate kinase and fructose diphosphatase. The enzyme activity of this fraction did not drop appreciably during storage at 4° for at least 6 months.

The activity assay was performed by measuring enzymatically the amount of fructose 1, 6-diphosphate formed, using NADH, aldolase, triphosphate isomerase and α-glycerophosphate dehydrogenase. In this system, the oxidation of NADH was followed by the absorbance at 340 nm using a Gilford model 240 recording spectrophotometer.

Results and Discussion. It was observed that PFK from F. thermophilum demonstrated an extreme thermostability. Figure 1 shows that no detectable inactivation occurred in 1 hour at 80° and that incubation for 1 hour at 90° resulted in only 10% decrease of the activity under the conditions described in legend.

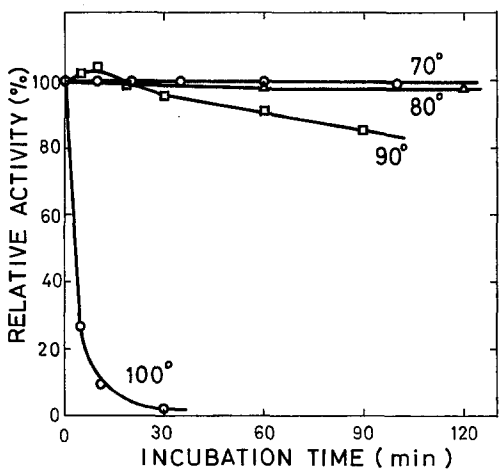

Fig. 1. Heat inactivation of F. thermophilum PFK.
Enzyme solutions, containing 0.51 mg/ml protein, in 50 mM Glycine -NaOH buffer, pH 8.3 (at 75°), were incubated at each temperature. At appropriate intervals, an aliquot of the enzyme solution was taken and the activity was measured at 30° .

The reaction rates of the enzyme for fructose 6-phosphate (F-6-P) and ATP followed Michaelis-Menten kinetics, and there was no evidence for sigmoidicity nor substrate inhibition at 30° or 75°, pH 7.0 or 8.5, in the presence of 1mM or 5mM $MgCl_2$, when 0.2mM or 5.0mM of the second substrate was used. K_m was estimated to be 2-3 X 10^{-5}M for ATP and 6-10 X 10^{-5}M for F-6-P under the optimal conditions. Citrate, succinate, pyruvate, inorganic phosphate, creatine phosphate, N-acetylglucosamine, 3',5'-AMP, glucose 1-phosphate, and twenty amino acids had no significant effect on the PFK activity. Pyrophosphate was a potent inhibitor, but this inhibition seemed to be abolished in the presence of high concentration of $MgCl_2$. ADP and AMP were competitive inhibitors of ATP. 2,3-diphosphoglycerate was also a potent inhibitor. It was found that phosphoenolpyruvate (PEP) strongly inhibited PFK in the presence of low concentrations of F-6-P (Fig. 2). The apparent Ki was estimated to be below 8 X 10^{-6}M. In the presence of PEP, the enzyme activity showed sigmoidal kinetics with respect to F-6-P concentration. The Hill-coefficient was estimated to be 2.8. ADP relieved inhibition by PEP, changing sigmoidal kinetics into normal one. The concentration of ATP had little effect on this inhibition. Such effects of PEP and ADP on the PFK activity were observed both at 30° and 75°.

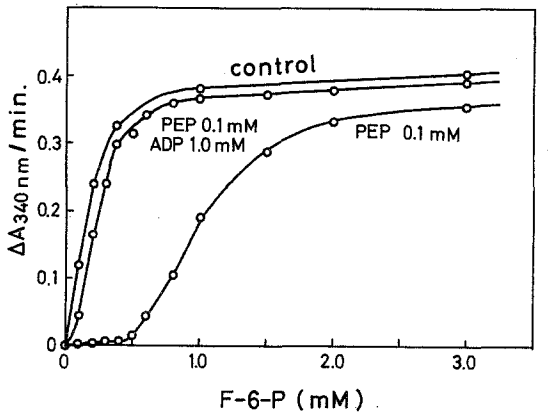

Fig. 2. Effects of PEP and ADP on the kinetics of F. thermophilum PFK at 75°.

Reaction mixture contained 50 mM Glycine-NaOH buffer, pH 8.3 (at 75°), 1.0 mM ATP, 1.0 mM $MgCl_2$, 5 mM β-mercaptoethanol and 10 mM KCl. After 48 seconds, reaction was stopped by the addition of cyclohexanediaminotetraacetate to the final concentration 40 mM. Then fructose 1, 6-diphosphate formed was measured by the corresponding NADH oxidation. Control contained reaction mixture without PEP or ADP.

The F. thermophilum PFK seems to be different from any of other various organisms, though the inhibition by PEP has been observed for PFK

from several organisms (4-8). Its distinct characteristics are the extreme themostability and the sigmoidal kinetics for F-6-P in the presence of PEP Kinetic natures of the enzyme suggest its important role in the regulation of carbohydrate metabolism as an allosteric enzyme in the cells growing at such elevated temperature. Preliminary examination in our laboratory revealed that the activity of partially purified fructose diphosphatase from the same bacterium, which catalyzes the hydrolysis of fructose 1, 6-diphosphate to F-6-P, was activated by PEP and inhibited by ADP. Then the concentrations of PEP and ADP in the cellular environment will be essential parameters in the regulation of the carbohydrate metabolism.

Acknowledgement. The work was in part aided by a grant from the Ministry Education of Japan.

References.

1. T. Oshima, in preparation.
2. F. Yoshizaki, T. Oshima and K. Imahori, J. Biochem. in press.
3. E.R. Stadtman, Advan. Enzymol. 28 (1967) 41.
4. K. Uyeda and E. Racker, J. Biol. Chem. 240 (1965) 4682.
5. J. Krzanowski and F. Mathinsky, Biochem. and Biophys. Research Communs. 34 (1969) 816.
6. G.J. Kelly and J.F. Turner, Biochem. and Biophys. Research Communs. 30 (1968) 195.
7. L.S. Mil'man and Yu.G. Yurovitskii, Dokl. Acad. Nauk SSSR 185 (1969) 1175.
8. D. Blangy, H. Buc and J. Monod, J. Mol. Biol. 31 (1968) 13.