Allosteric Nature of Thermostable Phosphofructokinase from an Extreme Thermophilic Bacterium†

Masasuke Yoshida

ABSTRACT: Phosphofructokinase from an extreme thermophilic bacterium was partially purified and some characteristics were examined. The enzyme was shown to be extremely thermostable. Unlike the analogous enzyme from other organisms, the reaction rate of the enzyme for fructose 6-phosphate and ATP followed Michaelis–Menten kinetics. ITP, GTP, UTP, and CTP were able to be phosphate doner in place of ATP. Citrate, creatine phosphate, 3′,5′-AMP, and P_i had no significant effect on the enzyme activity. ADP, IDP, GDP, UDP, CDP, and AMP were competitive inhibitors for ATP and fructose 1,6-diphosphate was a competitive inhibitor for fructose 6-phosphate. Phosphoenolpyruvate was a strong inhibitor at a low concentration of fructose 6-phosphate; in the presence of phosphoenolpyruvate, the activity of the enzyme varied as a sigmoidal function with respect to fructose 6-phosphate. Inhibition by phosphoenolpyruvate was relieved by ADP, reverting kinetics to Michaelis–Menten type, however IDP, GDP, CDP, UDP, and two analogs of ADP had no such effect. The effects of phosphoenolpyruvate and ADP were observed both at 30 and 75°, independent of the ATP concentration. These properties of thermostable phosphofructokinase suggest that it plays an important role in the regulation of carbohydrate metabolism as an allosteric enzyme even at high temperature. The author discusses the importance of the concentrations of phosphoenolpyruvate and ADP in the regulation of carbohydrate metabolism.

An extreme thermophile has been isolated from a hot spring. The maximum temperature for the growth of this bacterium was observed to be 85° (Oshima and Imahori, 1971). In order to elucidate the biological mechanisms which enable the bacterium to grow at such high temperature, investigations on the structure 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 (T. Oshima, 1971, personal communication; Yoshizaki *et al.*, 1971; Yoshida and Oshima, 1971).

Phosphofructokinase[1] (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) was extracted from the microorganism. Its properties have been studied to discern whether or not the PFK of this bacterium is a thermostable allosteric enzyme and its action on the glycolytic pathway. In various organisms, PFK is believed to play an essential role in the regulation of glycolytic metabolism and exhibits typical allosteric nature (see reviews by Atkinson, 1966, Stadtman, 1966, Wood, 1966, and Sanwal, 1970).

In this report, thermostability of the enzyme and allosteric effects of PEP and ADP will be presented.[2]

Experimental Section

Materials. NADH, F-6-P, PEP, nucleotides, adenosine 5′-phosphosulfate, and enzymes used in the assays were obtained from Boehringer Mannheim Japan K. K. α,β-Methyleneadenosine diphosphate was purchased from the Miles Laboratories, Inc., Kankakee, Ill. DEAE-cellulose was a gift of the Jujo Paper Co., Tokyo, Japan.

Assay of PFK Activity. Unless otherwise mentioned, PFK activity was determined by measuring the amount of FDP formed, dependent upon the observation of NADH oxidation followed at 340 nm in a simultaneous coupling manner (assay I), or in a successive manner (assay II) with a Gilford recording spectrophotometer.

The standard reaction mixture of assay I contained the following at a final volume of 0.4 ml: triethanolamine hydrochloride–NaOH buffer (50 mM, pH 8.4), NADH (0.15 mM), $MgCl_2$ (5 mM), KCl (10 mM), β-mercaptoethanol (5 mM), aldoase (8 μg), triose phosphate isomerase and α-glycerophosphate dehydrogenase mixture (4 μg), ATP (0.2 mM), and F-6-P (0.2 mM). The reaction was initiated by the addition of enzyme, and the rate of NADH oxidation was measured at 30°.

When auxiliary enzymes were inactive under the condition of PKF assay, assay II was used. In assay II, the standard reaction mixture contained the following: glycine–NaOH buffer (50 mM, pH 8.4), $MgCl_2$ (5 mM); KCl (10 mM), β-mercaptoethanol (5 mM), ATP (2.9 mM), and F-6-P (2 mM). The reaction was initiated by the addition of enzyme. After a given time, the reaction was terminated by addition of cyclohexanediaminotetraacetate at a final concentration of 40 mM. An aliquot of the reaction mixture was removed out and the amount of FDP formed was measured enzymatically by adding an assay mixture consisting of the following: NADH (0.15 mM), cyclohexanediaminotetraacetate (40 mM), aldolase (10 μg/ml), and triosephosphate isomerase and α-glycerophosphate dehydrogenase mixture (5 μg/ml). One unit of enzyme activity is defined as the amount of the enzyme that catalyzes the formation of 1 μmole of FDP per min under these conditions. Specific activity is expressed as units per mg of protein.

Bacteria and Growth of Cells. The microorganism used was isolated from a hot spring of Mine, Shizuoka-ken, Japan,

† From the Department of Agricultural Chemistry, Faculty of Agriculture, the University of Tokyo, Bunkyo-ku, Tokyo, Japan. *Received May 17, 1971.*

[1] Abbreviations used are: PFK, phosphofructokinase; PEP, phosphoenolpyruvate; F-6-P, fructose 6-phosphate; FDP, fructose 1, 6-diphosphate.

[2] A brief preliminary report of some of these experiments has already been published (Yoshida *et al.*, 1971).

TABLE I: Enzyme Purification.[a]

Fraction	Vol (ml)	Protein (mg/ml)	Sp Act.	Purifcn Factor	% Recov
Crude extract	410	17.5	0.015	1.0	100
First ammonium sulfate precipitate	240	12.5	0.028	1.9	77
DEAE-cellulose eluate	1080	0.55	0.062	4.1	34
Second ammonium sulfate precipitate	5.6	5.12	0.490	33	13

[a] All assays were performed at 30° as described in Experimental Section.

by T. Oshima. This thermophilic bacterium is a nonsporulating, yellow pigment producing, gram-negative rod, and has been named *Flavobacterium thermophilum* HB8 sp. n. (Oshima and Imahori, 1971). This thermophile was grown at 75° with continuous stirring and aeration. The culture medium contained 0.8% of polypeptone (Daigoeiyo Chemical Co., Ltd., Osaka, Japan), 0.4% of yeast extract (Kyokuto Seiyaku Co., Ltd., Tokyo, Japan), 0.3% of sodium chloride, and was adjusted to pH 7.0 with sodium hydroxide. When absorbancy of the culture medium at 600 nm attained a value of about 1.0, cells were harvested by centrifugation at 15,000*g* for 15 min, and stored in a frozen state at −20° until use.

Enzyme Preparation. The frozen cells (160 g) were thawed and resuspended in 320 ml of Tris-HCl buffer (50 mM, pH 7.6) containing dithiothreitol (0.1 mM) and EDTA (0.02 mM) buffer A) and sonicated for 10 min.

The following operations were performed at 6° or lower. The disrupted cells were centrifuged at 20,000*g* for 20 min. Solid ammonium sulfate was slowly added to the supernatant fraction with stirring. The precipitate formed (between 200 and 300 g per l.) was collected by centrifugation, and dissolved in 200 ml of buffer A (first ammonium sulfate precipitate). After extensive dialysis against buffer A, the enzyme solution was adsorbed on to DEAE-cellulose equilibrated with the same buffer. The adsorbed enzyme was washed with 250 ml of buffer A, then with 750 ml of buffer A containing 0.15 M NaCl using suction filtration. The enzyme was then eluted with 1100 ml of buffer A containing 0.30 M NaCl. Solid ammonium sulfate (300 g/l.) was added to the filtrate; the precipitate was collected by centrifugation and redissolved in 13 ml of buffer A (DEAE fraction). In order to remove excess salt, the enzyme solution was applied to a Sephadex G-25 column which had been equilibrated with buffer A and then eluted from the column with buffer A. To the enzyme fraction solid ammonium sulfate was added slowly with stirring. The precipitate formed (between 210 and 270 g per l.) was collected by centrifugation and subsequently dissolved in 3.0 ml of buffer A (second ammonium sulfate fraction). At this stage, the specific activity increased 33 times that of the crude extract. This fraction contained neither a detectable amount of pyruvate kinase nor fructose diphosphatase ac-

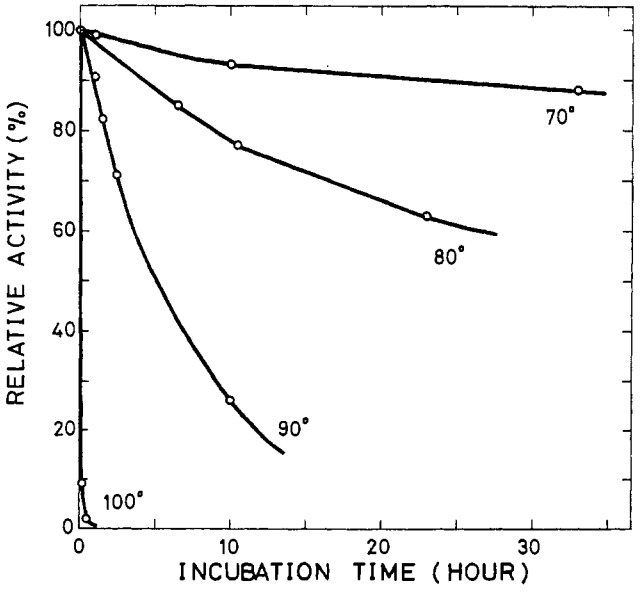

FIGURE 1: Heat inactivation of *F. thermophilum* PFK. Enzyme solutions, containing 0.51-mg/ml protein concentration, in 50 mM glycine–NaOH buffer (pH 8.3) at 75°, were incubated at each temperature. At appropriate intervals, an aliquot of the enzyme solution was taken and chilled rapidly. Then the activity was measured by assay I.

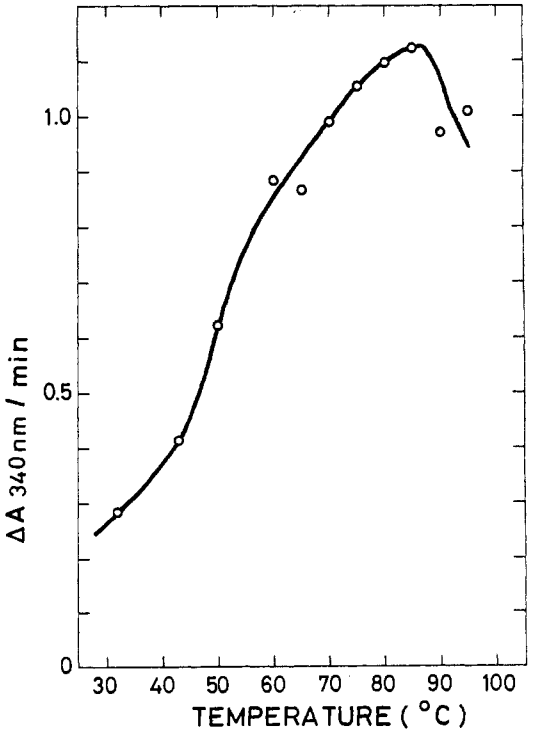

FIGURE 2: Effect of temperature on *F. thermophilum* PFK activity. PFK activities were measured at different temperatures and reaction time was 2 min. The reaction mixture contained 50 mM glycine–NaOH buffer, adjusted at pH 8.4 at each temperature, and other conditions followed assay II as described in the Experimental Section.

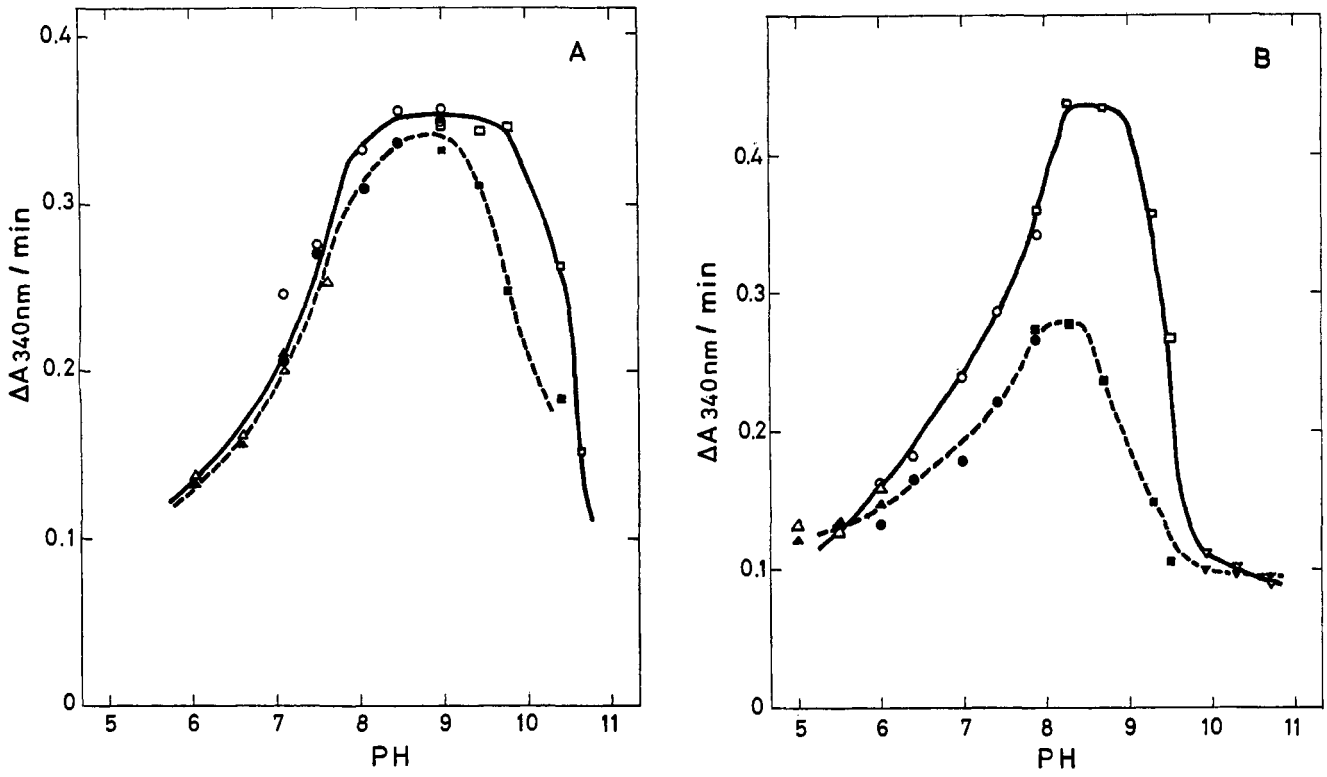

FIGURE 3: Effect of pH on *F. thermophilum* PFK activity at 30° (A) and 75° (B). Reaction mixture contained 0.4 mM F-6-P, 0.2 mM ATP (– – – –, closed signals), or 4.0 mM ATP (——, open signals). The buffers used were 75 mM Tris-acetate (Δ, ▲), Tris-HCl (○, ●), glycine–NaOH (□, ■), and Na_2HPO_4–NaOH (▽, ▼). Reaction time was 2 min. Other conditions were the same as assay II.

tivity. The enzyme activity in this fraction did not drop appreciably during storage at 4° for a 6-month period. The purification procedures are summarized in Table I. Protein concentrations were assayed by the method of Lowry *et al.* (1951).

Results

Thermostability of the Enzyme. The enzyme showed extreme thermostability. There was no detectable loss of activity at 80° for 1 hr, and only a 10% loss was apparent after incubation at 70° for 30 hr or at 90° for 1 hr (Figure 1). The effect of temperature on the velocity of the reaction was examined under low concentration of protein (3 μg/ml) and rather short reaction time (2 min) (Figure 2). The experiments revealed that the maximum velocity of the reaction was obtained at 85° and the velocity at 95° was 90% of that at 85°.

Effect of pH on the Enzyme Activity. The enzyme showed maximum activity around pH 9.0 at 30° (Figure 3A). At high ATP concentration (4.0 mM), the optimum pH seemed to be slightly higher and broader than at a low ATP concentration (0.2 mM). At 75° maximum activity was obtained at pH 8.4 (Figure 3B). There was also a slight shift of optimum pH at the high concentration of ATP. It was evident that the optimum pH of the enzyme was 0.5 unit lower at 75° than at 30°, suggesting the possibility that the inactivation of the enzyme at high temperatures proceeded more rapidly in a basic solution.

Kinetics with Respects to ATP and F-6-P. The PFK of *F. thermophilum* was found to follow simple Michaelis–Menten kinetics with respect to each substrate and demonstrated no substrate inhibition for ATP nor sigmoidal kinetics for F-6-P. When concentrations of ATP were varied against several fixed concentrations of F-6-P, Lineweaver–Burk plots were linear and gave a series of apparently parallel lines (Figure 4). When F-6-P concentrations were varied at fixed concentrations of ATP, double-reciprocal plots also exhibited essentially parallel lines (Figure 5). Parallel lines of Lineweaver–Burk plots were also obtained at 75°. These results seem to suggest that the two substrates, ATP and F-6-P, are not present simultaneously on the enzyme. This type of data is consistent with a "Ping-Pong" mechanism (Cleland, 1963). As the concentration of ATP was raised, the V_{max} and K_m for F-6-P increased; an increase in F-6-P resulted in a higher

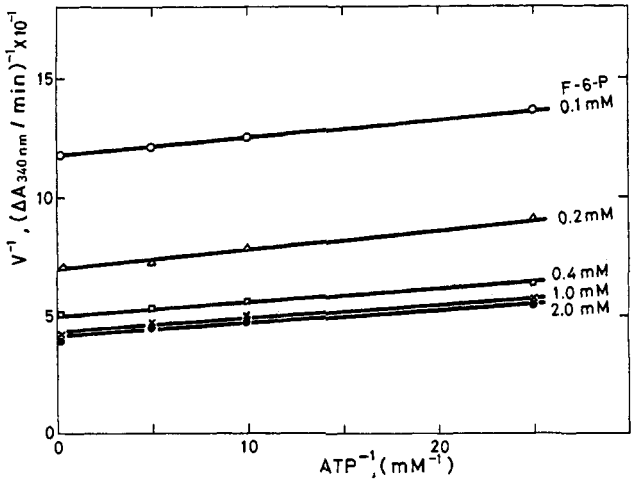

FIGURE 4: Effect of F-6-P on kinetics of ATP utilization. The activities were measured by assay I. 8×10^{-4} unit of the enzyme was added to the assay solution.

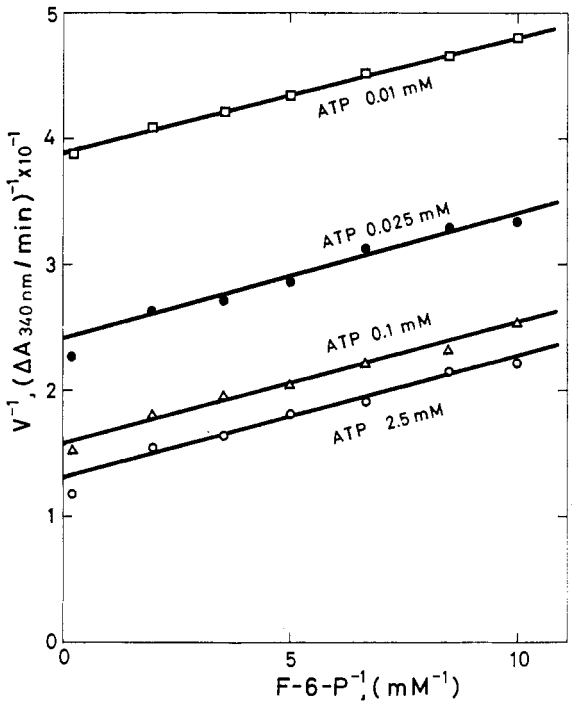

FIGURE 5: Effect of ATP on kinetics of F-6-P utilization. The activities were measured by assay I. 25×10^{-4} unit of enzymes was added to the assay solution.

V_{max} and K_m for ATP. K_m was estimated to be (2.0–3.0) $\times 10^{-5}$ M for ATP and (0.6–1.0) $\times 10^{-4}$ M for F-6-P under optimal conditions at 30°.

GTP, ITP, UTP, and CTP were all good substrates, showing similar hyperbolic kinetics (Table II).

Effects of Various Compounds on the Enzyme Activity. The effects of various compounds on the activity were tested. P_i, 3′,5′-AMP, creatine phosphate, glucose 1-phosphate, *N*-acetylglucosamine, pyruvate, succinate, citrate (each 2 mM), 2-phosphoglycerate, 3-phosphoglycerate (each 1 mM), and a mixture of twenty amino acids (each 0.5 mM) had no significant effect on the activity of the enzyme. PP_i demonstrated considerably strong inhibition, which was practically abolished at the elevated concentration of ATP or $MgCl_2$. Therefore, the mechanism of the inhibition was supposedly attributed to the Mg^{2+}-chelating action of PP_i.

TABLE II: K_m and V_{max} of Nucleotide Triphosphates Used as Substrates by PFK.[a]

Nucleotide	K_m (mM)	% of V_{max} Obtained with ATP
ATP	0.015	100
ITP	0.040	76
GTP	0.040	28
UTP	0.060	93
CTP	0.120	89

[a] Assays were performed at 1 mM F-6-P and 1 mM $MgCl_2$. Other conditions were described in the Experimental Section as assay I.

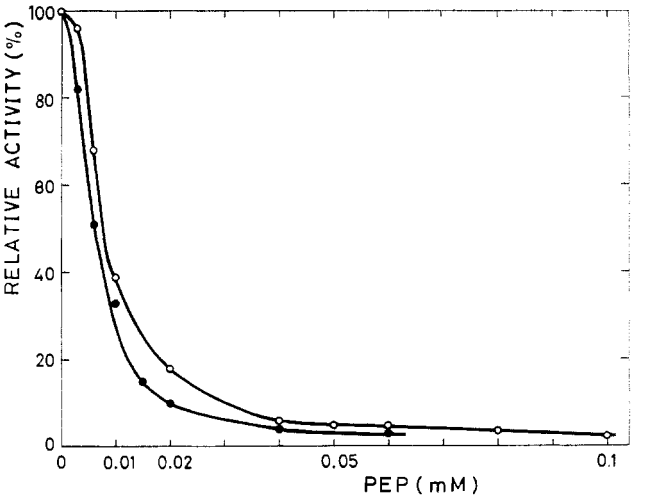

FIGURE 6: Effect of PEP concentration on the inhibition of PFK activity. Reaction mixtures contained 0.1 mM F-6-P, 0.2 mM ATP (●), or 4.0 mM ATP (○). Other conditions were the same as assay I.

ADP was a competitive inhibitor for ATP, and its K_i was estimated to be 0.1 mM. AMP was also a competitive inhibitor for ATP, but to a lesser degree. Both compounds decreased the V_{max} for F-6-P at a fixed concentration of ATP. Other purine nucleotide diphosphates, GDP and IDP were stronger competitive inhibitors than ADP, whereas pyrimidine nucleotide diphosphates, CDP and UDP, inhibited the reaction slightly (Table III). FDP was a competitive inhibitor for F-6-P of which K_i was 1.0 mM (Figure 9).

Effects of PEP and ADP on the Enzyme Activity. It was found that PEP was a strong inhibitor (Figure 6). The slopes of Hill plots of the results shown in Figure 6 were calculated to be 2.0 at both concentrations of ATP. The concentration of ATP had little effect on this inhibition; the apparent K_i of PEP was 4 μM at 0.2 mM ATP and 8 μM at 4.0 mM ATP, in the presence of 0.1 mM F-6-P. When PEP in the assay solution was rapidly changed into pyruvate by the addition of

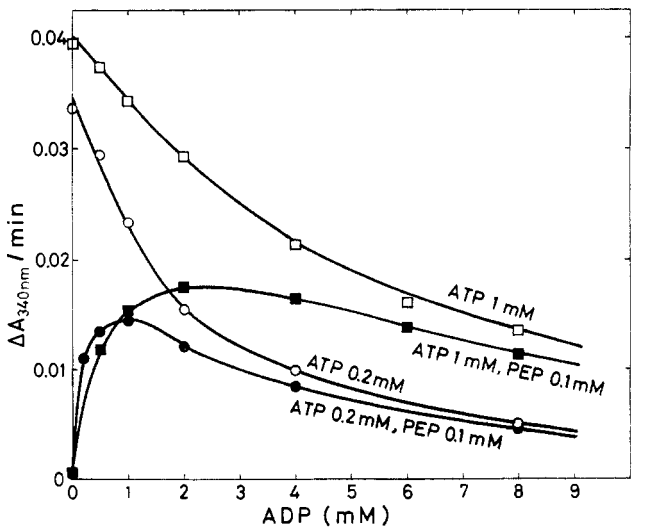

FIGURE 7: Effect of ADP concentration on PEP inhibition. Reaction mixtures contained 0.1 mM F-6-P and concentrations of ATP and PEP are indicated along the curves. Other conditions were the same as assay I.

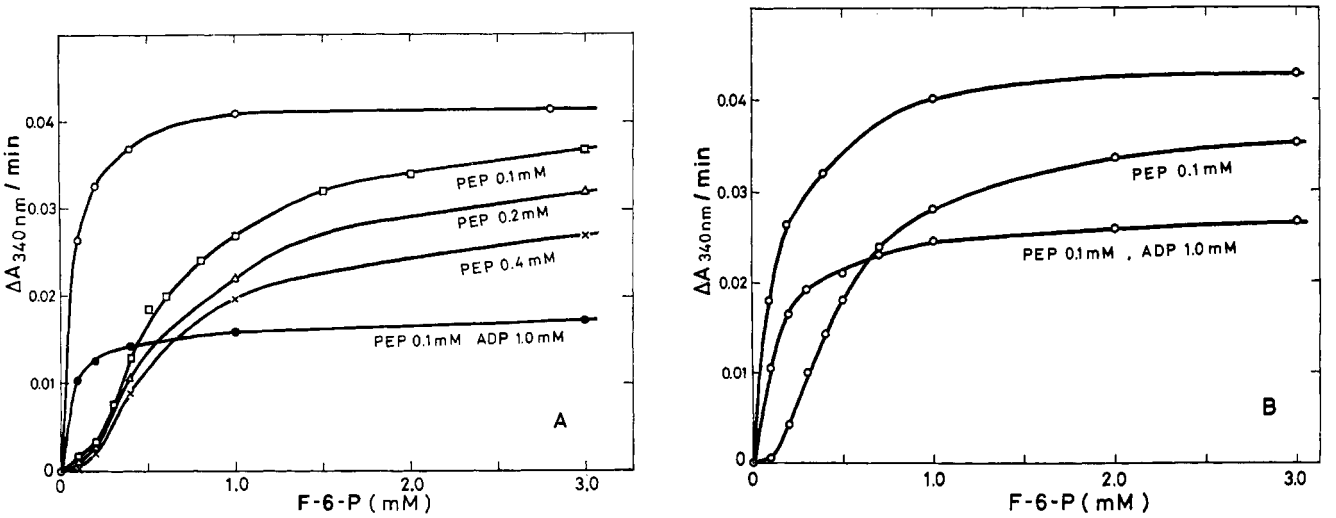

FIGURE 8: Effect of PEP on kinetics for F-6-P at 30°. Reaction mixtures contained 0.2 mM ATP (A) or 4.0 mM ATP (B) and indicated compounds along the curves. Other conditions were the same as assay I.

pyruvate kinase and a noninhibitory concentration of IDP, the velocity of the enzyme action immediately increased to nearly the original level in the absence of PEP.

PEP inhibition was partially relieved by the addition of ADP, and the effect of ADP concentration on PFK activity in the presence or absence of PEP was shown in Figure 7. In the presence of PEP, ADP restored PEP inhibition but higher concentrations of ADP inhibited the enzyme activity with nearly competitive manner. Evident is the dual effect of ADP; reversal of PEP inhibition and competitive inhibition for ATP.

With the addition of PEP, the normal kinetic curve for F-6-P was changed into a sigmoidal curve (Figure 8A,B). The Hill coefficients of all sigmoidal curves in the presence of PEP shown in Figure 7A,B were estimated to be 2.0. The cooperative nature of the enzyme in the presence of PEP was abolished by the addition of ADP and therefore activity of the enzyme was markedly stimulated at low concentrations of F-6-P. FDP had little effect on PEP inhibition, whereas it acted as a competitive inhibitor for F-6-P in the absence of PEP (Figure 9).

At 75° the particular effects of PEP and ADP were more pronounced than at 30° (Figure 10). The Hill coefficient was calculated to be 2.8 in the presence of PEP and higher concentration of F-6-P was required to overcome PEP inhibition. At 75° 1 mM ADP almost completely restored the enzyme activity from the inhibition by PEP.

TABLE III: K_i of Nucleotide Diphosphates.[a]

Nucleotide Diphosphate	K_i (mM)
ADP	0.10
GDP	0.03
IDP	0.09
CDP	>2.0
UDP	>2.0

[a] All assays were performed at 0.1 mM F-6-P and other conditions were the same as assay I described in the Experimental Section.

When other nucleotide triphosphates were used as substrates instead of ATP, the same cooperative kinetics were observed in the presence of PEP.

Specificity of ADP as Allosteric Effectors. No other compounds so far examined have exhibited the same allosteric effects as shown by PEP and ADP. Creatine phosphate, P_i, PP_i, AMP, 3′,5′-AMP, GDP, IDP, UDP, CDP, adenosine 5′-phosphosulfate, and α,β-methyleneadenosine diphosphate could not relieve PEP inhibition (Table IV). Consequently *F. thermophilum* PFK seems to distinguish both the adenine base and the precise electrostatic condition of the phosphate ester of the allosteric site.

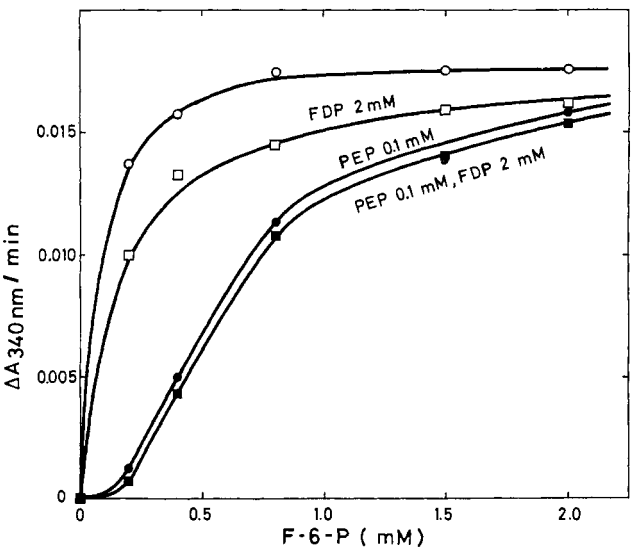

FIGURE 9: Effect of FDP on PEP inhibition. The enzyme activity was assayed by measuring ADP formed. Reaction mixtures (final volume, 0.4 ml) contained 1 mM ATP and other components were the same as assay II except indicated compound(s) along the curves in the figure. Reaction was initiated by addition of the enzyme. After incubation at 30° for 30 min it was terminated by addition of 0.1 ml of 1 N sodium hydroxide. Sulfic acid was added to neutralize the solution and subsequently NADH and PEP were added to final concentrations of 0.15 mM NADH and 0.5 mM PEP. The decrease of absorbancy at 340 nm was measured with adding excess amount of pyruvate kinase and lactate dehydrogenase.

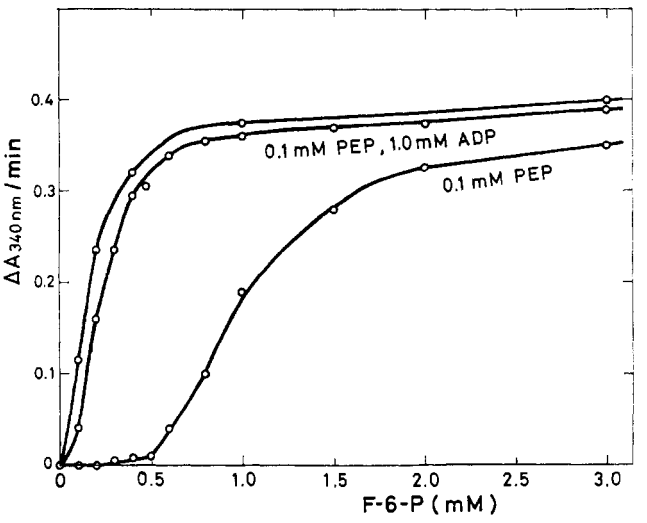

FIGURE 10: Effect of PEP on kinetics for F-6-P at 75°. The reaction mixtures contained 1.0 mM ATP and reactions were performed at 75° for 48 sec. Other conditions were the same as assay II described in the Experimental Section.

TABLE IV: Effects of Some Compounds on the Inhibition by PEP.[a]

	Effector	Act. (%)
A	None	100
	0.1 mM PEP	2
	0.1 mM PEP, 1.0 mM ADP	26
	0.1 mM PEP, 4.0 mM AMP	2
	0.1 mM PEP, 1.0 mM 3′,5′-AMP	4
	0.1 mM PEP, 10 mM P_i	4
	0.1 mM PEP, 1.0 mM PP_i	4
B	None	100
	0.1 mM PEP	2
	0.1 mM PEP, 1.0 mM ADP	29
	0.1 mM PEP, 1.0 mM GDP	4
	0.1 mM PEP, 1.0 mM IDP	4
	0.1 mM PEP, 1.0 mM CDP	4
	0.1 mM PEP, 1.0 mM adenosine 5′-phosphosulfate	2
	0.1 mM PEP, 1.0 mM α,β-methyleneadenosine diphosphate	3

[a] Reaction mixtures contained 0.1 mM F-6-P, 0.2 mM (A) or 4.0 mM (B) ATP. Other conditions were the same as assay I.

Discussion

This enzyme seems to be one of the most thermostable enzymes which have been found in cells of extremely thermophilic bacteria, such as aldolase of *Thermus aquaticus* (Freeze and Brock, 1970), aspartokinase of thermophile AT-62 (Saiki and Arima, 1970), phosphoglucomutase (Yoshizaki *et al.*, 1971), fructose diphosphatase (Yoshida and Oshima, 1971), glyceroaldehyde-3-phosphate dehydrogenase, aldolase, and DNA-dependent RNA polymerase of *F. thermophilum* (T. Oshima, 1971, personal communication). These enzymes have their optimum temperatures above 70° and are quite stable at 70° or above. But few are known about molecular basis of their extreme thermostabilities. Physicochemical study of purified PFK has been started to elucidate its thermostability and allosterism.

In the absence of PEP, the reaction rate of PFK from *F. thermophilum* followed simple Michaelis–Menten kinetics. The effect of concentration of substrate on the rate of the reaction varied with the concentration of the other substrate in such a manner as to give rise to parallel lines in a double-reciprocal plot. Similar kinetics has been observed with PFK from yeast (Viñuela *et al.*, 1963), muscle (Sols and Salas, 1966), calf lens (Lou and Kinoshita, 1967), *Dictyostelium discoideum* (Baumann and Wright, 1968), human muscle, erythrocytes (Layzer *et al.*, 1969), and rabbit skeletal muscle (Uyeda, 1970). This type of kinetics is consistent with, but not proof of, a "Ping-Pong" mechanism, *i.e.*, the first product is released from the enzyme before the second substrate combines (Cleland, 1963).

In the presence of PEP, the activity of *F. thermophilum* PFK varied as a sigmoidal function of F-6-P and ADP reverted kinetics from sigmoidal to hyperbolic. Recently concerted feedback inhibition of aspartokinases from *Bacillus stearothermophilus* (Kuramitsu, 1970) and an extreme thermophile AT-62 (Saiki and Arima, 1970) were reported. These enzymes completely lose their allosteric nature at temperatures above 60° (the former) or 75° (the latter) which correspond to the upper growth temperatures of each thermophile. *F. thermophilum* PFK exhibited an allosterism at 75° which is near the optimum growth temperature for this organism; it seems to be one of the most thermostable allosteric enzymes so far reported. Considering the low concentrations required for effects of ADP and PEP, it appears reasonable to assume that the enzyme actually plays an important role in the regulation of the Embden–Meyerhof pathway in this organism.

PFK usually exhibits complex allosteric properties. The degree of the complexity, however, depends on the source of the enzyme. For example, the enzymes from higher organisms and yeast show sigmoidal kinetics with respect to F-6-P and substrate inhibition by ATP, and this inhibition is counteracted by 3′,5′-AMP, ADP, P_i, F-6-P or FDP. ATP does not inhibit PFK from *Escherichia coli* (Blangy *et al.*, 1968), *Clostridium pasteurianum* (Uyeda and Kurooka, 1970), *Aerobacter aerogenes* (Sapico and Anderson, 1969), *Dictyostelium discoideum* (Baumann and Wright, 1968), or *Arthrobacter crystallopoietes* (Ferdinandus and Clark, 1969). The latter two enzymes seem to lack any of the allosteric properties so far reported. Since *F. thermophilum* PFK is not inhibited by ATP, it seems reasonable to conclude that the concentration of ATP is probably not important in the regulation of the Embden–Meyerhof pathway in this organism. Perhaps more significant in regulation is the observation that the activity of *F. thermophilum* PFK is affected by the concentration of F-6-P, FDP, PEP, and ADP. Furthermore it has been revealed that the activity of partially purified fructose diphosphatase from the same bacterium, which catalyzes the hydrolysis of FDP to F-6-P, is activated by PEP and inhibited by AMP (Yoshida and Oshima, 1971). These observations are consistent with the interpretation that the concentrations of PEP, ADP, and AMP in the cells are among essential parameters which determine the direction and the velocity of the Embden–Meyerhof pathway in this organism.

The *F. thermophilum* PFK seems to be different from that of various other organisms. One significant difference is its extreme thermostability and another is the sigmoidal kinetics

for F-6-P in the presence of PEP. Activity of allosteric PFK from other organisms follows sigmoidal curve with respect to F-6-P concentration, and effectors of the enzyme vary the apparent K_m value for F-6-P. For example, mammalian PFK is inhibited strongly by a high concentration of ATP and this inhibition can be attributed to the decreased affinity of the enzymes for F-6-P, as shown by the sigmoidal kinetics. At low concentrations of ATP, however, the kinetic pattern is nearly normal. *E. coli* PFK exhibits a sigmoidal curve with respect to F-6-P at any concentrations of ATP. In contrast, *F. thermophilum* PFK is inhibited strongly by PEP, as shown by the sigmoidal curve with respect to F-6-P. In the absence of PEP, however, the kinetic pattern is normal at any concentrations of ATP. Therefore it seems likely that the role of ATP as an allosteric inhibitor of the enzymes from other organisms are replaced by PEP in *F. thermophilum* PFK.

ADP acts as a competitive inhibitor for ATP and an allosteric effector in the presence of PEP which increases the affinity of F-6-P to the enzyme and also lowers the Hill coefficient for F-6-P. The specificity of allosteric sites on the enzyme seems to be more precise than that of catalytic sites, since no other compounds so far tested have shown the same allosteric effect as ADP whereas some other nucleotide diphosphates act as competitive inhibitors.

It is possible to consider that the distinct natures of *F. thermophilum* PFK may reflect one or both of two physiological necessities for the growth of this bacterium, that is, high temperature and gluconeogenesis. Sanwal proposed that the effects of PEP and ADP on the *E. coli* PFK are involved in the regulation of the Embden–Meyerhof pathway to fulfill both an anabolic and a catabolic function, that is, an "amphibolic" pathway (Sanwal, 1970). Krzanowski and Matschinsky discussed the possibility that increasing concentrations of PEP, 3-phosphoglycerate, and citrate in liver tissue caused the inhibition of PFK, turned off glycolysis, and switched on gluconeogenesis (Krzanowski and Matschinsky, 1969). Since the cells of *F. thermophilum* grow well using Casamino Acids as a single carbon source, it seems possible that the inhibition of PFK activity by PEP is more or less a common feature of this enzyme from tissues in which the Embden–Meyerhof pathway is regulated as an amphibolic one. The observations that PFK from the pea seed is inhibited strongly by PEP are consistent with this possibility, for in these tissues the Embden–Meyerhof pathway may be used as an amphibolic one (Kelly and Turner, 1970). There is also another possibility that the features of *F. thermophilum* PFK will be commonly observed in any extreme thermophile, since the same characteristics of PFK were observed in another extreme thermophile isolated from a hot spring, in Atagawa, Shizuoka-ken, by T. Saiki (M. Yoshida, 1971, unpublished data). Nevertheless more detailed studies of the comparative biochemistry of carbohydrate metabolism will be necessary in order to test for the hypothetical conclusions mentioned above.

Acknowledgment

The author thanks Dr. T. Oshima for supplying the strain used in this work and for his invaluable constant advice. Grateful appreciation is also acknowledged to Professor K. Imahori for stimulating discussions and encouragements.

References

Atkinson, D. E. (1966), *Annu. Rev. Biochem. 35*, 85.
Baumann, P., and Wright, B. E. (1968), *Biochemistry 7*, 3653.
Blangy, D., Buc, H., and Monod, J. (1968), *J. Mol. Biol. 31*, 13.
Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 104.
Ferdinandus, J., and Clark, J. B. (1969), *Biochem. J. 113*, 735.
Freeze, H., and Brock, T. D. (1970), *J. Bacteriol. 101*, 541.
Kelly, G. J., and Turner, J. F. (1968), *Biochem. Biophys. Res. Commun. 30*, 195.
Krzanowski, J., and Matschinsky, F. (1969), *Biochem. Biophys. Res. Commun. 34*, 816.
Kuramitsu, H. K. (1970), *J. Biol. Chem. 245*, 2991.
Layzer, R. B., Rowland, L. P., and Bank, W. J. (1969), *J. Biol. Chem. 244*, 3823.
Lou, M. F., and Kinoshita, J. H. (1967), *Biochim. Biophys. Acta 141*, 547.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Oshima, T., and Imahori, K. (1971), *J. Gen. Appl. Microbiol. 17*, 491.
Saiki, T., and Arima, K. (1970), *Agr. Biol. Chem. 34*, 1762.
Sanwal, B. D. (1970), *Bacteriol. Rev. 34*, 20.
Sapico, V., and Anderson, R. L. (1969), *J. Biol. Chem. 244*, 6280.
Sols, A., and Salas, M. L. (1966), *Methods Enzymol. 9*, 440.
Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.
Uyeda, K. (1970), *J. Biol. Chem. 245*, 2268.
Uyeda, K., and Kurooka, S. (1970), *J. Biol. Chem. 245*, 3315.
Viñuela, E., Salas, M. L., and Sols, A. (1963), *Biochem. Biophys. Res. Commun. 12*, 140.
Wood, W. A. (1966), *Annu. Rev. Biochem. 35*, 521.
Yoshida, M., and Oshima, T. (1971), *Biochem. Biophys. Res. Commun. 45*, 495.
Yoshida, M., Oshima, T., and Imahori, K. (1971), *Biochem. Biophys. Res. Commun. 43*, 36.
Yoshizaki, F., Oshima, T., and Imahori, K. (1971), *J. Biochem. (Tokyo) 69*, 1083.