Key Role of Phosphoenolpyruvate in the Regulation of Glycolysis-Gluconeogenesis in *Thermus thermophilus* HB 8

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The *in vivo* concentrations of metabolites and the level of enzymes were measured to elucidate the regulatory mechanism by phosphoenolpyruvate of glycolysis-gluconeogenesis in an extreme thermophile, *Thermus thermophilus* HB8. Glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate were low, and phosphoenolpyruvate was high in the cells growing on polypeptone-yeast extract (PY cells). On the contrary, phosphoenolpyruvate was low and hexose phosphates were high in the cells growing on glucose-polypeptone-yeast extract (GPY cells). The difference in the values of adenylate energy charge was not observed between PY and GPY cells. Among various enzymes, phosphofructokinase and fructose 1,6-bisphosphatase level changed markedly; the former was high in GPY cells and the latter in PY cells. It is reasonably considered that PY cells may turn on gluconeogenesis and GPY cells on glycolysis. The *in vivo* regulatory functions of key enzymes, phosphofructokinase, fructose 1,6-bisphosphatase, and pyruvate kinase, and the important roles of phosphoenolpyruvate and fructose 6-phosphate were discussed from these findings.

An important role of PEP in the regulation of allosteric enzymes, PFK (EC 2.7.1.11) and FBPa (EC 3.1.3.11), of an extreme thermophile, *Thermus thermophilus* HB8,^{1,2)} was reported by Yoshida *et al.*^{3~6)} In many organisms, PFK⁷⁾ and PK⁸⁾ are activated by AMP and inhibited by ATP, while FBPa⁹⁾ is inhibited by AMP. It is generally accepted that the glycolytic-gluconeogenetic pathway is amphibolic¹⁰⁾ and functions both catabolically and anabolically depending on the *in vivo* concentration ratio of ATP to AMP, but not on the change of PEP concentration. The properties of two enzymes, which catalyze the conversion of PEP, have been reported in our previous papers^{11,12)}: the thermophile PK (EC 2.7.1.40) shows a sigmoidal rate curve for PEP and is allosterically activated by sugar

phosphates; while the PEP carboxykinase (EC 4.1.1.49) is not a regulatory enzyme.

To confirm the *in vivo* regulatory functions of these allosteric enzymes, first, the concentration changes of intermediates including PEP and sugar phosphates were examined in this study. The *T. thermophilus* cells were reported to metabolize glucose, galactose, maltose, and lactose, but not to grow on glucose as a single carbon source.²⁾ In this paper, the metabolite concentrations in the cells grown on polypeptone-yeast extract (PY medium, commonly used for the cultivation of the thermophile) were compared with those in the cells grown on glucose and half the concentration of polypeptone-yeast extract (GPY medium).

Second, the enzyme level in the cells grown in PY and GPY media was compared with each other for various enzymes of the glycolytic pathway and the tricarboxylic acid cycle.

MATERIALS AND METHODS

Preparation of crude extract for assay of metabolite level
Centrifugation method. The preculture (2 ml, PY

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Abbreviations used: PEP, phosphoenolpyruvate; PFK, phosphofructokinase; FBPa, fructose 1,6-bisphosphatase; PK, pyruvate kinase.

medium) of *T. thermophilus* was added to 100 ml of PY medium, and the thermophile was grown at 75°C with shaking. The culture was rapidly chilled to 0°C in an ice bath when absorbance at 600 nm reached 0.43, and the cells were harvested by centrifugation at $15,000 \times g$ for 15 min. To a centrifuge tube was added 3 ml of 0.3 N HClO₄ containing 1 mM EDTA, and the cells were mixed thoroughly with pipettes. The suspension was centrifuged at $15,000 \times g$ for 25 min, and 0.3 ml of 1.5 N K₂CO₃ was added to the supernatant (final pH, 6.9). The precipitate formed was removed by centrifugation at $15,000 \times g$ for 15 min. The supernatant (PY extract, 3.31 ml) was divided into several parts and stored at -80°C until use. All operations were performed at 0°C. GPY extract (3.2 ml) was obtained by the same method as described above except that GPY medium was used for the cultivation.

TABLE I. COMPOSITION OF MEDIA

Solutions A (adjusted to pH 7.0 with NaOH), B, and C were autoclaved separately.

Component	Concentration (%)		
	PY	GPY	G
A. Polypeptone	0.8	0.4	—
Yeast extract	0.4	0.2	—
NaCl	0.3	0.3	0.3
Metal salt mixture A ^a	0.0025	0.0025	0.0025
Metal salt mixture B ^a	0.0025	0.0025	0.0025
B. Glucose	—	0.5	0.5
C. K ₂ HPO ₄	—	0.3	0.3
KH ₂ PO ₄	—	0.1	0.1
Vitamine mixture ^b	—	—	1.0
(NH ₄) ₂ SO ₄	—	—	0.2

^a Compositions of metal salt mixtures A and B were described previously.¹²⁾

^b Vitamine mixture contained the following components in the volume of 100 ml: thiamine·HCl, riboflavin, pyridoxine·HCl, pyridoxal·HCl, calcium pantothenate, and nicotinic acid, 10 mg each; *p*-aminobenzoic acid, 2 mg; biotin and folic acid, 1 mg each.

Table I shows the compositions of PY, GPY, and G media. About 90% of carbon is derived from amino acids and nucleotides for PY medium, and about 50% for GPY medium; on the contrary, all carbon from glucose for G medium.

Filtration method. Crude extracts were prepared by a modification of the method of Lowry *et al.*¹³⁾ The preculture (0.7 ml, PY medium) of *T. thermophilus* was added to 20 ml of PY medium, and the thermophile was grown at 75°C with shaking. When absorbance at 600 nm reached 0.4, the cells were collected rapidly

on a 47-mm diameter 8.0 μ m membrane filter (Sartorius Type SM 12801) with suction. As soon as the liquid had been removed (about 90 sec), the filter was frozen in a Petri dish containing liquid nitrogen, and then put in a centrifuge tube previously chilled to 0°C. To this tube was added 2 ml of 0.3 N HClO₄ containing 1 mM EDTA, and the tube was mixed and centrifuged at $15,000 \times g$ for 25 min. The supernatant was neutralized by the addition of 0.3 ml of 1.5 N K₂CO₃ (final pH, 7.0), and the precipitate formed was removed by centrifugation at $15,000 \times g$ for 15 min. The supernatant (PY extract, 1.7 ml) was obtained and stored at -80°C until use. GPY extract (1.2 ml) was prepared by the same method as described above except that GPY medium was used for the cultivation and the cells were collected at 0.27 of A_{600} .

Attempts to filtrate the culture with membrane filters ranging in pore size from 0.65 to 3.0 μ m failed because of the unknown viscous substances the thermophile produced. Practically all the cells in the culture were retained on a 8.0- μ m membrane filter, judging by A_{600} of the filtrate.

Measurement of metabolite level. The concentrations of various intermediates were determined by measuring NADH or NADPH concentration with a Hitachi Model MPF-3 fluorescence spectrophotometer. Table II shows the assay systems of metabolites. PY or GPY extract (50 μ l) was added to a quartz cuvette (3 $\times$ 10 mm) containing 450 μ l of the assay mixture, and mixed. After the cuvette was incubated for about 10 min in a cuvette room, fluorescence intensity at 450 nm (slit width, 16 nm) was recorded (excitation wavelength, 340 nm; slit width, 16 nm). Then 0.5 μ l of the coupling enzyme was added, and fluorescence intensity was recorded after the intensity reached a constant value. The increase or decrease in fluorescence intensity was corrected by the second addition of 0.5 μ l of the coupling enzyme. All assays were performed at 25.0°C with deionized and glass-distilled water.

The plots of the fluorescence intensities vs. NADH concentrations gave a straight line below 9 μ M, and intensity was converted into NADH (NADPH) concentration. The volume of cell water in 1 ml of the culture was assumed to be 0.25 μ l when A_{600} was 0.3 according to the procedure with *Escherichia coli*.¹³⁾ Losses of metabolites in the course of preparation of PY or GPY extract were neglected.

Preparation of crude extract for measurement of enzyme level. To 500 ml of PY medium was added 3 ml of the preculture (PY medium), and the *T. thermophilus* cells were cultivated at 75°C with shaking. When absorbance at 600 nm reached about 0.7, the cells were harvested by centrifugation at $15,000 \times g$ for 40 min. The cells were suspended in 50 mM Tris-HCl

TABLE II. ASSAY SYSTEMS OF METABOLITES

The abbreviations used: P, phosphate group; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; DHAP, dihydroxyacetone phosphate; GA3P, D-glyceraldehyde 3-phosphate; FBP, fructose 1,6-bisphosphate; Pyr, pyruvate.

Metabolites	Composition of assay mixture ^a	Enzymes
G6P, F6P, G1P	2 mM MgCl ₂ , 15 mM KCl, 0.08 mM NADP ⁺	G6P dehydrogenase, phosphoglucose isomerase, phosphoglucomutase
DHAP plus GA3P, FBP	4 μ M NADH	Glycerol 3-P dehydrogenase, triose P isomerase, aldolase
PEP	0.2 mM ADP, 2 mM MgCl ₂ , 75 mM KCl, 4 μ M NADH	Lactate dehydrogenase, PK
Pyr, AMP	0.01 mM PEP, 3 μ M ATP, 2 mM MgCl ₂ , 75 mM KCl, 4 μ M NADH	Lactate dehydrogenase, PK, myokinase
ATP	0.1 mM glucose, 5 mM MgCl ₂ , 0.2 mM EDTA, 0.03 mM NADP ⁺	G6P dehydrogenase, hexokinase
ADP	0.01 mM PEP, 2 mM MgCl ₂ , 75 mM KCl, 4 μ M NADH	Lactate dehydrogenase, PK

^a All assay mixtures contained 200 mM imidazole-HCl buffer (pH 7.0).

buffer (pH 7.5) containing 10 mM MgCl₂, 100 mM KCl, 0.2 mM EDTA, and 0.1 mM dithiothreitol, washed, and collected by centrifugation. The cells were resuspended in 10 ml of the same buffer, and disrupted by sonic oscillation at 20 kHz, 100 W for 1 min with a Branson Model B-12 Sonifier cell disruptor. Cell debris precipitated by centrifugation at 18,000 $\times g$ for 40 min was discarded, and the supernatant was used as PY extract. The protein concentration of PY extract, estimated by the method of Warburg and Christian,¹⁴ was 3.63 mg/ml ($A_{280}/A_{260}=0.66$). GPY extract was prepared by the same method as described above except that the cells were harvested at 0.5 of A_{600} . The protein concentration of GPY extract was estimated to be 1.92 mg/ml ($A_{280}/A_{260}=0.66$). After the addition of glycerol (50%), both PY and GPY extracts were stored at -20°C until use.

Assay of various enzymes. Table III shows the compositions of the assay mixtures. Enzyme activity was measured under similar conditions to those described in the preceding paper¹² unless otherwise noted in the footnote of Table III. The reaction was started by the addition of 0.5–10 μ l of the extract containing below 0.002 unit of the enzyme. One unit of enzyme activity is defined as the amount of the enzyme that catalyzes the formation of 1 μ mol of the product per min.

Reagents. Glucose 1,6-bisphosphate (tetracyclohexylammonium salt), glycerate 2,3-bisphosphate (pentacyclohexylammonium salt), NAD⁺, NADP⁺ (Na₂ salt), hexokinase (yeast), glucose 6-phosphate dehydrogenase (yeast), phosphoglucomutase (rabbit muscle), phosphoglucose isomerase (yeast), aldolase (rabbit muscle), triose phosphate isomerase (rabbit

muscle), glycerol 3-phosphate dehydrogenase (rabbit muscle), glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle), enolase (rabbit muscle), PK (rabbit muscle), myokinase (hog muscle), malate dehydrogenase (pig heart), and isocitrate dehydrogenase (pig heart) were the products of Boehringer Mannheim GmbH. Grade III imidazole of Sigma Chemical Co. was used for fluorometric assays. Thiamine pyrophosphate, 5,5'-dithiobis (2-nitrobenzoic acid), pyridoxal·HCl, and calcium pantothenate were purchased from Nakarai Chemicals, Ltd. Riboflavin and pyridoxine·HCl were obtained from Wako Pure Chemical Industries, Ltd., and thiamine·HCl, nicotinic acid, biotin, and folic acid from Daiichi Pure Chemicals Co., Ltd. *p*-Aminobenzoic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. Other reagents used were of the same maker as noted in the preceding paper.¹²

RESULTS AND DISCUSSION

Comparison of growth curves

Figure 1 shows the growth curves of *T. thermophilus* in PY, GPY, and G media. The cells grew logarithmically in PY medium, and the final absorbance at 600 nm approached 2.0. The growth curve in GPY medium resembled that in PY medium except for a longer period of a lag phase. The mean generation time under these conditions was calculated to be about 1 hr for both PY and GPY media. On the contrary, no growth was observed for G medium. The rate of growth decreased with

TABLE III. ASSAY SYSTEMS OF ENZYMES
The abbreviations used are shown in the legend of Table II.

Enzyme	Composition of assay mixture (0.5 ml)
Phosphoglucomutase	100 mM glycine-NaOH buffer (pH 9.0), 2 mM G1P, 0.2 mM glucose 1,6-P ₂ , 10 mM MgCl ₂ , 1 mM dithiothreitol, 0.5 mM NADP ⁺ , 0.35 unit of G6P dehydrogenase
Hexokinase	100 mM Tris-HCl buffer (pH 7.5), 50 mM glucose, 2 mM ATP, 10 mM MgCl ₂ , 0.5 mM NADP ⁺ , 0.35 unit of G6P dehydrogenase
G6P dehydrogenase	86 mM triethanolamine-HCl buffer (pH 7.6), 1 mM G6P, 10 mM MgCl ₂ , 0.4 mM NADP ⁺
Phosphoglucose isomerase	100 mM Tris-HCl buffer (pH 7.5), 2 mM F6P, 10 mM MgCl ₂ , 0.5 mM NADP ⁺ , 0.35 unit of G6P dehydrogenase
PFK	100 mM Tris-HCl buffer (pH 8.5), 1 mM F6P, 1 mM ATP, 10 mM MgCl ₂ , 10 mM KCl, 1 mM dithiothreitol, 0.24 mM NADH, 0.09 unit of aldolase, 4.5 units of triose P isomerase, 0.36 unit of glycerol 3-P dehydrogenase
FBPase	100 mM Tris-HCl buffer (pH 8.5), 2 mM FBP, 10 mM MgCl ₂ , 0.5 mM EDTA, 0.5 mM NADP ⁺ , 0.7 unit of phosphoglucose isomerase, 0.35 unit of G6P dehydrogenase
Aldolase	100 mM Tris-HCl buffer (pH 7.5), 2 mM FBP, 0.24 mM NADH, 4.5 units of triose P isomerase, 0.36 unit of glycerol 3-P dehydrogenase
Triose P isomerase ^a	228 mM triethanolamine-HCl buffer (pH 7.9), 1.5 mM GA3P, 0.24 mM NADH, 0.08 unit of glycerol 3-P dehydrogenase
GA3P dehydrogenase ^a	100 mM Tris-HCl buffer (pH 8.5), 3 mM GA3P, 8.5 mM arsenate (Na ₂ salt), 5 mM NAD ⁺ , 5 mM cysteine
3-Phosphoglycerate kinase	100 mM Tris-HCl buffer (pH 7.5), 5 mM glycerate 3-P, 2 mM ATP, 10 mM MgCl ₂ , 1.6 mM EDTA, 0.24 mM NADH, 1.6 units of GA3P dehydrogenase
Phosphoglyceromutase	100 mM Tris-HCl buffer (pH 7.5), 10 mM glycerate 3-P, 0.5 mM glycerate 2,3-P ₂ , 1 mM ADP, 10 mM MgCl ₂ , 50 mM KCl, 0.24 mM NADH, 0.08 unit of enolase, 0.4 unit of PK, 5.5 units of lactate dehydrogenase
Enolase	100 mM Tris-HCl buffer (pH 7.5), 4 mM glycerate 2-P, 1 mM ADP, 10 mM MgCl ₂ , 50 mM KCl, 0.24 mM NADH, 0.4 unit of PK, 5.5 units of lactate dehydrogenase
PK ^b	
Pyr dehydrogenase	100 mM Tris-HCl buffer (pH 7.5), 2 mM Pyr, 0.1 mM CoA, 1 mM NAD ⁺ , 10 mM MgCl ₂ , 0.5 mM thiamine pyrophosphate, 1 mM cysteine
PEP carboxylase ^c	100 mM Tris-HCl buffer (pH 8.5), 2 mM PEP, 10 mM NaHCO ₃ , 10 mM MgCl ₂ , 0.02 mM acetyl-CoA, 0.24 mM NADH, 6.0 units of malate dehydrogenase
Pyr carboxylase ^d	100 mM Tris-HCl buffer (pH 8.0), 4 mM Pyr, 2 mM ATP, 20 mM KHCO ₃ , 10 mM MgCl ₂ , 0.24 mM NADH, 6.0 units of malate dehydrogenase
PEP carboxykinase ^e	
Fumarase ^f	100 mM Tris-HCl buffer (pH 7.3), 20 mM malate
Malate dehydrogenase	100 mM Tris-HCl buffer (pH 8.5), 0.2 mM oxalacetate, ^g 0.24 mM NADH
Citrate synthase ^h	100 mM Tris-HCl buffer (pH 8.0), 0.1 mM acetyl-CoA, 0.2 mM oxalacetate, ^g 0.2 mM 5,5'-dithiobis(2-nitrobenzoic acid) ⁱ
Aconitase	100 mM Tris-HCl buffer (pH 7.5), 2 mM citrate, 10 mM MnCl ₂ , 0.25 mM NADP ⁺ , 0.02 unit of isocitrate dehydrogenase
Isocitrate dehydrogenase	100 mM Tris-HCl buffer (pH 7.5), 1 mM isocitrate, 10 mM MnCl ₂ , 0.25 mM NADP ⁺

^a The reaction was started by the addition of GA3P.

^b Experimental conditions followed assay method A described previously.¹²⁾

^c The control reaction was carried out in the absence of PEP.

^d The control reaction was carried out in the absence of ATP.

^e The activity of PEP formation was measured by the same method as described previously.¹¹⁾ The control reaction was carried out in the absence of ATP.

^f The activity was determined by measuring the rate of increase in absorbance at 250 nm due to fumarate ($\epsilon = 1450 \text{ M}^{-1} \cdot \text{cm}^{-1}$).¹⁵⁾

^g Oxalacetate solution was freshly prepared by the method of Lane *et al.*¹⁶⁾

^h The activity was determined by measuring the rate of increase in absorbance at 412 nm due to mercaptide ion ($\epsilon = 13,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$).¹⁷⁾ The control reaction was carried out in the absence of oxalacetate.

ⁱ Freshly prepared solution (250 mM Tris-HCl buffer, pH 8.0) was used.

FIG. 1. Growth Curves of *T. thermophilus* in PY (○), GPY (△), and G (□) Media.

When the absorbance at 600 nm reached about 1.0, a sample (0.15 ml) of the preculture (PY medium) was transferred to a 500-ml Sakaguchi flask containing 200 ml of PY, GPY, or G medium. The thermophile was cultivated at 75°C with shaking (about 100 strokes per min). At intervals, the bacterial growth was followed by measuring the absorbance at 600 nm with a spectrophotometer (water in reference cell). The composition of each medium is shown in Table I.

an increase in a ratio of glucose to polypeptone plus yeast extract. These findings are consistent with those reported by Oshima and Imahori²⁾ except for the slower rate of growth because of the differences in growth conditions. In the subsequent experiments, the levels of enzymes and metabolites were measured for the cells collected in the logarithmic phase.

Changes of metabolite level

The intracellular concentrations of metabolites were measured for both the cells growing in PY and GPY media, and the results are shown in Figs. 2 and 3. The whole patterns of metabolite level obtained by the two methods resembled each other, except for several metabolites. Hexose phosphates such as glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate, and pyruvate were high in the *T. thermophilus* cells growing in GPY medium, while PEP was high in those in PY medium. Among various intermediates, PEP and fructose 1,6-bisphosphate changed markedly in concentration. The values of adenylate energy charge¹⁸⁾ in the cells grow-

FIG. 2. Metabolite Level Measured by Centrifugation Method.

The abbreviations used are shown in the legend of Table II.

FIG. 3. Metabolite Level Measured by Filtration Method.

The abbreviations used are shown in the legend of Table II.

ing logarithmically in PY and GPY media were calculated to be the same value of 0.99 (filtration method).

Two methods were used for the collection of the cells, and some differences were observed. Centrifugation method is considered to give more accurate concentrations than filtration method, because only a small volume of culture

could be filtrated by the latter method and hence the metabolite concentrations in a cuvette was low. The main disadvantage of centrifugation method is the possibility that the *in vivo* metabolite concentrations may vary during centrifugation. For example, Lowry *et al.*¹³⁾ reported in *E. coli* that ATP, glucose 6-phosphate, and fructose 1,6-bisphosphate obtained by centrifugation method were 0, 5, and 2%, respectively, of those obtained by rapid filtration. The far more valid data, however, were obtained for the thermophile than for *E. coli*.

The intracellular concentrations of metabolites in *T. thermophilus* were compared with those reported in other bacteria (Table IV). The metabolite patterns of PY and GPY extracts resemble those for *E. coli* grown on amino acids and glucose, respectively, and it seems probable that the *T. thermophilus* cells growing in PY medium switch on gluconeogenesis, and in GPY medium on glycolysis. The common features of metabolite changes in several microorganisms reported^{13,21)} are pointed out as follows: first, hexose phosphates and PEP change inversely; second, the adenylate energy charge is kept constant when the conversion between glycolysis and gluconeogenesis occurs.

Changes of enzyme level

Enzyme activities per g of protein were measured for both PY and GPY extracts, and Table V shows the comparison of enzyme level. Among various enzymes of the glycolytic pathway and the tricarboxylic acid cycle, the FBPase activity was high in PY extract, while the activities of PFK, enolase, triose phosphate isomerase, *etc.* were high in GPY extract. In particular, the changes of FBPase and PFK level were great. Similar reciprocal effect of carbon sources on the level of these two enzymes was also reported in *Saccharomyces cerevisiae*.²²⁾ The *E. coli* FBPase was reported to be induced under gluconeogenic conditions²³⁾ and repressed under glycolytic conditions.²⁴⁾

No changes were observed for PK, PEP carboxykinase, PEP carboxylase, and so on. Constitutive PK's were reported in *E. coli* K12 (the ribose 5-phosphate activated enzyme),²⁵⁾ *Acetobacter xylinum*,²⁶⁾ *Bacillus subtilis*,²⁷⁾ *B. licheniformis*,²⁸⁾ *Rhodospirillum rubrum*,²⁹⁾ and *S. cerevisiae*.³⁰⁾ Inducible PK's, on the contrary, were reported in *E. coli* K12 (the fructose 1,6-bisphosphate activated enzyme),^{25,31)} *Alcaligenes eutrophus*,³²⁾ *Rhodopseudomonas sphaeroides*,³³⁾ *Candida utilis*,³⁴⁾ *Mucor rouxii*,^{34,35)}

TABLE IV. COMPARISON OF INTRACELLULAR METABOLITE CONCENTRATIONS

Carbon sources are as follows: G, glucose; AA, amino acids; T5, mixture of lactose, polypeptone, beef extract, phyton peptone, and yeast extract. The abbreviations used are shown in the legend of Table II.

Metabolite	Intracellular concentration (mm)					
	<i>E. coli</i> ^a		<i>E. coli</i> ^b	<i>Streptococcus lactis</i> ^c	<i>T. thermophilus</i>	
	G	G	AA	T5	PY	GPY
G1P	—	—	—	—	0.07	0.19
G6P	0.74	—	—	—	0.31	1.25
F6P	—	—	—	—	0.07	0.25
FBP	2.64	1.68~2.06	0.25	18.3	0.03	0.68
DHAP plus GA3P	0.19	—	—	—	~0	0.07
PEP	0.08	0.18~0.30	0.62	0.76	0.17	~0
Pyr	0.36	—	—	—	0.64	1.07
ATP	2.44	—	—	—	2.74	2.88
ADP	0.76	—	—	2.4	0.04	0.03
AMP	0.14	—	—	—	~0	~0

^a Calculated from the data of Lowry *et al.*¹³⁾

^b Morikawa *et al.*, reference¹⁹⁾ and personal communication.

^c Collins and Thomas, reference.²⁰⁾

TABLE V. COMPARISON OF ENZYME LEVEL

The abbreviations used are shown in the legend of Table II.

Enzyme	Activity (units/g of protein)		Ratio (GPY/ PY)
	GPY	PY	
Phosphoglucumutase	53.8	53.3	1.01
Hexokinase	72.2	73.1	0.99
G6P dehydrogenase	0	0	—
Phosphoglucose isomerase	27.3	28.4	0.96
PFK	27.6	15.3	1.80
FBPase	4.84	7.33	0.66
Aldolase	10.1	9.94	1.02
Triose P isomerase	1621	1089	1.49
GA3P dehydrogenase	75.6	68.9	1.10
3-Phosphoglycerate kinase	294	351	0.84
Phosphoglyceromutase	9.90	11.1	0.89
Enolase	723	428	1.69
PK	302	261	1.16
Pyr dehydrogenase ^a	10.8	8.90	1.21
PEP carboxylase ^b	50.2	41.2	1.22
Pyr carboxylase ^c	0	0	—
PEP carboxykinase	189	167	1.13
Fumarase	228	210	1.09
Malate dehydrogenase	798	686	1.16
Citrate synthase	93.3	90.4	1.03
Aconitase	16.1	14.8	1.09
Isocitrate dehydrogenase	26.0	24.0	1.08

^a The activity was not detected in the absence of pyruvate or CoA.

^b The activity was very low in the absence of acetyl-CoA. Acetyl-CoA may not be the substrate, since the concentration of NAD⁺ finally formed was several times that of acetyl-CoA initially added.

^c The activity was not detected even in the presence of 0.1 mM acetyl-CoA.

Coprinus lagopus,³⁶⁾ and *Euglena gracilis*.³⁷⁾ Inverse relationship between PEP carboxykinase and PEP carboxylase was reported in *E. coli* W grown on various carbon sources.³⁸⁾ PEP carboxykinases of animal tissues were also reported to be induced under gluconeogenic conditions.³⁹⁾

All the enzymes of the glycolytic pathway were present in the *T. thermophilus* cells, but glucose 6-phosphate dehydrogenase, one of the enzymes of the pentose phosphate cycle, was absent. Yamada and Carlsson⁴⁰⁾ reported that streptococci with the pentose phosphate cycle had PK activated by fructose 1,6-bisphosphate,

whereas those without the oxidative portion of the pentose phosphate cycle had PK activated by glucose 6-phosphate. This fact is consistent with our findings for *T. thermophilus*, though the physiological significance remains unclear.

Functions of regulatory enzymes in vivo

The regulatory functions *in vivo* of the three allosteric enzymes, PFK, FBPase, and PK, are considered as follows (see Fig. 4). When *T. thermophilus* grows in PY medium, relatively high concentration of PEP accumulates; FBPase is activated and PFK is inhibited by PEP, and so gluconeogenesis is switched on. As high concentration of fructose 6-phosphate accumulates, PK will be activated, and the rate of gluconeogenesis will be decreased by lowered concentration of PEP. On the contrary, relatively high concentration of fructose 6-phosphate accumulates when the cells grow in GPY medium; PK is activated by fructose 6-phosphate, and little amount of PEP accumu-

FIG. 4. Regulation of the Glycolytic-Gluconeogenic Pathway of *T. thermophilus*.

The abbreviations used: Glu, glucose; GlyBP, glyceraldehyde 1,3-bisphosphate; Gly3P, glyceraldehyde 3-phosphate; Gly2P, glyceraldehyde 2-phosphate; OAA, oxaloacetate; Mal, malate; Fum, fumarate; Cit, citrate; IC, isocitrate; KG, 2-ketoglutarate; (A), activator; (I), inhibitor. The others are shown in the legend of Table II.

lates. Therefore PFK is active, while FBPase is inactive; glycolysis is switched on. As high concentration of PEP accumulates, the rate of glycolysis will be decreased. The glycolytic-gluconeogenic pathway of *T. thermophilus* is considered to be controlled at the two steps: first, PFK and FBPase; second, PK. PEP is a substrate of PK, and also an allosteric effector of PFK and FBPase; fructose 6-phosphate, on the contrary, is a substrate of PFK and a product of FBPase, and also a potent allosteric activator of PK. Therefore we wish to name this control mechanism "coupled regulation." It is indispensable for "coupled regulation" that fructose 6-phosphate, not fructose 1,6-bisphosphate, functions as a potent activator of PK. It is probable that the concentration changes of PEP, fructose 6-phosphate, and glucose 6-phosphate act as the main regulatory parameters for the thermophile. The adenylate energy charge, on the other hand, is presumed not to be a main but a secondary signal for the following reasons: first, adenine nucleotides have relatively weak effect on the thermophile PK,¹²⁾ PFK,^{3,5)} and FBPase^{4,6)} compared with glycolytic intermediates such as PEP and fructose 6-phosphate; second, the *in vivo* concentrations of adenine nucleotides remain relatively constant during the transition from glycolysis to gluconeogenesis. In recent years, PEP activation of FBPase was reported in Alaskan king-crab muscle⁴¹⁾ and *B. licheniformis*,⁴²⁾ but the regulation mechanisms appear to differ from that in *T. thermophilus*.

The *T. thermophilus* PK is activated by various sugar phosphates as reported previously,¹²⁾ and hence the enzyme is assumed not to be regulated strictly. Since the *in vitro* activator concentrations giving the half-maximal activation are 0.004 and 0.02 mM at 75°C for glucose 6-phosphate and fructose 6-phosphate, respectively,¹²⁾ and at least one order smaller than the *in vivo* concentrations measured, PK may be activated to some degree even when the net flow of carbon is in the direction of glucose formation. It may be presented as one of the reasons why PK is loosely regulated that the enzyme plays a role in the supply of the

biosynthetic materials such as pyruvate and nucleoside triphosphates even under gluconeogenic conditions.¹²⁾ It is presumed that PK determines mainly the rate of glycolysis, while PFK and FBPase determine both the direction and the rate of glycolysis-gluconeogenesis.

The changes of enzyme concentrations well correspond to those of enzyme activities in the cases of PFK and FBPase. Therefore, it would appear the cells regulate PFK and FBPase activities by modulation of the activities and also by the alterations of the absolute amount of enzymes. The expressions of PFK and FBPase activities are considered to be controlled strictly as compared with that of PK activity.

This work shows a unique regulatory mechanism of glycolysis-gluconeogenesis in an extreme thermophile, *T. thermophilus* HB8, and other organisms having "coupled regulation" may be discovered in the wide biological world.

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