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Extremely Thermostable Phosphoenolpyruvate Carboxylase from an Extreme Thermophile, *Rhodothermus obamensis*[1]

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Phosphoenolpyruvate carboxylase (PEPC) was purified from an extremely thermophilic bacterium, *Rhodothermus obamensis*, growing optimally at 80°C, which had recently been isolated from a shallow marine hydrothermal vent in Japan. The native enzyme was a homotetramer of 400 kDa in molecular mass, as estimated by gel filtration chromatography, and the subunit exhibited an apparent molecular mass of 100 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimum temperature for enzyme activity was 75°C. The enzyme exhibited an absolute requirement for divalent cations and a pH optimum of 8.0. The enzyme was extremely thermostable and there was no loss of enzyme activity on incubation for 2 h at 85°C. The enzyme exhibited a positive allosteric property with acetyl-CoA and fructose 1,6-bisphosphate, and a negative one with L-aspartate and L-malate. These effectors affected not only the thermophilicity but also the thermostability of the enzyme, and the substrate, co-factors, and salts increased the thermostability as well. The extrinsic thermostabilization might be a possible mechanism for adaptation of the enzyme to high temperature.

Key words: extreme thermophile, extrinsic thermostabilization factor, phosphoenolpyruvate carboxylase, *Rhodothermus obamensis*, thermostability.

Recently, there has been increasing interest in thermophilic organisms due to their novel biochemical machinery that must be required for them to survive at extraordinary temperatures (*1, 2*). A number of thermophilic enzymes have been isolated from various thermophiles, and the enzymes from a few representative thermophiles, such as *Thermus, Thermotoga, Sulfolobus*, and *Pyrococcus*, have been well studied and focused on (*1-5*). However, the mechanisms underlying their thermostability are largely unknown.

The first step for elucidating the mechanisms is to accumulate knowledge on thermophilic enzymes, and to compare their properties with those of the thermophilic and mesophilic counterparts. In addition, different types of enzymes from various thermophilic sources should be further considered. For this purpose, we isolated and characterized phosphoenolpyruvate carboxylase (PEPC) [EC 4.1.1.31] from a hyperthermophilic autotrophic archaeon, *Methanothermus sociabilis*, and compared it with other thermophilic and mesophilic homologs (*6*).

PEPC catalyzes the reaction that fixes HCO_3^- on phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) and inorganic phosphate with Mg^{2+} as a cofactor (*7*). The enzyme is distributed widely in bacteria, protozoa, algae, and plants, and a large number of the enzymes from various sources, including *Escherichia coli* (*8*), *Zea mays* (*9*), and *Thermus* sp. (*10*), have been purified, cloned, and sequenced. In the previous study, we purified PEPC for the first time from the domain of Archaea (*11*), and reported that the enzyme from an autotrophic hyperthermophile was quite different from the counterparts in the other domains, Bacteria and Eucarya, with respect to structure and allosteric properties (*6*). Moreover, the archaeal PEPC from *M. sociabilis* was quite thermostable, similar to all known hyperthermophilic enzymes. However, the encountered difficulty in the manipulation of the hyperthermophilic methanogen and its enzymes has limited biochemical and structural studies due to the strong oxygen sensitivity.

Therefore, we explored another thermophilic PEPC from an extremely thermophilic bacterium, *Rhodothermus obamensis*, which was isolated from a shallow hydrothermal vent environment in Japan (*12*). This is an aerobic heterotroph, capable of growth up to 85°C, and suitable for biochemical and biotechnological investigation of thermophilic proteins due to the advantages of easy cultivation and a high growth yield. We report in this paper the purification and characterization of PEPC from an extreme thermophile, *R. obamensis*. Its enzymological properties were compared with those of thermophilic and mesophilic entities. In addition, the effects of the substances associated with the enzyme on the thermostability were examined as extrinsic thermostabilization factors.

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Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; OAA, oxaloacetate; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; CAM, crassulacean acid metabolism.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—The bacterial strain used in this study was *Rhodothermus obamensis* OKD7 (JCM 9785), which was isolated by Sako, Y. *et al.* from a shallow marine hydrothermal vent in Tachibana Bay, Nagasaki (*12*). For the cultivation of *R. obamensis*, Jx medium was used (*12*). *R. obamensis* was grown at 76°C, and harvested at the late exponential growth phase by centrifugation and washed three times with 50 mM Tris-HCl (pH 7.5). The cell pellet was frozen at −90°C prior to enzyme purification.

Enzyme Assays—PEPC activity was routinely coupled to the malate dehydrogenase (MDH) reaction and assayed in duplicate by the previously described method (*6*). For measurement of the activity, a heat-stable MDH from *Thermus flavus* (SIGMA) was used as a coupling enzyme. All assays were initiated by the addition of the enzyme preparation to the preheated reaction mixture containing 50 mM Tris-H_2SO_4 (pH 8.0), 10 mM $NaHCO_3$, 5 mM PEP, 10 mM $MgSO_4$, 0.15 mM NADH, and 2 U *T. flavus* MDH, in a final volume of 1.0 ml unless otherwise noted (standard assay conditions). In an attempt to determine the effect of pH on the enzyme activity, 50 mM Tris-H_2SO_4 was used in the range of 7.5-9.5 and 50 mM KH_2PO_4-Na_2HPO_4 (phosphate buffer) in the range of pH 5.5-7.0, as buffers for the reaction mixture. In order to examine the requirement by PEPC for divalent cations, various concentrations of $MgSO_4$ or $MnSO_4$ were added to the reaction mixture instead of 10 mM $MgSO_4$. To examine the effect of metabolites on the activity, various concentrations of acetyl-CoA, fructose 1,6-bisphosphate, L-aspartate, and L-malate were added to the reaction mixture. These metabolites were expected to have effects on the activity of the coupling enzyme, *T. flavus* MDH, but the effects were negligible for the measurement of PEPC activity due to the relatively excessive amount of MDH in the reaction mixture. One unit of PEPC activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADH per min.

Purification—All procedures were carried out at 0 to 4°C. Thawed cell paste of *R. obamensis* (150 g) was suspended in 300 ml of 50 mM Tris-HCl (pH 7.5). The cells were disrupted by seven passages through a French Press 5501-N (Ohtake Seisakusho, Tokyo) at 1,500 kg/cm^2 and then centrifuged at 24,000 × *g* for 20 min. The supernatant was used as the crude extract. Solid ammonium sulfate was added slowly to the crude extract to obtain 35% saturation. After stirring for 30 min, the extract was centrifuged at 24,000 × *g* for 20 min. The supernatant was brought to 80% saturation. After centrifugation, the pellet was dissolved in 150 ml of 50 mM Tris-HCl (pH 7.5) buffer and then dialyzed against a 100-fold volume of the same buffer twice. The 35-80% saturated fraction exhibiting PEPC activity was subjected to the next purification step.

The active fraction was loaded on a DEAE-cellulose DE-52 (Whatman, Kent, UK) column (2.5 × 30 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), and then washed with the same buffer. The enzyme was eluted with a 1,200 ml linear gradient of NaCl (0-0.5 M) in the buffer. The bulk of the active fractions eluted with the buffer between 0.05 and 0.15 M NaCl was pooled and then dialyzed twice against a 100-fold volume of 10 mM Tris-HCl (pH 8.0).

The dialyzed enzyme solution was put on a column of Phenyl-Sepharose 6FF (Pharmacia) (1.5 × 30 cm) equilibrated with the same buffer as for the dialysis. The enzyme was stepwisely eluted with 300 ml of 10 mM Tris-HCl (pH 8.0) buffer containing 25 and 50% (v/v) of ethylene glycol, respectively. The active fraction eluted with the buffer containing 50% of ethylene glycol was dialyzed twice against a 100-fold volume of 50 mM Tris-HCl (pH 7.5).

The dialyzed enzyme solution was put on a column of Mono Q (Pharmacia) (bed volume, 1 ml) equilibrated with 50 mM Tris-HCl (pH 7.5), and then eluted with a 10 ml linear gradient of NaCl (0-0.5 M) using a FPLC system (Pharmacia). The pooled active fractions were concentrated 5-fold with an Amicon ultrafiltration apparatus with a YM-30 ultrafilter (Amicon). The concentrated enzyme solution was subjected to the final step on a column of Superdex 200HR (Pharmacia) (bed volume, 8 ml) in the FPLC system equilibrated with 200 mM Tris-HCl (pH 7.5).

The gel filtration column was used not only for the final purification step, but also for determining the molecular mass of the enzyme. As molecular weight standards, a HMW gel filtration calibration kit was used (Pharmacia). The purified enzyme was dialyzed twice against a 100-fold volume of 50 mM Tris-HCl (pH 7.5), and then stored at −90°C.

Other Methods—Polyacrylamide gel electrophoresis (PAGE) of the purified enzyme was performed with a 7.5% (w/v) polyacrylamide gel in the absence of sodium dodecylsulfate (SDS) to confirm the enzyme purity or to examine the change in the quaternary structure during the thermoinactivation, and with a 10% (w/v) polyacrylamide gel in the presence of SDS to determine the molecular mass of the subunit by the method of Laemmli (*13*). The molecular weight markers for SDS-PAGE were from Bio-Rad. Protein concentrations were routinely estimated by the method of Bradford (*14*) with bovine serum albumin as the standard.

Thermostability Studies—For measurement of the thermostability, the enzyme was usually incubated at various temperatures in 50 mM Tris-HCl (pH 8.0) containing 10% (v/v) of glycerol at a concentration of 9 μg PEPC/ml for different periods of time, unless otherwise noted. Then, the thermoinactivation was stopped by cooling aliquots on ice and the residual PEPC activity was measured at 75°C under the standard assay conditions. The effects of pH, salts, and the enzyme concentration on thermostability were also examined. In these experiments, thermoinactivation was carried out at various pHs with 50 mM Tris-HCl (pH 7.5-9.0), with 50 mM Tris-HCl (pH 8.0) containing a variety of salts at various concentrations, or with different enzyme concentrations (9, 18, or 36 μg PEPC/ml). To examine the effects of the substrate, co-factors and metabolites on the thermostability, each substance (5 mM PEP, 0.5 mM $MgSO_4$, 0.5 mM $MnSO_4$, 1 mM acetyl-CoA, 10 mM fructose 1,6-bisphosphate, 0.5 mM L-aspartate, 5 mM L-malate) was added to the incubation buffer described above. Residual activity after thermoinactivation was expressed relative to the initial enzyme activity under the same conditions without the thermoinactivation. The thermoinactivation rate, k, was calculated with the equation, $k = -t^{-1}\ln(A_{(t)}/A_0)$, based on the concept that the residual

activity exponentially decreased during the incubation at high temperature, and on the equation, $A_{(t)} = A_0 e^{-kt}$: t, time (min); $A_{(t)}$, residual activity after thermoinactivation (μmol NADH/min); and A_0, initial enzyme activity (μmol NADH/min). The thermostabilization effect was defined as k/k_f: k, thermoinactivation rate in the absence of extrinsic thermostabilization factors; and k_f, thermoinactivation rate in the presence of extrinsic thermostabilization factors.

RESULTS

Purification of PEPC from Rhodothermus obamensis—As shown in Table I, PEPC of *R. obamensis* was purified 1,563-fold with a final specific activity of 100 μmol NADH/min per mg, under the standard assay conditions, and an overall yield of about 17.3%. The purity of the purified enzyme was confirmed by native PAGE and SDS-PAGE. The sample obtained on gel filtration on a Superdex 200HR column gave a single protein band on both native PAGE and SDS-PAGE (Fig. 1), and the band position on native PAGE corresponded to an active fraction among the fractions of gel slices, every 1 mm from top to bottom. Therefore, the purification of the enzyme was completed on a column of Superdex 200HR (Fig. 1 and Table I). The most notable property observed in the course of purification was that the enzyme was strongly bound on hydrophobic interaction chromatography. The enzyme was eluted with 10 mM Tris-HCl (pH 8.0) containing 50% (v/v) ethylene glycol. This extreme hydrophobicity allowed the enzyme to be successfully separated from the preparation.

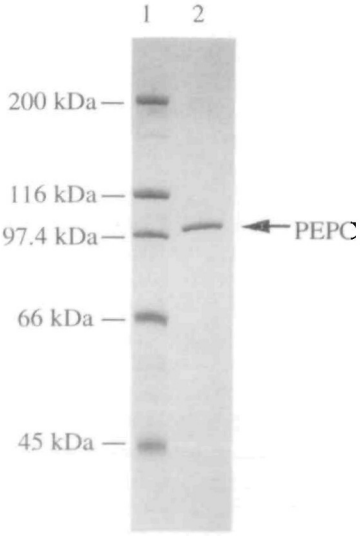

Fig. 1. **SDS-polyacrylamide gel (10% of acrylamide) electrophoresis of the purified *R. obamensis* PEPC.** Approximately 5 μg of protein was applied to a lane. After electrophoresis, the gel was stained with 40% (v/v) methanol-10% (v/v) acetate containing 0.2% (w/v) Coomassie Brilliant Blue (CBB) R250. Lane 1, molecular weight marker; lane 2, the active fraction obtained from FPLC Superdex 200 HR.

Molecular Properties of PEPC—The molecular mass of PEPC from *R. obamensis* was estimated by gel filtration to be 400 kDa. On SDS-PAGE, the purified enzyme gave a single protein band corresponding to a molecular mass of 100 kDa (Fig. 1). These results suggested that the enzyme was a 400 kDa homotetramer consisting of 100 kDa subunits. On the basis of its molecular mass, the *R. obamensis* PEPC is similar in size to other known PEPCs from the domains Bacteria and Eucarya.

The Effects of Temperature, pH, and Cations on PEPC—The effect of temperature on the enzyme activity is shown in Fig. 2. The enzyme indicated the highest activity at ambient temperature of around 75°C. The optimum pH for activity was approximately 8.0. The enzyme absolutely required divalent cations for its activity (Fig. 3). It utilized not only Mg^{2+}, which is the most common cofactor for PEPC, but also a low concentration of Mn^{2+} instead of Mg^{2+}. The concentrations which resulted in half the maximum enzyme activity were 0.25 mM for Mg^{2+} and 0.1 mM for Mn^{2+}. When the concentrations of both cations were increased, however, the enzyme activity gradually decreased, and it was completely inhibited by 15 mM Mn^{2+} or 100 mM Mg^{2+}.

The Effects of Metabolites on PEPC—The effects of several metabolites, which are major positive or negative

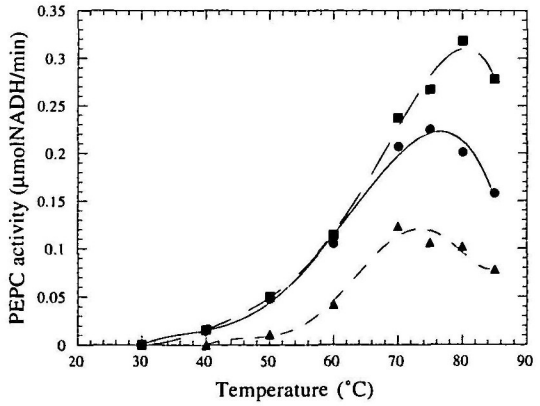

Fig. 2. **The effect of temperature on the activity of *R. obamensis* PEPC.** The activity was measured at various temperatures with an enzyme concentration of 3.6 μg PEPC/ml under the standard assay conditions in the absence (●) or presence of 1 mM acetyl-CoA (■), or 0.5 mM L-aspartate (▲). With allosteric effectors, the optimum temperature for activity was shifted from 75 to 80°C for a positive effector, *e.g.* acetyl-CoA, or to 70°C for a negative effector, *e.g.* L-aspartate.

TABLE I. **Purification of PEPC from *R. obamensis*.**

Preparation	Volume (ml)	Total protein (mg)	Total activity (μmol NADH/min)	Yield (%)	Specific activity (μmol NADH/min per mg)	Purification fold
Crude extract	400	10,470				
35-80% $(NH_4)_2SO_4$	185	5,790	370	100	0.064	1
DEAE-Cellulose DE-52	175	723	284	76.8	0.393	6
Phenyl-Sepharose 6FF	72.0	23.4	162	43.8	6.92	108
FPLC MonoQ	13.1	1.83	160	43.2	87.4	1,366
FPLC Superdex 200HR	16.0	0.64	64	17.3	100	1,563

effectors for mesophilic PEPCs, were investigated. The relative activities with various concentrations of metabolites were 143.2% (1 mM), 155.8% (2 mM), and 157.1% (5 mM) with acetyl-CoA, 126.6% (2 mM), 141.8% (5 mM), and 143.7% (10 mM) with fructose 1,6-bisphosphate, 72.4% (0.5 mM), 23.7% (1 mM), 9.1% (2 mM), and 0% (2.5 mM) with L-aspartate, and 22.3% (0.5 mM), 9.5% (1 mM), and 0% (2 mM) with L-malate. As positive effectors, acetyl-CoA and fructose 1,6-bisphosphate significantly enhanced the enzyme activity (maximally 157 and 144%, respectively). The concentrations of the effectors which resulted in half the maximum effectiveness was 0.35 mM for acetyl-CoA and 1.2 mM for fructose 1,6-bisphosphate. On the other hand, the enzyme activity was completely inhibited by 2.5 mM L-aspartate and 2.0 mM L-malate. 50% inhibition required concentrations of 0.7 mM for L-aspartate, and 0.3 mM for L-malate. In addition, the half saturation concentration of the substrate ($S_{0.5}$) was reduced in the presence of 1 mM acetyl-CoA, and increased in the

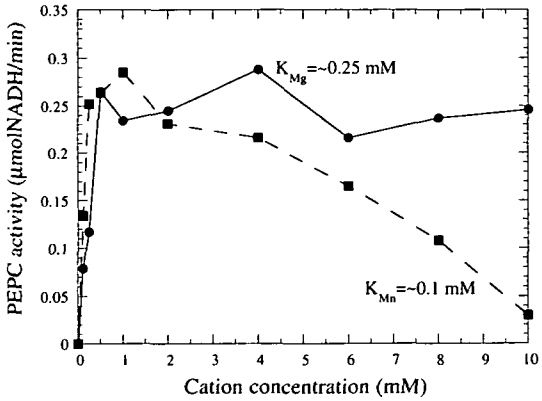

Fig. 3. **The effects of divalent cations on the activity of *R. obamensis* PEPC.** The activity was measured in the presence of various concentrations of Mg^{2+} (●), or Mn^{2+} (■) at 75°C and pH 8.0, at a concentration of 3.6 μg PEPC/ml. K_{Mg} and K_{Mn} indicate the concentrations of Mg^{2+} and Mn^{2+}, respectively, that result in half the maximum activity.

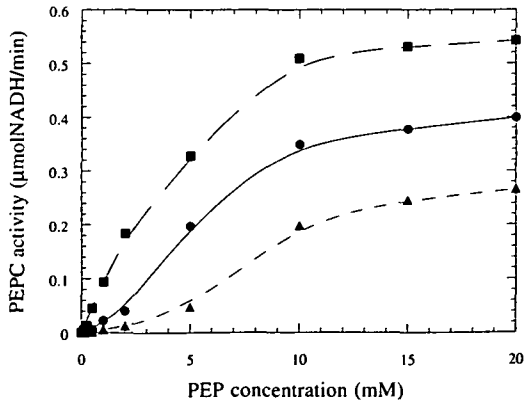

Fig. 4. **The effect of the substrate concentration on the activity of *R. obamensis* PEPC.** The enzyme activity was measured with various concentrations of PEP in the absence (●) or presence of 1 mM acetyl-CoA (■), or 1 mM L-aspartate (▲) at 75°C and pH 8.0, at a concentration of 3.6 μg PEPC/ml. The activity exhibited a typical sigmoid relationship with the PEP concentration.

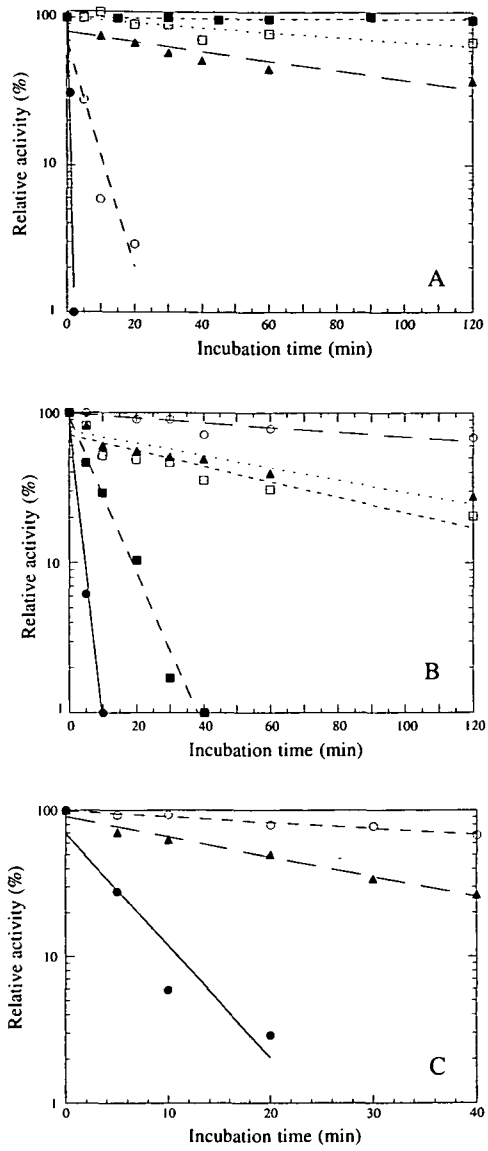

Fig. 5. **The effects of temperature, pH, and the enzyme concentration on the thermostability.** (A) The thermoinactivation of *R. obamensis* PEPC at various temperatures. The enzyme was incubated at a concentration of 9 μg PEPC/ml in the incubation buffer (50 mM Tris-HCl containing 10% of glycerol, pH 8.0) for different periods of time at 85°C (■), 90°C (□), 91°C (▲), 93°C (○), or 100°C (●). The thermoinactivation was terminated by cooling aliquots on ice, and then the residual activity was measured at 75°C and pH 8.0. The activity before the thermoinactivation was set at 100%. (B) The effect of pH on the thermostability of *R. obamensis* PEPC. The enzyme was incubated at 91°C and a concentration of 9 μg PEPC/ml in incubation buffer of pH 7.0 (■), 7.5 (□), 8.0 (○), 8.5 (▲), or 9.0 (●). The pH of the incubation buffer indicated in this figure was the value adjusted at 25°C, and the value was found to decrease to the extent of 1.3 at 91°C. The activity at each pH before the thermoinactivation was set at 100%. (C) The effect of the enzyme concentration on the thermostability of *R. obamensis* PEPC. The enzyme was incubated at 93°C and pH 8.0 at 9 μg PEPC/ml (●), 18 μg PEPC/ml (▲), or 36 μg PEPC/ml (○). In this experiment, the total protein concentration was standardized as to a concentration of 72 μg/ml in all cases by means of the compensation with bovine serum albumin (BSA). The activity at each enzyme concentration before the thermoinactivation was set at 100%.

presence of 1 mM L-aspartate under the standard conditions for activity (Fig. 4). These results indicated that *R. obamensis* PEPC was an allosteric enzyme.

These allosteric effectors affected the thermophilicity of the enzyme (Fig. 2). The effects were strengthened in a high temperature range, and hence the optimum temperature for activity was shifted higher with a positive effector (80°C) and lower with a negative one (70°C). Acetyl-CoA activated the enzyme above 60°C, while L-aspartate inhibited the enzyme activity in all the temperature range (Fig. 2).

Thermostability of PEPC—R. obamensis PEPC was extremely stable at high temperatures. No loss of activity was observed on incubation for 2 h at 85°C (Fig. 5A). The times required for 50% loss of activity were about 240 min at 90°C, 60 min at 91°C, and 10 min at 93°C, respectively (Fig. 5A). These half lives were not altered in the presence or absence of glycerol in the incubation buffer. However, the extreme thermostability of the enzyme was strongly influenced by pH, salts, and the enzyme concentration. As shown in Fig. 5B, the enzyme was most stable at pH 8.0. In a buffer of pH 7.5 or 9.0, the enzyme was rapidly inactivated. These pH values were adjusted at 25°C. Around 90°C, the pH of the incubation buffer (50 mM Tris-HCl containing 10% glycerol) was found to decrease to an extent of 1.3 or 1.4 from the value adjusted at 25°C. Therefore, the enzyme was likely to be stable at around pH 6.5-7.0 at ambient high temperatures.

When the enzyme was incubated at 93°C at several protein concentrations, the thermoinactivation rate decreased with increasing enzyme concentration (Fig. 5C). In other words, an elevated enzyme concentration enhanced

TABLE II. **The effects of various cations on the thermostability of *R. obamensis* PEPC.**[a]

Cation	Conc. (mM)	Anion				
		None	SO_4^{2-}	HPO_4^{2-}	Cl^-	NO_3^-
None		1.00[b]				
Mg^{2+}	50		4.45[b]	N.D.[c]	2.28	N.D.
	5		4.32	N.D.	1.95	N.D.
	0.5		5.51	N.D.	3.02	N.D.
Ca^{2+}	50		N.D.	N.D.	0.772	0.639
	5		N.D.	N.D.	1.08	1.68
	0.5		N.D.	N.D.	3.25	2.81
Mn^{2+}	50		0	N.D.	0	N.D.
	5		0.587	N.D.	0.786	N.D.
	0.5		5.72	N.D.	4.32	N.D.
Na^+	100(50)[d]		6.81	4.40	4.11	2.60
	10(5)		4.36	4.11	3.36	2.32
	1(0.5)		4.66	1.58	2.38	2.19
K^+	100(50)		4.19	3.89	2.98	2.34
	10(5)		4.11	3.25	1.22	1.67
	1(0.5)		3.77	2.91	0.867	0.907
NH_4^+	100(50)		3.62	3.17	2.13	1.90
	10(5)		3.62	2.49	1.67	1.78
	1(0.5)		2.49	2.28	0.923	0.789

[a]The pH of the incubation buffer (50 mM Tris-HCl, pH 8.0, containing 10% of glycerol) was readjusted to 8.0 with HCl or NaOH when the presence of some salt resulted in a significant change in pH of the buffer. [b]The enzyme was incubated at 93°C at a concentration of 9 μg PEPC/ml in the buffer (50 mM Tris-HCl, pH 8.0, containing 10% of glycerol) with or without various concentrations of salts. Each value indicates a thermostabilization effect, as described under "MATERIALS AND METHODS." [c]N.D. indicates "not determined." [d]The concentrations of monovalent cations in the buffer were doubled as coupled to the divalent anions because each ion was supplied as a salt.

TABLE III. **The effects of various anions on the thermostability of *R. obamensis* PEPC.**[a]

Anion	Conc. (mM)	Cation			
		None	Na^+	K^+	NH_4^+
None		1.00[b]			
SO_4^{2-}	50		6.81[b]	4.19	3.62
	5		4.36	4.11	3.62
	0.5		4.66	3.77	2.49
HPO_4^{2-}	50		4.40	3.89	3.17
	5		4.11	3.25	2.49
	0.5		1.58	2.91	2.28
Cl^-	50		4.11	2.98	2.13
	5		3.36	1.22	1.67
	0.5		2.38	0.867	0.923
NO_3^-	50		2.60	2.34	1.90
	5		2.32	1.67	1.78
	0.5		2.19	0.907	0.789

[a]The pH of the incubation buffer (50 mM Tris-HCl, pH 8.0, containing 10% of glycerol) was readjusted to 8.0 with HCl or NaOH when the presence of some salts resulted in a significant change in pH of the buffer. [b]The enzyme was incubated at 93°C at a concentration of 9 μg PEPC/ml in the buffer (50 mM Tris-HCl, pH 8.0, containing 10% of glycerol) with or without various concentrations of salts. Each value indicates a thermostabilization effect as described under "MATERIALS AND METHODS."

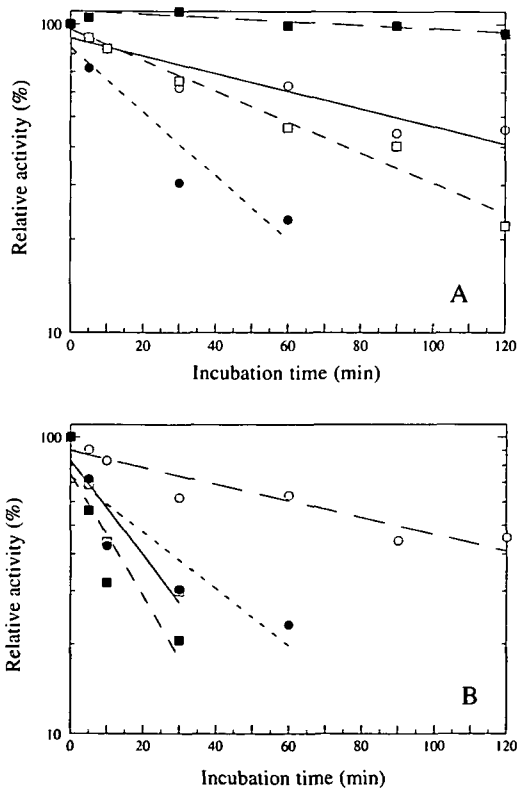

Fig. 6. **The effects of the substrate and allosteric effectors on the thermostability of *R. obamensis* PEPC.** The enzyme was incubated at 93°C and pH 8.0 at a concentration of 18 μg PEPC/ml in the absence or presence of each substance. (A) The thermoinactivation rates in the absence (●) or presence of 5 mM PEP (○), 1 mM acetyl-CoA (■), or 10 mM fructose-1,6 bisphosphate (□). (B) Those in the absence (●) or presence of 5 mM PEP (○), 0.5 mM L-aspartate (□), or 0.5 mM L-malate (■). No change of pH was observed in the incubation buffer in the presence of each substance, and the activity with each substance before the thermoinactivation was set at 100%.

TABLE IV. **The combined effects of extrinsic thermostabilization factors.**[a]

Factor	Thermostabilization effect[b] (Multiplications indicate the calculated values)
(1) +5 mM PEP	**3.14**
(2) +0.5 mM $MgSO_4$	**4.37**
(3) +50 mM Na_2SO_4	**5.58**
(4) +1 mM Acetyl-CoA	**8.81**
(5) +5 mM PEP,	**16.1**
0.5 mM $MgSO_4$	(1)×(2)=13.7
(6) +50 mM Na_2SO_4	**39.9**
1 mM Acetyl-CoA	(3)×(4)=49.2
(7) +5 mM PEP,	**50.6**
0.5 mM $MgSO_4$,	(1)×(2)×(3)=76.6
50 mM Na_2SO_4	(3)×(5)=89.8
(8) +5 mM PEP,	**106.4**[c]
0.5 mM $MgSO_4$,	(1)×(2)×(4)=121
1 mM Acetyl-CoA	(4)×(5)=141.8
(9) +5 mM PEP,	**106.3**[c]
0.5 mM $MgSO_4$,	(1)×(2)×(3)×(4)=673.5
50 mM Na_2SO_4,	(3)×(4)×(5)=791.4 (1)×(2)×(6)=547.5
1 mM Acetyl-CoA	(3)×(8)=593.7 (4)×(7)=445.8

[a]The enzyme was incubated at 95°C in the buffer (50 mM Tris-HCl, pH 8.0, containing 10% of glycerol) at a concentration of 9 μg PEPC/ml. [b]The thermostabilization effects were defined as described under "MATERIALS AND METHODS." [c]The combined effects of cases (8) and (9) were so strong that little loss of activity was observed at 95°C for 2 h. Hence, the thermostabilization effects of cases (8) and (9) can be underestimated.

the thermostability of the enzyme. Furthermore, the effects of salts on the thermostability were investigated (Tables II and III). With divalent cations including co-factors of the enzyme, the thermostabilization effect decreased with increasing concentration (Table II). On the contrary, with monovalent cations and anions, the thermostabilization effect increased with increasing concentration (Tables II and III). In addition, the thermostabilization effect was generally in the order, $Na^+ > K^+ > NH_4^+$, for monovalent cations, and in the order, $SO_4^{2-} > HPO_4^{2-} > Cl^- > NO_3^-$, for anions, as compared at the same concentration. These orders as to stabilization of PEPC were consistent with the order of the water structuring effects of ions (*15, 16*).

Not only salts, but also the substrate and allosteric effectors had effects on the thermostability of PEPC (Fig. 6). The substrate, PEP, and a positive effector, acetyl-CoA, strongly enhanced the thermostability of the enzyme, while another positive effector, fructose 1,6-bisphosphate, had little effect on the enzyme thermostability (Fig. 6A). On the other hand, negative effectors reduced the enzyme thermostability, the effectiveness being consistent with the inhibitory effectiveness of the effectors as to enzyme activity (Fig. 6B). In addition, the combined effects of the substrate, co-factors, allosteric effectors, and salt on the thermostability were examined (Table IV). The thermostabilization effect of each factor in the course of thermoinactivation at 95°C was 3.14 for PEP, 4.37 for $MgSO_4$, 5.58 for Na_2SO_4, and 8.81 for acetyl-CoA. The thermostabilization effect was in the order, acetyl-CoA > Na_2SO_4 > $MgSO_4$ > PEP, at temperatures of 93, 95, and 100°C. When the enzyme was incubated with combinations of these factors, the thermostabilization effects were significantly higher than with each factor, and also cumulative (Table IV).

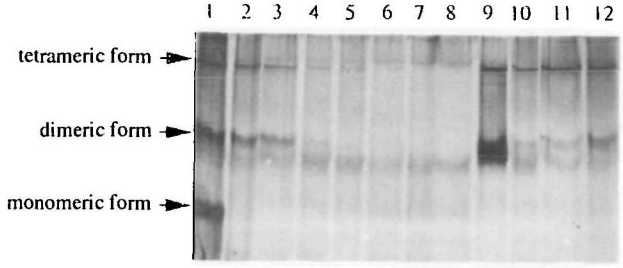

Fig. 7. **Native-polyacrylamide gel electrophoresis (native-PAGE) of the enzyme during thermodenaturation.** The enzyme was incubated at a concentration of 9 μg PEPC/ml in incubation buffer (50 mM Tris-HCl containing 10% of glycerol, pH 8.0) at 80°C for 30 h, at 93°C for 5, 15, and 30 min, at 100°C for 3, 5, and 10 min in the absence of an extrinsic thermostabilization factor, and at 93°C for 30 min in the presence of 5 mM PEP, 0.5 mM $MgSO_4$, 50 mM Na_2SO_4, or 1 mM acetyl-CoA. The same amount of enzyme (1 μg) was loaded for 7.5% (w/v) PAGE and then silver-stained. Lane 1, the protein pattern of the sample before thermoinactivation; lane 2, sample denatured at 80°C for 30 h; lanes 3, 4, and 5, samples denatured at 93°C for 5, 15, and 30 min, respectively; lanes 6, 7, and 8, samples denatured at 100°C for 3, 5, and 10 min, respectively; lanes 9, 10, 11, and 12, samples denatured at 93°C for 30 min in the presence of 5 mM PEP, 0.5 mM $MgSO_4$, 50 mM Na_2SO_4, and 1 mM acetyl-CoA, respectively. In order to check the different forms of PEPC, the non-denatured enzyme was loaded in two lanes. One was silver-stained (lane 1 in the figure), and the other was sliced every 1 mm from top to bottom. The enzyme activity was measured with respect to each fraction electrophoretically eluted from the gel slices, and the active fractions were thought to correspond to the tetrameric and dimeric forms. The monomeric forms were expected from the relative molecular size. The reproductivity of the electrophoretic patterns was confirmed at least three times.

Electrophoretical Analysis of the Quaternary Structure during Thermoinactivation—The structural change of the enzyme during thermoinactivation was determined by native polyacrylamide gel electrophoresis (native-PAGE). On native-PAGE, the non-denatured enzyme gave three major bands (lane 1 in Fig. 7). As described for purification of the enzyme, the protein sample obtained from a column of Superdex 200HR gave a single band stained with Coomassie Brilliant Blue (CBB) R250 on both native- and SDS-PAGE. The sample used in this experiment was the same, but was frozen at −90°C and then thawed for this experiment. This procedure was likely to induce dissociation of the tetrameric form. The upper major band and the middle one appeared to correspond tetrameric and dimeric forms, respectively, since the enzyme activity was found at the positions with fractions of gel slices cut every 1 mm. The lower band did not exhibit enzyme activity, but was likely to be a monomeric form because the sample was highly purified and gave a single band on SDS-PAGE. During thermoinactivation at 80°C, although the putative monomeric form decreased, no apparent change in other structures was seen after 30 h incubation (lane 2 in Fig. 7). However, on thermoinactivation at higher temperatures, the tetrameric and dimeric forms decreased with the incubation time and disappeared after 5 min incubation at 100°C (lane 7 in Fig. 7).

The extrinsic thermostabilization factors had effects on the maintenance of the quaternary structure during thermoinactivation at 93°C for 30 min (lanes 9–12 in Fig. 7). In the presence of the factors, the protein patterns before and

after 30 min thermoinactivation at 93°C were almost identical. Of these factors, PEP strongly maintained the tetrameric and dimeric structures of the enzyme (lane 9 in Fig. 7).

DISCUSSION

An extremely thermostable phosphoenolpyruvate carboxylase (PEPC) was purified from an extreme thermophile, *R. obamensis*, recently isolated from a shallow marine hydrothermal vent in Japan (*12*). The enzyme was a homotetramer with a molecular mass of 400 kDa consisting of a 100 kDa subunit. To date, a number of PEPCs have been purified from the domains Bacteria and Eucarya, such as *E. coli* (*8*), *Brevibacterium flavum* (*17*), *Thermus* sp. (*10*), *Anabaena* sp. (*18*), *Mesembryanthemum crystallinum* (*19*), and *Zea mays* (*9, 20*). Most of them are homotetramers with molecular masses of about 400 kDa, but the homolog from *M. sociabilis* belonging to the domain Archaea exhibited a molecular mass of 240 kDa (*6*). In addition, the enzymological properties of *R. obamensis* PEPC were similar to those of bacterial counterparts. Of the bacterial PEPCs, *R. obamensis* PEPC most resembled the thermophilic PEPC from an extreme thermophile, *Thermus* sp. (*10, 21*). However, the sensitivity to the negative allosteric effectors and the thermostability significantly differed between these PEPCs. These results indicated that the *R. obamensis* PEPC was a bacterial type of PEPC, but a new version of the extremely thermophilic PEPC.

R. obamensis PEPC is extremely thermostable. The enzyme remained active even after it had been exposed to a high temperature that was above the upper limit for growth for a few hours. As compared to other thermophilic PEPCs from *M. sociabilis* and *Thermus* sp. (*6, 10, 21*), *R. obamensis* PEPC is one of the most thermostable PEPCs reported to date. Moreover, the thermostability of the enzyme increased in proportion to the enzyme concentration (Fig. 5C). It has been shown and postulated that the protein concentration influenced the enzyme activity, and the interconversion of the quaternary structure of PEPC in C_4 and CAM (crassulacean acid metabolism) plants, and the high concentration of the enzyme induced the formation and maintenance of the more active tetrameric structure, while dilution induced the less active dimeric formation in these plants (*22-24*). The elevated thermostability with a higher concentration of *R. obamensis* PEPC may be due to the interconversion of the quaternary structure, which suggested that the intrinsic thermostability of the enzyme strongly depended on the formation and maintenance of the tetrameric structure.

Recently, there has been increasing interest in thermophilic organisms and their thermostable biochemical molecules associated with their extraordinary habitats (*1, 2, 25, 26*). Accordingly, an increasing number of thermophilic enzymes have been studied by comparison of the amino acid sequences between thermophilic and mesophilic homologous proteins (*27, 28*), analyses of protein folding (*29*), and structural studies on the proteins including their three-dimensional structures (*30, 31*). Another approach to the mechanism of protein thermostability is to explore the extrinsic factors that lie not in the protein itself but outside of it, and that might maintain it at a high temperature. In fact, possible extrinsic thermostabilization mechanisms have been discovered in various thermophiles (*32-34*). The thermostabilization of allosteric enzymes by positive effectors is likely to be one of such mechanisms (*35*). Taguchi *et al.* reported that fructose 1,6-bisphosphate stabilized the L-lactate dehydrogenase (LDH) of *Thermus caldophilus* GK24 as to heat and that the stabilization might reflect the stabilization of the tetrameric form (*35*). Likewise, in this study, PEPC of *R. obamensis* was strongly stabilized by the positive effector, acetyl-CoA (Fig. 6). The enhanced subunit-subunit interaction on the binding of fructose 1,6-bisphosphate has been also postulated from the results of structural analysis of *Bifidobacterium longum* LDH (*36*). Hence, one of the possible explanations for the enhanced thermostability of *R. obamensis* PEPC may be the enhanced subunit-subunit hydrophobic interaction due to the binding of acetyl-CoA to the enzyme.

R. obamensis PEPC was also stabilized by the substrate, cofactors, and various salts (Tables II and III, Fig. 6A). The presence of PEP or $MgSO_4$ significantly decreased the thermoinactivation rate of the enzyme. This implied that the substrate and cofactors as well as allosteric effectors were possible thermostabilization factors for the enzyme. It was acceptable that the binding of the substrate and cofactors altered the enzyme conformation to the active state, and the active conformation was more stable than the native one. Moreover, the thermostabilization by various salts and the variation in the extent led us not only to recognize the contribution of salts as important thermostabilization factors, but also to expect a mechanism for the intrinsic thermostability of *R. obamensis* PEPC. As can be seen in Tables II and III, the thermostabilization effect increased generally with increasing concentrations of the monovalent cations and anions. In contrast, the thermostabilization effect decreased with increasing concentrations of divalent cations. With regard to the divalent cations, Mg^{2+} or Mn^{2+} was a cofactor for the enzyme and necessary for the enzyme activity, nevertheless it was shown that excess amounts of these ions inhibited the enzyme activity (Fig. 3). Therefore, excess amounts of the divalent cations were expected to have some harmful effects on the enzyme activity and stability. On the other hand, the thermostabilization by the monovalent cations and anions was thought to result from a different interaction from that in the case of the divalent cations. In a stability study on maize leaf PEPC, Jensen *et al.* found that the enzyme was stabilized by kosmotropic (water structuring) anions such as HPO_4^{2-} and SO_4^{2-}, and suggested that the stabilization effect was due to promotion of the intersubunit hydrophobic interaction due to the solvent-mediated effects of these anions (*37*). This suggestion was supported by the concept of the solvophobic theory, *i.e.* that the water-structuring effectiveness of these anions with high charge densities increases the surface tension of the solvent, which subsequently increases the free energy of cavity formation in the protein surface area, and, as the result the increase in the cavity free energy will induce stronger intersubunit hydrophobic interactions (*38-40*). In *R. obamensis* PEPC, the thermostabilization effect was mostly in the order, $Na^+ > K^+ > NH_4^+$, for monovalent cations, and in the order, $SO_4^{2-} > HPO_4^{2-} > Cl^- > NO_3^-$, for anions, which are in good agreement with the order of water-structuring effectiveness (*15, 16*). Moreover, the

thermostabilization effects were consistent with increasing concentrations of these ions. These results indicated that the ions with high water-structuring effectiveness and the increased concentrations of such ions had stronger effects in enhancing the enzyme thermostability. According to the concept of the solvophobic theory (*38-40*), the enhanced thermostability due to the salts of monovalent cations and anions was expected to be due to the stronger intersubunit hydrophobic interactions, resulting in the maintenance of the tetrameric structure of the enzyme.

On the basis of the results presented herein, we propose that the substrate (PEP), a cofactor (Mg^{2+}), an allosteric effector (acetyl-CoA), and salts (*e.g.* Na_2SO_4) are possible extrinsic thermostabilization factors. In general, these substances are constitutional components of bacterial cells, and PEP, Mg^{2+}, and acetyl-CoA are essential for PEPC activity or its regulation. In this study, *R. obamensis* PEPC was shown to be extremely thermostable in itself (Fig. 5), but it was also shown that the thermostability of the enzyme was considerably increased by these substances. Therefore, we came to the conclusion that the thermostability of *R. obamensis* PEPC *in vivo* might be maintained due to the extrinsic mechanisms in addition to the intrinsic thermostability.

The possible function of these extrinsic thermostabilization factors was thought to be maintenance of the quaternary structure, the active tetrameric form, of the enzyme. During thermoinactivation, the tetrameric form was found to decrease on native-PAGE (Fig. 7). This might imply that the active tetramer was dissociated into the dimer and monomer, and that the dissociated monomers immediately formed an irreversible aggregate. The presence of the extrinsic thermostabilization factors apparently suppressed the dissociation of the tetrameric form and maintained the quaternary structure during thermoinactivation (Fig. 7). From the enzyme concentration-dependent thermostability and the thermostabilization by the salts, it was also suggested that the intersubunit hydrophobic interaction was strongly involved in the thermostability of this enzyme. Hence, one possible mechanism for the thermostabilization by the extrinsic factors is promotion of the intersubunit hydrophobic interaction.

The combined effects of the extrinsic thermostabilization factors was investigated at high temperatures, at which the enzyme was rapidly inactivated (Table IV). When the *R. obamensis* PEPC was incubated with each of the extrinsic thermostabilization factors at 95°C, the thermostabilization effect was in the order, acetyl-CoA > Na_2SO_4 > $MgSO_4$ > PEP (Table IV). The order of the thermostabilization effects was the same at all temperatures tested, and acetyl-CoA appeared to be the most effective factor for the enzyme thermostabilization. Furthermore, when various combinations of the factors were added to the enzyme solution, the effects were cumulative (Table IV). The thermostabilization effect of each factor represents the simple proportion of the thermoinactivation rate in the absence/presence of the factors at a certain temperature. Since the thermoinactivation rate is strongly dependent on the free energy of activation ($\Delta G^{\neq}$) during the thermoinactivation, the thermostabilization effect can be described as the simple proportion of the free energy of activation in the presence/absence of the factors at a certain temperature. Therefore, the combined effects of extrinsic thermostabilization factors indicated that the free energy of activation during the thermoinactivation was cumulatively increased with the addition of the factors, and that each of the factors enhanced the thermostability of the enzyme without interference from others. Although it is still unclear how the extrinsic thermostabilization factors interact with the enzyme and elevate its free energy of activation during thermoinactivation, the extrinsic thermostabilization mechanism proposed in this study provides new insight for elucidating the thermostability of the proteins from thermophiles. In addition, the structural approach for the thermostability and the molecular basis of the extrinsic thermostabilization mechanism should be further considered when determining how the intrinsic thermostability of the enzyme is established and how the extrinsic thermostabilization mechanism works. A study on the folding or conformation of the enzyme in relation to the extrinsic thermostabilization factors, and gene cloning and expression for elucidation of the three-dimensional structure of the enzyme are underway. Such attempts might shed light on the understanding of both the intrinsic and extrinsic thermostabilization mechanisms in *R. obamensis* PEPC.

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