J. Biochem. 120, 518-524 (1996)

Purification and Characterization of Recombinant Phosphoenolpyruvate Carboxylase of *Thermus* sp.[1]

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Received for publication, March 14, 1996

Recombinant phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) of an extreme thermophile, *Thermus* sp., which was expressed in *Escherichia coli* cells, was purified and its enzymological properties were investigated and compared with native *Thermus* PEPC. The enzyme activity was strongly dependent on acetyl-CoA, an allosteric activator, and inhibited by malate or aspartate. Contrary to the other known PEPCs, *Thermus* PEPC was not activated but rather inhibited by phosphorylated compounds such as fructose 1,6-bisphosphate and GTP. The specific activity in the presence of 0.3 mM acetyl-CoA and 2 mM phosphoenolpyruvate was highest at 70°C. The half-saturation concentrations for both substrates at 70°C were about twice those at 30°C. Half-lives of the enzyme at 85, 90, and 95°C were 220, 110, and 50 min, respectively. *Thermus* PEPC was highly tolerant also to guanidine hydrochloride (Gdn-HCl): the concentrations required for complete inactivation of *Thermus* and *E. coli* PEPCs after incubation at 30°C for 20 h were 3.5 and 0.6 M, respectively. The properties of recombinant and native enzyme were similar to each other except for the catalytic activity after incubation with 1-2 M Gdn-HCl.

Key words: allosteric effectors, guanidine hydrochloride, phosphoenolpyruvate carboxylase, thermostable enzyme, *Thermus* sp.

Phosphoenolpyruvate carboxylase (PEPC) [EC 4.1.1.31] catalyzes the reaction of phosphoenolpyruvate (PEP) with HCO_3^- to form oxaloacetate and orthophosphate using Mg^{2+} as a cofactor (*1*). PEPC is widespread in all higher plants and many kinds of bacteria, and plays an anaplerotic role by replenishing C_4 dicarboxylic acids in the citric acid cycle. In addition, in C_4- and CAM plants, a specific molecular species of PEPC plays a key role in photosynthetic CO_2 assimilation (*2*).

Several PEPCs have been purified from bacteria and plants and characterized. PEPC is composed of four identical subunits with a molecular mass of about 100 kDa. The activities of most PEPCs from various sources are regulated by a wide variety of allosteric effectors (*1, 2*) or by reversible phosphorylation (*3*). As in the case of carbamoyl phosphate synthetase (*4*) and biotin-containing carboxylases (*5*), PEPC utilizes HCO_3^-, which is chemically less reactive than CO_2, as a substrate. The reaction mechanism of PEPC has been studied mostly using the enzymes from *Escherichia coli* (*6-8*) and *Zea mays* (*9-11*), and the stepwise reaction model has been generally accepted. The genes for PEPCs from various sources have been cloned and their primary structures deduced (*12*). Crystallization of *E. coli* PEPC has been achieved (*13*), but its three-dimensional structure has not yet been elucidated.

Proteins from thermophiles are usually more tolerant to high temperatures than their counterparts from mesophilic organisms. In many cases, the former are more readily crystallized than the latter, thus allowing pioneering X-ray crystallographic analyses (*14, 15*). Since enzymes of thermophiles are often tolerant not only to heat but also to various severe chemical and physical conditions, they are often used for industrial applications. Thus, it seems worthwhile to extend enzymological studies to a thermostable PEPC of an extreme thermophile.

We previously cloned the gene for thermostable PEPC (*ppc*) for the first time from an extreme thermophile, *Thermus* sp. (*16*). The deduced polypeptide of *Thermus* PEPC is composed of 857 amino acid residues with a molecular mass of 95,632 Da. Comparison of the amino acid composition and sequence of *Thermus* PEPC with other mesophile PEPCs revealed several features which must reflect the stability of the enzyme. The gene for *Thermus* PEPC was expressed and thermostable PEPC was obtained from *E. coli* JM109 harboring the expression plasmid pTHPPC. In this study, the recombinant *Thermus* PEPC was purified and its enzymatic properties were investigated. The tolerance of the enzyme to heat and guanidine hydrochloride (Gdn-HCl) was also examined.

[1] This study was supported in part by Grants from Kansai Research Foundation for Technology Promotion, Asahi Chemical Industry Co. Inc., and Ajinomoto Co. Inc.

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Abbreviations: PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; *ppc*, the gene for phosphoenolpyruvate carboxylase; OAA, oxaloacetate; Pyr, pyruvate; CoASAc, acetyl-CoA; Fru-1,6-P_2, fructose 1,6-bisphosphate; Gdn-HCl, guanidine hydrochloride; *P*-compounds, phosphorylated compounds; Ches, 2-(cyclohexylamino)-ethanesulfonic acid; Bicine, *N,N*-(bis-2-hydroxyethyl)-glycine.

MATERIALS AND METHODS

Materials—Malate dehydrogenase from *Thermus* sp. was purchased from Sigma. All other materials and organisms were obtained as reported elsewhere (*7, 17*). The *E. coli* strains JM109 and F15 (*7*) were used. The latter strain is a deletion mutant of the *ppc* gene. The plasmid used was pTHPPC (*16*) constructed for the expression of *Thermus* PEPC in *E. coli*.

Purification of PEPC—Recombinant *Thermus* PEPC was purified as follows. Cells of *E. coli* JM109/pTHPPC were grown in a medium (2 liters) of Terrific Broth (*18*) supplemented with 1 mM isopropyl-1-thio-β-D-galactoside and 50 μg/ml ampicillin. The crude extract was prepared by disrupting the cells suspended in buffer A [50 mM Tris-H_2SO_4, pH 7.4, 10% (v/v) glycerol, 1 mM DTT, and 1 mM phenylmethanesulfonyl fluoride] with a French press (Ohtake Seisakusho), and nucleic acids were removed by 2% (w/v) streptomycin sulfate treatment as described (*19*). The proteins precipitated between 30 and 60% saturation of ammonium sulfate were collected by centrifugation at 20,000 × *g* for 30 min. The precipitates were dissolved in buffer B [50 mM Tris-H_2SO_4, pH 7.4, 5% (v/v) glycerol, 10 mM sodium aspartate, and 1 mM DTT] and dialyzed against the same buffer. The protein concentration was adjusted to 10 mg/ml by the addition of buffer B, and the protein solution was heat-treated at 65°C for 10 min. Aggregated proteins were removed by centrifugation at 17,000 × *g* for 20 min. The remaining proteins were precipitated with ammonium sulfate (60%-saturation) and gel-filtered through PD-10 (Pharmacia) using buffer B containing 0.5 M ammonium sulfate. The proteins (about 280 mg) were then applied to a column (1 × 11 cm) of Butyl-Toyopearl 650S (Tosoh) equilibrated with buffer B containing 0.5 M ammonium sulfate. The column was washed with 30 ml of the equilibration buffer and PEPC activity was eluted from the column with a 50-ml linearly decreasing concentration gradient of 0.5–0 M ammonium sulfate in buffer B. Active fractions were collected and the buffer was changed to buffer C [50 mM Tris-HCl, pH 7.4, 5% (v/v) glycerol, 10 mM sodium aspartate, and 1 mM DTT] by ammonium sulfate precipitation and gel filtration as described above. Then, the proteins (about 17 mg) were applied to a Mono-Q column (1 ml) (Pharmacia) equilibrated with buffer C. The column was washed with 10 ml of buffer C and PEPC activity was eluted from the column with a 20-ml linear concentration gradient of 0–0.5 M NaCl in buffer C. Active fractions were collected and subjected to gel filtration with a Superose 12 HR 10/30 column (Pharmacia) equilibrated with buffer D [0.1 M potassium phosphate, pH 7.4]. Elution of PEPC was carried out using the same buffer. Native PEPC purified from *Thermus* sp. was obtained as described (*16*). *Escherichia coli* PEPC was purified from F15/pT3 (*20*) by the method described previously (*13*).

Enzyme Assay—The enzyme activity was assayed by a coupled spectrophotometric method. For assay at temperatures below 40°C, the standard assay mixture contained, in a total volume of 1.0 ml, 2 mM cyclohexylammonium or potassium PEP, 10 mM $KHCO_3$, 10 mM $MgSO_4$, 0.1 mM NADH, 0.3 mM acetyl-CoA (CoASAc, an allosteric activator), 0.1 M Ches-KOH, pH 8.6, 1.0 U malate dehydrogenase from pig heart, and the enzyme. For the assay of *E. coli* PEPC, only potassium salt of PEP was used. The rate of NADH oxidation was followed by use of a recording spectrophotometer. At temperatures above 40°C, the enzyme activity was assayed as follows. The standard assay mixture contained, in a total volume of 90 μl, 2 mM cyclohexylammonium or potassium PEP, 10 mM $KHCO_3$, 10 mM $MgSO_4$, 1 mM NADH, 0.3 mM CoASAc, 0.1 M Ches-KOH, pH 8.6, 0.1 U malate dehydrogenase from *Thermus* sp., and the enzyme. The mixtures were incubated in a 1.5-ml plastic tubes at the indicated temperatures for various periods. The reaction was terminated by the addition of EDTA to a final concentration of 90 mM and residual NADH was determined spectrophotometrically. The activity was obtained from the slope of the plot of residual NADH *versus* reaction time. Malate dehydrogenase from *Thermus* sp. had sufficient activity for assay at temperatures below 90°C. The phosphate-liberating activity of PEPC was also assayed by determining orthophosphate by the molybdophosphate method (*21*). One unit of the enzyme activity was defined as that producing 1 μmol oxaloacetate per min. The protein concentration was determined using a Protein Assay Kit (Bio-Rad) with crystalline bovine serum albumin as a standard.

Heat Treatment of Thermus PEPC—*Thermus* PEPC in buffer D (0.1 mg/ml) was incubated at 85, 90, or 95°C. Aliquots were withdrawn at indicated times and chilled at 0°C for 5 min. The residual enzyme activities were assayed at 30°C as described above.

Gdn-HCl Treatment of PEPC—Enzyme samples (0.23 and 0.46 mg/ml for *Thermus* and *E. coli* PEPCs, respectively) were incubated at 30°C in a buffer containing 100 mM Tris-H_2SO_4, pH 8.0, and various concentrations of Gdn-HCl. After incubation for 20 h, residual activity was assayed at 30°C. In each enzyme assay, the final concentration of Gdn-HCl in the reaction mixture was adjusted to 40 and 5 mM for *Thermus* and *E. coli* PEPCs, respectively.

RESULTS

Expression of Thermus PEPC in E. coli—As described previously (*16*), JM109 harboring the expression plasmid pTHPPC produced active and thermostable PEPC. Unexpectedly, however, pTHPPC was not able to complement the glutamate-requiring phenotype of *E. coli* strain F15, a deletion mutant of the *ppc* gene. When the cells of F15/pTHPPC were grown in a medium supplemented with glutamate, neither PEPC activity nor the corresponding protein band on SDS-PAGE was detected from the crude extract. It seems that *Thermus* PEPC polypeptide was degraded in the cells of strain F15 for an unknown reason. Therefore, recombinant *Thermus* PEPC was purified from JM109/pTHPPC. During the purification procedures, recombinant *Thermus* PEPC and endogenous *E. coli* PEPC were completely separated from each other as described below.

Purification and Molecular Properties of Thermus PEPC—Recombinant *Thermus* PEPC was purified about 130-fold, with a final yield of 20%, by the sequential procedure described in "MATERIALS AND METHODS." Purification of the enzyme is summarized in Table I. As judged by SDS-PAGE (Fig. 1), the preparation obtained was more than 95% homogeneous. The mobility of recombinant

PEPC was the same as that of native PEPC. The molecular mass of *Thermus* PEPC estimated by SDS-PAGE was a little smaller than that of the *E. coli* enzyme, being consistent with the difference between calculated molecular masses of *Thermus* and *E. coli* PEPCs (95,632 and 99,061 Da, respectively). Gel-filtration chromatography, the last step of the purification, indicated that the apparent molecular mass of *Thermus* PEPC was 400 kDa (data not shown). Since the molecular mass of the subunit is about 100 kDa, the native enzyme supposedly consists of four identical subunits like other PEPCs.

The behavior of *Thermus* PEPC markedly differed from that of the *E. coli* enzyme in the hydrophobic-interaction chromatography (Butyl-Toyopearl). The *E. coli* enzyme was eluted from the column at $(NH_4)_2SO_4$ concentrations of 0.3 and 0.5 M in the absence and presence of 10 mM aspartate (an allosteric inhibitor), respectively (*7*). On the other hand, *Thermus* PEPC was eluted from the column only when $(NH_4)_2SO_4$ was almost absent (data not shown), suggesting that it has a stronger hydrophobic interaction than the *E. coli* enzyme. Because the bulk of *E. coli* proteins were not retained in the column of Butyl-Toyopearl during washing with 0.5 M $(NH_4)_2SO_4$, *Thermus* PEPC was purified by 10-fold at this step. This hydrophobic-interaction chromatography, together with heat treatment, completely separated recombinant PEPC from endogenous *E. coli* PEPC.

TABLE I. **Purification of recombinant *Thermus* PEPC.** The enzyme was purified from 19 g in fresh weight of JM109/pTHPPC. The enzyme assay was performed at 30°C as described in "MATERIALS AND METHODS."

Purification step	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude extract	31	2,110	327	0.155	100
$(NH_4)_2SO_4$ (30-60% sat.)	21	1,500	311	0.207	95.2
Heat treatment (65°C, 10 min)	10	280	146	0.520	44.7
Butyl-Toyopearl	15	17.0	89.2	5.25	27.3
Mono-Q	3.5	4.52	89.5	19.8	27.4
Superose 12	4.0	2.26	66.4	29.4	20.3

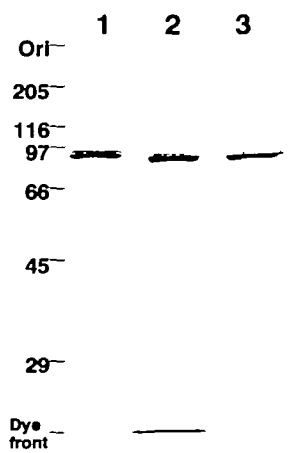

Fig. 1. **SDS-PAGE of 0.5 μg purified PEPCs.** *E. coli* (lane 1), native *Thermus* (lane 2), and recombinant *Thermus* (lane 3) PEPCs were subjected to 10% SDS-PAGE. Molecular masses (kDa) are shown at left.

Catalytic and Regulatory Properties of Thermus PEPC—The activities of *Thermus* PEPC in the presence of various effectors are shown in Table II. The activity was strongly dependent on the presence of CoASAc when assayed with 2 mM PEP. The half-saturation concentrations of CoASAc were 0.05 and 0.14 mM at 30 and 70°C, respectively (data not shown). On the other hand, it was inhibited by malate and aspartate. Although these effectors were the same as those for the *E. coli* enzyme (*22*), *Thermus* PEPC was more sensitive to malate than to aspartate, unlike the *E. coli* enzyme. Unexpectedly, the activity of *Thermus* PEPC was inhibited by various phosphorylated compounds (*P*-compounds). Fructose 1,6-bisphosphate (Fru-1,6-P_2) and GTP, which are known to be activators of *E. coli* PEPC (*23*), inhibited *Thermus* PEPC. In addition, ATP, ADP, glucose 6-phosphate, and fructose 6-phosphate were also inhibitory (data not shown), as was orthophosphate. The inhibition by these *P*-compounds showed a tendency to be released by increasing CoASAc.

Figure 2 shows saturation curves of PEP at 70°C. Acetyl-CoA affected not only maximum velocity (V_{max}) but also half-saturation concentration ($S_{0.5}$) of PEP. Half-saturation concentrations of the substrates, metal cofactor, and inhibitors in the presence of 0.05 and 0.3 mM CoASAc are summarized in Table III. Half-saturation concentrations of PEP and Mg^{2+} were decreased by increasing concentrations of CoASAc, whereas those of inhibitors, malate and aspartate, were increased at both 30 and 70°C. The $S_{0.5}$ values of

TABLE II. **Modulation of the activity of *Thermus* PEPC by various effectors.** Assays were performed at 30°C as described in "MATERIALS AND METHODS" except for the concentration of CoASAc. All effectors were added to the concentration of 10 mM.

Additions	Enzyme [CoASAc] (mM)	Relative activity (%)			
		Recombinant		Native	
		0.3	0.05	0.3	0.05
None		100	100	100	100
Aspartate		84	58	82	67
Malate		46	0	43	0
Fru-1,6-P_2[a]		70	28	77	43
GTP[a]		100	67	94	77
ATP[a]		58	11	61	19
P_i[a]		92	70	98	77

[a]Assayed in the presence of 50 mM $MgSO_4$.

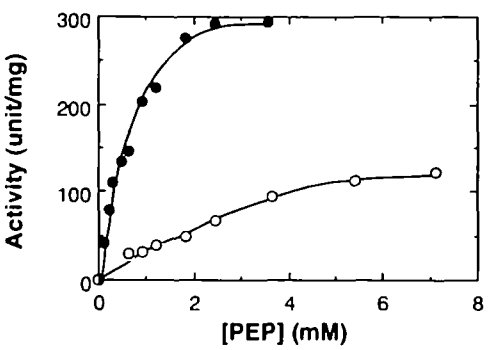

Fig. 2. **Variation of the enzyme activity of recombinant *Thermus* PEPC with increasing concentration of PEP at 70°C.** Assays were performed at 70°C as described in "MATERIALS AND METHODS" except for the concentrations of PEP and CoASAc. The concentrations of CoASAc were 0.3 mM (●) and 0.05 mM (○).

TABLE III. **Half-saturation concentrations of substrates, cofactor, and inhibitors.** Assays were performed as described in "MATERIALS AND METHODS" except for the concentrations of ligands.

Temperature	Enzyme	[CoASAc]	V_{max} (unit/mg)	$S_{0.5}$ (mM)			$I_{0.5}$ (mM)	
				PEP	HCO_3^-	Mg^{2+}	Aspartate	Malate
30°C	Recombinant	0.3 mM	35	0.4	0.8	1.1	64	9
		0.05 mM	23	0.6	0.5	2.5	12	1
	Native	0.3 mM	40	0.4	0.6	1.0	80	8
		0.05 mM	30	0.6	0.5	2.1	20	2
	E. coli	0.3 mM	75	2.0	0.3	2.9	0.7	0.8
		0.05 mM	57	4.2	0.3	6.9	0.3	0.4
70°C	Recombinant	0.3 mM	360	1.0	1.1	1.3	70	21
		0.05 mM	170	2.7	0.6	3.0	47	10
	Native	0.3 mM	400	1.4	0.9	1.3	75	14
		0.05 mM	160	2.8	0.5	2.2	40	8

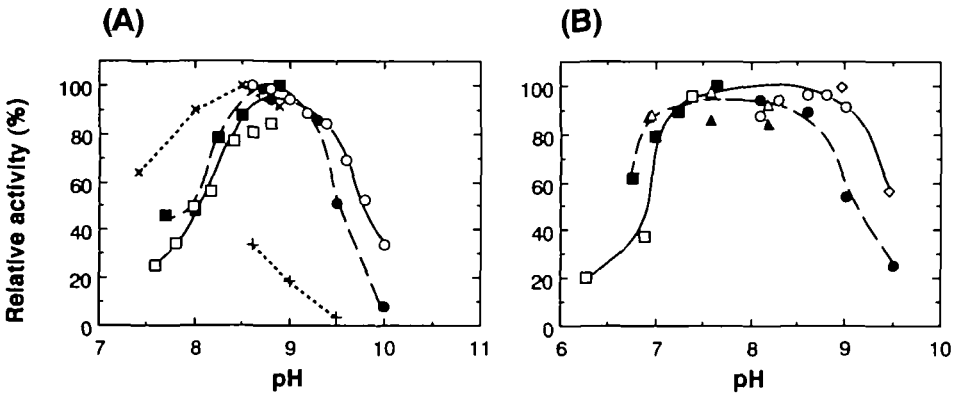

Fig. 3. **Effect of pH on the activity of PEPCs.** The enzymes examined were recombinant *Thermus* PEPC (□, ○, ◇, △ with solid lines), native *Thermus* PEPC (■, ●, ▲ with dashed lines), and *E. coli* PEPC (×, + with dotted lines). Assays were performed at 30°C (A) and 70°C (B) as described in "MATERIALS AND METHODS" except for the buffers. The buffers used were 0.1 M Tris-H_2SO_4 (■, □, ×), 0.1 M Ches-KOH (●, ○, +), 0.1 M Gly-NaOH (◇), and 0.1 M Bicine-KOH (▲, △). The highest activities were designated as 100%. The pH of buffers at 70°C was estimated according to the value of $\Delta pH/\Delta t$ (pH units per °C) summarized in Ref. 27.

PEP and Mg^{2+} for *E. coli* PEPC were higher than those for the *Thermus* enzyme when assayed under the same conditions as those for the *Thermus* enzyme. When the *E. coli* enzyme was assayed using 0.1 M Tris-acetate buffer (pH 8.5) in the presence of 2 mM CoASAc, the $S_{0.5}$ values of PEP and Mg^{2+} were 0.15 and 0.20 mM, respectively (*8*). No substantial difference in the properties described above was observed between recombinant and native *Thermus* PEPCs.

Dependencies of Thermus PEPC on pH and Temperature—The activities of recombinant and native *Thermus* PEPCs at 30°C were highest at pH 8.6 (Fig. 3). Optimum pH of *E. coli* PEPC was almost the same as that of *Thermus* PEPC. Unlike *Thermus* PEPC, the activity of *E. coli* PEPC in 0.1 M Ches-KOH buffer was significantly lower than that in 0.1 M Tris buffer. When the assay was performed using the latter buffer in the presence of 20 mM PEP and 2.0 mM CoASAc, the *E. coli* enzyme showed almost constant activity in the range from pH 7.0 to 9.0 (*8*). The pH profile of *Thermus* PEPC at 70°C showed a broad peak, and activity near the maximum was observed even at pH 7.0. The temperature-dependence of the enzyme activity was measured using 0.1 M Ches-KOH buffer (pH 8.6). The activity of *Thermus* PEPC in the standard reaction mixture in which concentrations of PEP and CoASAc were 2 and 0.3 mM, respectively, increased with increasing temperature up to 70°C and then sharply decreased (Fig. 4). The OAA-forming activity and P_i-liberating activity of *Thermus* PEPC showed similar variations with increasing temperature (discussed in a later section). When the recombinant

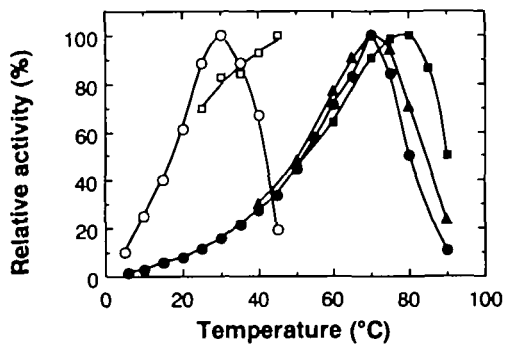

Fig. 4. **The effect of temperature on the activity of PEPCs.** The enzymes examined were recombinant *Thermus* PEPC (●, ■), native *Thermus* PEPC (▲), and *E. coli* PEPC (○, □). Assays were performed in the standard assay mixture (●, ▲, ○), modified standard assay mixture which contained 1 mM CoASAc and 18 mM PEP (■), or a buffer which contained 0.1 M Tris-H_2SO_4, pH 8.5, 16 mM PEP, 10 mM $KHCO_3$, 10 mM $MgSO_4$, 0.1 mM NADH, 1.0 mM CoASAc, and 1.0 U malate dehydrogenase (□). The buffers were prepared so as to adjust the pH to 8.6 at each assay temperature. The pH values at each temperatures were estimated as described in the legend to Fig. 3. The highest activities of each enzyme were designated as 100%.

enzyme was assayed in the presence of 18 mM PEP and 1 mM CoASAc, the activity reached maximum at 80°C and decreased at higher temperature. The decrease of the activity at higher temperature was not due to irreversible heat inactivation during assay, as will be discussed in a later section. Similar tendencies were also observed with *E. coli*

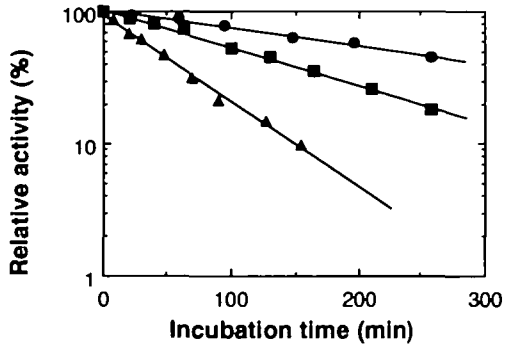

Fig. 5. **Heat inactivation of recombinant *Thermus* PEPC at 85 (●), 90 (■), and 95°C (▲).** The experiments were performed as described in "MATERIALS AND METHODS." The control values (100%) are the activities before the incubation.

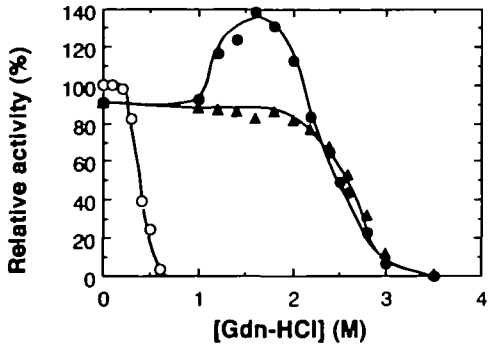

Fig. 6. **The activities of PEPCs after incubation with Gdn-HCl.** The enzymes examined were recombinant *Thermus* PEPC (●), native *Thermus* PEPC (▲), and *E. coli* PEPC (○). Experiments were performed as described in "MATERIALS AND METHODS." The control values (100%) for both enzymes are the activities in the absence of Gdn-HCl in both incubation and assay mixtures. Final concentrations of Gdn-HCl in the assay mixture were 40 mM (*Thermus* PEPC) and 5 mM (*E. coli* PEPC). The apparent activity of the *Thermus* enzyme incubated without Gdn-HCl was 90%, since Gdn-HCl in the assay mixture slightly inhibited the enzyme (see text).

PEPC. When the enzyme was assayed in the standard assay mixture, the decreased activities were observed above 30°C. Since the Ches-KOH buffer was inhibitory for *E. coli* PEPC (see Fig. 3), the activities at temperatures between 25 and 45°C were measured in 0.1 M Tris-H_2SO_4 buffer (pH 8.5) containing 16 mM PEP and 1 mM CoASAc. Under these conditions, the activity increased with increasing temperature up to 45°C (Fig. 4).

Stability of Thermus PEPC—Inactivation of recombinant *Thermus* PEPC at high temperature (above 85°C) proceeded very slowly, while *E. coli* enzyme was completely inactivated by incubation at 65°C for 10 min (data not shown). The inactivation kinetics was of first-order and the times required for half-inactivation of *Thermus* PEPC at 85, 90, and 95°C were 220, 110, and 50 min, respectively (Fig. 5). The inactivation kinetics at 90°C was almost the same as that of native *Thermus* PEPC studied previously (*16*).

Both recombinant and native *Thermus* PEPCs were highly tolerant to Gdn-HCl, a protein denaturant. Treatment with 1 M Gdn-HCl had no effect on *Thermus* PEPCs, whereas it caused instantaneous inactivation of the *E. coli* enzyme. Figure 6 shows residual activities of *Thermus* and *E. coli* PEPCs after incubation with various concentrations of Gdn-HCl at 30°C for 20 h. The residual activities of recombinant *Thermus* PEPC incubated with 1-2 M Gdn-HCl and assayed at both 30 and 70°C were reproducibly higher than the original activity. However, this phenomenon was not observed with native *Thermus* PEPC. In the presence of 2.5 M Gdn-HCl, half of the original activities were retained after 20 h. Entire loss of the activities was brought about by incubation with 3 M Gdn-HCl. In Fig. 6, activities of *Thermus* PEPCs incubated without Gdn-HCl were about 90% of the original ones because of inclusion of 40 mM Gdn-HCl. These decreased activities are not due to a denaturing effect of Gdn-HCl but rather to inhibition by the accompanying chloride ion, as reported with the *E. coli* enzyme (*23*), because addition of 40 mM NaCl depressed the activity of *Thermus* PEPC to the same extent (data not shown). When the rate of inactivation was measured at 3 M Gdn-HCl, the time required for half-inactivation was 1 h at 30°C (data not shown).

In both experiments with *Thermus* PEPCs, no recovery of the activity was detected for fully or partially inactivated samples during enzyme assay in which the temperature was lowered or Gdn-HCl was diluted.

DISCUSSION

In this work, several properties of *Thermus* PEPC were compared with those of the *E. coli* enzyme, since a phylogenetic tree constructed with 19 PEPCs including *Thermus* PEPC showed that *Thermus* PEPC has the closest relationship with the *E. coli* enzyme (Ref. *16* and Toh, H. *et al.*, unpublished data).

As described in "RESULTS," pTHPPC, an expression plasmid for *Thermus* PEPC, did not complement the phenotype of *E. coli* F15, a deletion mutant of the *ppc* gene. This is not because *Thermus* PEPC was inactive in the cells of F15 but because the polypeptide of the recombinant enzyme was not accumulated in F15 cells. This is supported by the fact that neither PEPC activity nor the corresponding polypeptide band on SDS-PAGE was detected from F15/pTHPPC cells grown in a medium containing glutamate. In the case of expression of cDNA for maize PEPC, in which the same *lac* promoter was used, the enzyme was accumulated and complemented the phenotype of *ppc* deletion (Ref. *24* and Ueno, Y. *et al.*, unpublished data). It is still unknown why *Thermus* PEPC was expressed in JM109 but not in F15. When the properties of *Thermus* PEPC purified from *E. coli* and from *Thermus* sp. were compared, they were found to be similar but not identical: a significant difference was observed when the enzymes were incubated with 1-2 M Gdn-HCl. The reason of the difference is under investigation.

Most PEPCs of various species are regulated by a wide variety of allosteric effectors. Phosphoenolpyruvate carboxylases have been assigned into four classes according to their regulatory behavior (*1*): (i) enzymes activated by CoASAc, (ii) those inhibited by C_4 dicarboxylic acids or intermediates of the citric acid cycle, (iii) those regulated with no effectors, and (iv) those belonging to no classes described above. *Thermus* PEPC belongs to class (i) because it is activated by CoASAc, like the *E. coli* enzyme. Inhibition by aspartate or malate was also observed,

although the sensitivity to aspartate was lower than that of the *E. coli* enzyme. The regulatory properties of *Thermus* PEPC were characterized by the inhibition by several *P*-compounds and orthophosphate. *Thermus* PEPC was inhibited by multiple *P*-compounds such as Fru-1,6-P_2 and GTP, which are known to activate the *E. coli* enzyme. The manner and physiological importance of the inhibition by *P*-compounds remain to be elucidated. *E. coli* PEPC is also activated by non-physiological organic solvents such as dioxane (*25*). However, such activation was not observed with *Thermus* PEPC, although the enzyme was not inactivated by high concentrations (up to 4 M) of dioxane (Abe, K. *et al.*, unpublished data).

During the purification procedures, a marked difference between *Thermus* and *E. coli* PEPCs was found in the behavior in the hydrophobic-interaction chromatography with Butyl-Toyopearl, as mentioned in "RESULTS." The observation suggests that *Thermus* PEPC interacts more strongly with the hydrophobic-interaction column than does the *E. coli* enzyme, which is consistent with the amino acid composition of *Thermus* PEPC reported previously (*16*): in *Thermus* PEPC, the content of aliphatic residues is higher and that of hydrophilic and neutral residues is lower than those in PEPCs from mesophilic organisms.

The activity of *Thermus* PEPC began to decrease above 70°C when assayed in the standard assay mixture. When the activity was measured in the presence of higher concentrations of ligands where the concentration of PEP was brought near to complete saturation, the so-called optimum temperature shifted to 80°C, but the activity still decreased at higher temperature. The results of the heat inactivation experiment (Fig. 5) indicated that decrease of activity at higher temperature was not the consequence of irreversible heat inactivation. To confirm this, recombinant *Thermus* PEPC in the PEP-omitted reaction mixture was incubated at 80°C for 10 min (twice the time taken for the enzyme assay at high temperature) and then assayed at 70°C. The activity after incubation at 80°C, where the activity had been 50% of that at 70°C, recovered to 90% at 70°C. Thus, the decrease of activity at high temperature may be caused by a marked change of kinetic parameters. There is also a possibility of temperature-facilitated reversible unfolding of the enzyme at these temperatures. Further characterization of this behavior remains to be carried out.

Since the OAA-forming activity and P_i-liberating activity varied in parallel with increasing temperature (data not shown), catalytic activity in the standard assay mixture for the whole reaction, but not for the partial reaction, was impaired above 70°C as discussed below. According to the stepwise model of the PEPC-catalyzed reaction, the first step is a reaction that forms carboxyphosphate and the enolate anion of pyruvate (Pyr), and the second step is a nucleophilic attack of the enolate anion on carboxyphosphate or on CO_2 derived from the carboxyphosphate. Impairment of only the latter reaction leads to the formation of Pyr, HCO_3^-, and P_i due to hydrolysis of carboxyphosphate by the occasional attack of water in the surrounding medium. In maize PEPC, the activity of bicarbonate-dependent PEP-hydrolysis is inherent and constitutes about 5% of the total activity (*26*). In the case of *E. coli* PEPC, the activity of PEP-hydrolysis was observed only for the mutant enzymes in which His138 (*7*) or Arg587 (*8*) was replaced by Asn or Ser, respectively. In *Thermus* PEPC, no difference was observed between OAA-forming and P_i-liberating activities at all temperatures examined (data not shown). Thus, the decreased activities at temperatures above 70°C reflect the impairment of the whole reaction or the first partial reaction, but not the latter partial reaction(s).

Proteins of thermophiles often show tolerance not only to heat but also to denaturants. In this work, tolerance of *Thermus* and *E. coli* PEPCs to Gdn-HCl was examined. Like other proteins from thermophiles, the concentration of Gdn-HCl required for inactivation of *Thermus* PEPC was higher than that of *E. coli* PEPC. It is of interest that incubation with 1–2 M Gdn-HCl enhanced the activity of recombinant *Thermus* PEPC but not that of the native enzyme. Details of the behavior of recombinant and native *Thermus* PEPCs in the presence of 1–2 M Gdn-HCl and what differentiates these enzymes remain to be elucidated.

We express our sincere thanks to Mr. I. Yoshioka and Mr. M. Takahashi (Asahi Chemical Industry) for partial characterization of PEPC purified from *Thermus* sp. and their generous gift of purified native PEPC from *Thermus* sp. and to Dr. S. Hata (Kyoto University) for useful suggestions and critical reading of the manuscript.

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