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Purification and characterization of phosphoenolpyruvate carboxylase from the hyperthermophilic archaeon *Methanothermus sociabilis*

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Abstract Phosphoenolpyruvate carboxylase (PEPC) was purified for the first time from hyperthermophilic archaeon *Methanothermus sociabilis*, growing autotrophically with an optimum at 88°C. The optimum temperature for enzyme activity was similar to that for growth and was 85°C. The native enzyme was a homotetramer of 240 kDa molecular mass and the subunit displayed an apparent molecular mass of 60 kDa. The archaeal PEPC was insensitive to various metabolites which are known as allosteric effectors for most bacterial and eucaryal counterparts. The enzyme showed extreme thermostability such that there remained 80% of the enzyme activity after incubation for 2 h at 80°C. These results implied that archaeal PEPC was significantly different from bacterial and eucaryal entities.

Key words: Phosphoenolpyruvate carboxylase; Hyperthermophilic archaeon; *Methanothermus sociabilis*; Central metabolism; Non-allosteric property; Thermostability

1. Introduction

Phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) catalyzes the reaction that fixes HCO_3^- on phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) and inorganic phosphate using Mg^{2+} as a cofactor [1]. The enzyme is distributed widely in mesophilic bacteria, protozoa, algae and plants, and a large number of the enzymes have been purified, cloned, and sequenced from various sources [2–6]. In these organisms, PEPC primarily plays an anaplerotic role by replenishing C_4-dicarboxylic acids to the citric acid cycle or a key role in photosynthetic CO_2 assimilation [1]. These regulatory functions are also associated with the allosteric properties of the enzymes [7].

A distinct group of microorganisms called hyperthermophiles, capable of growing up to 90°C [8–11], are known. Most of them have been classified within the domain of Archaea [12]. These microorganisms adapt their biochemical machinery to extraordinary temperatures close to and even above 100°C. Therefore, their enzymes and metabolic systems are largely different from other mesophilic counterparts with respect to structures and functions [8–10,13]. However, understanding of such novel biochemistry is limited except for several well studied hyperthermophiles such as *Thermotoga maritima* [14] and *Pyrococcus furiosus* [15] and no hyperthermophilic PEPC has been reported to date.

As a possible source of the hyperthermophilic PEPC, we chose the hyperthermophilic methanogen *Methanothermus sociabilis*, which grows at temperatures up to 97°C [16]. This organism is an anaerobic sulfur-independent autotroph utilizing H_2 and CO_2 as sole carbon and energy source. It has been suggested that the central metabolism of methanogens is quite different from that of other archaeal members with respect to hexose metabolism and the citric acid cycle [17–19]. Fuchs and Stupperich [20] also suggested that the novel incomplete reductive citric acid cycle of the methanogens plays an important role in autotrophic CO_2 fixation. In the methanogenic archaea, PEPC is thought to catalyze the first step of the reductive citric acid cycle [21]. However, the enzymatic properties and physiological roles remain unknown.

We report here the purification and characterization of PEPC from the hyperthermophilic archaeon *M. sociabilis*. The enzyme was compared with bacterial and eucaryal homologs with respect to enzymological properties. This is the first report on the purification of archaeal PEPC and can provide new insight into the diversity and evolution of the enzyme.

2. Materials and methods

2.1. Bacterial strains and growth conditions

M. sociabilis (DSM3496) was purchased from the Deutsche Sammlung von Mikroorganismen (Germany). For cultivation of *M. sociabilis*, the medium and procedure of Lauerer et al. [16] were used except for modification of the gas pressure (200 kPa). For enzyme purification, *M. sociabilis* was grown at 85°C, harvested at the late exponential growth phase by centrifugation and washed three times with 50 mM Tris-HCl (pH 7.5). The pellet was stored at −85°C prior to purification.

2.2. Enzyme assays

PEPC activity was routinely coupled to the malate dehydrogenase (MDH) reaction and assayed by monitoring NADH oxidation at 340 nm using a UV-240 recording spectrophotometer (Shimadzu, Kyoto, Japan) at 85°C. For measurement of the activity at high temperature, a heat-stable MDH from *Thermus flavas* (Sigma) was used as a coupling enzyme. All assays were initiated by addition of enzyme preparation to the preheated reaction mixture containing 50 mM Tris-H_2SO_4 (pH 8.0), 10 mM $NaHCO_3$, 5 mM PEP, 2 mM $MgSO_4$, 0.15 mM NADH, and 2 U *T. flavas* MDH in a final volume of 1.0 ml unless otherwise noted. In an attempt to examine the effect of pH on enzyme activity, 50 mM Tris-H_2SO_4 was used in the range pH 7.0–9.5 and 50 mM KH_2PO_4-Na_2HPO_4 (phosphate buffer) was employed in the range pH 5.5–7.0, as a buffer of the reaction mixture. In order to test the requirement of PEPC for divalent cations, various concentrations of $MgSO_4$ or $MnSO_4$ were added to the reaction mixture in place of 2 mM $MgSO_4$. One unit of PEPC activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADH per min.

2.3. Purification

All procedures were carried out aerobically at 0–4°C. Thawed cell paste of *M. sociabilis* (50 g wet wt) was suspended in 100 ml of 50 mM Tris-HCl (pH 7.5) buffer. The cells were disrupted by seven passages through a French Press 5501-N (Ohtake Seisakusho, Tokyo, Japan) at

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Abbreviations: PEPC, phosphoenolpyruvate carboxylase; PEP, phosphoenolpyruvate; OAA, oxaloacetate; MDH, malate dehydrogenase; SDS, sodium dodecyl sulfate

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1500 kg/cm^2 and centrifuged at 24000×g for 20 min. The supernatant was used as the cell-free extract. Solid ammonium sulfate was added to the cell-free extract to reach 80% saturation. After stirring for 30 min, the extract was centrifuged at 24000×g for 20 min. The pellet was dissolved in 50 mM Tris-HCl (pH 7.5) and dialyzed against the same buffer twice. This dialyzed fraction after ammonium sulfate precipitation was applied to the next purification step.

The fraction was loaded onto a DEAE-cellulose DE-52 (Whatmann, Kent, UK) column (1.5×22 cm) equilibrated with 50 mM Tris-HCl (pH 7.5), and then washed with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0–0.5 M) in the buffer. The active fractions were pooled and dialyzed against 10 mM Tris-HCl (pH 8.0) containing ammonium sulfate at 40% saturation.

The dialyzed enzyme solution was applied to a column of phenyl-Sepharose 6FF (Pharmacia) (1.5×14 cm) equilibrated with the same buffer as for the dialysis. The enzyme was eluted with a co-linear gradient of ammonium sulfate (40–0% saturation) and ethylene glycol (0–50%, v/v) in the buffer. The active fractions was pooled and dialyzed against 50 mM Tris-HCl (pH 7.5) and then concentrated 10-fold with an Amicon YM-30 ultrafilter (Amicon).

The concentrated enzyme solution was applied to a column of Mono Q (bed volume 1 ml) equilibrated with 50 mM Tris-HCl (pH 7.5) that had been attached to the FPLC system (Pharmacia). The enzyme was eluted with a linear gradient of NaCl (0–0.5 M). The pooled active fractions were concentrated 5-fold with the Amicon YM-30 ultrafilter and diluted 5-fold with 200 mM Tris-HCl (pH 7.5). This procedure was repeated twice, and finally, the 5-fold concentrated enzyme solution was applied to the final step, the column of Superdex 200HR in the FPLC system pre-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 *M. sociabilis* enzyme. As molecular weight standards, a HMW gel filtration calibration kit was used (Pharmacia). The purified enzyme was frozen and stored at −90°C, being stable for at least 6 months.

2.4. Other methods

Polyacrylamide gel electrophoresis of the native enzyme was performed with a 7.5% (w/v) polyacrylamide gel for confirming the enzyme purity, and with a 10% (w/v) polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) for determining the molecular mass of the subunit by the method of Laemmli [22]. Molecular weight markers for SDS-PAGE were from Bio-Rad. Protein concentrations were routinely estimated according to Bradford [23] with bovine serum albumin as the standard.

2.5. Thermostability studies

The enzyme was incubated at various temperatures in 50 mM Tris-HCl (pH 8.0) for different periods of time. Thermoinactivation was stopped by cooling the aliquots on ice and then the residual PEPC activity was measured at 85°C.

3. Results

3.1. Purification of PEPC from hyperthermophilic methanogen

As shown in Table 1, PEPC of *M. sociabilis* was purified 69-fold with a final specific activity of 2.70 μmol NADH/min per mg, and the overall yield of the activity was 9.5%. Although the Superdex 200HR column was used for further purification of the enzyme, this treatment decreased the yield from 9.5 to 0.4% due to the strong affinity for the enzyme, but

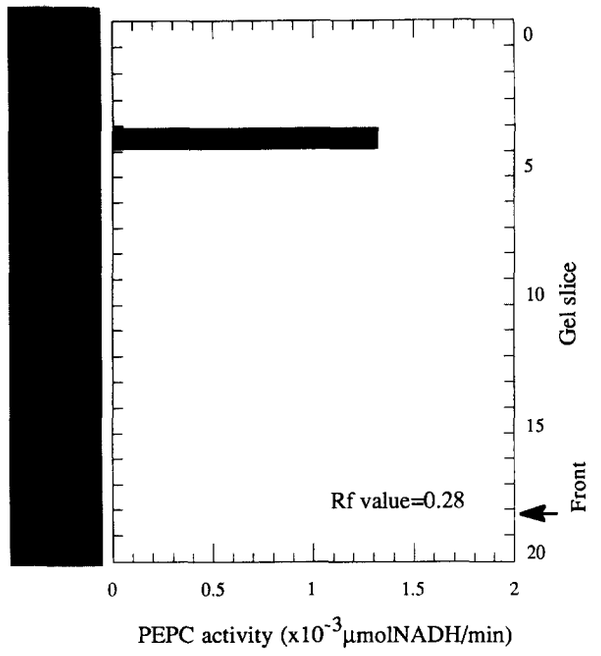

Fig. 1. Native polyacrylamide gel electrophoresis of purified PEPC from *M. sociabilis* (left) and PEPC activity of the gel fractions sliced every 3 mm (right). Purified PEPC from MonoQ (5 μg) was loaded on 7.5% PAGE and stained with Coomassie brilliant blue R-250.

led to 704-fold purification. The gel filtration column was then used only for the determination of the molecular mass. The purity of the enzyme was confirmed by native PAGE (Fig. 1). The sample which passed through MonoQ showed a single protein band corresponding to the active fractions sliced every 3 mm from top to bottom. The most notable property observed in the course of purification was that the enzyme strongly bound on hydrophobic interaction chromatography. This caused the enzyme to be successfully separated in the preparation.

3.2. Molecular properties of PEPC

The molecular mass of the native enzyme from *M. sociabilis* was estimated by gel filtration to be about 240 kDa (Fig. 2A). On SDS-polyacrylamide gel electrophoresis, the purified enzyme gave rise to a single protein band with a molecular mass of about 60 kDa (Fig. 2B). These results suggested that the enzyme was a 240 kDa homotetramer consisting of a 60 kDa subunit.

3.3. Effect of temperature, pH, and cations on PEPC

The purified enzyme had the highest activity at ambient temperature of around 85°C (Fig. 3). The optimum pH value for the activity was approx. 8.5 (Fig. 4). The enzyme absolutely required divalent cations for activity (Fig. 5). It utilized

Table 1
Purification of PEPC from *M. sociabilis*

Preparation	Volume (ml)	Total protein (mg)	Total activity (μmol NADH/min)	Yield (%)	Specific activity (μmol NADH/min per mg)	Purification (-fold)
Cell-free extract	140.0	18900	303	100	0.016	1
0–80% saturation of $(NH_4)_2SO_4$	139.2	16008	288	95.2	0.018	1.1
DEAE-cellulose DE-52	66.0	376.4	56.5	18.7	0.15	9.4
phenyl-Sepharose 6FF	72.0	23.8	38.9	12.8	1.64	103
FPLC Mono Q	24.0	1.04	28.6	9.5	2.70	169

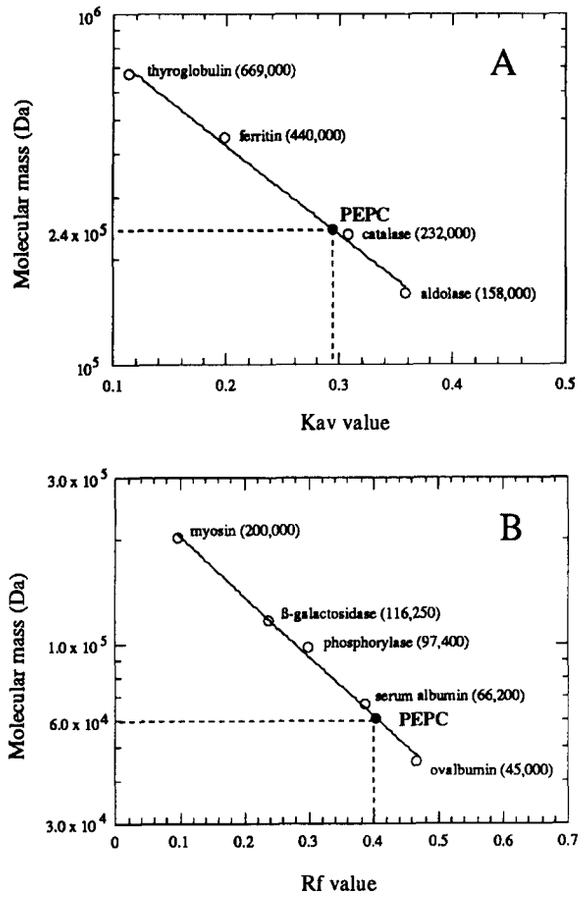

Fig. 2. Determination of the molecular mass of PEPC from *M. sociabilis*. (A) Molecular mass of the native enzyme determined using a gel filtration column (Superdex 200HR). As molecular weight standards, a HMW gel filtration calibration kit was used (Pharmacia). (B) Molecular mass of the subunit determined by SDS-polyacrylamide gel electrophoresis. Molecular weight markers for SDS-PAGE were from Bio-Rad.

not only Mg^{2+} which is the most common cation for PEPC, but also a low concentration of Mn^{2+} instead of Mg^{2+}. The kinetic constant for PEP at 85°C at pH 8.5 was calculated to be 1.3 mM from a Lineweaver-Burk plot.

3.4. Effect of metabolites on PEPC

The effect of several metabolites on PEPC activity which are major positive or negative effectors for the mesophilic PEPCs were investigated. The relative activities with various concentrations of metabolites were 102.2% (5 mM), 95.8% (10 mM) with acetyl-CoA, and 97.4% (2 mM), 98.8% (5 mM), 103.3% (10 mM) with glucose 6-phosphate, and 99.6% (2 mM), 100.8% (5 mM), 99.7% (10 mM) with fructose 1,6-diphosphate, and 96.4% (2 mM), 94.7% (5 mM), 92.1% (10 mM) with L-aspartic acid, and 105.3% (2 mM), 106.5% (5 mM), 107.2% (10 mM) with L-malic acid. All substances tested had no effect on the activity at any concentration.

3.5. Thermostability of PEPC

M. sociabilis PEPC was quite stable at high temperatures. No loss of activity was observed after incubation for 2 h at 75°C (Fig. 6). Moreover, the times required for 50% loss

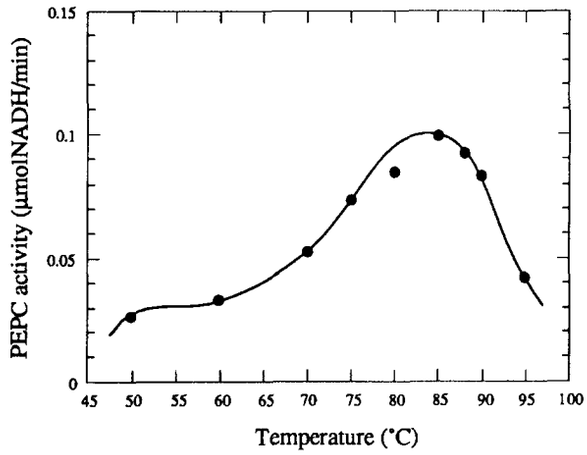

Fig. 3. Effect of temperature on PEPC activity from *M. sociabilis*. Enzyme assay conditions are described in Section 2. The activity was measured with a concentration of 10 μg PEPC/ml.

of activity were about 240 min at 80°C, 20 min at 90°C and 1 min at 100°C (Fig. 6).

4. Discussion

During recent years, a number of studies of the novel carbohydrate and tricarboxylic acid metabolisms of archaea have been published [17,24–26]. The most extensively investigated metabolic pathway is the saccharolytic pathway of *P. furiosus*, called 'pyroglycolysis' and the enzymes involved have been purified and characterized [8]. These are new type of enzymes as well as modified versions of known mesophilic enzymes. They are hyperthermostable and good models for understanding the thermostability of the central metabolic enzymes. However, the biochemical diversity within the hyperthermophilic archaea except for archaeal sulfur-reducing heterotrophic species is largely unknown.

PEPC catalyzes the reaction that fixes HCO_3^- on PEP to form OAA and inorganic phosphate using Mg^{2+} as a cofactor [1]. The enzyme is widely distributed in the domain Bacteria

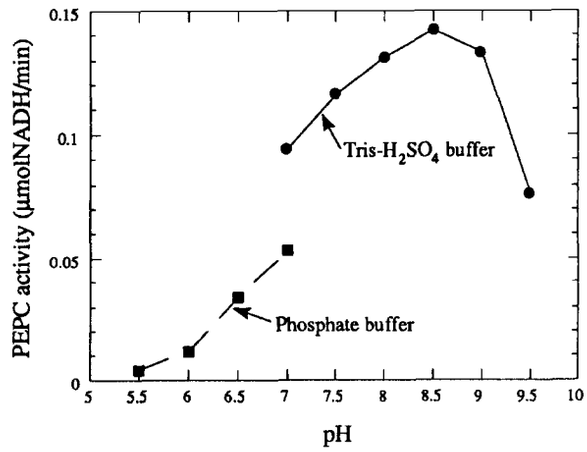

Fig. 4. Effect of pH on PEPC activity from *M. sociabilis*. The activity was measured at 85°C with a concentration of 10 μg PEPC/ml. As a buffer of the reaction mixture, 50 mM Tris-H_2SO_4 was used in the range pH 7.0–9.5 and 50 mM phosphate buffer in the range pH 5.5–7.0.

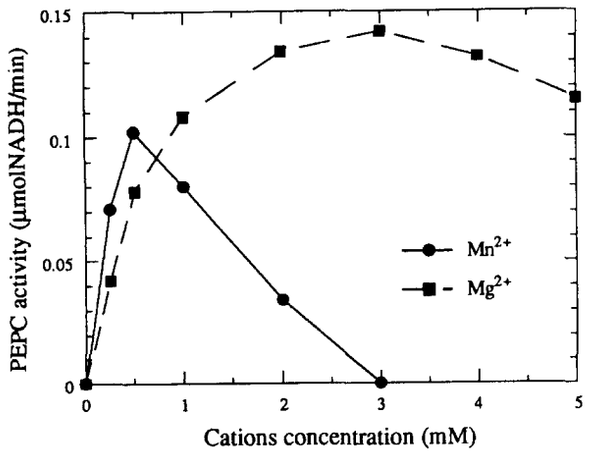

Fig. 5. Effect of divalent cations on PEPC activity from *M. sociabilis*. The activity was measured at 85°C with a concentration of 10 μg PEPC/ml. Both ions were supplied in the form of sulfate salts.

and Eucarya, and primarily acts anaplerotically by replenishing C_4-dicarboxylic acids to the citric acid cycle. Nevertheless, the archaeal entity is little known not only regarding the molecular and catalytic properties, but also its physiological function and regulation. Hence, we describe here the purification and characterization of PEPC from the hyperthermophilic methanogen *M. sociabilis* for the first time and compare the molecular and enzymatic properties with those of the mesophilic homologs from the domain Bacteria and Eucarya.

In comparison with the bacterial and eucaryal homologs, PEPC purified from *M. sociabilis* indicated a unique molecular size and a novel non-allosteric property (Table 2). The enzyme was a homotetramer of about 240 kDa consisting of subunits with a molecular mass of 60 kDa. Considering that almost all PEPCs reported to date are homotetramers of approx. 400 kDa consisting of about 100 kDa subunits, the *M. sociabilis* enzyme is considerably smaller than its bacterial and eucaryal counterparts. Conversely, a 60 kDa subunit might be sufficient for the primary catalytic activity of PEPC. Moreover, on the basis of the non-allosteric property of *M. sociabilis* PEPC that the enzyme was not affected by any metabolites tested, it was acceptable that a considerable part of the allosteric PEPC body participated in allosteric regulation. From a comparison of the amino acid sequences between PEPCs from *Escherichia coli* (allosteric) and *Anacystis nidulans* (non-allosteric), Ishijima et al. [27] suggested that the conserved region of the C-terminal half was the active core of the enzymes and the variable region of the N-terminal half was involved in the allosteric regulation. Hence, the lack of the N-terminal half might account for the smaller size and non-allosteric property in the *M. sociabilis* enzyme to a large extent.

The non-allosteric property of *M. sociabilis* PEPC also implied that the physiological role of PEPC in the hyperthermophilic methanogen was different from both the anaplerotic function in most bacteria and plants and the CO_2 assimilation in C_4 and CAM (crassulacean acid metabolism) plants. Fuchs and Stupperich [20] suggested that the novel incomplete reductive citric acid cycle of the methanogens played an important role in autotrophic CO_2 fixation and that PEPC was one of the key CO_2-fixing enzymes. For *M. sociabilis*, CO_2 is the sole carbon source and must be taken up vigorously during growth. Therefore, it was speculated that *M. sociabilis* PEPC functioned for CO_2 assimilation in vivo and for that reason, it almost lacked the N-terminal half of the enzyme which might be involved in the allosteric property, in order to elevate the CO_2 fixation efficiency.

On the other hand, the *M. sociabilis* enzyme exhibited extreme thermostability in response to temperature. The enzyme had an optimum temperature for activity around ambient, and possessed about 50% of activity after incubation at 80°C for 240 min (Fig. 6). However, *M. sociabilis* PEPC was relatively less thermostable as compared to not only PEPC of an extreme thermophile, *Thermus* sp. [3], but also other intracellular enzymes of hyperthermophiles [9]. This reduced thermostability may be coupled to the sensitivity to oxygen since a decrease in enzyme activity was observed according to exposure to air in liquid buffer. In order to prove the proper thermostability of *M. sociabilis* PEPC, anaerobic manipulation including a purification procedure may be required.

In proportion to the accumulation of phylogenetic studies on thermophilic bacteria and archaea, speculation on the thermophilic origin of life has been widely accepted [28]. Therefore, the discovery of PEPC from the hyperthermophilic archaeon *M. sociabilis* is consistent with the ancient origin of this enzyme, i.e. that it might have arisen before the division between the domain Bacteria and Archaea. In relation to the ancient form of PEPC, the small size (60 kDa subunit) and non-allosteric property of *M. sociabilis* PEPC are important clues and comparable to the full size (100 kDa subunit) and allosteric property of the thermophilic counterpart from *Thermus* sp. ([3]; personal communication). Besides, the mechanism of the extreme thermostability of the enzyme and its physiological function in methanogenic archaea are the focus of future research.

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Table 2
Comparison of *M. sociabilis* PEPC with bacterial and eucaryal homologs

Properties	Archaea *M. sociabilis*	Bacteria *E. coli*[a]	Eucarya *Z. mays*[b]
Molecular mass (kDa)	240	360	400
Subunit	Homotetramer	Homotetramer	Homotetramer
Optimum temperature for activity (°C)	85	35–38	40
Optimum pH for activity	8.5	7.5	7.5–8.0
K_{PEP} value (mM)	1.3	~20	~0.1
Positive effectors	–	Acetyl-CoA, fructose 1,6-bisphosphate, fatty acids, etc.	Glucose 6-phosphate
Negative effectors	–	L-asparatate, L-malate	L-malate

[a]Taken from Teraoka et al. [5].
[b]Taken from Ting and Osmond [6].

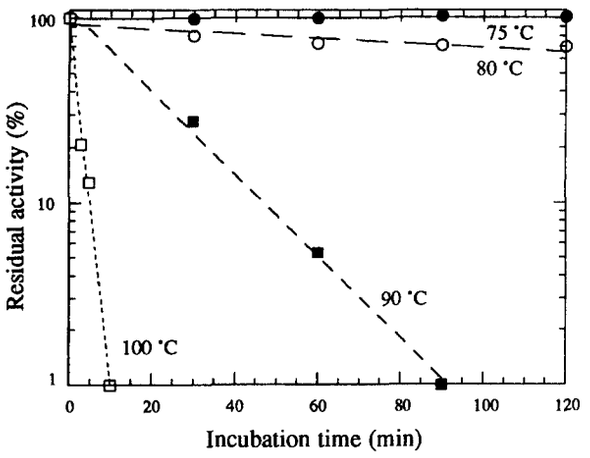

Fig. 6. Thermostability of *M. sociabilis* PEPC. Enzyme solution at a concentration of 100 μg PEPC/ml was incubated at various temperatures and cooled on ice. The residual activity was measured at 85°C with a concentration of 10 μg PEPC/ml.

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