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ORIGINAL PAPER

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A stable archaeal pyruvate carboxylase from the hyperthermophile *Methanococcus jannaschii*

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Abstract The pyruvate carboxylase (PYC) of the hyperthermophilic, strictly hydrogenotrophic, autotrophic and marine methanarchaeon *Methanococcus jannaschii* was purified to homogeneity. Optimal activity was at pH 8.5, ≥80 °C, and a KCl concentration of 0.175 M. This enzyme is the most thermophilic PYC so far studied. Unlike the *Methanobacterium thermoautotrophicum* enzyme, *Mc. jannaschii* PYC was expressed in cells grown without an external source of biotin and in the purified form was stable during storage at 4, –20 and –80 °C. However, it was rapidly inactivated at 80 °C. The enzyme was insensitive to aspartate and glutamate, mildly inhibited by α-ketoglutarate, and was strongly inhibited by ATP and ADP (apparent K_m for ATP, 0.374±0.039 mM; apparent K_i for ATP, 5.34±2.14 mM; K_i for ADP, 0.89±0.18 mM). It was also strongly inhibited when the Mg^{2+} concentration in the assay exceeded that of ATP. Thus, this stable PYC could serve as a model for mechanistic studies on archaeal PYCs. It was apparently an $\alpha_4\beta_4$-type PYC composed of a non-biotinylated 55.5-kDa subunit (PYCA) and a 64.2-kDa biotinylated subunit (PYCB). The determined NH_2-terminal sequences for these subunits provided additional support for our earlier proposal to rename the ORFs MJ1229 and MJ1231 in the NCBI *Mc. jannaschii* genome sequence database as PYCA and PYCB, respectively; even very recently, these have been misidentified as a subunit of acetyl-CoA carbxoylase (AccC) and the α-subunit of ion-pumping oxaloacetate decarboxylase (OADα), respectively.

Keywords Methanarchaea · *Methanococcus jannaschii* · Pyruvate carboxylase · Extreme thermophile · Storage stable · Thermolability · *pyc* genes

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Abbreviations *OAA* Oxaloacetate · *α-KGA* α-Ketoglutarate · *OADα* α-Subunit of ion-pumping oxaloacetate decarboxylase · *oadA* Gene for OADα · *PYC* Pyruvate carboxylase · *PPC* Phosphoenolpyruvate carboxylase · *BC* Biotin carboxylase · *ACC* Acetyl-CoA carboxylase · *accC* Gene for the biotin carboxylase subunit of ACC · *PYCA* Subunit A of PYC carboxylase (composed of the ATP/bicarbonate binding or the BC domain) · *PYCB* Subunit B of PYC carboxylase (composed of carboxytransferase and biotinylated domains) · *Mc. Methanococcus* · *Mb. Methanobacterium*

Introduction

Biosynthesis of oxaloacetate (OAA) is one of the major CO_2-fixation reactions in methanarchaea (Simpson and Whitman 1993). Of the known OAA-biosynthesis enzymes, methanogens have been found to possess pyruvate carboxylase (PYC) and phosphoenolpyruvate carboxylase (PPC) (Simpson and Whitman 1993).

$$\text{Pyruvate} + HCO_3^- + \text{ATP} \xrightarrow{\text{PYC}} \text{OAA} + \text{ADP} + P_i$$

$$\text{PEP} + HCO_3^- \xrightarrow{\text{PPC}} \text{OAA} + P_i$$

Methanococcus (*Mc.*) *maripaludis* uses a PYC for OAA synthesis (Shieh and Whitman 1987). *Methanosarcina* is devoid of PPC activity (Weimer and Zeikus 1979), and the OAA biosynthesis route in these organisms remains unknown. Kenealy and Zeikus (1982) and Jansen et al. (1982) have shown that *Methanobacterium* (*Mb.*) *thermoautotrophicum* (basonym; newly classified as *Methanothermobacter thermoautotrophicus*; Wasserfallen et al. 2000) is devoid of PYC and makes OAA by use of PPC. The same conclusion has been re-iterated recently by Menendez et al. (1999), although we have demonstrated that *Mb. thermoautotrophicum* possesses a PYC (Mukhopadhyay et al. 1998). Similar to several bacteria, this methanarchaeon needs exogenously supplied biotin for expressing this activity (Mukhopadhyay et al. 1998) even though it can synthesize this vitamin (Noll and Barber

1988). We have described the genes for the subunits PYCA and PYCB of *Mb. thermoautotrophicum* PYC and provided the experimental evidence for these assignments (Mukhopadhyay et al. 1998). From sequence similarity searches, we also identified the *pycA* and *pycB* genes in *Methanococcus jannaschii* (Jones et al. 1983; Bult et al. 1996). Previous to our work, ORFs corresponding to the *pycA* and *pycB* genes of *Mc. jannaschii* and *Mb. thermoautotrophicum* were assigned the following protein functions: *pycA*, biotin carboxylase or BC (AccC), and *pycB*, the α-subunit of ion-pumping oxaloacetate decarboxylase (OADα) (Bult et al. 1996). Although these assignments are misleading or wrong, they have been emphasized in two recent reports (Burton et al. 1999; Menendez et al. 1999). For this reason and in the search for an archaeal PYC that would be relatively stable and thus suitable for detailed functional analysis, we have purified and characterized the PYC of *Mc. jannaschii*. The purified PYC of *Mb. thermoautotrophicum* loses its activity very rapidly (Mukhopadhyay et al. 1998). We also show that the use of tools based on biotin-avidin interaction for identification and purification of biotinylated proteins from methanarchaea could yield erroneous or ambiguous results.

Materials and methods

Purification of PYC from *Mc. jannaschii*

The organism was grown in a stirred-tank reactor by using the previously recommended conditions for best cell yield (Mukhopadhyay et al. 1999), except the Se level was 50 μM. The harvested cells were stored frozen at –70 °C. The enzyme was purified by use of a modified version of the procedure that has been used for the PYC from *Mb. thermoautotrophicum* strain ΔH (Mukhopadhyay et al. 1998); only the changes are detailed below. Each individual step was carried out aerobically at 4 °C. The lysis buffer contained 75 mM Tris-HCl (pH 8), 7.5 mM $MgCl_2$, 7.5% inositol, 1.5 M KCl, 3 mM DTT, and 0.75 mM phenylmethyl sulfonylfluoride. Typically, 10 g of wet cell paste was mixed with 20 ml of lysis buffer; the cells were then lysed and the lysate was centrifuged as described previously (Mukhopadhyay et al. 1998). About 25 ml of supernatant was recovered from the ultracentrifugation step and was passed through four layers of cheesecloth to remove material floating on the surface. The clarified extract was diluted and fortified with pyruvate and bicarbonate, but not with ATP. The fortified extract was loaded onto a regenerated 1-ml column bed of monomerized avidin-Sepharose that was pre-equilibrated with a high-salt buffer (contained 1 M KCl and previously called column buffer; Mukhopadhyay et al. 1998). Then the matrix was washed with 20 bed volumes of high-salt buffer and with same volume of a low-salt buffer (high-salt buffer without KCl), in that sequence. The bound enzyme was eluted with 10 mM D-biotin in the low-salt buffer.

Size-exclusion chromatography, gel electrophoresis, activity staining, Western blot analysis, and MALDI mass spectrometry

For the size-exclusion chromatography, a 7.8 mm×30 cm TSK-GEL G3000SWXL column, a 6 mm×4 cm SWxl guard column (Toso-Haas, Montgomeryville, Pa.) and a HPLC system (System Gold, Beckman Instrument, Fullerton, Calif.) were used. The development was isocratic at a flow rate of 1 ml min^{-1} and with a mobile phase of 100 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and 5 mM $MgCl_2$. The elution was monitored at 214 and 280 nm. The enzyme from avidin-Sepharose chromatography was concentrated to 0.6 mg protein ml^{-1} on a Centricon-10 concentrator (Amicon, Beverly, Mass.) and a 10-μl sample of this concentrate was chromatographed. The location of the PYC peak in the elution profile was confirmed by assaying the corresponding fraction for activity. The molecular size standards (Bio-Rad Laboratories, Hercules, Calif.) (mass in kDa; Stokes radius in Å) were bovine thyroglobulin (670; 85), bovine gammaglobulin (158; 52.5), chicken ovalbumin (44; 30.5), horse myoglobin (17; 19), and vitamin B_{12} (1.357 kDa); an application of 20 μl solution contained these components in amounts of 33, 33, 33, 16.7, and 3 μg, respectively. The Stokes radii and the apparent molecular masses of the eluting proteins were estimated by fitting the corresponding elution volume data to, respectively, the plots of the log[Stokes radius] and log[molecular mass value] vs elution volume for the standard proteins.

SDS-PAGE was performed with a slab gel according to Laemli (1970). For non-denaturing gel electrophoresis, Tris-HCl Ready Gels, containing 7.5% polyacrylamide, and the accompanying protocols from the Bio-Rad Laboratories were used. An avidin-blot analysis was performed as described previously (Mukhopadhyay et al. 1998).

For determining whether a protein band appearing in a non-denaturing gel possessed PYC activity, the in-gel detection protocol of Scrutton and Fatebene (1975) with the following modifications was used. After the electrophoresis was complete, the gel was incubated in a PYC assay buffer (described below) at 40 °C for 90 min. The OAA generated from this incubation was detected by following its reaction with fast violet B (1 mg ml^{-1}) (Sigma), which produced a red band.

MALDI measurements for intact PYC polypeptides were performed at the University of Illinois Mass Spectrometry Laboratory by use of a PerSeptive Biosystems (Framingham, Mass.) Voyager-DE STR time-of-flight spectrometer operating in positive linear mode. Before analysis, an enzyme sample was concentrated and desalted (by washing extensively with water) on a Microcon-10 concentrator, dried under vacuum, dissolved in 0.1% aqueous trifluoroacetic acid (TFA) and mixed with an equal volume of the matrix solution (a saturated solution of sinapinic acid in acetonitrile/water, 50:50, v/v).

Determination of the NH_2-terminal amino acid sequence and protein identification by use of in-gel tryptic digestion, mass spectrometry and database searches

The NH_2-terminal amino acid sequence of a polypeptide of interest was determined by use of the Edman degradation technique at the Protein Sciences Facility, Biotechnology Center, University of Illinois at Urbana-Champaign.

The general approach of Henzel et al. (1993) was used for obtaining the primary structure identity for a polypeptide band from a denaturing gel. A Coomassie-blue-stained gel piece containing the band (approx. 10 μl gel containing 2 μg of protein) was the starting material; a similar piece of gel, but from a lane that was not loaded with a protein sample, acted as the control. The proteolytic fragments of the polypeptide were generated by use of the in-gel digestion technique (Rosenfeld et al. 1992; Sheer 1994), but with modifications. The polypeptide was reduced by incubating the water-washed gel piece in 100 μl of 10 mM DTT (in 100 mM Tris-HCl, pH 8.5) at 55 °C for 2 h. It was then alkylated by incubating the cooled gel piece in 100 μl of 15 mM iodoacetamide (in 100 mM Tris-HCl, pH 8.5) for 30 min at room temperature in the dark. The gel piece containing alkylated polypeptide was washed twice with shaking (each time for 20 min with 200 μl 50% acetonitrile in 50 mM Tris-HCl, pH 8.5) and dried under vacuum. The dried gel piece was covered with a solution of 0.05 μg sequencing grade trypsin (Roche Molecular Biochemicals, Indianapolis, Ind.) in a minimum volume of 25 mM Tris-HCl (pH 8.5), and the mixture was incubated at 32 °C overnight. The resultant peptides were extracted from the gel piece with 50% acetonitrile containing 2% TFA; two extractions were performed, each with 200 μl acetonitrile/ TFA solution. These extracts were combined and dried under vacuum. The dried material was dissolved in 2% TFA. This solution was desalted by use of a C_{18} ZipTip (Millipore, Bedford, Mass.). The desalted product was recovered in 50% acetonitrile

containing 2% TFA and dried under vacuum. The dried material was resuspended in a matrix solution of the following composition: 4-hydroxy-α-cyanocinnamic acid (10 mg/ml) and two internal standards (angiotensin and bovine insulin) in 50% acetonitrile/0.1% TFA. This solution was spotted onto a MALDI sample plate, and the spot was allowed to dry completely. MALDI-TOF mass spectrometric analysis was then performed by use of a PerSeptive Voyager DE-RP mass spectrometer in the linear mode. Both the in-gel digestion and the mass spectrometric analyses of the unseparated digests were performed in the Protein Chemistry Core Facility of the Howard Hughes Medical Institute at Columbia University. The programs MS-Fit and ProFound (P.R. Baker and K.R. Clauser, http://prospector.ucsf.edu; http://prowl.rockefeller.edu/cgi-bin/ProFound) were used to search the NCBI non-redundant (nr) database.

Enzyme and protein assays, and data analysis

Protein was assayed according to Bradford (1976) by using the dye reagent purchased from Bio-Rad Laboratories. PYC was assayed as described previously (Mukhopadhyay et al. 1998). Unless otherwise indicated, an assay was conducted at 80 °C and by use of a reaction mixture that contained 100 mM Tris-HCl (pH 8.5), 175 mM KCl, 1 mM $MgCl_2$, 20 mM sodium pyruvate, 20 mM potassium bicarbonate, 1 mM disodium ATP, 0.2 mM disodium NADH, and 2 U thermophilic malate dehydrogenase from *Thermus flavus* (Sigma St. Louis, Mo.) per ml. For pH studies, the Tris buffer was adjusted to desired pH values with HCl and the assays were performed at 80 °C. All initial rate data were analyzed by using the KinetAsyst program version 1.01 (Intellikinetics, State College, Pa.).

Stability studies with purified enzyme

Two enzyme preparations were used for the stability studies. One was purified as described above and called the regular preparation. The other was eluted in a low-salt buffer that lacked inositol (see Results) and called the no-inositol preparation. For the storage stability studies, an enzyme solution was brought to a desired composition by diluting the starting material with an equal volume of an appropriate diluent. If the starting material was the regular preparation, the diluent was 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 10% inositol±1 M KCl. The same diluent but without inositol was used if the no-inositol preparation was used. For studying thermal stability, the no-inositol enzyme preparation was used. Here, one of the enzyme stock was diluted with four parts of a stock solution of the test compound in 100 mM Tris-HCl (pH 7); dilution with 100 mM Tris-HCl (pH 7) provided the control. These diluted enzyme solutions were then incubated at 80 °C prior to being assayed for PYC activity. In addition to various chemically defined compounds, a refined *Mc. jannaschii* cell extract preparation was also examined for its effect on the thermostability of the purified enzyme. This extract was prepared as follows: 1 g of *Mc. jannaschii* cell paste was resuspended in 1 ml 200 mM Tris-HCl (pH 7), and the cells in this suspension were lysed as described above. From the resulting slurry, a supernatant was recovered through two successive centrifugation steps (14,000×*g* for 35 min and at 160,000×*g* for 1 h). It was refined further via passage though a filter in a Microcon-10 concentrator to remove the larger components. As described above for other compounds, four parts of this filtrate were then mixed with one part of the enzyme stock; the filtrate did not possess PYC activity.

Results

Purification of PYC from *Mc. jannaschii*

Starting from 10 g of wet cells and by use of 1 ml avidin-Sepharose matrix, approximately 1 mg of enzyme, with a specific activity in the range of 20–35 U/mg, was recovered in approximately 6 ml eluent. The entire purification procedure could be completed in 6–7 h. The yield in terms of the amount of homogeneous protein was independent of whether the cells were grown autotrophically or in a medium supplemented with tryptone, yeast extract and vitamins. A native-PAGE gel (Fig. 1A) for the enzyme preparation exhibited a single Coomassie-blue-staining band. Hence, it was considered homogeneous. The corresponding avidin blot showed that the enzyme was biotinylated (Fig. 1B).When the native-PAGE gel was processed for PYC activity-dependent staining, the above mentioned protein band produced a faint red color (data not shown). The SDS-PAGE pattern for the enzyme preparation exhibited a 65-kDa and a 55-kDa band in an apparent 1:1 stoichiometric ratio and a faint 45-kDa band (Fig. 1C). Although it was found in every SDS-PAGE pattern examined, the intensity of the 45-kDa band varied from one preparation to another and was also dependent on the sample preparation method; omission of SDS in the sample buffer reduced the relative amount of the 45-kDa band, but it gave a poor separation for the other two bands (data not shown). An avidin blot with the denatured enzyme showed that the 65- and 45-kDa polypeptides were biotinylated (Fig. 1D); a few minor avidin-reacting polypeptide bands were also observed, but the 55-kDa polypeptide did not produce a signal. Attempts to obtain a PYC preparation free of the 45-kDa polypeptide via further fractionation of the avidin-Sepharose chromatography-derived enzyme were unsuccessful. For example, the PYC-activity-containing fractions from gel filtration chromatography produced the same SDS-PAGE pattern as shown in Fig. 1C. An anion-exchange chromatography step employing a KCl gradient with POROS 20 HQ matrix (PerSeptive Biosystems) inactivated the enzyme, but it did not alter the SDS-PAGE pattern for the corresponding protein peak that appeared at a KCl concentration of ~380 mM (details not shown). The PYC protein did not bind to the phenyl-group-based hydrophobic interaction chromatography material POROS 20 HP2 under a mobile phase containing 1 M $(NH_4)_2SO_4$, but an interaction with this matrix inactivated the enzyme (details not shown).

When an inositol-free enzyme was desired, a low-salt buffer without inositol was used for the last wash and the elution at the avidin-Sepharose chromatography step. These changes did not influence the polypeptide composition of the product. On the other hand, when KCl was eliminated from all buffers, the enzyme preparation was found to contain two additional polypeptides (110 and 92 kDa; Fig. 1E); the specific activity was not adversely affected.

Primary structures identities for the polypeptides in the PYC preparations

The NH_2-terminal sequences for four of the five polypeptides described above were found to be as follows: 65 kDa, VKIVDTTFRDAQ; 55 kDa, MFNKVLIANRGE; 110 kDa, VKELKVAEAYQG; and 92 kDa, EVT-SGFSDYKE. The

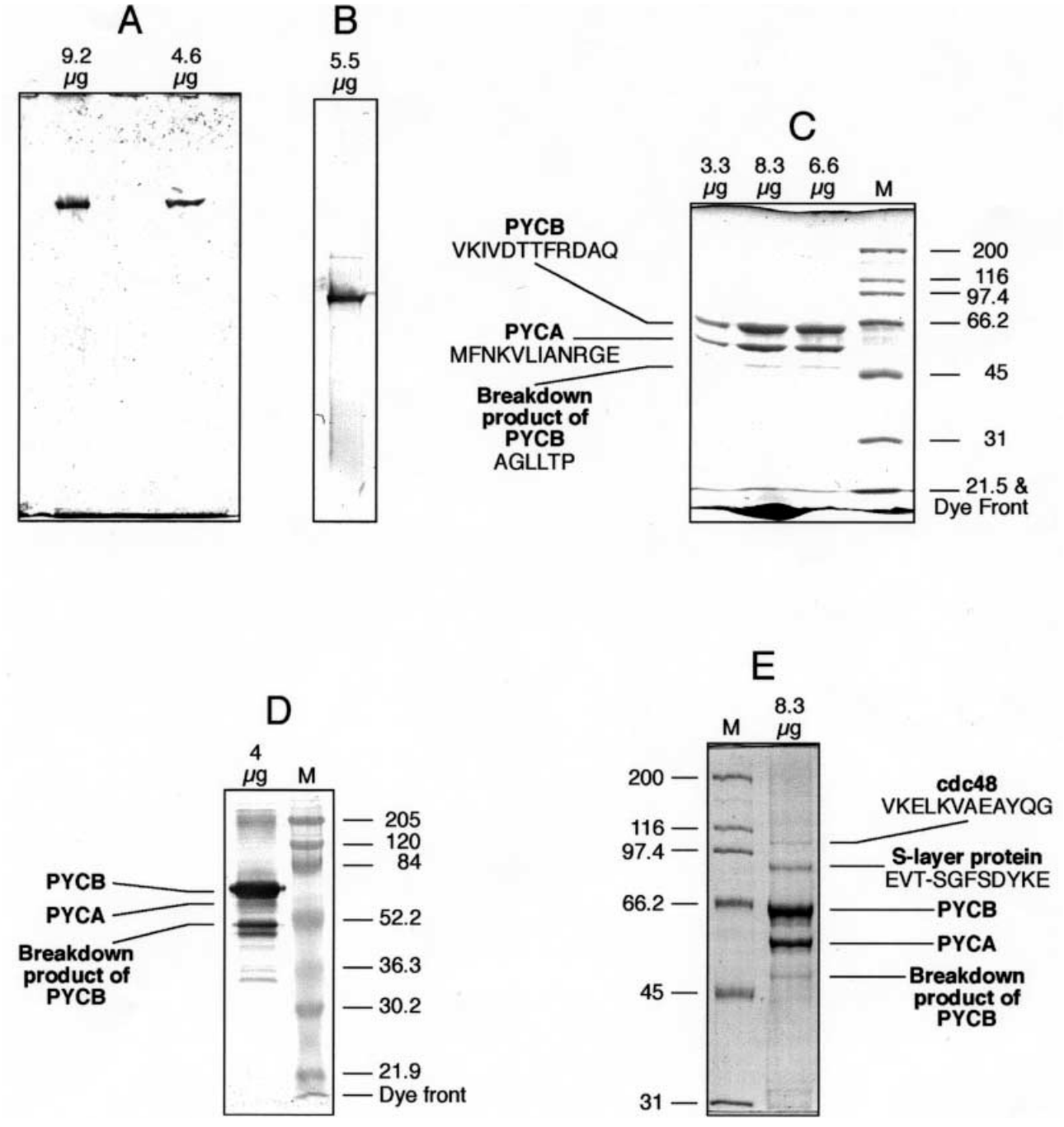

Fig. 1A–E Nondenaturing-PAGE, SDS-PAGE and avidin blot of the purified pyruvate carboxylase (PYC) from *Methanococcus jannaschii*. When known, the name and the experimentally determined NH_2-terminal sequence for a polypeptide are shown next to the corresponding gel band; the name is shown in *bold face* and the NH_2-terminal sequence is in single letter codes for the amino acid residues. PYCA and PYCB are the PYC subunits. The value (in μg) appearing above a lane corresponds to the total amount of protein loaded onto that lane. The values of the molecular masses for the standards (in the lanes marked as *M*) are in kDa. **A** Nondenaturing-PAGE (7.5% acrylamide). **B** Avidin blot with a gel similar to that shown in **A**. **C** SDS-PAGE (10% acrylamide). **D** Avidin blot with a gel similar to that shown in **C**, except acrylamide concentration was 12% and pre-stained standards were used. **E** SDS-PAGE (10% acrylamide) with an enzyme preparation that was obtained by using buffers lacking KCl

BLAST searches (Altschul et al. 1997) with these sequences gave the following results. The NH_2-terminal sequences for the 55 and 65 kDa proteins corresponded, respectively, to two ORFs of *Mc. jannaschii* designated as the biotin carboxylase or AccC (MJ1229; accession number U67563) and oxaloacetate decarboxylase α-chain or OADα (MJ1231; accession number U67563) (Bult et al. 1996). But, based on the primary structure data for the purified *Mb. thermoutotrophicum* PYC, we have proposed that the MJ1229 and MJ1231 polypeptides be considered, respectively, as the PYCA and PYCB subunits of an $\alpha_4\beta_4$-type PYC (Mukhopadhyay et al. 1998). The 92-kDa polypeptide was identified as a NH_2-terminally processed form

of an S-layer structural protein (MJ0822; accession number U67526) (Bult et al. 1996); the deduced molecular mass for this polypeptide was 60.5 kDa. A discrepancy between the SDS-PAGE-derived and nucleic-acid-sequence-deduced molecular masses has also been found for the *Methanococcus voltae* S-layer protein (Konisky et al. 1994) and could be an indication of protein glycosylation or some other modification. The NH_2-terminal sequence of the 110-kDa polypeptide corresponded to an ORF (MJ1156; accession number U67557) (Bult et al. 1996) that has been annotated as a putative cell division control protein 48 (cdc48), AAA family (ATPase associated with diverse cellular activities).

Edman degradation of the 45-kDa polypeptide band provided very weak signals; only the first few residues could be interpreted unequivocally. The corresponding sequence was AGLLTP and it was not sufficient to identify the polypeptide. A MALDI-TOF mass spectrometric analysis of the corresponding unseparated tryptic digest and database searches revealed that the 45-kDa polypeptide was a fragment of the *Mc. jannaschii* PYCB (MJ1231). The data set that led to this conclusion is as follows (averaged mass of a fragment ion [$M+H^+$], identified peptide sequence, sequence position for this peptide in MJ1231, calculated averaged mass): 3303.38, DLGYPPLVTPTSQIVGTQAVLNVLTEERYK, 329–358, 3303.780; 3140.45, KDLGYPPLVTPTSQIVGTQAVLNVLTEER, 328–356, 3140.604; 3012.09, DLGYPPLVTPTSQIVGTQAVLNVLTEER, 329–356, 3012.43; 2940.81, LKEEISLPIDVHSHCTSGLAPMTYLK (Cys alkylated), 190–215, 2941.430; 2850.1, RVLDEGEKPITCRPADLLPPEWEK (Cys alkylated), 386–409, 2850.262; 2693.76, VLDEGEKPITCRPADLLPPEWEK (Cys alkylated), 387–409, 2694.074; 2001.91, EQGALDKFEEVLQEIPR, 309–325, 2002.231; 1995.23, KEEDILTYALYPQIAVK, 422–438, 1995.323; 1898.000, GFYGKPPAPINPELLKR, 370–386, 1898.258; 1741.76, GFYGKPPAPINPELLK, 370–385, 1742.070; 1738.07, EVITAEMEGAVTSPFR; 491–506, 1737.965; 1477.51, MLFSPISQIVDAR, 277–289, 1477.761; 1442.54, LLNEIRDYFMK, 260–270, 1442.716.

With the identification of the polypeptide as a fragment of PYCB, the NH_2-terminal sequence could be readily assigned to residues 175–180 of PYCB. The calculated molecular mass of the larger fragment that would be generated if the PYCB is cleaved after the Met^{174} is 43,872 Da, and this value was consistent with the observed mobility in SDS-PAGE.

Quarternary structure of *Mc. jannaschii* PYC

The identification of the 45-kDa band as a breakdown product of PYCB (generated plausibly during the preparation of a sample for electrophoresis) helped to conclude that the enzyme was composed of two kinds of polypeptides, the larger of which was biotinylated. The MALDI mass spectrum for the enzyme showed two major peaks at the *m/z* value of 55,500 and 64,160 mass units. From the gel filtration chromatography data, the Stokes radius and the apparent native molecular mass of the native PYC were determined to be 74Å and 480 kDa, respectively. Thus, the enzyme appeared to be a $\alpha_4\beta_4$-type PYC. However, a final conclusion must await an accurate determination of the native molecular mass for the *Mc. jannaschii* PYC by use of a more appropriate method; the molecular mass data derived solely from a gel filtration data set are highly unreliable (Cantor and Schimmel 1980).

Storage and thermal stability

Room temperature storage was found to be unsuitable for this hyperthermophilic enzyme. The most convenient and the best way to store the purified enzyme for up to 36 h was at 4 °C in a solution of 50 mM Tris-HCl (pH 8) with or without 10% inositol; under these conditions 83–85% of the original activity was retained. Inclusion of KCl (1 M) in the storage solution (with or without inositol) caused a 10–15% reduction in residual activity. For a 5-day storage, temperatures of –20 and –80 °C provided the best retention (up to 75%) of enzymatic activity; the preservative effect of inositol was small and amounted to only a 6–9% increase in residual activities. In the absence of added Mg^{2+}, the enzyme lost 20% of its activity in 4 h at 4 °C.

The purified enzyme was rapidly inactivated upon incubation in 100 mM Tris-HCl (pH 7) at 80 °C; the apparent $t_{1/2}$ was about 1 h. Of various compounds tested, none provided full or substantial protection against thermal inactivation. Sulfate salts provided partial protection. About 60% of the original activity was retained after 1 h incubation in the presence of 0.4 M K_2SO_4, 0.8 M Na_2SO_4, or 0.1 M $MgSO_4$, and about 40% remained after 3 h. No protection was seen with 10% inositol. The stability was lowered by 0.8 M KCl, 1 M KNO_3, 0.4 M K_2HPO_4, 0.4 M $KHCO_3$, 1 M $(NH_4)_2SO_4$, 0.4 M Na-pyruvate and a refined *Mc. jannaschii* cell extract.

Catalytic properties of *Mc. jannaschii* PYC

The activity of the purified enzyme was strictly dependent on the presence of ATP, pyruvate, bicarbonate, and Mg^{2+}. Pyruvate could not be replaced with phosphoenolpyruvate. GTP or ADP did not substitute for ATP. Acetyl-CoA (50 μM) neither was required nor did it enhance or inhibit activity of the enzyme. Incubation of purified enzyme for 5 min with avidin at 100 molar excess amount (with respect to the biotin content of the PYC protein) completely inhibited the activity, establishing the typical dependence of PYC activity on protein-bound biotin for this enzyme. No activity was lost if avidin was incubated for 10 min with 10-fold molar excess biotin prior to its addition to the enzyme. If an avidin-inactivated enzyme preparation was incubated for 5 min with biotin, one-third of the original enzymatic activity was restored.

The purified enzyme exhibited maximum activity at pH 8.5 (Fig. 2). Measurable activities of the enzyme were seen throughout the range of 25–90 °C and the maximum

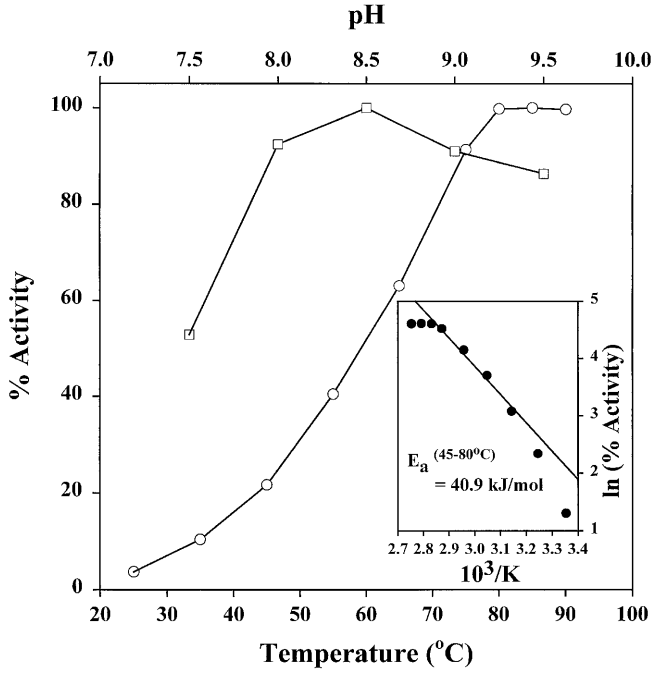

Fig. 2 Effects of temperature and pH on the activity of PYC from *Mc. jannaschii*. For each set (temperature or pH study) the activities are reported as percentages of the corresponding peak value. Temperature studies (❍): The assays were performed at pH 8; at pH 8.5 essentially the same profile was seen. pH studies (❑): The Tris-HCl buffer was adjusted to the desired pH values. The assays were conducted at 80 °C. *Inset* (●): A replot of the data from temperature studies according to the Arrhenius relationship

specific activity was recorded at 80–90 °C (Fig. 2). Activities at even higher temperatures were not determined due to technical limitations. From the linear portion of the Arrhenius plot (45–80 °C; Fig. 2, inset), a value of 40.9 kJ/mol was obtained for the activation energy.

At lower concentrations, KCl stimulated the enzyme and at higher concentrations it was inhibitory; the maximum specific activity was exhibited at 0.175 M KCl. Although Mg^{2+} was absolutely required for both stability and activity, it inhibited activity when it was present in molar excess amounts with respect to ATP (Fig. 3). Maximum activity was obtained only when the concentration of Mg^{2+} was equal to that of ATP. Albeit poorly, Mn^{2+} and Co^{2+} could replace Mg^{2+}. In assays with 1 mM divalent cation and 1 mM ATP, Mn^{2+} and Co^{2+} provided, respectively, 16% and 54% of the activity recorded with Mg^{2+}. In an assay in which both the ATP and Mg^{2+} concentrations were 1 mM, addition of Co^{2+} to 1 mM reduced the activity by 65%, and similarly Mn^{2+} lowered the activity by 85%.

The initial velocity data over a range of pyruvate concentrations (0.23–18 mM pyruvate; 1 mM ATP; 1 mM Mg^{2+}; and 20 mM HCO_3^-) and a range of HCO_3^- concentrations (0.23–18 mM HCO_3^-; 1 mM ATP; 1 mM Mg^{2+}; and 20 mM pyruvate) fit well the Henri-Michaelis-Menten relationship. From these analyses, the values of apparent K_m values for pyruvate and HCO_3^- were found to be 0.53±0.002 mM and 0.22±0.002 mM, respectively.

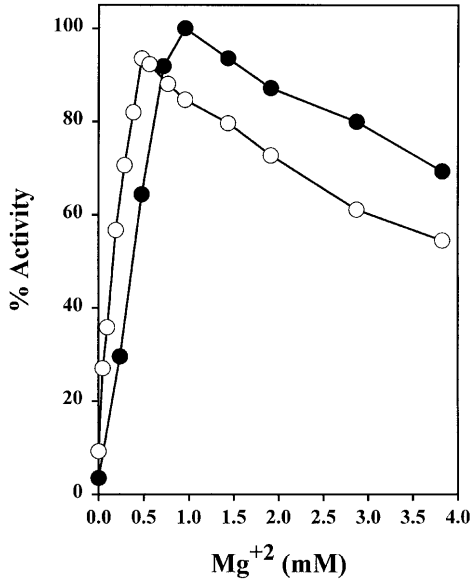

Fig. 3 Effect $MgCl_2$ on the activity of PYC from *Mc. jannaschii*. The standard reaction mixture with 0.5 or 1 mM Na_2ATP and a desired concentration of $MgCl_2$ was used. Each activity value is reported as a percentage of that determined with 1 mM Na_2ATP and 1 mM $MgCl_2$, which was 21.3 U/mg protein. ❍ 0.5 M ATP, ● 1 mM ATP

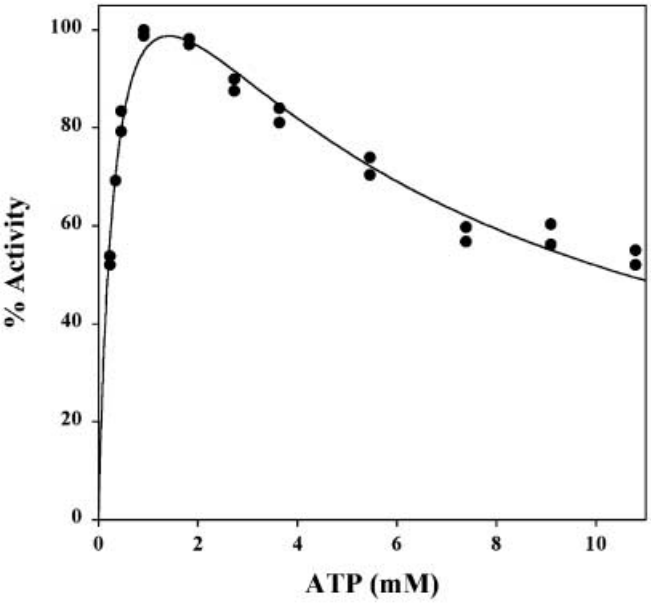

Fig. 4 Inhibition of the activity of *Mc. jannaschii* PYC by ATP. Each assay was conducted at 80 °C in the standard assay mixture with equimolar amounts (in the 0.23–10.8 mM range) of Na_2ATP and $MgCl_2$. The activities are presented as percentages of the maximum value (25 U/mg protein) which was attained at an ATP concentration of 1 mM. The *line* represents a fit to the substrate inhibition relationship $v=V_{max}S/\{K_m+S+(S^2/K_i)\}$ which provided the following values: K_m for ATP, 0.374±0.039 mM; apparent K_i for ATP, 5.34±2.14 mM

ATP inhibited the enzyme at higher concentrations. The initial velocity vs ATP concentration data (0.23–10.8 mM ATP; 20 mM pyruvate; 20 mM HCO_3^-; concentration of Mg^{2+} being equal to that of ATP) fit the substrate inhi-

bition relationship $v=V_{max}S/\{K_m+S+(S^2/K_i)\}$ (Fig. 4) and provided the following values: apparent K_m for ATP, 0.374±0.039 mM; apparent K_i for ATP, 5.34±2.14 mM.

When tested at a concentration of 9 mM in the assays, aspartate and glutamate had no effect on the activity of the enzyme, and α-ketoglutarate reduced the activity by 21%. In a complete assay mixture, the presence of AMP did not influence the activity. ADP was found to be a competitive inhibitor and the corresponding K_i value was 0.89± 0.18 mM.

Discussion

We were interested in studying the PYC of methanarchaea for two specific reasons. The methanarchaea derive very meager amounts of energy from their catabolic activities (Thauer 1990). Thus, it would be interesting to know how efficiently they use their energy resources for cell material biosynthesis. This aspect is of paramount importance in the submarine hydrothermal vent environments, since here autotrophic microorganisms such as *Mc. jannaschii* are the primary producers, and they help to sustain the overall living community through chemosynthesis (Jannasch 1989). PYC plays a major role in the methanarchaeal CO_2 fixation process. The published data (Simpson and Whitman 1993; Mukhopadhyay et al. 1998) and this report, in conjunction with our unpublished observations, suggest that this enzyme is probably present in all methanogens. Also, the archaeal PYCs offer an excellent opportunity to study the evolution of various attributes of biotin-dependent enzymes.

Unlike *Mb. thermoautotrophicum* (Mukhopadhyay et al. 1998), *Mc. jannaschii* did not require exogenously supplied biotin to express PYC activity. The PYC of *Mb. thermoautotrophicum* is a very unstable enzyme (Mukhopadhyay et al. 1998). In contrast, the enzyme from *Mc. jannaschii* was found to be much more stable. Thus, this enzyme will allow meaningful mechanistic studies on the archaeal PYC reaction. Its selection as a model is justified, since each of the primary structure features (including the putative PLP-binding motif) of the only other characterized archaeal PYC are present in this methanococcal enzyme (Mukhopadhyay et al. 1998). In the current work, a similar trend was seen in the kinetic properties.

A modification of the method that was used for the *Mb. thermoautotrophicum* PYC (Mukhopadhyay et al. 1998) provided the best conditions for the purification of *Mc. jannaschii* PYC. The presence of KCl at each step except the last two assured purity of the final product (see below for the reason). This enzyme from a marine methanarchaeon did not require KCl or NaCl or other salts for low-temperature stability. Rather, KCl affected the activity during storage, and the recovery of the enzyme in a KCl-free buffer helped to avoid this harmful effect. The specific activity of the purified enzyme was found to vary, and the lowest observed value was 57% of the maximum. Since the homogenous protein yield did not vary and because the purification method was based on avidin-biotin interaction, the variability in specific activities was probably not linked to the biotinylation of apo-PYC. We are currently pursuing this topic.

The determined NH_2-terminal sequences for the *Mc. jannaschii* PYC subunits unequivocally supported our earlier proposal to rename the ORFs MJ1229 and MJ1231 in the NCBI data base as PYCA and PYCB, respectively (Mukhopadhyay et al. 1998).

The *Mc. jannaschii* enzyme was found to be the most thermophilic PYC known. But, at the optimal temperature for activity it was rapidly inactivated. This effect was seen in the Arrhenius plot (Fig. 2, inset) which formed a plateau at higher temperatures; the observed deviation from linearity at lower temperatures was probably due to changes in the enzyme's structure or kinetic mechanism or both. Our screen failed to find a condition in which the purified enzyme would be stable at the optimal growth temperature for the host (85 °C; Jones et al. 1983), and even the low molecular weight (<10 kDa) components of *Mc. jannaschii* cells were ineffective. It would be interesting to investigate the mechanism by which *Mc. jannaschii* protects its PYC from thermal inactivation. The fact that the PYC from *Mb. thermoautotrophicum*, a moderate thermophile, is even more unstable indicates that protecting PYC from thermal inactivation could be a common requirement for all methanarchaea or at least for the thermophilic members. Here, the discovery of a binding protein from *Mb. thermoautotrophicum* that protects other proteins from thermal denaturation serves as a guiding example (Ideno et al. 2000).

The *Mc. jannaschii* enzyme belongs to the class of PYCs that do not require acetyl-CoA for activation and that are composed of two dissimilar subunits, of which the larger (PYCB) carries the biotin and the smaller (PYCA) carries the ATP-binding domain (Mukhopadhyay et al. 1998). It was also insensitive to aspartate and glutamate, which often act as allosteric inhibitors for the α_4-type PYCs (Scrutton and White 1974a, b; Modak and Kelly 1995). But, unlike the bacterial $\alpha_4\beta_4$ enzymes and similar to many α_4 PYCs (Scrutton and Taylor 1974; Scrutton and White 1974a, b; Modak and Kelly 1995) and *Mb. thermoautotrophicum* PYC (Mukhopadhyay et al. 1998), the methanococcal enzyme was inhibited by α-KGA. Another similarity between the two archaeal enzymes was that both were stabilized by Mg^{2+}, but their activities were inhibited by this divalent cation when its concentration exceeded that of ATP. This effect was seen with Co^{2+} and Mn^{2+} as well. In contrast, for most other PYCs, excess Mg^{2+} relieves the inhibition by ATP by keeping most of this nucleotide in a complexed form (Mukhopadhyay et al. 1998). The *Mc. jannaschii* enzyme was also inhibited by ATP, and this effect followed the classical substrate inhibition pattern (Fig. 4). The physiological relevance of this inhibition is hard to conjecture. One could speculate that *Mc. jannaschii* does not use PYC under an energy-rich state. But, it probably does not have an alternate pathway for OAA generation. *Methanococcus maripaludis*, a close relative of *Mc. jannaschii*, is devoid of PPC and

phosphoenolpyruvate carboxykinase (Shieh and Whitman 1987), and a similar situation is expected for the latter.

The purification experiments revealed a general and serious problem in research on the biotin-containing proteins from the methanarchaea and their relatives. Since KCl affected stability of the enzyme, in an experiment this salt was eliminated from the buffers. The result was the recovery of a PYC preparation that also contained the S-layer structural protein and putative cdc48 protein (Fig. 1E). These additional proteins do not possess the signature biotin-binding motif (the AMKM sequence; Samols et al. 1988) in their primary structures, yet they bound to the avidin-Sepharose. In fact, if the cell extract was supplemented with biotin at a concentration of 20 μM, PYC did not bind to the matrix, but the above mentioned polypeptides did (data not shown); the bound polypeptides could be eluted with 10 mM biotin. A similar problem was encountered in avidin blotting experiments. In spite of using avidin-AP at a very low concentration (0.01 mg l^{-1}) in the hybridization solution, a blot with cell extracts of *Mc. jannaschii* gave many positive signals (B. Mukhopadhyay, unpublished observations). Avidin-AP levels of 0.1–10 mg l^{-1} are routinely used for blots with bacterial and eukaryotic biotin-containing proteins (Hurley and Finkelstein 1990; Bayer at al. 1990; Brewster et al. 1994). These observations contrasted with the fact that *Mc. jannaschii* possesses only one ORF (MJ1231; Bult et al. 1996) with the biotin-binding motif, and the current work identified it as the PYCB. It is very likely that the non-biotinylated polypeptides bound to avidin through charge-charge interactions. Avidin is a highly basic protein (pI 10.5; Bailey et al. 1969) and under the fractionation and hybridization conditions (pH 8 and 7.4, respectively) it was positively charged. On the other hand, many methanarchaea possess large number of acidic proteins (B. Mukhopadhyay, unpublished observations) and the calculated pI values for the S-layer structural protein and putative cdc48 protein were, 4.27 and 5.4, respectively. These properties set up an ideal condition for strong ionic interactions. Such interactions not only pose a problem in the purification of biotinylated proteins from a methanarchaeon, but also would create confusion when one uses avidin blots to determine the number or the levels of biotinylated proteins in such an organism. It is possible that the problem of non-specific interactions was more acute for *Mc. jannaschii*, because this organism lives in a saline environment, and organisms from such an environment possess an abundance of negatively charged proteins (Lanyi 1974). Shieh and Whitman (1987) reported that the PYC activity in cell extracts of *Mc. maripaludis* is not inhibited by avidin, whereas we found that the purified PYC from *Mc. jannaschii* was inhibited by avidin. It is plausible that the lack of inhibition of PYC activity in the cell extracts of *Mc. maripaludis* was due to sequestering of avidin by negatively charged non-biotinylated proteins; our data showed that the affinities of such non-biotinylated proteins for avidin could exceed that of the PYC. Inclusion of KCl in the buffers that were used in enzyme purification helped to avoid non-specific charge-charge interactions and to obtain a homogeneous enzyme from the avidin-Sepharose chromatography.

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