Purification, Regulation, and Molecular and Biochemical Characterization of Pyruvate Carboxylase from 
*Methanobacterium thermoautotrophicum* Strain ΔH*

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We discovered that *Methanobacterium thermoautotrophicum* strain ΔH possessed pyruvate carboxylase (PYC), and this biotin prototroph required exogenously supplied biotin to exhibit detectable amounts of PYC activity. The enzyme was highly labile and was stabilized by 10% inositol in buffers to an extent that allowed purification to homogeneity and characterization. The purified enzyme was absolutely dependent on ATP, Mg2+ (or Mn2+ or Co2+), pyruvate, and bicarbonate for activity; phosphoenolpyruvate could not replace pyruvate, and acetyl-CoA was not required. The enzyme was inhibited by ADP and α-ketoglutarate but not by asparagine or glutamate. ATP was inhibitory at high concentrations. The enzyme, unlike other PYCs, exhibited non-linear kinetics with respect to bicarbonate and was inhibited by excess Mg2+, Mn2+, or Co2+. The 540-kDa enzyme of ΔB composition contained a non-biotinylated 52-kDa subunit (PYCA) and a 75-kDa biotinylated subunit (PYCB). The pycB gene was probably monocistronic and followed by a putative gene of a DNA-binding protein on the opposite strand. The pycA was about 727 kilobase pairs away from pycB on the chromosome and was probably co-transcribed with the biotin ligase gene (bira). PYCA and PYCB showed substantial sequence identities (33–62%) to, respectively, the biotin carboxylase and biotin carboxyl carrier + carboxyltransferase domains or subunits of known biotin-dependent carboxylases/decarboxylases. We discovered that PYC and probably the equivalent domains or subunits of all biotin-dependent carboxylases harbored the serine/threonine dehydratase types of pyridoxal-phosphate attachment site. Our results and the existence of an alternative oxaloacetate synthesizing enzyme phosphoenolpyruvate carboxylase in *M. thermoautotrophicum* strain ΔH (Kenealy, W. R., and Zeikus, J. G. (1982) *FEMS Microbiol. Lett.* 14, 7–10) raise several questions for future investigations.

Puruvate carboxylase (PYC) catalyzes ATP-dependent carboxylation of pyruvate to generate oxaloacetate. The other possible routes for oxaloacetate synthesis are carboxylation of phosphoenolpyruvate (PEP) by PEP carboxylase (PPC) and PEP carboxytransphosphorylase, splitting of citrate by citrate lyase and ATP-citrate lyase, and the reversal of the PEP carboxykinase reaction (1). Of these enzymes, PYC and PPC are more commonly employed enzymes for generating oxaloacetate (1–3), and only rarely do they co-exist in a given organism (2, 4–6). A similar pattern has been found in methanogenic archaea. *Methanococcus* possesses PYC but not PPC (7). Enzyme assays and isotope labeling studies suggest that *Methanobacterium* possesses PPC and is devoid of PYC and PPC carboxytransphosphorylase activities (8, 9). In *Methanosarcina*, PPC is absent (10), and the PYC activity has yet to be demonstrated.

In mammals and yeast, PYC activity is responsible for replenishing oxaloacetate for continued operation of the tricarboxylic acid cycle (3). In the absence of this anaplerotic function, consumption in cell material biosynthesis depletes the oxaloacetate pool. PYC, in conjunction with PEP carboxykinase, also provides PEP that is needed for gluconeogenesis, since the pyruvate kinase reaction of the glycolytic pathway is irreversible (1–3). PYC is also present in plants, where its role has yet to be established (11). In *Escherichia coli*, PPC provides oxaloacetate during growth on glucose (1). Since *E. coli* does not possess PYC, during growth on acetate it employs the glyoxalate cycle to generate oxaloacetate for gluconeogenesis. Depending on the growth conditions, *Pseudomonas citronellolisol* uses either PYC or PPC as the anaplerotic enzyme (5). In methanogens, PYC and PPC activities serve anabolic functions. In *Methanococcus*, *Methanobacterium*, and *Methanospirillum*, oxaloacetate is the starting point of an incomplete reductive tricarboxylic acid cycle that terminates at α-ketoglutarate and provides several precursors for cell material and coenzyme biosynthesis (7, 12–14). In *Methanosarcina*, oxaloacetate initiates an incomplete oxidative tricarboxylic acid cycle to generate α-ketoglutarate (10).

PYC belongs to a large family of biotinylated enzymes that carry out carboxyl-group transfer in a variety of reactions (3, 15). These enzymes use biotin as a “swinging arm” to transfer a −COO− group between active sites and show strong conservation at the amino acid sequence level (15, 16). They also carry

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1 The abbreviations used are: PYC, pyruvate carboxylase; PPC, phosphoenolpyruvate; PPC, PEP, carboxylase; PYCA, a subunit of PYC; PYCB, B subunit of PYC; ORF, open reading frame; OAD, oxaloacetate deacrylase; TC, (S)-methylmalonyl-CoA-pyruvate transcarboxylase; PCC, propionyl-CoA carboxylase; ACC, acetyl-CoA carboxylase; BC, biotin carboxylase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; bp, base pairs; kb, kilobase pairs.
out analogous partial reactions, which for PYCs are as indicated in Reactions 1 and 2.

**Enzyme-biotin + MgATP2– + HCO3–***

\[ \text{enzyme-biotin-COO}^- + \text{MgADP}^- + P_i \]

\[ \text{REACTION 1} \]

**Enzyme-biotin-COO– + pyruvate = enzyme-biotin-oxaloacetate***

\[ \text{REACTION 2} \]

Since previous work in our laboratory has shown the presence of biotin in *Methanobacterium thermoautotrophicum* strain D (17), efforts were made to detect biotinylated peptides in this organism. SDS-PAGE with cells of grown with exogenously supplied [3H]biotin showed the presence of a strain of *M. thermoautotrophicum* if D-biotin is added to the growth medium. The cloning of the biotinylated enzyme was achieved using the cloning vector. Plasmids from *Thermus flavus* were used as the cloning vector. Plasmids from *M. thermoauto-

**Purification of Pyruvate Carboxylase—**

**Organisms and Culture Conditions—** *M. thermoautotrophicum* strain DH (19) was grown in tubes, bottles, and in a 14-liter stainless steel fermentor (Microferm, New Brunswick Scientific, New Brunswick, NJ) in Medium 1 of Balch and Wolfe (20) as described previously (20, 21). For studying the effect of medium compositions on the expression of PYC, the growth medium was modified as described under "Results." The cells grown in the fermentor were harvested by using a continuous flow centrifuge, frozen in liquid nitrogen, and stored at −70 °C (21). *E. coli* DH5α was used as the cloning host, and this strain was grown in LB medium at 37 °C. Wherever needed, the LB medium was supplemented with ampicillin (100 μg/ml), 5-bromo-4-chloro-3-indolyl β-galactoside (40 μg/ml), and isopropyl-β-thiogalactopyranoside (100 μM).

**Preparation of Enzyme-Biotin**—This affinity matrix was prepared according to previously published protocols (22). Before use, the matrix was regenerated with washing by with 0.1 M glycine-HCl at pH 2. The regenerated column was washed with 20 mM potassium phosphate buffer, pH 7, until the pH of the effluent was 7 and then was equilibrated with the column buffer of the following composition: 50 mM Tris, 1 mM KCl, 10% inositol, 5 mM MgCl2, 2 mM DTT, pH 8 (adjusted with HCl). The cell extract was diluted with an equal volume of column buffer and, following the protocols of Purell and Wallace (23), was supplemented with ATP, KHCO3, and sodium pyruvate to the final concentrations of 3, 10, and 10 mM, respectively, to improve accessibility to protein-bound biotin. The diluted cell extract was loaded onto the affinity column at a flow rate of 6-75 μl/min either under gravity or by using a Tri- peristaltic pump (Iscose, Inc., Lincoln, NE). The column bed was then washed with 10 bed volumes of column buffer to remove unbound material. Pyruvate carboxylase bound to the matrix very tightly and was eluted with 2 bed volumes of 1 mM b-biotin in column buffer.

**Assays and Data Analysis—** Protein was assayed according to Bradford (24) using the dye reagent purchased from Pierce. Pyruvate carboxylase was assayed in the direction of oxaloacetate formation by coupling the reaction with malate dehydrogenase. The oxidation of NADH in the malate dehydrogenase reaction was followed spectrophotometrically at 340 nm. Unless mentioned otherwise, the assay mixture contained 50-100 mM Tris-HCl buffer at pH 8, 4 mM MgCl2, 400 mM KCl, 50 mM sodium pyruvate, 50 mM KHCO3, 0.2 mM ATP, 0.2 mM NADH, and 1 unit of malate dehydrogenase from *Thermus flavus* (Sigma). The assays were initiated by the addition of 10-100 μl of pyruvate carboxylase preparations to 1 ml of pre-warmed assay mixture. For pH studies the Tris-HCl buffer was replaced with buffers containing 60 mM each of MES, Tris, and glacial acetic acid and adjusted to the desired pH (6.5-9.5) with HCl or NaOH; the values calculated (25) for the ionization strengths of these buffers were between 0.06 and 0.09 units. All initial rate data were analyzed by using the KinetAsyst program version 1.01 (IntelliGenetics, State College, PA), except those for bicarbonate, which were fitted to 2/1 function (s = KY(S2 + DS)BS + BS + BC) where B, C, and D are constants and KY = 0.5B + D + (0.5B - DY + C)1/2 (26). Gel Electrophoresis and Western Blot Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% polyacrylamide slab gels according to Laemmli (27). For non-denaturing PAGE, 5% polyacrylamide gels and a variety of gel and electrode buffers were used. The best results were obtained when 100 mM phosphate buffer, pH 7.1, with 10% inositol and 10 mM MgCl2 served as both the gel buffer and the electrode buffer (see "Results"). For Western blot analysis, the protein samples were electrophoresed and electroblotted onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). The membranes with blotted polypeptides were washed with phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl) and then blocked with 3% bovine serum albumin in PBS for 2 h. The blocked membranes were washed three times with PBS and shaken for 30 min at 4 °C while immersed in 0.01 mg/liter alkaline phosphatase-conjugated avidin (Sigma) in PBS. The membranes were then washed with PBS, equilibrated in a veronal buffer (32 mM veronal, 30 mM sodium acetate, 120 mM NaCl, pH 9.6) for 5 min prior to blocking with the triton x-turbulent (350 μg/ml) and bromochloroindolyl phosphate (175 μg/ml) in the veronal buffer. The use of VECTASTAIN ABC-AP reagent (Vector Laboratories, Burlingame, CA) in place of alkaline phosphatase-conjugated avidin gave very high background, with the majority of the protein bands reacting with avidin.

**Gel Filtration Chromatography—** An HR 10/30 Superose-6 column and a fast protein liquid chromatography system (Pharmacia Biotech Inc.) was used for this purpose. The mobile phase was 50 mM Tris-HCl buffer, pH 7.5, 100 mM KCl, and 5% glycerol, and the flow rate was 0.5 ml/min. The molecular mass standards in kDa were thyroglobulin (669), apoferritin (443), β-amylose (200), alcohol dehydrogenase (150), bovine serum albumin (60), carbonic anhydrase (29), and vitamin B12 (1.357). Before reapplying the purified enzyme, the column was equilibrated with the mobile phase supplemented with 1 mM DTT and 5 mM MgCl2. The eluted proteins were detected by their absorbance at 280 nm.

**Determination of the Amino-terminal Amino Acid Sequence—** The purified pyruvate carboxylase was electrophoresed in a 12.5% polyacrylamide slab gel under denaturing conditions, and the separated subunits were electrophoretically transferred to a Pro-Blott membrane (Applied Biosystems, Foster City, CA) according to manufacturer protocols using Tris/glycine/methanol as the blotting buffer. The pieces of membrane containing individual subunits, as visualized by staining with Coomassie Brilliant Blue, were used for sequencing by Edman degradation at the University of Illinois Genetic Engineering Facility.

**DNA Methods—** Generally, all manipulations were performed according to standard methods with genomic DNA from *M. thermoauto-

**APPENDIX II K S** (Stratagene, La Jolla, CA) was used as the cloning vector. Plasmids from *E. coli* cell lysates were purified by using Qiagen Tips and reagents from Qiagen (Chatsworth, CA). DNA fragments of interest were purified from agarose gels by using either Qiuaquick columns (Qiagen) or by digestion with β-garase

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2 A. A. DiMarco and J. E. Cronan, Jr., personal communication.
FIG. 1. Native-PAGE and Western blot of purified pyruvate carboxylase from M. thermoautotrophicum strain ΔH. The PAGE was performed at a polyacrylamide concentration of 5%. Wherever indicated, inositol and MgCl₂ were present in both the gel and the electrode buffer at 10% and 10 mM concentrations, respectively. The Western blot corresponds to the native PAGE performed without inositol and MgCl₂.

Western blot analysis in the nondenatured state using alkaline phosphatase-conjugated avidin and nitro blue tetrazolium/bromochloroindoyl phosphate. DNA sequencing was performed at the University of Iowa DNA Facility (Iowa City, IA) using an automated sequencer. The first round of sequencing was with plasmids pBM1 and pBM2 using the primers based on the vector sequences. Additional sequencing was from pBM1 using primers based on the accumulated sequences. Both strands were sequenced.

DNA and Protein Sequence Analysis—The DNA sequences were assembled, aligned, and analyzed using the DNA Star (DNASTAR Inc., Madison, WI) program. Data base searches were performed using the BLAST program (30) of National Center for Biotechnology Information (Genius 3) from Boehringer Mannheim according to manufacturer instructions. The pre-hybridization and hybridization were conducted at 55 °C and post-hybridization washes were at 24 °C. The hybridizing bands were detected by using alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) and the colorimetric substrates nitro blue tetrazolium and bromochloroindoyl phosphate. The PAGE was performed at a polyacrylamide concentration of 12.5% and with 20 μg of purified enzyme. Most enzyme preparations did not exhibit the 67-kDa band (data not shown). For the detection of biotinylated peptides by Western blot, VECTASTIN ABC-AP kit was used.

FIG. 2. SDS-PAGE and Western blot of purified pyruvate carboxylase from M. thermoautotrophicum strain ΔH. The PAGE was performed at a polyacrylamide concentration of 12.5% and with 20 μg of purified enzyme. Most enzyme preparations did not exhibit the 67-kDa band (data not shown). For the detection of biotinylated peptides by Western blot, VECTASTIN ABC-AP kit was used.

Pyruvate Carboxylase of M. thermoautotrophicum

The enzyme was greatly stimulated by KCl and to a lesser extent by NaCl (Fig. 3); optimum specific activity was exhibited at 0.1–0.4 mM KCl. As reported above, Mg²⁺ was required by the purified enzyme for activity. However, this divalent cation presence of inositol and MgCl₂ (data not shown), and in their absence a smear was observed (Fig. 1). Gel filtration was used to estimate the molecular mass of the native enzyme. The presence of Mg²⁺, DTT, and glycerol in the running buffer was essential for achieving a sharp elution of PYC. From the relative elution volume data, the apparent native molecular mass of PYC was estimated to be 540 kDa. Fig. 2 shows the SDS-PAGE pattern of the denatured PYC. In most cases only two polypeptide bands at 52- and 75-kDa locations were seen, and the corresponding subunits were designated as A and B, respectively. Some enzyme preparations gave an additional band of 67-kDa size (Fig. 2). Western blot analysis of the denatured protein showed that the 75- and 67-kDa bands, but not the 52-kDa band, reacted with avidin and thus carried biotin (Fig. 2). The NH₂-terminal sequence of the 75-kDa polypeptide was determined to be MKGIKVETHAFLIQSLLA and that of the 52-kDa was MFGKILVANGRLAIRV. The NH₂-terminal sequence of the 67-kDa polypeptide was determined to be AEGIDTITPAAAIKIVFHSSSVV. The 67-kDa band was either a breakdown product of the 75-kDa polypeptide or the 75-kDa band was a modified form of the 67-kDa polypeptide.

Catalytic Properties of M. thermoautotrophicum Strain ΔH Pyruvate Carboxylase—The activity of the purified enzyme was strictly dependent on the presence of ATP, pyruvate, bicarbonate, and Mg²⁺ (supplied either as MgCl₂, MgSO₄, or Mg-ATP). Pyruvate could not be replaced with PEP, GTP, CTP, UTP, ITP, or ADP did not substitute for ATP. Incubation of purified enzyme with excess avidin completely inhibited its activity (Table I), establishing the typical dependence of pyruvate carboxylase activity on protein-bound biotin for the Methanobacterium enzyme. This inactivation did not occur if avidin was incubated with excess biotin prior to its addition to the enzyme. Addition of biotin after avidin had acted on the enzyme restored only a very minor portion of the original activity. The purified enzyme exhibited maximum activity at pH 8. Measurable activities of the enzyme were seen throughout the range of 30–80 °C, and the maximum specific activity was recorded at 60 °C.

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Pyruvate Carboxylase of M. thermoautotrophicum

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Purification and Molecular Properties of Pyruvate Carboxylase from M. thermoautotrophicum Strain ΔH—Most of the pyruvate carboxylase activity was found in the 100,000 × g supernatant of the cell extract containing 1 mM KCl. Thus, it is a soluble and hydrophilic protein. The enzyme bound tightly to monomerized avidin-Sepharose and was eluted from this matrix with 1 mM b-biotin as a single sharp peak. As judged by nondenaturing PAGE patterns, the product was homogeneous (Fig. 1). The presence of 1 mM KCl in the buffer was critical for obtaining a good yield and purity, which was typically 1–1.5 mg of enzyme/200 g of wet cell paste. Inositol was required for maintaining activity. We were unable to recover active fractions when glycerol was used in place of inositol.

The enzyme preparation from affinity chromatography gave a sharp band in native polyacrylamide gel electrophoresis (Fig. 1). The use of 10% inositol and 10 mM MgCl₂ in the gel and electrode buffer was essential for obtaining this sharp band, which in the absence of inositol and MgCl₂ spread to form a smear (Fig. 1). The enzyme preparation was also examined by Western blot analysis in the nondenatured state using alkaline phosphatase-conjugated avidin and nitro blue tetrazolium/bromochloroindoyl phosphate. Again, a sharp avidin-reacting band was seen when the electrophoresis was conducted in the

M. thermoautotrophicum strain ΔH. The PAGE was performed at a polyacrylamide concentration of 12.5% and with 20 μg of purified enzyme. Most enzyme preparations did not exhibit the 67-kDa band (data not shown). For the detection of biotinylated peptides by Western blot, VECTASTIN ABC-AP kit was used.

Pyruvate Carboxylase of M. thermoautotrophicum

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Catalytic Properties of M. thermoautotrophicum Strain ΔH Pyruvate Carboxylase—The activity of the purified enzyme was strictly dependent on the presence of ATP, pyruvate, bicarbonate, and Mg²⁺ (supplied either as MgCl₂, MgSO₄, or Mg-ATP). Pyruvate could not be replaced with PEP, GTP, CTP, UTP, ITP, or ADP did not substitute for ATP. Incubation of purified enzyme with excess avidin completely inhibited its activity (Table I), establishing the typical dependence of pyruvate carboxylase activity on protein-bound biotin for the Methanobacterium enzyme. This inactivation did not occur if avidin was incubated with excess biotin prior to its addition to the enzyme. Addition of biotin after avidin had acted on the enzyme restored only a very minor portion of the original activity. The purified enzyme exhibited maximum activity at pH 8. Measurable activities of the enzyme were seen throughout the range of 30–80 °C, and the maximum specific activity was recorded at 60 °C.

The enzyme was greatly stimulated by KCl and to a lesser extent by NaCl (Fig. 3); optimum specific activity was exhibited at 0.1–0.4 mM KCl. As reported above, Mg²⁺ was required by the purified enzyme for activity. However, this divalent cation
Pyruvate Carboxylase of M. thermoautotrophicum

TABLE I

Effect of various compounds on pyruvate carboxylase activity

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 % control</td>
</tr>
<tr>
<td>Avidin (25-fold molar excess)</td>
<td>0</td>
</tr>
<tr>
<td>Avidin (25-fold molar excess) + biotin (4000-fold molar excess)</td>
<td>10</td>
</tr>
<tr>
<td>Biotin (4000-fold molar excess) + avidin (25-fold molar excess)</td>
<td>77</td>
</tr>
<tr>
<td>Bovine serum albumin (25 mol/mol PYC tetramer)</td>
<td>90</td>
</tr>
</tbody>
</table>

Alternate nucleotide
- AMP, ADP, CTP, GTP, ITP, or UTP: 0

Additional nucleotide
- AMP: 104
- ADP: 33
- CTP: 205
- GTP: 94
- ITP: 80
- UTP: 105

Tricarboxylic acid cycle-related compounds
- Acetyl-CoA: 84
- Aspartate: 91
- Glutamate: 95
- α-Ketoglutaric acid: 73

Divalent cations replacing Mg$^{2+}$
- Mn$^{2+}$: 17
- Co$^{2+}$: 46
- Zn$^{2+}$: 0

Divalent cation in addition to Mg$^{2+}$
- Mn$^{2+}$: 9
- Co$^{2+}$: 0
- Zn$^{2+}$: 0

a Unless mentioned otherwise (see Footnotes c, e, and f below), effectors were added to the assay mixture at the concentrations indicated.

b Activity in 50 mM Tris-HCl buffer, pH 8.0, 800 mM KCl, 50 mM sodium pyruvate, 50 mM KHCO$_3$, 4 mM MgCl$_2$, 4 mM K$_2$ATP, 0.2 mM Na$_2$NADH, 4 mM K$_3$ATP, 1 unit/ml malate dehydrogenase from T. flavus, and desired amount of MgCl$_2$-6H$_2$O. The activities are reported as percent of the value determined at 4 mM MgCl$_2$. For the work on Mg$^{2+}$, the reaction mixtures contained 50 mM Tris-HCl buffer, pH 8.0, 15 mM sodium pyruvate, 15 mM NaHCO$_3$, 0.2 mM Na$_2$NADH, 2 mM Na$_3$ATP, 2 mM MgCl$_2$-6H$_2$O, 1 unit/ml malate dehydrogenase from T. flavus and desired amounts of KCl and NaCl. The activities are reported as percent of the value determined at 0.2 mM KCl.

c Activity in 50 mM Tris-HCl buffer, pH 8, with 10% inositol. This enzyme preparation did not exhibit any activity in the assay mixture at optimal levels. However, preincubation of the desalted enzyme preparation at 4 °C with 10 mM MgCl$_2$ for 30–60 min restored the activity to 60–70% of the original value that was recorded before desalting.

d The activity of the enzyme was neither inhibited nor enhanced by the presence of acetyl-CoA at 0.2 or 2 mM concentrations (Table I). The enzyme was insensitive to aspartate and glutamate and was slightly affected by α-ketoglutarate (Table I).

e The nucleotides tested only ADP and ITP offered mild inhibition (Table I).

For obtaining preliminary information on the kinetic characteristics of the enzyme, we performed initial rate studies. The initial velocity data for varied pyruvate concentration (0.5–38 mM pyruvate; 4 mM ATP; 4 mM Mg$^{2+}$; and 50 mM HCO$_3$) fit well the Henri-Michaelis-Menten relationship, and from this analysis the values of apparent $K_m$ for pyruvate and apparent $V_m$ were found to be $7.5 \pm 0.06$ mM and $134 \pm 1$ μmol min$^{-1}$ mg$^{-1}$, respectively. ATP inhibited the enzyme at higher concentrations. When Mg$^{2+}$ concentrations were equal to that of ATP and both pyruvate and HCO$_3$ were 50 mM, the initial velocity versus ATP concentration (0.5–12 mM) data fit the substrate inhibition relationship $v = V_m s(K_m s + (s^2/K_m s))$ and provided the following values: apparent $K_m$ for ATP, 1.1 ± 0.1 mM; apparent $K_i$ for ATP, 8.8 ± 2.8 mM; and apparent $V_m$ of 267 ± 14 μmol min$^{-1}$ mg$^{-1}$. A similar study on inhibition by ATP, where the Mg$^{2+}$ concentrations were higher than that of ATP by 4 mM, provided a different set of values: apparent $K_m$ for ATP, 0.21 ± 0.01 mM; apparent $K_i$ for ATP, 15.4 ± 4.7 mM; and apparent $V_m$ of 149 ± 2.5 μmol min$^{-1}$ mg$^{-1}$.

The Eadie-Hofstee ($v/S$ versus $v$) plot of the data for bicarbonate as the varied substrate (0.4–19 mM HCO$_3$; 4 mM ATP; 4 mM Mg$^{2+}$; and 50 mM pyruvate) was nonlinear and indicative of negative cooperativity. These data were fitted to a 2/1 function $v = V_m s^2 + d (s^2 + B s + C)$ where $B$, $C$, and $D$ are constants and $K_m = 0.5B - D + ((0.5B - D)^2 + C)^{1/2}$. From this fit the following values were obtained: apparent $K_m$, 6.5 ± 0.8 mM; apparent $V_m$, 115 ± 0.05 μmol min$^{-1}$ mg$^{-1}$; $B$, 10.6 ± 2.2 mM; $C$, 4.3 ± 2.5 μmol$^2$; $D$, 2.4 ± 0.9 mM.

FIG. 3. Effect of MgCl$_2$, KCl, and NaCl on the activity of pyruvate carboxylase from M. thermoautotrophicum strain DHE. For the work on Mg$^{2+}$, the reaction mixtures contained 50 mM Tris-HCl buffer, pH 8.0, 400 mM KCl, 50 mM sodium pyruvate, 50 mM HCO$_3$, 0.2 mM Na$_2$NADH, 4 mM K$_3$ATP, 1 unit/ml malate dehydrogenase from T. flavus, and desired amount of MgCl$_2$-6H$_2$O. The activities are reported as percent of the value determined at 4 mM MgCl$_2$. For the work on KCl and NaCl concentration, the reaction mixtures contained 50 mM Tris-HCl buffer, pH 8.0, 15 mM sodium pyruvate, 15 mM NaHCO$_3$, 0.2 mM Na$_2$NADH, 2 mM Na$_3$ATP, 2 mM MgCl$_2$-6H$_2$O, 1 unit/ml malate dehydrogenase from T. flavus and desired amounts of KCl and NaCl. The activities are reported as percent of the value determined at 0.2 mM KCl.
Cloning and Sequencing of the Gene for the Biotinylated Subunit of PYC—Southern blot analysis of EcoRI-digested M. thermoautotrophicum ΔH DNA with a 100% degenerate (at all wobble positions) oligonucleotide 5’ RAA NGT NGT YAC NAC NAC YTT DAT NCC YTT 3’, corresponding to the NH2-terminal amino acid sequence (residue 2–11) of the 75-kDa subunit of purified PYC, showed a hybridization signal at 3–4 kb position. Accordingly, using pBluescript II SK+ as the vector, a limited library of 3–5-kb EcoRI fragments of M. thermoautotrophicum ΔH genomic DNA was constructed in E. coli DH5α. This library was screened by colony hybridization using the degenerate oligonucleotide as the probe. Ten of these colonies showed positive hybridization signals, and the corresponding recombinant plasmids had inserts of size ~3.5 kb. One of these strains, bearing the recombinant plasmid designated pBM1, was preserved. Fig. 4A shows the restriction map of the M. thermoautotrophicum ΔH DNA insert in pBM1. The 2.1-kb EcoRI-XhoI fragment of this insert was subcloned into pBluescript II SK+ giving the plasmid pBM2 (Fig. 4A). Fig. 4B shows the DNA sequence of the entire clone in pBM1 and the deduced amino acid sequence of the biotinylated subunit of PYC.

Cloning and sequencing of regions of the M. thermoautotrophicum ΔH chromosome that are adjacent to the insert in pBM1 showed that the gene for the non-biotinylated subunit of PYC was at least 1 kb away from the termini of the clone in pBM1 (data not shown). As further efforts of cloning this gene was in progress, the tentative and unpublished sequence of the pBM1 (data not shown). As further efforts of cloning this gene were discontinued.

DNA Sequence Analysis—The DNA sequence shown in Fig. 4B harbored three open reading frames of significant lengths. From comparison with the determined NH2-terminal amino acid sequence as reported above, the largest of these open reading frames was identified as the gene for the biotinylated or B subunit of pyruvate carboxylase of M. thermoautotrophicum strain ΔH (Fig. 4B). This gene was designated as pycB and the corresponding gene product as PYCB. For the pycB gene, ATG was the start codon and TAA was the stop codon (Fig. 4B). The pycB gene sequence was 54 mol % G + C, and this value was comparable to the overall mol % G + C content (48%) of M. thermoautotrophicum ΔH genome (31). The initiation codon of the pycB gene was preceded by the sequence AGAGG (position −11 to −7), which could serve as a ribosome-binding site. The available sequence upstream of pycB did not harbor any open reading frame of significant length but several AT-rich stretches resembling TATA box component of methanogen promoters (32). Several oligo(dT) sequences that might provide transcription termination signals (33) were found downstream of the pycB gene (underlined sequences in Fig. 4B). This downstream region also contained an inverted repeat CATAATAAAGCTTTAATTGT that included last 10 bases of the pycB gene including the termination codon and could form a stem and loop structure.

The other two open reading frames of significant lengths in the DNA insert of pBM1 were designated as ORF1 and ORF2. ORF1F was located 865 bp downstream of pycB, and in pBM1 it was incomplete. ORF2R originated at 832 bp downstream and was in the opposite orientation of pycB. It was also preceded by a canonical ribosome-binding sequence (GGAGG; sequence position 2546–2542) and several stretches of BoxA-like sequences (32) and was followed by several oligo(dT) sequences (shown as doubly underlined in Fig. 4B) and the inverted repeat described above. It was 669 bp in length and had the potential of coding for a 222-residue hydrophilic (hydrophobicity, −0.118) polypeptide of calculated molecular mass and pI of 25,008 Da and 4.63, respectively. The ORF2R polypeptide was found to be highly similar to an open reading frame of unknown function in Methanococcus jannaschii (34) and the exsB gene product of Rhizobium meliloti (35) and was fairly similar to a putative ExsB protein of Synechocystis sp. PCC 6803 (36) and a putative protein (YbaX) of unknown function in E. coli (37). The exsB gene product of R. meliloti is probably a regulator for the biosynthesis of acidic exopolysaccharide succino-glycan (35).

Deduced Properties of PYCB Polypeptide—A comparison of the deduced PYCB sequence with the determined NH2-terminal amino acid sequence of the 75-kDa subunit of purified enzyme suggested that the initiator methionine was retained in the matured PYCB peptide. The calculated molecular mass of the PYCB peptide was 63961 daltons, about 11-kDa smaller than the value obtained from SDS-PAGE with purified enzyme. The theoretical pI of the protein was 4.66 and the aliphatic index was 90.25. The net charge of the protein at pH 7 and 9, as calculated by using the ISOELECTRIC program of the GCG package (Genetic Computer Group Inc., Madison, WI), were −39.87 and −50.16, respectively. An analysis by using the SOUSI program (Mitaku Laboratory, University of Tokyo Agricultural and Technology, Tokyo) predicted that PYC was devoid of potential transmembrane segments and was a soluble protein with a hydrophobicity of −0.926. A PROSITE search (University of Geneva) revealed that the sequence EALCEDSVAIK174DMAG (residues 164–178) of PYCB could represent a serine/threonine dehydratase type pyridoxal-phosphate attachment site (accession number PS00165).

PYCB shared high degrees of sequence similarities with the putative oxaloacetate decarboxylase of M. jannaschii (34) and a large number of biotin containing enzymes of bacterial and eukaryotic origin. In particular PYCB showed substantial identities to the COOH-terminal halves of several eukaryotic (for example: rat (38), yeast (16), and human (39); average identities, 37%) and bacterial (for example: Bacillus stearothermophilus (40), Rhizobium etli (41), and Mycobacterium tuberculosis (accession no. 560527); average identities, 35%) PYCs, to the entire a subunit of one archaeal oxaloacetate decarboxylase or OAD (M. jannaschii OADα (34); 61% identity), several bacterial OADs (for example: Klebsiella pneumoniae (42) 48% identity) and the 5 S subunit of the transcarboxylase (TC) from Propionibacterium shermanii (Ref. 43; 43% identity). An alignment of PYCB with these sequences revealed several regions that were strongly conserved across phylogenetic lines (Fig. 5); note that we renamed the M. jannaschii OADs as M. jannaschii PYCB for the reasons given under “Discussion.” Based on this comparison and previously reported sequence features of biotin-dependent enzymes (15, 16, 38, 41, 43, 44), the following functional domains were identified in PYCB. The sequence EAWGGATFDTCIRYLNEDPW66ERLNE near the NH2-terminal region of PYCB (residues 44–69) corresponded to a consensussen sequence motif EAWGGATDXDDXRFLXECWPXR that has been implicated in binding the ketoacid substrates (43, 44) and forms a part of the region suggested to be involved in metal ion coordination (metal ion coordination site 1, Fig. 5; (43)). The residue Trp64 of PYCB was found to be equivalent to Trp73 of the 5 S subunit of P. shermanii TC that has been shown to be protected by pyruvate from chemical modification with 2,4-dinitrophenylsulfenyl chloride (44). The HXHXX motif, which is conserved in several biotin containing enzymes, was found in part as NLCNH at residues 201–205 in PYCB (putative metal ion coordination site 2 in Fig. 5). It has recently been pointed

8 Sequence is available on-line at the following address: http://WWW.genomecorp.com/htdocs/sequences/methanobacter/abstract.html.
out that this motif is similar to those found in other metalloenzymes and could play important roles in binding Zn\(^{2+}\) or Mn\(^{2+}\) (38, 45) and in rare cases Co\(^{2+}\) (38). The consensus biotin attachment site AMKM with the conserved Lys residue, which covalently links biotin to the protein (15), was located in PYCB at position 534 and characteristically 33 residues away from COOH terminus. In several eukaryotic PYCs and in \textit{R. etli} PYC, the sequence PX(P/A) is found -29 residues upstream of...
this biotinylation site (15, 41), and this region is thought to act as a hinge allowing the biotinylated domain to move from biotin carboxylation site to ketoacid carboxylation site (15). In PYCB this sequence was either absent or corresponded to the PEP at location 493–495. As seen with OADs, TC, and other PYCs, several highly conserved residues surrounded the biotinylation site of PYCB as follows: Gly500, Gly508, Val510, Val515, Gly518, Val521, Gly524, Val529, Glu531, Glu536, Ile539, Pro542, and Gly545. PYCB generally showed higher identities with the OADs than with PYCs. In Fig. 5, the regions of the M. thermoautotrophicum D H PYC that are marked as OAD/TC did not match with the corresponding region of PYCs but were highly similar to the OAD and TC sequences. PYCB lacked the sequence features that have been implicated in the carboxylation of biotin and in the transfer of carboxyl group to the ketoacids (see below). The Lys174 of PYCB, which was identified as the active residue of a putative serine/threonine dehydratase type pyridoxal-phosphate attachment site (see above), was conserved in each of the sequences shown in Fig. 5.

Fig. 6 shows the patterns of functional domain distribution in various parts or subunits of several biotin containing enzymes. Based on these patterns PYCB fell into a distinct class of its own. PYCB carried both pyruvate or ketoacid-binding site and the biotin-binding site. Although this pattern is found in the a subunit of OAD from K. pneumoniae, in the complete enzyme complex this subunit is not accompanied by a BC type subunit.

**The pycA Gene and Deduced Properties of PYCA Polypeptide**—Since a comparison of the NH2-terminal sequence of the 52-kDa subunit to the available sequences in the database showed high degrees of similarities to bacterial biotin carboxylases and the putative biotin carboxylase of M. jannaschii (34), this subunit was assumed to be the biotin carboxylase unit of PYC. Since the biotin carboxylases bind ATP, this subunit was named PYCA. A search in the recently available unpublished and tentative sequence of the entire genome of M. thermoautotrophicum strain D H3 revealed that the pycA gene was 727 kilobase pairs or about half a genome away from pycB and was immediately (4 bp downstream) followed by a putative biotin ligase (birA) gene. Analysis of the sequences upstream of pycA and downstream of putative birA suggested that these two genes are probably co-transcribed. The calculated molecular mass of the PYCA peptide was 54,656 daltons and the aliphatic index was 86.07. The theoretical pI of the protein was 6.15 and the net charges at pH 7 and 9 were +2.72 and +9.72, respectively, making PYCA a neutral polypeptide at physiological pH.
FIG. 5. Primary structure alignment for the biotinylated or B subunit of pyruvate carboxylase from *M. thermoautotrophicum* strain DH and the relevant portions of other biotin-dependent carboxylases. Polypeptide abbreviations used are as follows: PYC, pyruvate carboxylase; OAD, oxaloacetate decarboxylase; TC, (S)-methylmalonyl-CoA-pyruvate transcarboxylase. The sequences shown are: *MtDH*/PYCB
Pyruvate Carboxylase of M. thermoautotrophicum

**Fig. 6. Patterns of functional domain distribution in biotin-dependent carboxylases of various origin and substrate specificity.** The names within boxes and ovals indicate the locations of sequences that have been implicated or shown to interact with the corresponding substrates or effectors. The abbreviations used are as follows: Pyr, pyruvate-binding site (43, 44); ATP, ATP-binding site (16, 38, 47); CBBS, carboxy biotin-binding site (62); RECS or RDCS, a sequence stretch harboring a Cys residue believed to be involved in CO₂ fixation (41, 47); PMA, biotinylated Lys (15); , biotin attached to a conserved lysine residue (15). The source of all sequences used here are given in the legends of Figs. 5 and 7 except that of human PCCβ (63), E. coli ACCs and ACCβ (62), and 12 S and 1.3 S subunits of P. shermanii TC (64, 65).

PYCA was predicted to be a soluble protein with a hydrophobicity of −0.286. The PYCA sequence was found to be highly similar to the NH₂-terminal half of the α₂ PYCs from eukaryotes (human (39) and yeast (16); average identity, 45%) and bacteria (R. etli (41), 43% identity) and to the entire sequences of biotin carboxylase subunits of several multi-subunit biotin-dependent carboxylases (human propionyl-CoA carboxylase α subunit or PCCA (46), 48% identity; M. jannaschii biotin carboxylase (34), 62% identity). A multiple alignment of these sequences (Fig. 7) revealed that the ATP binding motif and other sequence features, which are usually associated with the biotin carboxylases, were present in PYCA; note that we renamed the M. jannaschii BC as M. jannaschii PYCA for the reasons given under “Discussion.” The sequence GGGGGIGMRU(x)U (44) that is implicated in binding ATP by biotin carboxylases (39, 41, 47). This consensus sequence is similar to the “P loop” motif GXXXXGK(TS) that is involved in binding ATP or the nucleotide portion of nicotinamides in several ATP- or nicotinamide-dependent enzymes (48, 49). The Cys₅₃₉ of PYCA, which was located 62 residues downstream of the last Gly of the putative ATP binding motif, was also highly conserved in all biotin carboxylases. This Cys residue is commonly found as a part of the sequence DCS (ECS at location 228–230 in PYCA) and is believed to be involved in the CO₂ fixation reaction by biotin-dependent enzymes (47).

From x-ray crystallographic studies Waldrop et al. (50) showed that in the biotin carboxylase subunit of E. coli acetyl-CoA carboxylase, the residues His₂₀₉-Glu₂₁¹, His₂₉₆-Glu₂₄₁, Glu₂₇₆, Ile²⁵⁷-Glu²⁹₆, and Arg³₂⁸ probably form part of the active site pocket, and some of these residues (Lys²₁⁸, Arg²₉₂, Glu²₉₄, Glu²₉₆, and Arg³₂⁸) might bind a hydrogen phosphate ion. In the PYCA sequence (Fig. 7) the corresponding residues were probably His₁₀⁻⁶-Glu₁₉⁶, His₂₉₆-Glu₂₄₁, Glu₂₇₅, Leu₂₈₆-Glu₂₉₅, and Arg₃₂₇. Also the NH₂-terminal region (residue 1–103) of PYCA was found to be 50% identical to that of E. coli biotin carboxylase, and this region in the latter protein has been found by Waldrop et al. (50) to adopt a dinucleotide binding motif making it a possible candidate for binding ATP.

**Conditions for Expression of PYC in M. thermoautotrophicum Strain ΔH—**For routine purification of PYC, M. thermoautotrophicum strain ΔH cells grown in Medium 1 of Balch and Wolfe (20) were used as the starting material. Despite repeated attempts, we were unable to recover active PYC preparations from autotrophically grown cells, and the corresponding affinity column fractions from wash with 1 mMD-biotin did not show any protein band in SDS-PAGE or any avidin-reacting band in Western blots. To explore the reason for this observation, we performed Western blot analysis with extracts of cells grown in the following three media: Medium 1, autotrophic or minimal medium (Medium 1 without acetate, yeast extract, tryptone, and vitamins), and autotrophic medium supplemented with D-biotin to a final concentration of 100 μM. The results from these experiments are shown in Fig. 8. The autotrophically grown cells were either devoid of this protein band or possessed it below the detection limit of the system employed. A 75-kDa avidin reacting band was seen when the growth medium contained either D-biotin or yeast extract + acetate + tryptone + vitamins.

**DISCUSSION**

We discovered that the pyruvate carboxylase (PYC) activity was present in M. thermoautotrophicum strain ΔH when complex components and vitamin solutions that contained biotin or biotin itself were added to the growth medium. These results explain the failures by previous workers to find PYC in cells grown under autotrophic conditions (9). A similar situation probably exists with the closely related organism M. thermoautotrophicum Marburg (31), which has been reported to be devoid of PYC activity (8). The presence of both PYC and PPC...
activities and regulation of PYC activity by exogenously supplied biotin in the methanoarchaeon\textit{M. thermoautotrophicum} strain \textit{DH} raise several questions. These aspects as well as the characteristics of the purified enzyme are discussed below.

The bacterial and eukaryotic PYCs are homotetramers ($\alpha_4$) of 110–130-kDa subunits (3, 41, 51) with PYC of \textit{P. citronellolis} (52) and probably \textit{Azobacter vinelandii} PYC (6) being the only known exceptions. The \textit{P. citronellolis} PYC is of $\alpha_4\beta_4$ structure, where the biotinylated $\alpha$ subunits (65 kDa) are on the outside of the molecule and surround the 54-kDa $\beta$ subunits that form the core (52). Also, the $\alpha_4\beta_4$ PYCs, unlike the $\alpha_4$ enzymes, do not require acetyl-CoA for activity or stability and are insensitive to tricarboxylic acid cycle members or related metabolites. Thus, in respect to the quaternary structure and the requirement of or response to effectors, the PYC of \textit{M. thermoautotrophicum} \textit{DH} was found to be a typical $\alpha_4\beta_4$-type enzyme, except it was very mildly inhibited by $a$-ketoglutarate. Our analysis of published data (34) suggested that \textit{M. jannaschii} most likely possesses a \textit{M. thermoautotrophicum}-type PYC (see below).

Similar to all other PYCs, the PYC of \textit{M. thermoautotrophicum} was absolutely dependent on ATP and was inhibited by ADP. Our preliminary analysis revealed that the \textit{M. thermoautotrophicum} PYC possessed complex kinetic properties. Unlike other PYCs (6, 51, 53, 54), this enzyme showed negative cooperativity with respect to bicarbonate and followed Henri-Michaelis-Menten relationship with respect to pyruvate. Earlier studies with pyruvate carboxylases showed that the inhibition by ATP could be relieved by excess Mg$^{2+}$, or Co$^{2+}$ (6, 53–55). For the \textit{M. thermoautotrophicum} PYC, an increase in Mg$^{2+}$ concentration beyond that of ATP increased the apparent affinity for ATP but decreased the apparent $V_m$.

The inhibition of methanogen PYC by free Mg$^{2+}$ was in congruence with the observation that Mn$^{2+}$ or Co$^{2+}$ when supplied in place of Mg$^{2+}$ supported PYC activity but when present along with sufficient amount of Mg$^{2+}$ inhibited the reaction severely. On the other hand, activation of desalted enzyme preparations by Mg$^{2+}$ suggested that binding of this ion to the protein was necessary for attaining a catalytically active con-

\textbf{Fig. 7. Primary structure alignment for the non-biotinylated or A subunit of pyruvate carboxylase from \textit{M. thermoautotrophicum} strain \textit{DH} and the relevant portions of other biotin-dependent carboxylases.} The polypeptide abbreviations used are: PYC, pyruvate carboxylase; BC, biotin carboxylase; PCC, propionyl-CoA carboxylase; The sequences shown are: Mt\textit{DH}/PYCA, non-biotinylated subunit of \textit{M. thermoautotrophicum} strain \textit{DH} PYC; Mt/PYCA, non-biotinylated subunit of putative \textit{M. jannaschii} PYC (34); Ec/BC, BC of \textit{E. coli} (47); Hs/PCCA, a subunit of \textit{H. sapiens} PCC (40); Mt/BCCP, \textit{M. tuberculosis} biotin carboxyl carrier protein (66); Bo/PYC, \textit{B. Orbis} PYC (41); Sc/PYC, \textit{S. cerevisiae} PYC (16). Letters in boldface indicate identity with Mt\textit{DH}/PYCA. The boxed letters in reverse contrast indicate residues that are conserved in other polypeptides but are not present in Mt\textit{DH}/PYCA. Cys$^{225}$ is marked with a * and Lys$^{239}$, Arg$^{292}$, Glu$^{296}$, and Arg$^{298}$ are marked with $\Pounds$. See “Results” and “Discussion” for the roles of marked residues and sequence stretches.
dependent enzymes in a biotin-producing organism by exogenously added biotin was intriguing, since the organism can synthesize apo-PYC and biotin ligase but cannot biotinylate the apoenzyme (58); the corresponding extract can be biotinylated to the apoform, and its level is not influenced by the addition of biotin to the growth medium. Rather, in this organism the level of expression of PYC is also not influenced by the addition of biotin to the growth medium; the organism can maintain intracellular biotin levels beyond what can be maintained from the growth medium. In contrast, in M. thermoautotrophicum makes oxaloacetate using pyruvate carboxylase and is devoid of phosphoenolpyruvate carboxylase (7). A similar situation is expected for M. jannaschii, as the organism is not able to biotinylate apo-PYC or BC (possessing ATP-binding and CO2-fixation sites), and carboxyltransferase (possessing ketoacid- and metal ion-binding sites) (15, 16, 38, 41). Several types of arrangements of these units within the primary and quaternary structures of enzymes have been found (Fig. 6 and references 4, 58–60). When grown in the absence of added biotin, B. coagulans synthesizes apo-PYC and biotin ligase but cannot biotinylate the apoenzyme (58); the corresponding extract can produce active holoenzyme if excess biotin is added. It is not known whether this observation is due to a general purpose ligase that shows low affinity for biotin while ligating biotin to PYC, but not for biotinylating ACC, or to the low affinity of a PYC-specific ligase.

Only on rare occasions both PPC and PYC are found in a given organism, and wherever they co-exist their activities are regulated differently. Subculturing in minimal medium with succinate and biotin increases PYC activity in R. etli, but its PPC activity remains unchanged (4). P. citronellolis possesses both of PPC and PYC (5, 61). In this organism PPC is constitutive, but its activity is modulated through inhibition by aspartate and activation by ADP and acetetyl-CoA (5). On the other hand its PYC is insensitive to these modulators, but both subunits of the enzyme are induced when a pyruvate-generating carbon source is in use and are repressed if a carbon source that converts readily to OAA is supplied (5, 61). A preliminary report on M. thermoautotrophicum PPC shows that, similar to the PYC of this organism, this enzyme is neither activated nor inhibited by acetetyl-CoA (9). Thus, our current knowledge is insufficient to predict if and how M. thermoautotrophicum \( \Delta H \) controls the expression and activity of PPC and PYC in response to physiological conditions.

We propose to rename the putative biotin carboxylase (BC) and oxaloacetate decarboxylase a subunit (OADa) of M. jannaschii (accession number, U67563) (34), respectively, as the A and B subunits of pyruvate carboxylase. The current names were derived solely from comparative analyses of sequences in a whole genome sequencing project (34) and seemed justified in the light of observed strong similarities (see “Results”). However, for the following reasons the proposed names describe the in vivo functions of these polypeptides properly. First, the putative BC and OADa polypeptides of M. jannaschii were, respectively, 62 and 61% identical to the M. thermoautotrophicum PYCA and PYCB. Second, a search of the entire genome sequence of M. jannaschii (34) did not show the presence of another putative pyruvate carboxylase or a putative PEP carboxylase. Methanococcus maripaludis, an organism closely related to M. jannaschii, makes oxaloacetate using pyruvate carboxylase and is devoid of phosphoenolpyruvate carboxylase (7). A similar situation is expected for M. jannaschii.

The PYCA and BirA (biotin ligase) polypeptides would interact with PYCB for biotinylatation and carboxylation reactions, respectively. In M. thermoautotrophicum the pyca and birA genes were found to be part of an operon located about half a genome away from pycB. It would be interesting to investigate how the regulation of expression of these polypeptides at two different chromosomal locations are coordinated to generate a functional oxaloacetate synthesizing system on demand. In contrast, in M. jannaschii the putative pyca and pycB genes along with an intervening open reading frame seem to form an operon, and the birA gene is about 422 kilobase pairs away from pycA (34). In E. coli the genes for the subunits of acetyl-CoA carboxylase are located at three different locations of the chromosome (62), and the system coordinating the regulation of expression for this enzyme is still unknown.

Our analysis of primary structures of two methanoarchaeal PYCs demonstrated that the previously recorded high degrees of conservation in the primary structures of bacterial and eukaryotic biotin-dependent carboxylases of diverse substrate specificity extend into the domain of archaea, although the methanogen enzyme possesses a unique structure. All biotin-dependent carboxylases are composed of the following functional units: biotin carrier or biotin carboxyl carrier, biotin carboxylase or BC (possessing ATP-binding and CO2- fixation site), and carboxytransferase (possessing ketoacid- and metal ion-binding sites) (15, 16, 38, 41). Several types of arrangements of these units within the primary and quaternary structures of enzymes have been found (Fig. 6 and references 4, 58–60). The denatured proteins were separated in a 12.5% polyacrylamide gel. Amount of cell extract protein per lane is 6 \( \mu \)g. The biotin containing polypeptide bands were detected by using 0.01 \( \mu \)g/ml alkaline-phosphatase-conjugated avidin. The labels above the lanes indicate the growth medium used. Complete, Medium 1 of Balch and Wolfe (20); Yeast Extract, Medium 1 without tryptone, acetate, and vitamins but with yeast extract; Minimal, Medium 1 without yeast extract, tryptone, acetate, and vitamins; Biotin, minimal medium + D-biotin (100 \( \mu \)M).

A detailed study to fully understand the basis of these unique properties of the M. thermoautotrophicum enzyme is underway.
The role of pyridoxal phosphate in modulating activities of these decarboxylases (Fig. 5.). This site has not been identified before, and the reshuffling of gene segments occurred differently for creatine kinase and the larger subunit of M. thermoautotrophicum. The larger subunit of M. thermoautotrophicum had all of the sequence characteristics of a BC domain. Thus, if both the eukaryotic PCCs and M. thermoautotrophicum PYC had originated either from fusion of genes for the E. coli ACC-type enzymes or through splitting of the gene for α4-type PYCs, the reshuffling of gene segments occurred differently for creating these two types of proteins.

Interestingly the M. thermoautotrophicum PYC was found to harbor a putative serine/threonine dehydratase type pyridoxal-phosphate attachment site, and the corresponding region is highly conserved in other biotin-dependent carboxylases. This site has not been identified before, and there is no report on the requirement of pyridoxal phosphate for the activity or regulation of biotin-dependent carboxylases/decarboxylases. We are currently investigating the role of pyridoxal phosphate in modulating activities of these enzymes.

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Note Added in Proof—After submission of this manuscript, the sequence of the complete genome of M. thermoautotrophicum ΔH was published (Smith, D. R., Doucette-Stamm, L. A., Deloughery, C. Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, C., Pothier, B., Qui, D., Spadarola, R., Vicaire, R., Wang, Y., Wierzbowski, J., and Putnam, D., 1999). The Yeast Pyruvate Carboxylase 2 Gene (PYC2) and the reshuffling of gene segments occurred differently for creatine kinase and the larger subunit of M. thermoautotrophicum.