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, Mg\(^{2+}\) (or Mn\(^{2+}\) or Co\(^{2+}\)), 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 aspartate 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 Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\). The 540-kDa enzyme of ΔH 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 proteins of known 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 Methanobacterium strain ΔH (Kenealy, W. R., and Zeikus, J. G. (1982) FEMS Microbiol. Lett. 14, 7–10) raise several questions for future investigations.

Pyruvate carboxylase (PYC)\(^1\) 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 PEP 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 glyoxylate cycle to generate oxaloacetate for gluconeogenesis. Depending on the growth conditions, Pseudomonas citronellololus 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 carbonyl-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

\(^1\) The abbreviations used are: PYC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PPC, PEP, carboxylase; PYCA, A subunit of PYC; PYCB, B subunit of PYC; ORF, open reading frame; OAD, oxaloacetate decarboxylase; 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.

\[ \text{Enzyme-biotin} + \text{MgATP}^- + \text{HCO}_3^- \rightarrow \text{enzyme-biotin-COO}^- + \text{MgADP}^- + P_i \]

**Reaction 1**

\[ \text{Enzyme-biotin-COO}^- + \text{pyruvate} \rightarrow \text{enzyme-biotin} + \text{oxaloacetate} \]

**Reaction 2**

Since previous work in our laboratory has shown the presence of biotin in *Methanobacterium thermooautotrophicum* strain \( \Delta H \) (17), efforts were made to detect biotinylated peptides in this organism. SDS-PAGE with extracts of cells grown with exogenously supplied \([3H]\)biotin showed the presence of a tides in this organism. SDS-PAGE with extracts of cells grown strain *E. coli* DH5\( \alpha \) was grown in tubes, bottles, and in a 14-liter stainless steel flow centrifuge, frozen in liquid nitrogen, and stored at \(-20^\circ C \). A. A. DiMarco and J. E. Cronan, Jr., personal communication.

**Purification of Pyruvate Carboxylase—**

*Pyruvate Carboxylase of M. thermoautotrophicum* \( \Delta H \) was grown in tubes, bottles, and in a 14-liter stainless steel flow centrifuge, frozen in liquid nitrogen, and stored at \(-20^\circ C \). A. A. DiMarco and J. E. Cronan, Jr., personal communication.

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Western blot corresponds to the native PAGE performed without inorganic phosphate. Again, a sharp avidin-reacting Western blot analysis in the nondenatured state using alkaline 

Dase from M. thermoautotrophicum Strain 

supernatant of the cell extract containing 1 M KCl. Thus, it is a pyruvate carboxylase activity was found in the 100,000 
electrode buffer at 10% and 10 mM concentrations, respectively. The Western blot corresponds to the native PAGE performed without inositol and MgCl₂.

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.

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.

resulted in a smear when the electrophoresis was conducted in the 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 MKGKVETFARQDHQQSLA and that of the 52 kDa was MFGKILVANREIYRV. The NH₂-terminal sequence of the 67-kDa polypeptide was determined for the first 17 residues, and it was the same as that of the 75-kDa band. From these results it was concluded that the M. thermoautotrophicum strain ΔH PYC was most likely of A₄B₄ structure, and 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.

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 M KCl. As reported above, Mg²⁺ was required by the purified enzyme for activity. However, this divalent cation
30 min. was added to the mixture, and the incubation was continued for another mixture.

In the assay. None of these proteins were directly added to the assay

Table I

| Effectors | Activity |
|-----------|----------|
| Control | 100 |
| Avidin (25-fold molar excess) | 0 |
| Avidin (25-fold molar excess) | 10 |
| Bovine serum albumin (25 mol/mol PYC tetramer) | 90 |
| Alternate nucleotide | 0 |
| AMP, ADP, GTP, ITP, or UTP | 0 |
| Additional nucleotide | 0 |
| AMP | 104 |
| ADP | 73 |
| GTP | 106 |
| ITP | 94 |
| UTP | 80 |
| UTP | 105 |

Tricarboxylic acid cycle-related compounds

| Acetyl-CoA | 84 |
| Aspartate | 91 |
| Glutamate | 95 |
| α-Ketoglutaric acid | 73 |

Divalent cations replacing Mg²⁺

| Mn²⁺ | 17 |
| Co²⁺ | 46 |
| Zn²⁺ | 0 |

Divalent cation in addition to Mg²⁺

| Mn²⁺ | 9 |
| Co²⁺ | 0 |
| Zn²⁺ | 0 |

Alternate nucleotide

AMP, ADP, GTP, ITP, or UTP

Activity in 50 mM Tris-HCl buffer, pH 8.0, 400 mM KCl, 50 mM sodium pyruvate, 50 mM KHCO₃, 0.2 mM Na₂NADH, 4 mM K₃ATP, 1 unit/ml malate dehydrogenase from T. flavus, and desired amount of MgCl₂·6H₂O. The activities are reported as percent of the value determined at 4 mM MgCl₂. For the work on Mg²⁺, the reaction mixtures contained 50 mM Tris-HCl buffer, pH 8.0, 15 mM sodium pyruvate, 15 mM NaHCO₃, 0.2 mM Na₂NADH, 2 mM Na₂ATP, 2 mM MgCl₂·6H₂O, 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.

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). Of 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 Mg²⁺; and 50 mM HCO₃⁻) fit well the Henri-Michaelis-Menten relationship, and from this analysis the values of apparent Vₘₐₓ for pyruvate and apparent Kᵣ for pyruvate and apparent Vₘₐₓ were found to be 1.75 ± 0.06 mM and 134 ± 1 μmol min⁻¹ mg⁻¹, respectively. ATP inhibited the enzyme at higher concentrations. When Mg²⁺ concentrations were equal to that of ATP and both pyruvate and HCO₃⁻ concentrations were 50 mM, the initial velocity versus ATP concentration data fit the substrate inhibition relationship v = Vₘₓ(S/Kᵣ + S + (S²/Kᵣ)) and provided the following values: apparent Kᵣ for ATP, 1.1 ± 0.1 mM; apparent Kᵣ for ATP, 0.8 ± 2.8 mM; and apparent Vₘₐₓ, 267 ± 14 μmol min⁻¹ mg⁻¹. A similar study on inhibition by ATP, with the Mg²⁺ concentrations were higher than that of ATP by 4 mM, provided a different set of values: apparent Kᵣ for ATP, 0.21 ± 0.01 mM; apparent Kᵣ for ATP, 15.4 ± 4.7 mM; and apparent Vₘₐₓ, 149 ± 2.5 μmol min⁻¹ mg⁻¹. The Eadie-Hofsette (v/S versus v) plot of the data for bicarbonate as the varied substrate (0.4–19 mM HCO₃⁻; 4 mM ATP; 4 mM Mg²⁺; and 50 mM pyruvate) was nonlinear and indicative of negative cooperativity. These data were fitted to a 2/1 function v = Vₘₓ[S² + DS(1/S² + BS + C)] where B, C, and D are constants and Kᵣₘₐₓ = 0.5B + D + ((0.5B + D)² + C²/2). From this fit the following values were obtained: apparent Kᵣₘₐₓ, 6.5 ± 0.8 mM; apparent Vₘₐₓ, 115 ± 0.05 μmol min⁻¹ mg⁻¹; B, 10.6 ± 2.2 mM; C, 4.3 ± 2.5 mM²; D, 2.4 ± 0.9 mM.

mM Tris-HCl buffer, pH 8, with 10% inositol. This enzyme preparation did not exhibit any activity even when KCl and MgCl₂ were present in the assay mixture at optimal levels. However, preincubation of the desalted enzyme preparation at 4 °C with 10 mM MgCl₂ for 30–60 min restored the activity to 60–70% of the original value that was recorded before desalting.

Acted as an inhibitor when it was present in molar excess with respect to ATP (Fig. 3). The requirement for a divalent cation was also fulfilled, albeit poorly, by Mg²⁺ and Mn²⁺, but Zn²⁺ was ineffective (Table I). However, in the presence of sufficient Mg²⁺ (equimolar to ATP), Co²⁺, Mn²⁺, and Zn²⁺ inhibited the reaction (Table I). In addition to its role in catalysis, Mg²⁺ had a stabilizing effect on the enzyme. This requirement was determined by using an enzyme preparation that was desalted by ultrafiltration (10-kDa molecular mass cut off) and placed in 50
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 pBlue-Script 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 entire M. thermoautotrophicum ΔH genome was released on the World Wide Web.3 Therefore, our efforts to clone the pyCA 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 CATAAAATATAAAacctcTTTTATTTTGT 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 ORF1F and ORF2R. 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 Synecocystis 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 succinoglycan (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 (Mitsaku Laboratory, University of Tokyo Agricultural and Technology, Tokyo) predicted that PYCB was devoid of potential transmembrane segments and was a soluble protein with a hydrophobicity of −0.286. A PROSITE search (University of Geneva) revealed that the sequence EALCDSDVAIK174DMAG (residues 164–178) of PYCB could represent a serine/threonine dehydratase type pyridoxal-phosphate attachment site (accession number P800165).

PYCB shared high degrees of sequence similarities with the putative oxalocacetate 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 oxalacetate 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 OADα 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 EAWGGATFDTCIRYLDNEPW5Creative Commons License Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0).
Fig. 4. Restriction map and nucleotide sequence of the 3.5-kb clone carrying the gene for the biotinylated or B subunit of M. thermoautotrophicum strain ΔH PYC. A, restriction map of the 3.5-kb EcoRI insert in plasmid pBM1 and the 2.1-kb EcoRI-XhoI insert in the subclone pBM2. The locations and orientations of the pycB gene, ORF1F, and ORF2R are shown. The dashed arrow indicates that ORF1F is incomplete in pBM1. B, nucleotide sequence of the EcoRI insert in pBM1. The horizontal arrows show the start sites and the orientations of the genes. The putative ribosome binding sequences are shown by asterisks. The nucleotide sequence is numbered from the first nucleotide of the translation initiation codon of the pycB gene. The deduced amino acid sequences are shown below the nucleotide sequence in single-letter codes. The inverted repeat sequence is indicated by converging arrows. The single underlined stretches of T residues are putative transcription termination signals for pycB, and the corresponding sequences for ORF2R are shown as doubly underlined. The portion of the NH2-terminal amino acid sequence of PYCB that was used to design the degenerate oligonucleotide probe for hybridization and screening is shown in italics and underlined.

out that this motif is similar to those found in other metalloenzymes and could play important roles in binding Zn2+ or Mn2+ (38, 45) and in rare cases Co2+ (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 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 α 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 NH$_2$-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 $-3.72$ 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 ΔH 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: *MtΔH/PYCB*, pyruvate carboxylase of *M. thermoautotrophicum* strain 5162.
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 α₃ 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 GGGGIGM-TC (43) 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 GGGGIGM-TC (43) 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 GGGGIGM-TC (43) 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 GGGGIGM-TC (43) 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.”

**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, ATP, PAP, or PLA, conserved PX(P)A sequence located ~29 residues upstream of the biotinylated Lys (15); , biotin attached to a conserved lysine residue (15). The sources 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).

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 PCCβ (63), E. coli ACCs and ACCβ (62), and 12 S and 1.3 S subunits of P. shermanii TC (64, 65).
activities and regulation of PYC activity by exogenously supplied biotin in the methanoarchaeon M. thermoautotrophicum strain 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 (α4) of 110–130-kDa subunits (3, 41, 51) with PYC of P. citronellolis (52) and probably Azobacter vinelandii PYC (6) being the only known exceptions. The P. citronellolis PYC is of α4β4 structure, where the biotinylated α subunits (65 kDa) are on the outside of the molecule and surround the 54-kDa β subunits that form the core (52). Also, the α4β4 PYCs, unlike the α4 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 M. thermoautotrophicum DH was found to be a typical α4β4-type enzyme, except it was very mildly inhibited by α-ketoglutarate. Our analysis of published data (34) suggested that M. jannaschii most likely possesses a M. thermoautotrophicum-type PYC (see below).

Similar to all other PYCs, the PYC of M. thermoautotrophicum was absolutely dependent on ATP and was inhibited by ADP. Our preliminary analysis revealed that the 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 Mg2+ or Co2+ (6, 53–55). For the M. thermoautotrophicum PYC, an increase in Mg2+ concentration beyond that of ATP increased the apparent affinity for ATP but decreased the apparent Vm.

The inhibition of methanogen PYC by free Mg2+ was in congruence with the observation that Mn2+ or Co2+ when supplied in place of Mg2+ supported PYC activity but when present along with sufficient amount of Mg2+ inhibited the reaction severely. On the other hand, activation of desalted enzyme preparations by Mg2+ suggested that binding of this ion to the protein was necessary for attaining a catalytically active con-
fication. A detailed study to fully understand the basis of these unique properties of the *M. thermoautotrophicum* enzyme is underway.

Since the PYCB peptide would be acidic at electrophoresis pH (calculated value of the charge, −50), the SDS-PAGE-derived molecular weight value was expected to be lower than the sequence-derived value. But our observation was the opposite. It was also difficult to explain how a biotinylated band of 67 kDa could arise from the cleavage of 75-kDa subunit retaining the same NH2-terminal sequence, because a cleavage at the COOH terminus that would retain the biotinylation site AMKM would account for a mass change of only 3.6 kDa.

The regulation of PYC activity in *M. thermoautotrophicum* ΔH by added biotin was intriguing, since the organism can synthesize biotin on its own (17). It seems that the elevation of intracellular biotin level beyond what can be maintained from biosynthesis was necessary for exhibiting detectable levels of PYC, and one or more of the following reasons could account for this requirement. 1) Higher biotin level was necessary for the (over)expression of PYC subunits and/or the biotinylating enzyme biotin ligase. 2) The biotinylation of PYC required higher biotin levels due to relatively low affinity of ligase for biotin; the low affinity was either an intrinsic property of the ligase or an effect imposed by a modulator. Regulation of activity for biotinylating ACC, or due 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. eili*, 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 acetyl-CoA (5). On the other hand its PPC in 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 acetyl-CoA (9). Thus, our current knowledge is insufficient to predict if and how *M. thermoautotrophicum* ΔH 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 (OADs) 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 OADs 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 biotinylating 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 possessed 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 carboxyltransferase (possessing ketoadid- 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
therein). The M. thermoaerotrophicum PYC possessed two types of subunits and therefore appeared to be similar to eukaryotic PCCs (Fig. 6). However, a comparative analysis of primary structures (Figs. 5 and 7) showed that it had a pattern of its own (Fig. 6). The larger subunit of M. thermoaerotrophicum PYC carried the putative biotin and pyruvate-binding sites and therefore harbored both the biotin carboxyl carrier and the carboxyltransferase domains, and the smaller subunit had all of the sequence characteristics of a BC domain. Thus, if both the eukaryotic PCCs and M. thermoaerotrophicum PYC had originated either from fusion of 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 enzymes.

Interestingly the M. thermoaerotrophicum PYC was found to harbor a putative serine/threonine dehydratases type pyridoxal-phosphate attachment site, and the corresponding region was highly conserved in other biotin-dependent carboxylases/decarboxylases (Fig. 5). 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. thermoaerotrophicum ∆H was published (Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, D., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Deloughery, C., Plapp and Endang Purwantini for help in kinetic analysis.

REFERENCES

1. Gottschalk, G. (1986) Bacterial Metabolism, 2nd Ed., Springer-Verlag Inc., New York.
2. Kornberg, H. L. (1966) in Essays in Biochemistry (Campbell, P. N. and Greville, G. D., eds) Vol. 2, pp. 1–31, Academic Press, New York.
3. Wallace, J. C. (1985) in Pyruvate Carboxylase (Keech, D. V., and Wallace, J. C., eds) pp. 6–63, CRC Press, Inc., Boca Raton, FL.
4. Encarnacion, S., Dunn, M., Willms, K., and Mora, J. (1995) J. Bacteriol. 177, 2958–2960.
5. O'Brien, R., Chuang, D. T., Taylor, B. L., and Utter, M. F. (1977) J. Biol. Chem. 252, 1257–1263.
6. Scrutton, M. C., and Taylor, B. L. (1974) Arch. Biochem. Biophys. 164, 641–654.
7. Shieh, J., and Whitman, W. B. (1987) J. Bacteriol. 169, 5327–5329.
8. Jansen, K., Stupperich, E., and Fuchs, G. (1982) Arch. Microbiol. 132, 355–364.
9. Kenealy, W. R., and Zeigler, J. G. (1982) FEMS Microbiol. Lett. 14, 7–10.
10. Weimer, P. J., and Zeigler, J. G. (1979) J. Bacteriol. 137, 332–339.
11. Wurtele, E. S., Nikolau, B. J. (1990) J. Bacteriol. 1257–1263.
12. Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Haase, F. C. and Wood, H. G. (1988) J. Biol. Chem. 263, 6461–6464.
13. Lim, F., Morris, C. P., Occhiodoro, F., and Wallace, J. C. (1988) J. Bacteriol. 263, 11493–11497.
14. Lenn, K. M., and Barber, T. S. (1988) J. Bacteriol. 170, 4315–4321.
15. Stoddard, S. F., and Wolfe, R. S. (1991) Abstr. Genl. Meeting American Society for Microbiology, Washington, D. C. (Abstr. I-120).
16. Zeigler, J. G., and Wolfe, R. S. (1972) J. Bacteriol. 109, 707–713.
17. Balch, W. E., and Wolfe, R. S. (1976) Appl. Environ. Microbiol. 32, 781–791.
18. Mukhopadhyay, B. (1993) Coenzyme F420-dependent Methanothrix HAMPT Dehydrogenases of Methanobacterium thermophilum Thermotoga maritima and Methanosaeta barteringi and Effectors of Methanogenic Substrates on the Levels of Three Catabolic Enzymes in Methanosaeta barteringi, Ph.D. thesis, University of Iowa, Iowa City, IA.
19. Kohara, K. A., and Lane, M. D. (1990) Methods Enzymol. 184, 195–200.
20. Puurell, A. W., and Wallace, J. C. (1996) Anal. Biochem. 238, 213–216.
21. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
22. Ellis, K. J., and Morrison, J. F. (1982) Methods Enzymol. 87, 405–426.
23. Celander, W. W. (1970) in The Enzymes (Boyer, P. D., ed) Vol. 2, pp. 1–65, Academic Press, New York.
24. Fuchs, G., Stupperich, E., and Fuchs, G. (1982) Arch. Microbiol. 132, 355–364.
25. Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Haase, F. C. and Wood, H. G. (1988) J. Biol. Chem. 263, 6461–6464.