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Pathway of Glycogen Metabolism in *Methanococcus maripaludis*

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***Methanococcus maripaludis*, a facultatively autotrophic archaebacterium that grows with H_2 or formate as the electron donor, does not assimilate sugars and other complex organic substrates. However, glycogen is biosynthesized intracellularly and commonly reaches values of 0.34% of the cellular dry weight in the early stationary phase. To determine the pathway of glycogen catabolism, specific enzymes of sugar metabolism were assayed in cell extracts. The following enzymes were found (specific activity in milliunits per milligram of protein): glycogen phosphorylase, 4.4; phosphoglucomutase, 10; glucose-6-phosphate isomerase, 9; 6-phosphofructokinase, 5.6, fructose-1,6-bisphosphatase, 10; fructose-1,6-bisphosphate aldolase, 4.2; triosephosphate isomerase, 44; glyceraldehyde-3-phosphate dehydrogenase, 26; phosphoglycerate kinase, 20; phosphoglycerate mutase, 78; enolase, 107; and pyruvate kinase, 4.0. Glyceraldehyde-3-phosphate dehydrogenase was $NADP^+$ dependent, and the pyruvate kinase required $MnCl_2$. The 6-phosphofructokinase had an unusually low pH optimum of 6.0. Four nonoxidative pentose-biosynthetic enzymes were found (specific activity in milliunits per milligram of protein): transketolase, 12; transaldolase, 24; ribulose-5-phosphate-3-epimerase, 55; and ribulose-5-phosphate isomerase, 100. However, the key enzymes of the oxidative pentose phosphate pathway, the reductive pentose phosphate pathway, and the classical and modified Entner-Doudoroff pathways were not detected. Thus, glycogen appears to be catabolized by the Embden-Meyerhoff-Parnas pathway. This result is in striking contrast to the nonmethanogenic archaebacteria that have been examined, among which the Entner-Doudoroff pathway is common. A dithiothreitol-specific $NADP^+$-reducing activity was also found (8.5 mU/mg of protein). Other thiol compounds, such as cysteine hydrochloride, reduced glutathione, and 2-mercaptoethanesulfonic acid, did not replace dithiothreitol for this activity. The physiological significance of this activity is not known.**

Methanogens are ubiquitous archaebacteria whose substrates for growth are limited to H_2, acetate, C_1 compounds like formate and methylamines, and a few alcohols (17). Although they do not assimilate sugars, glycogen is present in some methanogens as well as in other archaebacteria (26, 31, 34, 37). The pathway of glycogen catabolism is of evolutionary interest for comparison with sugar metabolism in heterotrophic archaebacteria. Although variations of the Entner-Doudoroff pathway are common in *Halobacterium* spp., *Sulfolobus* spp., *Thermoplasma acidophilum*, and *Pyrococcus furiosus* (8, 17, 43), two halobacteria, *Haloferax mediterranei* and *Haloarcular vallismortis*, utilize fructose by a modification of the Embden-Meyerhoff-Parnas (EMP) pathway (1, 2). In this pathway, fructose is phosphorylated to fructose-1-phosphate by ketohexokinase prior to its conversion to fructose-1,6-bisphosphate by 1-phosphofructokinase. In addition, the EMP pathway has been identified in the hyperthermophile *Thermoproteus tenax* (47). Thus, the pathway glycogen catabolism in methanogens is of special interest in determining the diversity of sugar catabolism pathways in the archaebacteria.

Although the gluconeogenic pathway has been demonstrated in a number of methanogens (reviewed in reference 48), sugar metabolism in *Methanococcus maripaludis* and other methanogens has not been examined in detail. In *Methanolobus* spp., glycogen can serve as an electron donor for methane production when exogenous substrates are not available (25). Similarly, *M. maripaludis* produces significant amounts of methane, possibly from a reserve material like glycogen, in the absence of exogenous substrates (59). Therefore, in this autotrophic archaebacterium, a catabolic pathway(s) could provide endogenous electron donors for survival under limited growth conditions.

MATERIALS AND METHODS

Bacterium and culture conditions. *M. maripaludis* JJ (Deutsche Sammlung von Mikroorganismen [DSM] 2067, Oregon Collection of Methanogens [OCM] 175) was obtained from W. J. Jones. *M. maripaludis* was grown at 40°C in the mineral medium McN under H_2 plus CO_2 (80:20 [vol/vol]) in a 10-liter fermentor (54). The mineral medium was autoclaved under H_2 plus CO_2 gas at a pressure of 35 kPa for 40 min. After cooling, the medium was sparged with H_2 plus CO_2. One hour before inoculation, 10 ml of a sterile solution of 20% $Na_2S \cdot 9H_2O$ was added. The inoculum was 2 to 5% of the working volume and was in the exponential growth phase. During growth, the gas pressure inside the vessel was maintained at 140 kPa with an H_2 plus CO_2 flow rate of 9 to 18 liters h^{-1}. The stirring rate was 240 to 300 rpm. An additional 10 ml of sterile 20% $Na_2S \cdot 9H_2O$) was added when the A_{660} of the culture reached 0.5. Cells were harvested in the early stationary phase with a Sharples continuous-flow centrifuge and stored under N_2 gas at −20°C. In some experiments, 1-liter portions of the culture were anaerobically harvested throughout growth. A 1-liter stoppered bottle that had been sitting in an anaerobic chamber overnight was connected to the fermentor outlet with tubing and a syringe needle. The culture was forced into the bottle by the pressure in the fermentor. During this procedure, pressure inside the bottle was vented by using a second syringe needle. Cells were then transferred to 500-ml centrifuge bottles in the anaerobic chamber and centrifuged at 11,000 × *g* for 20 min at

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4°C. Plastic centrifuge bottles were stored for at least 1 day in the anaerobic chamber to allow O_2 to diffuse from them.

Preparation of cell extracts. Extracts were prepared as described previously and stored under N_2 gas at −20°C (57). Extracts were dialyzed at 4°C for 18 h against 100 mM Tris-hydrochloride buffer (pH 7.5) containing 1 mM disodium EDTA, 1 mM dithiothreitol, and 20% (vol/vol) glycerol except as noted below. For the glycogen phosphorylase assay, undialyzed extracts were treated with 20 mM $MnCl_2$ to precipitate endogenous phosphate. This mixture was incubated at 4°C for 30 min before centrifugation at 60,000 × *g* in a 50Ti rotor for 30 min at 4°C.

Enzymatic assays. All assays were performed at 37°C under N_2 gas in 1.6-ml cuvettes (Uvonic Instruments Inc., Plainview, N.Y.) that were sealed with red rubber stoppers (Thomas Scientific, Swedesboro, N.J.). The final volume of the reactions was 1 ml. Care was taken to remove oxygen from the buffers and other assay components by sparging with N_2 gas for at least 30 min. All reactions were measured spectrophotometrically at 365 nm. One milliunit of enzyme activity is defined as 1 nmol of product formed min^{-1}. Specific activities are given in milliunits per milligram of protein.

Glycogen phosphorylase was measured in the direction of the phosphorolysis of glycogen by coupling to phosphoglucomutase and glucose-6-phosphate dehydrogenase by the method of Khandelwal et al. (23) except that 20 mM dithiothreitol and 50 mM Tris-hydrochloride (pH 7.0) were used.

Glucose-1-phosphate-dependent glucose-6-phosphate formation by phosphoglucomutase was coupled to glucose-6-phosphate dehydrogenase. Based on the method of Lowry and Passonneau (29), the assay mix contained 50 mM Tris-hydrochloride (pH 7.5), 20 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM AMP, 0.4 mM $NADP^+$, 3 U of baker's yeast glucose-6-phosphate dehydrogenase, and 10 μl of undialyzed cell extract. The reaction was initiated with 2 mM α-D-glucose-1-phosphate.

Glucose-6-phosphate dehydrogenase was measured by the formation of 6-phosphogluconate from glucose-6-phosphate with the reduction of either $NADP^+$ or NAD^+ (33). The assay mix contained 100 mM Tris-hydrochloride (pH 7.5), 20 mM cysteine hydrochloride, 5 mM $MgCl_2$, 1 mM AMP, 1 mM $NADP^+$ or NAD^+, and 10 μl of cell extract. The reaction was initiated with 2 mM glucose-6-phosphate.

Glucose dehydrogenase was measured by the formation of D-glucono-δ-lactone from D-glucose with the reduction of either $NADP^+$ or NAD^+. Based on the method of Sadoff (42), the reaction mix contained 100 mM Tris-hydrochloride (pH 8.0), 2 mM glutathione, 5 mM $MgCl_2$, 1 mM $NADP^+$ or NAD^+, and 10 μl of cell extract. The reaction was initiated with 10 mM D-glucose.

6-Phosphogluconate dehydrogenase was measured by the 6-phosphogluconate-dependent reduction of either $NADP^+$ or NAD^+. Modified from the method of Rippa et al. (39), the assay mix contained 100 mM Tris-hydrochloride (pH 7.5), 2 mM dithiothreitol, 10 mM $MgCl_2$, 0.4 mM $NADP^+$, and 10 μl of cell extract. The reaction was initiated with 5 mM 6-phosphogluconate. This enzyme was also measured by the formation of 6-phosphogluconate from ribulose-5-phosphate and sodium bicarbonate with oxidation of either NADPH or NADH. The assay mix contained 100 mM Tris-hydrochloride (pH 7.5), 2 mM glutathione, 1 mM $MgCl_2$, 0.5 mM NADPH, 50 mM $NaHCO_3$, and 10 μl of cell extract. The reaction was initiated with 0.5 mM ribulose-5-phosphate.

The combined activities of gluconate dehydratase and 2-keto-3-deoxygluconate aldolase were determined as discussed by Budgen and Danson (4). The assay mix contained 100 mM Tris-hydrochloride (pH 8.0), 2 mM glutathione, 5 mM $MgCl_2$, 0.25 mM NADH, 4 U of rabbit muscle lactate dehydrogenase, and 10 μl of cell extract. The reaction was initiated with 10 mM gluconate. The combined activities of 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase were measured in identical assays except that 2 mM 6-phosphogluconate was substituted for gluconate.

Glucose-6-phosphate isomerase was measured by the fructose-6-phosphate-dependent formation of glucose-6-phosphate (34). The complete assay mix contained 50 mM Tris-hydrochloride (pH 7.5), 20 mM dithiothreitol, 1 mM AMP, 10 mM $MgCl_2$, 3 U of baker's yeast glucose-6-phosphate dehydrogenase, 0.4 mM $NADP^+$, and 10 μl of cell extract. The reaction was initiated with 2 mM fructose-6-phosphate.

6-Phosphofructokinase was assayed by coupling to fructose-1,6-bisphosphate aldolase, triose isomerase, and α-glycerol-3-phosphate dehydrogenase (28). The assay mix contained 100 mM MES (2-[*N*-morpholine]ethanesulfonic acid)-Tris (pH 6.0), 5 mM dithiothreitol, 1 mM ATP, 2 mM $MgCl_2$, 0.25 mM NADH, 5 U of rabbit muscle α-glycerol-3-phosphate dehydrogenase, 30 U of rabbit muscle triose isomerase, 1 U of trout muscle aldolase, and 10 μl of cell extract. The reaction was initiated with 5 mM fructose-6-phosphate. In some assays, 1 mM AMP or 100 mM KH_2PO_4, pH 6.0, was also added.

Fructose-1,6-bisphosphate-dependent fructose-6-phosphate formation by fructose-1,6-bisphosphatase was coupled with glucose-6-phosphate isomerase to glucose-6-phosphate dehydrogenase (15). The assay mix contained 100 mM Tris-hydrochloride (pH 7.6), 20 mM cysteine hydrochloride, 5 mM $MgCl_2$, 1 mM $NADP^+$, 0.7 U of glucose-6-phosphate dehydrogenase, 0.97 U of rabbit muscle glucose-6-phosphate isomerase, and 10 μl of cell extract. The reaction was initiated with 2.5 mM fructose-1,6-bisphosphate.

Fructose-1,6-bisphosphate aldolase was assayed in both the anabolic and catabolic directions. For the anabolic activity, triosephosphate-dependent fructose-1,6-bisphosphate formation was coupled with fructose-1,6-bisphosphatase and glucose-6-phosphate isomerase to glucose-6-phosphate dehydrogenase (15). For the catabolic activity, fructose-1,6-bisphosphate-dependent glyceraldehyde-3-phosphate formation was coupled to glyceraldehyde-3-phosphate dehydrogenase. The catabolic assay mix contained 100 mM potassium Tricine (*N*-tris[hydroxymethyl]methylglycine) (pH 8.1), 2 mM glutathione, 2 mM KH_2PO_4, 5 mM KH_2ASO_4, 2 mM KCl, 1 mM NAD^+, 5 U of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and 10 μl of cell extract. The reaction was initiated with 2 mM fructose-1,6-bisphosphate.

Glyceraldehyde-3-phosphate dehydrogenase activity was determined in the direction of $NADP^+$ reduction as described by Zeikus et al. (60).

α-Glycerol-3-phosphate dehydrogenase activity was measured as discussed by White (53). The assay mix contained 100 mM Tris-hydrochloride (pH 8.0), 2 mM glutathione, 0.25 mM NADH or NADPH, and 10 μl of cell extract. The reaction was initiated by the addition of 2 mM dihydroxyacetone phosphate.

3-Phosphoglycerate dehydrogenase activity was determined by monitoring NAD^+ reduction (41). The assay mix contained 100 mM Tris-hydrochloride (pH 7.5), 20 mM dithiothreitol, 1 mM NAD^+, 1 mM hydrazine sulfate, 1 mM KH_2PO_4, and 10 μl of cell extract. The reaction was initiated with 2 mM 3-phosphoglycerate.

Glyceraldehyde-3-phosphate-dependent dihydroxyacetone phosphate formation by triosephosphate isomerase was coupled to α-glycerol-3-phosphate dehydrogenase (15).

Phosphoglycerate kinase activity was measured by the method of Jansen et al. (16). 3-Phosphoglycerate- and ATP-

dependent 1,3-biphosphoglycerate formation was coupled to glyceraldehyde-3-phosphate dehydrogenase. The assay mix contained 100 mM potassium Tricine (pH 8.1), 20 mM cysteine hydrochloride, 5 mM $MgCl_2$, 2 mM 3-phosphoglycerate, 0.25 mM NADH, 4.5 U of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and 10 μl of cell extract. The reaction was initiated with 2 mM ATP.

3-Phosphoglycerate-dependent 2-phosphoglycerate formation by phosphoglycerate mutase was coupled with enolase and pyruvate kinase to lactate dehydrogenase (16) except that the pH was 7.5, 20 mM dithiothreitol replaced 4 mM cysteine hydrochloride, and 2,3-bisphosphoglycerate was omitted.

2-Phosphoglycerate-dependent phosphoenolpyruvate formation by enolase was coupled with pyruvate kinase to lactate dehydrogenase (16). The assay mix contained 100 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 0.5 mM ADP, 0.25 mM NADH, 4 U of rabbit muscle lactate dehydrogenase, 3 U of rabbit muscle pyruvate kinase, and 10 μl of cell extract. The reaction was initiated with 5 mM 2-phosphoglycerate.

Phosphoenolpyruvate- and Mn^{2+}-dependent pyruvate formation by pyruvate kinase was coupled to lactate dehydrogenase or endogenous alanine dehydrogenase activity. When lactate dehydrogenase was used, the assay mix contained 100 mM Tris-hydrochloride (pH 7.6), 20 mM dithiothreitol, 2 mM ADP, 2 mM $MnCl_2$, 0.25 mM NADH, 4 U of rabbit muscle lactate dehydrogenase, and 10 μl of cell extract. For assay with the endogenous alanine dehydrogenase, lactate dehydrogenase was replaced with 30 mM $(NH_4)_2SO_4$. The reaction was initiated with 10 mM phosphoenolpyruvate.

Alanine dehydrogenase was measured by monitoring the oxidation of NADH (21). The assay mix contained 100 mM Tris-hydrochloride (pH 7.6), 20 mM dithiothreitol, 2 mM $MgCl_2$, 30 mM $(NH_4)_2SO_4$, 0.25 mM NADH, and 10 μl of cell extract. The reaction was initiated with 2 mM pyruvate.

Lactate dehydrogenase was determined by monitoring the oxidation of either NADH or NADPH (27). The assay mix contained 100 mM Tris-hydrochloride (pH 7.0), 0.25 mM NADH or NADPH, and 10 μl of cell extract. The reaction was initiated with 1 mM pyruvate.

The ribose-5-phosphate-, xylulose-5-phosphate-, and thiamine pyrophosphate-dependent formation of glyceraldehyde-3-phosphate catalyzed by transketolase (EC 2.2.1.1) was coupled with triose phosphate isomerase to α-glycerol-3-phosphate dehydrogenase (24). The assay mix contained 100 mM Tris-hydrochloride (pH 7.5), 2 mM glutathione, 10 mM $MgCl_2$, 0.25 mM NADH, 4 U of rabbit muscle α-glycerol-3-phosphate dehydrogenase, 30 U of rabbit muscle triosephosphate isomerase, and 10 μl of cell extract. The reaction was initiated with a 1 mM concentration of both ribose-5-phosphate and xylulose-5-phosphate.

The fructose-6-phosphate- and erythrose-4-phosphate-dependent formation of glyceraldehyde-3-phosphate catalyzed by transaldolase (EC 2.2.1.2) was coupled with triosephosphate to α-glycerol-3-phosphate dehydrogenase (51). The assay mix contained 100 mM Tris-hydrochloride (pH 7.6), 2 mM glutathione, 0.5 mM erythrose-4-phosphate, 0.25 mM NADH, 4 U of rabbit muscle α-glycerol-3-phosphate dehydrogenase, 30 U of rabbit muscle triosephosphate isomerase, and 10 μl of cell extract. The reaction was initiated with 1 mM fructose-6-phosphate.

Ribulose-5-phosphate-dependent xylulose-5-phosphate formation catalyzed by D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was coupled with phosphoriboisomerase, transketolase, and triose isomerase to α-glycerol dehydrogenase (55). The assay mix contained 100 mM Tris-hydrochloride (pH 7.6), 2 mM glutathione, 1 mM $MgCl_2$, 1 mM thiamine pyrophosphate, 0.25 mM NADH, 3 U of α-glycerol-3-phosphate dehydrogenase, 30 U of triose isomerase, 0.1 U of transketolase, 7 U of phosphoriboisomerase, and 10 μl of cell extract. The reaction was initiated with 1 mM ribulose-5-phosphate.

The ribulose-5-phosphate-dependent formation of ribose-5-phosphate catalyzed by D-ribulose-5-phosphate isomerase (EC 5.3.1.6) was coupled with ribulose-5-phosphate epimerase, transketolase, and triose isomerase to α-glycerol-3-phosphate dehydrogenase. The assay conditions were the same as for ribulose-5-phosphate 3-epimerase except that 0.5 U of ribulose-5-phosphate 3-epimerase was used instead of phosphoriboisomerase.

Ribulose-1,5-bisphosphate carboxylase was assayed spectrophotometrically as discussed by Racker (36).

To determine the pH optima of 6-phosphofructokinase and fructose-1,6-bisphosphatase, different pH buffers were made: MES-Tris buffer for pH 5.5 and 6.0; PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid])-Tris buffer for pH 6.0, 6.5, 7.0, and 7.5; Tris-PIPES buffer for pH 7.5, 8.0, 8.5, and 9.0; and CHES (2-[*N*-cyclohexylamino]ethanesulfonic acid)-Tris buffer for pH 9.0 and 9.5. The concentration of each buffer was 100 mM.

Analytical procedures. The protein content of cell extracts was measured by the method of Lowry et al. (30) after treatment of extracts with 0.1 N NaOH in a boiling-water bath for 30 min. Bovine serum albumin was the standard. The glycogen content was quantitatively determined by using amyloglucosidase, hexokinase, and glucose-6-phosphate dehydrogenase, as discussed by Keppler and Decker (22).

Materials. All chemicals, gases, and reagents were of analytical grade or better. Glycogen, other substrates, and enzymes were from Sigma Chemical Company (St. Louis, Mo.). N_2, H_2, and H_2 plus CO_2 (80:20, vol/vol) gases were supplied by Selox Co. (Gainesville, Ga.) and passed through heated copper filings to remove traces of oxygen.

RESULTS AND DISCUSSION

Glycogen content and gluconeogenic enzymes. The glycogen content of *M. maripaludis* was determined throughout growth with various modifications of the gassing regimen (Fig. 1 and data not shown). In general, the glycogen content increased during growth from 0.11% ± 0.05% cellular dry weight (mean ± standard deviation, $n = 3$) at low cell densities (A_{660}, ≤0.5) to 0.34% ± 0.19% ($n = 7$) at high cell densities (A_{660}, 1.0 to 1.6). After the switch from H_2 plus CO_2 to H_2 gas at high cell densities, the cellular glycogen content decreased concomitantly with an increase in the pH to 10. Because the glycogen content remained constant during the same treatment when the pH was controlled with buffer (data not shown), glycogen catabolism appeared to be a response to alkaline pH. Turning off the gas altogether also caused the glycogen content to decrease by twofold, although the pH did not change significantly. Because the replacement of H_2 plus CO_2 with N_2 plus CO_2 or H_2 alone did not cause a similar response, glycogen consumption appeared to depend on both substrates for methanogenesis.

To determine possible pathways of glycogen biosynthesis, the enzymes of gluconeogenesis were assayed. Cell extracts contained high levels of the five enzymes necessary for the conversion of phosphoenolpyruvate to dihydroxyacetone phosphate (Table 1). Phosphoglycerate mutase activity required the reductant dithiothreitol, cysteine hydrochloride, or glutathione for full activity. Phosphoglycerate kinase activity was found only when assays were initiated with ATP. The inability to initiate the assays with 3-phosphoglycerate is not common (44), and it could result from interference by ATPase activities in

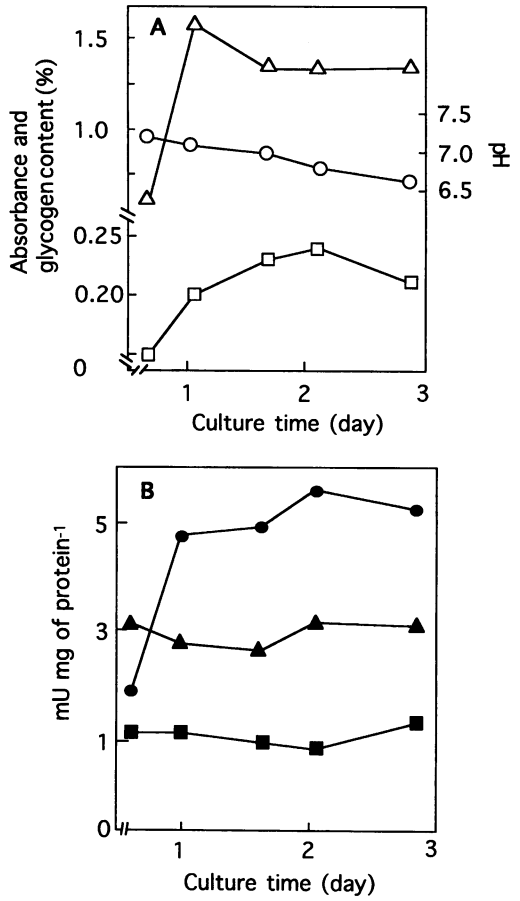

FIG. 1. Changes in the glycogen content and levels of glycolytic enzymes in *M. maripaludis* during growth on H_2 plus CO_2. (A) Changes in glycogen content (□, % cellular dry weight), A_{660} (△), and pH (○) during growth. (B) Changes in specific activities of 6-phosphofructokinase (●), fructose-1,6-bisphosphatase (▲), and pyruvate kinase (■) during growth. 6-Phosphofructokinase activity was measured as described in the text except that 1 mM AMP was added. In this 10-liter batch culture, H_2 plus CO_2 gas was replaced with N_2 plus CO_2 gas at 1.7 days.

cell extracts. Alternatively, the methanococcal enzyme could be unusual in this regard. The glyceraldehyde-3-phosphate dehydrogenase activity was specific for $NADP^+$ as an electron acceptor. In the direction of glyceraldehyde-3-phosphate oxidation, the specific activities with 6.5 mM $NADP^+$ or NAD^+ were 30 and 1 mU (mg of protein)$^{-1}$, respectively. In the direction of glyceraldehyde-3-phosphate formation, the specific activities with 0.25 mM NADPH or NADH were 25 and <0.1 mU (mg of protein)$^{-1}$, respectively. In contrast, the enzyme from *Methanobacterium thermoautotrophicum* has little specificity for either $NADP^+$ or NAD^+ (60). Finally, the triosephosphate isomerase activity was dependent on both dithiothreitol and $MgCl_2$.

The remaining enzymes required for formation of glucose-6-phosphate from dihydroxyacetone phosphate were also present (Table 1). Fructose-1,6-bisphosphate aldolase was dependent on dithiothreitol in methanococcal extracts, and both the anabolic and catabolic activities were readily detected. In contrast, only the anabolic reaction was detected in *M. thermoautotrophicum* (15). Glucose-6-phosphate isomerase activity was dependent on dithiothreitol. Because no activity was

TABLE 1. Enzymes of glycogen metabolism in *M. maripaludis*[a]

Enzyme (EC no.)	Sp act (mU [mg of protein]$^{-1}$)
Glycogen phosphorylase (EC 2.4.1.1)	4.4
Phosphoglucomutase (EC 2.7.5.1)	10.0
Glucose-6-phosphate isomerase (EC 5.3.1.9)	9.0[b]
6-Phosphofructokinase (EC 2.7.1.11)	5.6
Fructose-1,6-bisphosphatase (EC 3.1.3.11)	10[b]
Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13)	4.2 (1.0[b])
Triosephosphate isomerase (EC 5.3.1.1)	44
Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)	26
Phosphoglycerate kinase (EC 2.7.2.3)	20[b]
Phosphoglycerate mutase (EC 2.7.5.3)	78
Enolase (EC 4.2.1.11)	107
Pyruvate kinase (EC 2.7.1.40)	4.0

[a] Enzyme activities were measured in the catabolic direction as described in the text, except as noted.
[b] Enzyme activity was measured in the anabolic direction.

detected with fructose-1-phosphate, the glucose-6-phosphate isomerase activity was specific for fructose-6-phosphate. Phosphoglucose mutase activity was not influenced by the common effectors $MgCl_2$ and AMP. Because no activity was found with β-glucose-1-phosphate, the phosphoglucose mutase activity was specific for α-glucose-1-phosphate.

Fructose-1,6-bisphosphatase is a key enzyme in gluconeogenesis. In the methanococcal extracts, activity was dependent on $MgCl_2$ and cysteine, and the enzyme had a broad pH optimum from 7 to 8.5 (Fig. 2), similar to the enzymes from other sources (35). However, this enzyme was not inhibited by AMP, which is an inhibitor of the eubacterial and eucaryotic enzymes. Likewise, the phosphatase from *M. thermoautotrophicum* is insensitive to AMP (15).

Presence of the EMP pathway. To determine whether the EMP pathway in methanococci was glycolytic as well as gluconeogenic, the presence of specific catabolic enzymes associated with the pathway was examined. Glycogen phosphorylase was identified in undialyzed cell extracts of *M. maripaludis* (Table 1). This activity was dependent on dithiothreitol and was inhibited by oxygen. Because activity was lost upon dialy-

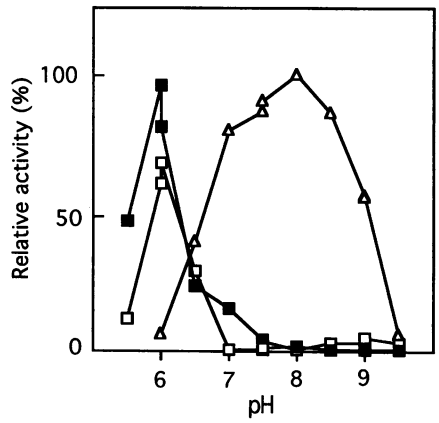

FIG. 2. pH optima of 6-phosphofructokinase and fructose-1,6-bisphosphatase. 6-Phosphofructokinase measured without KH_2PO_4 (□), 6-phosphofructokinase measured with 50 mM KH_2PO_4 (■), fructose-1,6-bisphosphatase (△). Full specific activities (100%) of the kinase and the phosphatase were 1.55 and 9.48 mU (mg of protein)$^{-1}$, respectively.

TABLE 2. Effectors of the methanococcal 6-phosphofructokinase

Addition (1 mM)	Sp act (mU [mg of protein]$^{-1}$)	
	No P_i	100 mM KH_2PO_4
None	0.7	1.5
AMP	5.7	5.5
ADP	4.9	3.4
cAMP	0.5	1.6
GDP	1.2	0.9
Citrate	1.3	0.9
F-2,6-P[a]	0.9	1.5

[a] F-2,6-P, fructose-2,6-bisphosphate.

sis, extracts were treated with $MnCl_2$ to precipitate endogenous phosphate. After treatment, only 36% of the activity remained in the absence of 10 mM KH_2PO_4, which confirmed a phosphate requirement for activity. AMP also stimulated activity by 45%.

During glycolysis, 6-phosphofructokinase is an important regulatory enzyme. In *M. maripaludis*, this activity had an optimum pH of 6.0 (Fig. 2). In contrast, the pH optima of the enzymes from other organisms are generally between 7 and 8 (3). AMP, ADP, and P_i activated the methanococcal 6-phosphofructokinase (Table 2). GDP and citrate activated the enzyme in the absence of P_i and inhibited the enzyme in the presence of P_i. Cyclic AMP (cAMP) and fructose-2,6-bisphosphate had only small effects in the presence or absence of P_i. Also, the enzyme activity was inhibited by more than 50 mM $(NH_4)_2SO_4$ or KCl.

The activation of 6-phosphofructokinase by AMP and ADP is a common feature in the enzymes from mammals, *Escherichia coli*, and *Saccharomyces cerevisiae* (3). In plants and some fungi, both are inhibitors. The insensitivity to cAMP is interesting because cAMP has little effect on the enzymes of plants, lower eucaryotes, and eubacteria, while it is an activator of the animal enzyme. The activation of the enzyme by P_i is also intriguing because this kind of activation is found only in the enzymes from mammals, plants, and one species of *Lactobacillus*. In *M. maripaludis*, citrate served as both an activator and inhibitor, depending on the presence of P_i (Table 2). Citrate is inhibitory to the enzymes from mammals, plants, *E. coli*, and *S. cerevisiae*. Little activation of the methanococcal enzyme was observed with fructose-2,6-bisphosphate, in contrast to the eucaryotic enzymes, which are greatly activated by this compound. Therefore, the regulatory features of the methanococcal 6-phosphofructokinase are distinct from those of the enzymes from the other organisms.

Pyruvate kinase, another key enzyme of glycolysis, was dependent on Mn^{2+} ion for its activity. Fe^{2+} and Mg^{2+} showed 70 and 20% of the Mn^{2+} activity, respectively. No activity was detected without metal or with Zn^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+}. Previously, this enzyme had not been detected in *M. maripaludis* when only the Mg^{2+}-dependent activity was examined (46).

Changes in the activities of key enzymes of both glycolysis and gluconeogenesis during growth were examined (Fig. 2). In this culture, H_2 plus CO_2 gas was replaced with N_2 plus CO_2 gas after 1.7 days in an attempt to induce glycogen mobilization. This attempt was unsuccessful, because the glycogen content was essentially unchanged. Although the specific activities of fructose-1,6-bisphosphatase and pyruvate kinase were constant throughout growth, the specific activity of 6-phosphofructokinase increased 2- to 2.5-fold. This result suggests that changes in the levels of 6-phosphofructokinase alone were insufficient to affect glycogen mobilization.

The presence of 6-phosphofructokinase and pyruvate kinase in *M. maripaludis* suggested that glycogen catabolism occurs by the EMP pathway. This conclusion was supported by the absence of activities of alternative pathways. For the Entner-Doudoroff pathway, specific activities for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrase plus 2-keto-3-deoxy-6-phosphogluconate aldolase were less than 0.1 and 0.04 mU (mg of protein)$^{-1}$, respectively. Because some archaebacteria contain modified Entner-Doudoroff pathways in which the intermediates are phosphorylated after the aldolase reaction (8, 17), the presence of glucose dehydrogenase and gluconate dehydratase plus 2-keto-3-deoxy-gluconate aldolase was examined. The specific activities were less than 0.1 mU (mg of protein)$^{-1}$. Similarly, the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the key enzymes of the oxidative pentose phosphate pathway, were less than 0.1 and 0.5 mU (mg of protein)$^{-1}$, respectively. These latter enzymes were examined under a variety of conditions, including replacement of $NADP^+$ with NAD^+ and examination of cells in the early exponential and late stationary phases. For 6-phosphogluconate dehydrogenase, ribulose-5-phosphate-dependent NADPH and NADH oxidation was also measured. In all cases, these experiments failed to detect either dehydrogenase.

Pentose biosynthesis. While searching for possible pathways of pentose biosynthesis, high activities of transketolase, transaldolase, D-ribose-5-phosphate 3-epimerase, and D-ribulose-5-phosphate isomerase were detected in cell extracts of *M. maripaludis* (specific activities, 12, 24, 55, and 100 mU/mg of protein, respectively). Fructose-6-phosphate and glyceraldehyde-3-phosphate, which are intermediates in the EMP pathway, were converted into xylulose-5-phosphate and erythrose-4-phosphate by transketolase. Likewise, xylulose-5-phosphate and ribose-5-phosphate were converted to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate by the same enzyme. The enzyme activity was dependent on the presence of thiamine pyrophosphate, glutathione, or both substrates. The conversion of fructose-6-phosphate and erythrose-4-phosphate to glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate by transaldolase was dependent on both substrates but not on glutathione. In addition, D-ribulose-5-phosphate 3-epimerase activity and D-ribulose-5-phosphate isomerase activities were abundant.

The high levels of the pentose-biosynthetic enzymes suggest that *M. maripaludis* utilizes a nonoxidative pathway of pentose biosynthesis (Fig. 3). In contrast, ^{13}C nuclear magnetic resonance studies of *M. thermoautotrophicum* and *Methanospirillum hungatei* suggest that pentoses are formed by the oxidative decarboxylation of hexoses in these methanogens (10, 11). While labeling studies have not been performed with the methanococci, the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were not detected under a variety of experimental conditions. Although it is possible that both dehydrogenases were determined under unfavorable conditions (e.g., the lack of a physiological cofactor such as coenzyme F_{420}), the high levels of transketolase and transaldolase tend to support the presence of a nonoxidative pathway and the conclusion that pentose biosynthesis in the methanococci differs from that in other methanogens.

In addition, a nonoxidative pathway of pentose biosynthesis could be beneficial for autotrophic growth with H_2 plus CO_2. A major role of the oxidative pentose pathway is to provide a reductant (NADPH). Under favorable growth conditions, methanococci could probably obtain sufficient reductant from H_2 oxidation. The nonoxidative pathway further avoids the release of CO_2, which is inefficient in an autotroph. Under H_2

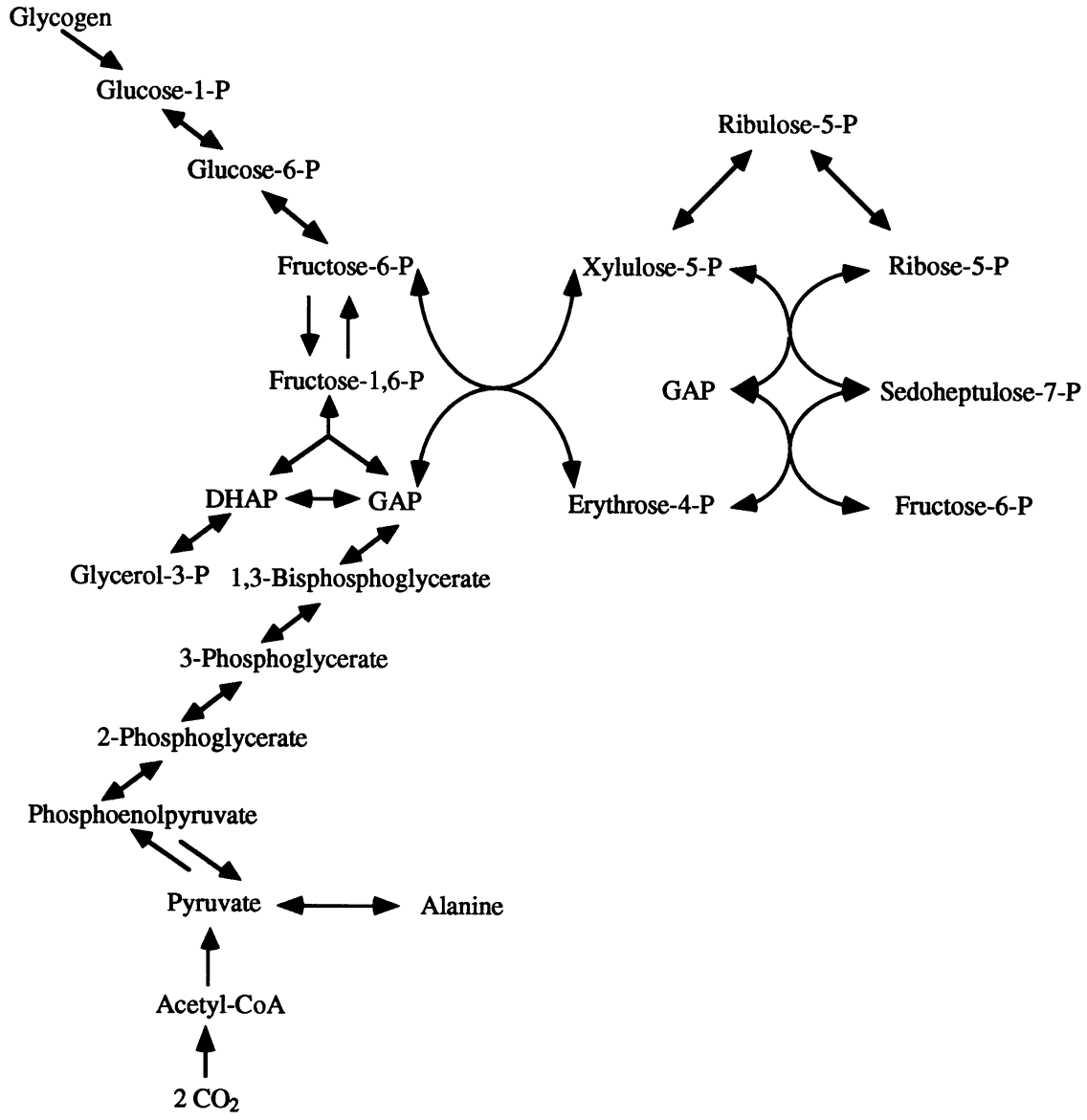

FIG. 3. Proposed pathway of sugar metabolism in *M. maripaludis*. DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; P, phosphate; CoA, coenzyme A.

limitation, NADPH could be formed by the EMP pathway via glyceraldehyde-3-phosphate dehydrogenase, which is specific for $NADP^+$ in methanococci. In contrast, the methanobacterial glyceraldehyde-3-phosphate dehydrogenase is not specific for $NADP^+$ (60), and the oxidative pentose phosphate pathway may be present in that organism.

Other enzymes examined in *M. maripaludis*. The activities of several enzymes which catalyze branch points in other pathways of cellular metabolism have also been determined. Low levels of α-glycerol-3-phosphate dehydrogenase for fatty acid and lipid biosynthesis were detected (0.12 mU [mg of protein]$^{-1}$) in extracts from early-logarithmic-phase cultures. This activity was dependent upon glutathione.

High levels of alanine dehydrogenase were found, 210 mU (mg of protein)$^{-1}$, with NADH and 4.3 mU (mg of protein)$^{-1}$ with NADPH as the electron donor. The apparent K_m for ammonium sulfate was 40 mM, which was close to the value found for *M. thermoautotrophicum* (21). Alanine dehydrogenase probably plays a role in ammonia incorporation and alanine biosynthesis (21, 58).

In contrast to *Methanospirillum hungatei* (11), evidence for 3-phosphoglycerate dehydrogenase for serine and glycine biosynthesis was not found (≤0.08 mU [mg of protein]$^{-1}$). Therefore, in *M. maripaludis*, serine biosynthesis may occur by a different pathway (e.g., by the reactions of serine dehydratase or 2-phosphoglycerate phosphatase).

Lactate dehydrogenase activity was also not detected (≤0.1 mU [mg of protein]$^{-1}$) with either NADH or NADPH as the electron donor. Ribulose-1,5-bisphosphate carboxylase, the key enzyme of the reductive pentose phosphate pathway, has been found in *Halobacterium mediterranei* (38). This enzyme activity was not found in the methanococci (≤0.1 mU [mg of protein]$^{-1}$).

During enzymatic assays, a dithiothreitol-specific $NADP^+$-

reducing activity was found (8.5 mU [mg of protein]$^{-1}$ at a 20 mM concentration of dithiothreitol). NAD^+ was not reduced ($\leq$0.28 mU [mg of protein]$^{-1}$). Cysteine hydrochloride, 2-mercaptoethanesulfonic acid, and reduced glutathione could not replace dithiothreitol with either $NADP^+$ or NAD^+ as the electron acceptor ($\leq$0.09 and $\leq$0.13 mU [mg of protein]$^{-1}$, respectively). While the physiological role of this enzyme activity was not identified, its specificity for $NADP^+$ resembles the electron-transferring flavoproteins of some glycine-utilizing bacteria (9).

Comparison of the enzyme activities of the EMP pathway among three different domains. Although in vitro specific activities do not fully represent the activity in the cell, some comparisons of the specific activities of the methanococcal enzymes with those of other organisms can be made. For this analysis, specific activities for the enzymes from rabbit muscle, a tissue specialized for sugar metabolism, and *E. coli*, a saccharolytic bacterium, were obtained from the literature (5–7, 12–14, 18–20, 32, 40, 45, 49, 50, 52, 56). Three differences between these tissues and the methanococcal extracts were striking. (i) The ratio of the specific activity of 6-phosphofructokinase to fructose-6-phosphatase was lowest in *M. maripaludis*:0.56 for *M. maripaludis*, 20 for *E. coli*, and 10 for rabbit muscle. The low ratio for the methanococcal enzymes probably reflects the autotrophic nature of the organism. Therefore, gluconeogenesis appears to be the major function of the pathway. (ii) The specific activities of the enzymes of sugar metabolism in methanococci are 10- to 100-fold lower than found in *E. coli* and 100- to 1,000-fold lower than found in rabbit muscle, which indicates the lesser importance of these pathways to methanococcal metabolism. (iii) The ratios of the average specific activities of reversible hexose phosphate conversions (phosphoglucomutase, glucose-6-phosphate isomerase, and fructose-1,6-bisphosphate aldolase) and the average specific activities of reversible triose phosphate conversions (enolase, phosphoglycerate mutase, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase) are 7.5, 4.8, and 3.2 for *M. maripaludis*, *E. coli*, and rabbit muscle, respectively. The higher ratio for the methanococcal enzymes could indicate the greater relative utilization of the triose portion of the pathway for pentose biosynthesis in methanococci than in rabbit muscle and *E. coli*. It may also reflect the greater importance of the anabolic role of the pathway in methanococci.

In conclusion, the four major pathways of sugar metabolism (e.g., the EMP, Entner-Doudoroff, oxidative, and reductive pentose phosphate pathways) are present in both of the procaryotic phylogenetic domains of organisms despite their early divergence. Even within the same phylogenetic group, such as the archaebacteria, carbohydrate metabolism is diverse. Although convergent evolution cannot be eliminated without molecular evidence, these results seem to imply that these pathways were present in the ancestor common to all modern organisms. If so, the evolutionary implications are profound. Assuming that the physiological properties of the common ancestor were at all comparable to those of modern organisms, it is unlikely that a single organism would express this degree of diversity. More plausible interpretations are that either some of these pathways were acquired late in evolution by horizontal transfer between unrelated groups or that there was no single common ancestor but a population of physiologically specialized organisms that readily exchanged genetic information.

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