Metabolite and enzyme profiles of glycogen metabolism in *Methanococcoides methylutens*

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Received 20 November 2000; received in revised form 21 February 2001; accepted 22 February 2001

Abstract

When a buffered anaerobic cell suspension of *Methanococcoides methylutens* was maintained under methanol-limited conditions, intracellular glycogen and hexose phosphates were consumed rapidly and a very small amount of methane formed at 4 h of a starvation period. When methanol was supplemented after a total of 20 h of starvation, a reverse pattern was observed: the glycogen level and the hexose phosphate pool increased, and formation of methane took place after a lag period of 90 min. A considerable amount of methane was formed in 120 min after its detection with a rate of 0.18 \( \mu \text{mol mg}^{-1} \text{protein min}^{-1} \). When methane formation decreased after 270 min of incubation and finally came to a halt, probably due to complete assimilation of supplemented methanol, the levels of glycogen and hexose monophosphates decreased once again. However fructose 1,6-diphosphate levels showed a continuous increase even after exhaustion of methane formation. In contrast to the hexose phosphate pool, levels of other metabolites showed a small increase after addition of methanol. The enzyme profile of glycogen metabolism showed relatively high levels of triose phosphate isomerase. Glyceraldehyde 3-phosphate dehydrogenase reacted with NADPH with a three-fold higher activity as compared to that with NADH. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Gluconeogenesis; Glycolysis; Glycolytic metabolite; Methanogen; *Methanococcoides methylutens*

1. Introduction

The occurrence of glycogen had been reported in methanogens [1–3]. Biosynthesis of carbohydrates from acetate and pyruvate through the gluconeogenic pathway was demonstrated in *Methanotrichs concilli* and *Methanococcus jannaschii* [4,5]. Presence of enzymes of glycogen metabolism was also demonstrated in cell-free extract of *Methanobacterium thermoautotrophicum* [6,7] and *Methanococcus maripaludis* [8]. The environmental modulations of methanogen central carbon metabolism in presence or absence of exogenous substrates with reversible gluconeogenic pathway are of interest. In this communication, we report the quantitative aspects of different metabolites and enzymes of glycogen metabolism of a methylotrophic marine methanogen, *Methanococcoides methylutens* [9]. This is the first in vivo study to measure the actual steady state concentrations of metabolites of the gluconeogenic pathway in methanogens.

2. Materials and methods

2.1. Materials

All chemicals and reagents were of analytical grade. Coenzymes and enzymes were purchased from Sigma chemical company or Boehringer Mannheim.

2.2. Organism and cultivation

*M. methylutens* strain (DSM 2657) was obtained from MACS collection of microorganisms (MCM B 708), MACS-ARI, Pune, India, and was grown anaerobically on methanol in a medium containing (g l\(^{-1}\)) \( \text{Na}_2\text{SO}_4, 1.0; \text{KCl}, 0.7; \text{MgCl}_2\cdot6\text{H}_2\text{O}, 10; \text{CaCl}_2\cdot2\text{H}_2\text{O}, 1.0; \text{NaHCO}_3, 0.2; \text{K}_2\text{HPO}_4, 0.1; \text{NH}_4\text{Cl}, 0.050; \text{Fe(NH}_3)_2\text{SO}_4, 0.002; \text{NaCl}, 30; \text{yeast extract}, 2.0; \text{tryptone}, 2.0; \text{cysteine+HCl}, 0.150; \text{vitamin and mineral}

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PII: S0378-1097(01)00118-5
solution 10 ml each at pH 7.0 under an atmosphere of N₂ as described by Balch et al. [10].

2.3. Preparation of cell suspension

All preparations were carried out at room temperature under strictly anaerobic conditions in an atmosphere of N₂. Cells were harvested at 48 h of active growth, washed anaerobically and resuspended in an anaerobically maintained medium (above), modified as: NaCl content was reduced to 10 g l⁻¹ and methanol, yeast extract, tryptone, vitamins and minerals were deleted, giving a cell concentration of 6–7 mg protein per ml and was incubated at 37°C under N₂ atmosphere. The cell suspension was incubated at 37°C under N₂ atmosphere in methanol-limited conditions for 20 h (−1200 min) and at 0 min methanol was supplemented to a concentration of 100 mM to develop methanol-sufficient conditions.

2.4. Analysis of gases

Samples were taken out from the gas phase for determination of methane and carbon dioxide by a gas chromatograph equipped with a Porapak Q column and thermal conductivity detector. Hydrogen was used as a carrier gas. The volume of gas other than head space was measured by displacement of water. The bottle was flushed out thoroughly at intervals and pressurized with N₂, after removal of the cell suspension for determination of glycogen and metabolites.

2.5. Extraction and determination of glycogen

Extraction and determination of glycogen were carried out as described by Gunja-Smith et al. [11] with slight modifications. At appropriate time intervals, 1 ml cell suspension was removed, centrifuged, washed and resuspended in 0.5 ml of 20% KOH. Tubes were immersed in a boiling water-bath for 1 h and after cooling, the suspensions were adjusted to pH 6–7 with 5 N HCl. The suspension was centrifuged at 5000 rpm for 10 min and washed with water. The volume of supernatant and washings was adjusted to 2 ml with water. The cell wall containing pellet was also suspended in 2 ml of water with gentle warming. For determination of glycogen, 0.2 ml of supernatant was incubated at 37°C for 2 h in a reaction mixture containing 0.8 ml of 50 mM citrate buffer, pH 5, 5 mM calcium chloride, 5 U of Aspergillus niger amyloglucosidase and 5 U of porcine pancreatic α-amylase. After complete digestion of glycogen, the liberated glucose was determined from a 0.2-ml aliquot by enzymic reactions of yeast hexokinase (2 U) and yeast glucose 6-phosphate (G6P) dehydrogenase (5 U) in presence of 0.4 mM NADP⁺ in 0.8 ml buffer containing 50 mM triethanolamine hydrochloride–20 mM KCl and 10 mM MgCl₂ (TRA-Mg-K buffer), pH 7.5. The reaction was monitored spectrophotometrically at 340 nm. Similarly glycogen content in the fraction containing cell wall was also measured.

2.6. Extraction and determination of metabolites and adenine nucleotides

The cell suspension described above was incubated at 37°C under nitrogen atmosphere. At appropriate time intervals, 2-ml samples were taken out with a syringe, before and after addition of methanol to a concentration of 100 mM, and immediately added to 1 ml of ice-cold 15% perchloric acid and mixed thoroughly. The samples were kept on ice for 30 min and then centrifuged. The supernatant was brought to pH 7.0 by the addition of triethanolamine hydrochloride–KOH solution as described by Maitra and Estabrook [12] and precipitated KClO₃ was removed by centrifugation. Supernatant was used for determination of different acid stable metabolites and adenine nucleotides [12,13]. Enzymic determinations of metabolites were performed at 37°C using 0.2 ml supernatant in 0.8 ml 50 mM TRA-Mg-K buffer, pH 7.5, by coupling appropriate enzymes with spectrophotometric detection of NADH, NADPH or related metabolites. NADH and NADPH levels have been raised to cope with the spectrophotometric method monitored at 340 nm. Pyruvate analysis was performed as earliest to avoid loss by polymerization. 1,3-Diphosphoglycerate was not determined as it was unstable during the acid extraction. Standards were run with known quantities of metabolites and adenine nucleotides.

2.7. Preparation of cell-free extracts and enzyme assays

Washed cell pellet was suspended in 50 mM phosphate buffer, pH 7.5, containing 5 mM dithiothreitol (DTT) and immediately sonicated at 4°C. Cell debris was removed by centrifugation at 38 000 × g for 20 min and the supernatant served as the enzyme solution. Extracts were always used on the same day of preparation. Enzymes were assayed by coupling the particular step to the appropriate NAD⁺- or NADP⁺-linked reaction, with the use of commercially available crystalline enzymes as coupling enzymes. Enzyme assays can be referred to largely in Maitra and Lobo [14] and also to Yu et al. [8]. The rate of production or disappearance of reduced nucleotides was followed continuously on a Shimadzu double beam spectrophotometer UV-2501 PC. Protein was determined by the biuret method [15]. One millimill (mU) of enzyme activity is defined as 1 nmol of product formed per min and specific activities are presented in mU per mg of protein. In most of the cases cell-free extract was diluted for enzyme assays in cold 50 mM phosphate buffer, pH 7.5, containing 2 mM EDTA and 2 mM β-mercaptoethanol. Addition of EDTA was avoided in case of enolase and aldolase. Assays of pyruvate kinase, phosphofructokinase and NADH-glyceraldehyde 3-phosphate dehydrogenase
were carried out with undiluted cell-free extract. G6P isomerase was assayed by the fructose 6-phosphate (F6P)-dependent formation of G6P and the reaction was initiated with 2 mM F6P that was freed from G6P. The produced G6P was measured in presence of 0.4 mM NADP+ and G6P dehydrogenase. 6-Phosphofructokinase was measured by coupling to fructose 1,6-diphosphate (F1,6-diP) aldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase. The enzyme assay was carried out in 50 mM phosphate buffer, pH 6, containing 0.25 mM NADH and 1 mM ATP. The reaction was initiated with 5 mM F6P. Fructose 1,6-diphosphatase was measured in presence of 0.4 mM NADP+, 3 U of yeast G6P isomerase and 2 U of yeast G6P dehydrogenase. The reaction was initiated with 2 mM F1,6-diP [7]. F1,6-diP aldolase was assayed in a catabolic direction in presence of 1 mM NADH, 2 U of rabbit muscle glycerol 3-phosphate dehydrogenase, 10 U of rabbit triose phosphate isomerase. The reaction was initiated with 2 mM F1,6-diP. Triose phosphate isomerase was measured in an assay mixture containing 0.4 mM NADH and 2 U of rabbit muscle glycerol 3-phosphate dehydrogenase. The reaction was initiated with 2 mM glyceraldehyde 3-phosphate [7].

NADH- and NADPH-dependent activity of glyceraldehyde 3-phosphate dehydrogenase was measured by glyceraldehyde 3-phosphate dehydrogenase–phosphoglycerate kinase coupled assay in presence of 20 mM cysteine, 0.4 mM NADH or NADPH, 50 mM ATP, 10 U of bakers yeast phosphoglycerate kinase. The reaction was initiated with 2 mM 3-phosphoglycerate. Phosphoglycerate kinase was assayed in presence of 20 mM cysteine hydrochloride, 2 mM 3-phosphoglycerate, 0.25 mM NADH, 2 U of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. The reaction was initiated with 2 mM ATP [6]. Phosphoglycerate mutase was assayed in a mixture containing 0.25 mM NADH, 2 mM ADP, 1 U of rabbit muscle enolase, 5 U of rabbit muscle lactate dehydrogenase, 2 U of rabbit muscle pyruvate kinase. The reaction was initiated with 2 mM 3-phosphoglycerate. Enolase assay was done in the same way as for phosphoglycerate mutase, 10 U of rabbit muscle phosphoglycerate kinase substituting for the crystalline enolase. Pyruvate kinase was measured in presence of 50 mM phosphate buffer, pH 7.5, 2 mM MnCl2, 0.25 mM NADH, 20 mM DTT, 2 mM ADP, 2 U of rabbit muscle lactate dehydrogenase. The reaction was initiated with 10 mM phosphoenol pyruvate.

3. Results and discussion

3.1. Glycogen metabolism and methanogenesis in M. methylutens

The glycogen content in extract was determined as glucose liberated after complete digestion of glycogen with amylglucosidase and a-amylase. No detectable glucose was released by digestion of resuspended pellet containing cell wall, obtained after cell breakage, with the above enzymes. Similarly glucose was not detected in glycogen-containing fractions unless amylglucosidase and a-amylase treatment was made. This rules out the presence of free glucose in the cells.

When M. methylutens was grown under nitrogen-limiting conditions (50 mg NH4Cl l−1) for 48 h, glycogen accumulated in cells. Cells grown under nitrogen-limiting conditions and suspended in methanol-limited conditions showed a rapid decrease of the glycogen content in the initial 240 min of incubation (Fig. 1). During the incubation period of 3–4 h (at −960 min) in methanol-limited conditions cells of M. methylutens produced methane and CO2, 6.4 and 0.1 nmol mg−1 protein respectively (data not presented in Fig. 1) following which the methane and carbon dioxide formation ceased. This indicates that methane and carbon dioxide could be formed by M. methylutens from intracellular glycogen as also from its other metabolites for a limited period of incubation in methanol-limited conditions. About 6.4 nmol of methane was produced, in the early starvation period of 4 h, from 72 nmol of glucose which indicates that about one carbon of glucose is converted to methane. König et al. [2] also showed a similar relationship of methane to glucose during glycogen degradation with M. tindarius. Murray and Zinder [3] observed that within 21 h most of the glycogen was metabolized. However in case of M. methylutens it was observed that about 50% glycogen was metabolized in 20 h of starvation period. After starvation of methanol for 20 h the

![Fig. 1. Effect of methanol on the glycogen metabolism and methanogenesis of M. methylutens. The cell suspension of 25 ml under N2 atmosphere had a protein content of 6–7 mg ml−1. At time zero methanol was added to a final concentration of 100 mM. Glycogen was measured as glucose liberated after amylglucosidase and α-amylase treatment. The data presented are the average of two separate experiments. Glycogen (□), methane (○) and carbon dioxide (△).](image-url)
cell suspension was supplemented with methanol (final concentration 100 mM). In the initial 10 min, no methane or CO₂ was detected in gas phase, however, a decrease in glycerogen content was observed. After 30 min of incubation carbon dioxide (0.2 μmol mg⁻¹ protein) was detected in the gas phase and it increased continuously. Methane formation was observed from 90 min of incubation period and it was 0.3 μmol mg⁻¹ protein. Then it steadily increased during the next 120 min to 20.28 μmol mg⁻¹ protein with a rate of 0.18 μmol mg⁻¹ protein min⁻¹ and then decreased rapidly (Fig. 1) possibly due to exhaustion of exogenous methanol as 20.28 μmol of methane produced requires about 27 μmol of methanol. About 24 μmol of methane and and 5 μmol of CO₂ were produced after supplementation of methanol which required about 32 μmol of methanol. Thus the methane and CO₂ were obtained from exogenous methanol. The exogenous methanol also appeared to be converted to glycerogen. After 60 min of incubation with methanol the glycerogen content increased gradually yielding about 38 nmol mg⁻¹ protein in the next 150 min of incubation in methanol-sufficient conditions indicating biosynthesis of glycerogen. The observed rate of glycogen synthesis was about 0.25 nmol mg⁻¹ protein min⁻¹. The glycerogen content reached a maximum at 210 min of incubation and then again showed a decreasing trend indicating its assimilation by the cells due to exhaustion of exogenous methanol due to formation of a considerable amount of methane at 210 min incubation period. This showed that there is a definite metabolic process in *M. methylutens* to utilize and biosynthesize glycerogen which functions depending upon the availability of exogenous substrate.

### 3.2. Steady state levels of glycolytic intermediates

The procedure employed has been used to regulate, in vivo, formation of metabolites in methanol-limited (starvation of substrate), methanol-sufficient and again methanol-limited conditions formed due to assimilation of supplemented methanol. Cells from methanol-limited conditions clearly showed a different pattern of metabolites from that of the methanol-sufficient conditions. The results in Figs. 2-4 illustrate results of experiments in which cells were starved for 20 h and then 100 mM methanol was supplemented. When cells of *M. methylutens* were suspended in methanol-limited conditions, hexose phosphates assimilated (Fig. 2). The concentration of F6P decreased to an immeasurably low level indicating possibility of activation of 6-phosphofructokinase in methanol-limited conditions. Soon after addition of methanol, the hexose phosphate pool got filled. There was about 40-fold increase in steady state level of G6P within 80 min after 10 min of supplementation of methanol and the observed rate of synthesis of G6P was 0.26 nmol mg⁻¹ protein min⁻¹. The level of F6P which was below the detectable level also recorded an increase of 6.6 nmol mg⁻¹ protein during the same period, possibly due to activation of fructose 1,6-diphosphatase. Similarly F1,6-diP also showed an increase of 3.5-fold in its concentration after supplementation of methanol in 330 min. This indicates after addition of methanol an upward flow of metabolites took place from methanol to glycerogen possibly via gluconeogenesis. After exhaustion of methane formation, after 270 min of incubation with methanol, the hexose monophosphates recorded a sharp decrease again as 6-phosphofructokinase was activated. However the level of F1,6-diP recorded a continuous increasing trend even after exhaustion of methane formation, as the reversible enzyme system operating in gluconeogenesis between phosphoenolpyruvate and F1,6-diP remained active. In contrast to the hexose phosphate pool, levels of other metabolites showed a small increase, after addition of methanol at 0 min (Fig. 3). Possibly these intermediates might have utilized a high rate for synthesis of amino acids, pentoses etc. Triose phosphate pool, phosphoglycerate pool and pyruvate concentration decreased during a starvation period of 20 h. However after supplementation of methanol at 0 min, levels of triose phosphates, phosphoglycerates, pyruvate as well as phosphoenolpyruvate increased (Fig. 3A–C). This profile of metabolites before and after addition of methanol definitely indicates that in presence of methanol gluconeogenesis is active. Interestingly it was observed that the triose phosphate pool became active before meth-
formation in methanol-sufficient conditions and as the methane formation was initiated the triose phosphate pool decreased. Whereas phosphoglycerate pool and pyruvates showed an increasing trend during methane formation. Jansen et al. [6] showed that the radioactivity of 14C first incorporated into 3-PGA and only then into carbohydrates. It was also further shown that the earliest labeled ketosugar was F6P. These conclusions were supported by 14CO2 pulse-labeling experiments with *M. thermoautotrophicum*. Similar to these observations, in *M. methylutens* also it is observed that synthesis of metabolites of reversible enzymes of gluconeogenesis takes place earlier than methane formation.

### 3.3. Adenine nucleotides

In freshly harvested cells, ATP and ADP contents (more than 10 nmol mg⁻¹ protein) and AMP (6.4 nmol mg⁻¹ protein) content were resulting in a low energy charge (Fig. 4A). During the methanol-limited condition ATP levels decreased, however, ADP and AMP levels remained more or less constant in the early 4 h of starvation period and then decreased. Immediately after addition of methanol and during the active formation of methane, levels of ATP increased, indicating the ATP synthesis is possibly related to methane formation. Apparently it was observed that there was a correlation between methanogenesis, gluconeogenesis and intracellular ATP content; the higher the ATP content, the higher the rate of methanogenesis and of gluconeogenesis. The amount of adenine nucleotides (27.3 nmol mg⁻¹ protein) was very high as compared to that in *Methanohalophilus soehngenii* (2.5 nmol mg⁻¹ protein) [16] and in *Methanosarcina barkeri* (10 nmol mg⁻¹ protein) [17]. Generally the amount found in bacteria varies between 6 and 14 nmol mg⁻¹ protein [18]. The higher amount of adenine nucleotide in *M. methylutens* is noteworthy.

The energy charge or the adenylate charge in freshly harvested cells of *M. methylutens* was 0.57, and in starva-
tion conditions it decreased to 0.48; after addition of methanol, it increased and reached a value of 0.67 and then decreased to its original level as the exogenous methanol was almost consumed (Fig. 4B). The maximum energy charge observed in M. methylutens was 0.67 which is lower as compared to the energy charge, 0.85–0.90, assumed necessary for maintaining metabolic stability [19]. However this low energy charge appeared to be sufficient to control energy generating system in M. methylutens.

### 3.4. Enzymes of glycogen metabolism

With the above metabolite studies and indications of reversible gluconeogenesis in M. methylutens, it is pertinent to assess the levels of enzymes taking part in reversible gluconeogenesis. The most interesting part observed in case of M. methylutens is that the bacterium is strictly anaerobic and yet the enzymes are fairly stable in aerobic conditions. Cell-free extracts of M. methylutens contained relatively high levels of triose phosphate isomerase, G6P isomerase, fructose 1,6-diphosphatase, phosphoglycerate kinase and enolase (Table 1). Such respectable levels are not reported earlier in case of other methanogens [6–8]. Phosphoglycerate mutase and NADPH-glyceraldehyde 3-phosphate dehydrogenase were also present in considerable amounts. The presence of NADPH-glyceraldehyde 3-phosphate dehydrogenase in significant levels is interesting as it suggests the operation of gluconeogenesis. This enzyme is more related to chloroplast enzyme from green algae and higher plants sharing the same coenzyme specificity. Similar observations were also reported in case of this enzyme from Methanothermus fervidus [20].

The higher affinity of this enzyme to NADPH than to NADH under physiological conditions for building up carbohydrates as does the chloroplast enzyme from green algae and higher plants in the dark reactions during photosynthesis [21]. On the contrary low levels of NADH-glyceraldehyde 3-phosphate dehydrogenase indicate the presence of normal glycolysis like in eubacteria and eukaryotes. Pyruvate kinase, 6-phosphofructokinase and aldolase were present in low levels, similar to those reported earlier in M. thermoautotrophicum [7] and M. maripaludis [8]. Fructose 1,6-diphosphatase is a key enzyme in gluconeogenesis and its activity is dependent on Mg$^{2+}$ and thiol reagents. Further, 6-phosphofructokinase and pyruvate kinase are also important regulatory enzymes of glycolysis. 6-Phosphofructokinase had optimum activity at pH 6.0 and not at pH 7–8.5 [22]. The activity of pyruvate kinase is dependent on Mn$^{2+}$ and not on Mg$^{2+}$. The presence of 6-phosphofructokinase and pyruvate kinase gives an indication of the glycolytic pathway whereas presence of fructose 1,6-diphosphatase and NADPH-glyceraldehyde 3-phosphate dehydrogenase supports the gluconeogenic pathway in M. methylutens.

In brief our data show that there is a remarkable robustness and rigidity in central carbon metabolism of M. methylutens. More significant physiological changes were seen in glycolen and hexose phosphates in response to altered environmental conditions. For example, in methanol-limited conditions, the glycolytic pathway was active resulting in catabolism of glycogen. In methanol-sufficient conditions, the gluconeogenic pathway was active resulting in synthesis of hexose phosphates and glycogen. It also appeared that the gluconeogenic pathway becomes active earlier as compared to methanogenesis. However much more information would be necessary to understand metabolic regulation in methanogens.

### References


