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

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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 μmol mg^{-1} 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 *Methanothrix 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}), Na_2SO_4, 1.0; KCl, 0.7; $MgCl_2·6H_2O$, 10; $CaCl_2·2H_2O$, 1.0; $NaHCO_3$, 0.2; K_2HPO_4, 0.1; NH_4Cl, 0.050; $Fe(NH_4)_2SO_4$, 0.002; NaCl, 30; yeast extract, 2.0; tryptone, 2.0; cysteine·HCl·H_2O, 0.150; vitamin and mineral

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solution 10 ml each at pH 7.0 under an atmosphere of N_2 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 N_2. 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 N_2 atmosphere. The cell suspension was incubated at 37°C under N_2 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 N_2, 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 $MgCl_2$ (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 $KClO_4$ 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 $38\,000\times g$ 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 milliunit (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 muscle 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. Phoshoglycerate 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 mutase substituting for the crystalline enolase. Pyruvate kinase was measured in presence of 50 mM phosphate buffer, pH 7.5, 2 mM $MnCl_2$, 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 amyloglucosidase and α-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 amyloglucosidase and α-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 $NH_4Cl\ l^{-1}$) 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 CO_2, 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. Konig 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

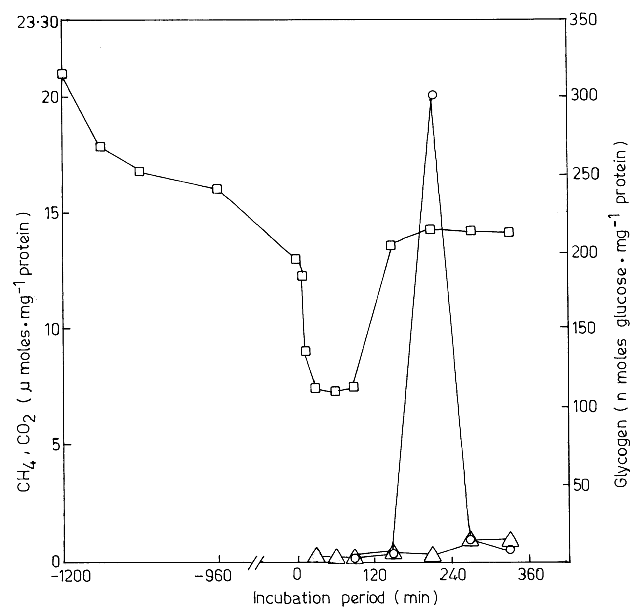

Fig. 1. Effect of methanol on the glycogen metabolism and methanogenesis of *M. methylutens*. The cell suspension of 25 ml under N_2 atmosphere had a protein content of 6–7 mg ml^{-1}. At time zero methanol was added to a final concentration of 100 mM. Glycogen was measured as glucose liberated after amyloglucosidase and α-amylase treatment. The data presented are the average of two separate experiments. Glycogen (□), methane (○) and carbon dioxide (△).

cell suspension was supplemented with methanol (final concentration 100 mM). In the initial 10 min, no methane or CO_2 was detected in gas phase, however, a decrease in glycogen 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 CO_2 were produced after supplementation of methanol which required about 32 μmol of methanol. Thus the methane and CO_2 were obtained from exogenous methanol. The exogenous methanol also appeared to be converted to glycogen. After 60 min of incubation with methanol the glycogen content increased gradually yielding about 38 nmol mg^{-1} protein in the next 150 min of incubation in methanol-sufficient conditions indicating biosynthesis of glycogen. The observed rate of glycogen synthesis was about 0.25 nmol mg^{-1} protein min^{-1}. The glycogen 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 glycogen 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

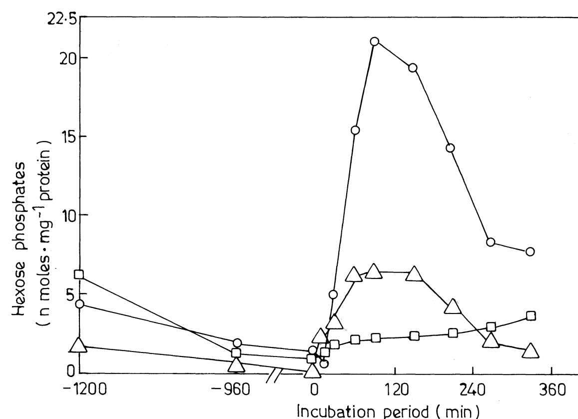

Fig. 2. Effect of methanol on steady state concentrations of hexose phosphates of *M. methylutens*. Metabolites were measured by enzymic determinations. One ml assay mixture contained 0.8 ml 50 mM TRA-Mg-K buffer, pH 7.5, having 2 mM DTT, 0.4 mM $NADP^+$ and 0.2 ml metabolite extract. The reaction was initiated by the addition of 5 U of yeast G6P dehydrogenase to measure G6P and after completion of the reaction 3 U of yeast glucose 6-phosphate isomerase was added to measure F6P. F1,6-diP was measured in 1 ml assay mixture containing 0.8 ml 50 mM TRA-Mg-K buffer, pH 7.5, having 1 mM NADH and 0.2 ml metabolite extract. The reaction was initiated with 2 U of rabbit muscle α-glycerol 3-phosphate dehydrogenase to measure dihydroxyacetone phosphate. After completion of the reaction to measure glyceraldehyde 3-phosphate, 10 U of rabbit muscle triose phosphate isomerase was added and then to measure F1,6-diP, 1 U of rabbit muscle aldolase was added. In this assay two equivalents of NADH were oxidized per mol of F1,6-diP. The data presented are the average of two separate experiments. G6P (○), F6P (△), and F1,6-diP (□).

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 glycogen 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-

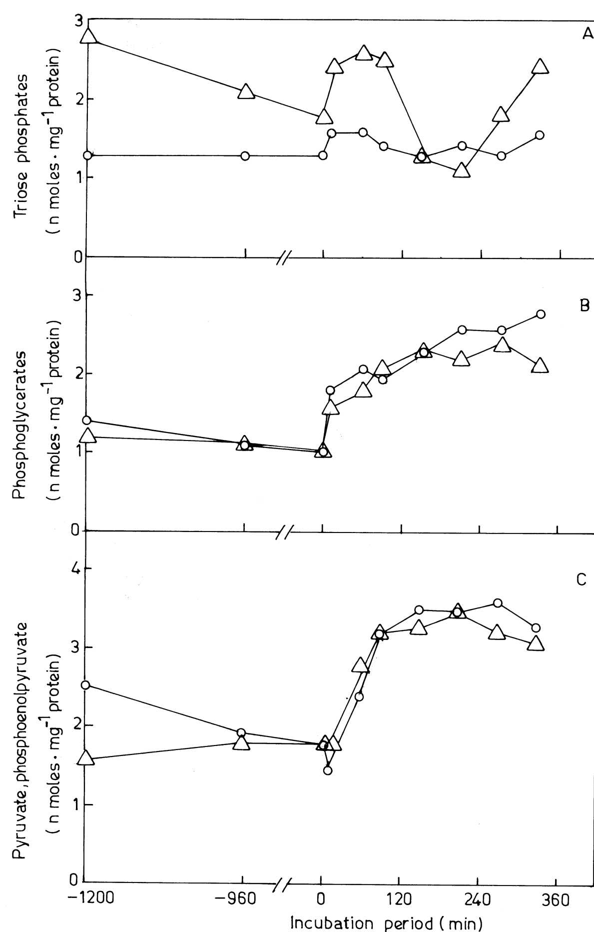

Fig. 3. Effect of methanol on steady state concentrations of triose phosphates (A), phosphoglycerates (B) and of pyruvates (C) of *M. methylutens*. Triose phosphates were determined as described in Fig. 2. Pyruvates and phosphoglycerates were measured in a 1-ml assay mixture containing 0.8 ml of 50 mM TRA-Mg-K buffer, pH 7.5, having 1 mM NADH, 2 mM ADP and 0.2 ml metabolite extract. Pyruvate was measured by addition of 2 U of rabbit muscle lactate dehydrogenase. After completion of reaction, 5 U of rabbit muscle pyruvate kinase was added to measure phosphoenolpyruvate. Then to measure 2-phosphoglycerate, 1 U of rabbit muscle enolase was added and after completion of the reaction, 10 U of rabbit muscle phosphoglycerate mutase was added to measure 3-phosphoglycerate. The data presented are the average of two separate experiments. A: Dihydroxyacetone phosphate (○), glyceraldehyde 3-phosphate (△), B: 3-phosphoglycerate (△), 2-phosphoglycerate (○) and C: phosphoenolpyruvate (△), pyruvate (○).

ane 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 ^{14}C 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 $^{14}CO_2$ 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 *Methanothrix 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-

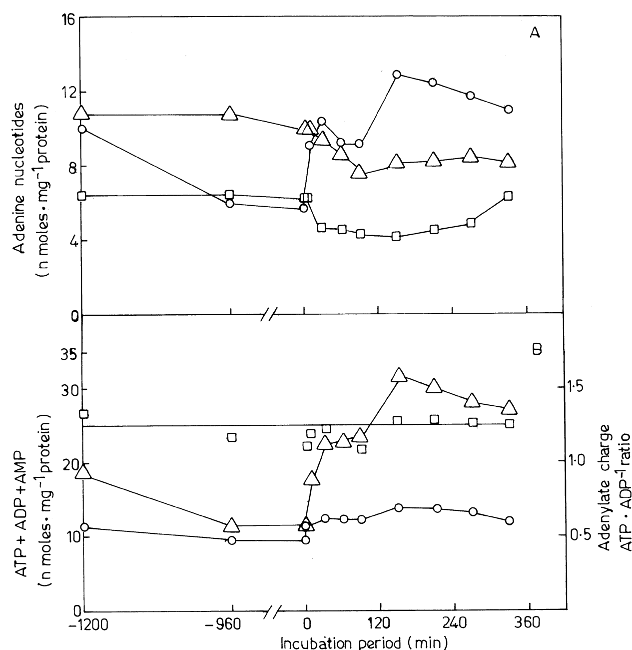

Fig. 4. Effect of methanol on adenine nucleotide content and adenylate charge of *M. methylutens*. ATP was measured by the enzyme-catalyzed reactions of G6P dehydrogenase and hexokinase in presence of 1 mM glucose and 0.4 mM NADP^{+}. ADP was measured by the enzyme-catalyzed reactions of pyruvate kinase and lactate dehydrogenase in presence of 1 mM NADH and 10 mM phosphoenolpyruvate. AMP was measured by the enzyme-catalyzed reaction of myokinase in presence of 1 mM NADH and 1 mM ATP. The data presented are the average of two separate experiments. A: ATP (○), ADP (△) and AMP (□). B: Total adenylate (□) is a sum of ATP, ADP and AMP. Adenylate charge (○) was calculated as (ATP+0.5 ADP). (ATP+ADP+AMP)$^{-1}$, ATP ADP^{-1} ratios (△) were calculated from the data of A.

Table 1
Enzymes of glycogen metabolism in *M. methylutens*

Enzymes	Specific activity (mU mg^{-1} protein)
G6P isomerase[a] (EC 5.3.1.9)	85
6-Phosphofructokinase (EC 2.7.1.11)	5
Fructose 1,6-diphosphatase[a] (EC 3.1.3.11)	97
F1,6-diP aldolase (EC 4.1.2.13)	8
Triose phosphate isomerase (EC 5.3.1.1)	3872
Glyceraldehyde 3-phosphate dehydrogenase-$NADP^+$ (EC 1.2.1.13)	57
Glyceraldehyde 3-phosphate dehydrogenase-NAD^+ (EC 1.2.1.13)	15
Phosphoglycerate kinase[a] (EC 2.7.2.3)	102
Phosphoglycerate mutase (EC 2.7.5.3)	92
Enolase (EC 4.2.1.11)	215
Pyruvate kinase (EC 2.7.1.40)	5

Cells of *M. methylutens* were grown on methanol for 48 h and cell-free extracts were prepared. Enzyme assay mixture, 1 ml, contained 50 mM TRA-Mg-K buffer, pH 7.5, 2 mM DTT, 2 mM EDTA, 0.4 mM NADH or NADPH and substrates. The enzyme assays were carried out with 10 μl of cell-free extracts by coupling with crystalline enzymes.
[a]Enzyme activity was measured in the gluconeogenic direction.

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 low 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 were 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 glycogen 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.

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