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Na^+-driven ATP synthesis in *Methanobacterium thermoautotrophicum* can be modulated with sodium ion concentrations in the growth medium

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Methane formation by nongrowing cells of *Methanobacterium thermoautotrophicum* ΔH can be enhanced with sodium ions only when the cells were originally grown in the presence of a low (5 mM) concentration of NaCl. ATP synthesis in the cells driven by an artificially imposed Na^+ gradient can be modulated by changing the sodium ion concentration in the growth medium while H^+ gradient-driven ATP synthesis does not depend on it. Stimulation of the entire ATPase activity with Na^+ in permeabilized cells depends also on the Na^+ concentration in the growth medium. The results obtained might indicate the presence of an inducible Na^+-translocating ATPase in *Methanobacterium thermoautotrophicum*.

Methanogen; ATP synthesis; Na^+-translocating ATPase; (*Methanobacterium thermoautotrophicum*)

1. INTRODUCTION

Recently, many important facts on the bioenergetic function of sodium ions in procaryotes have been obtained [1,2]. Sodium ions also play an important role in the very specialized energy metabolism of methanogenic bacteria [3–5] but despite the numerous attempts made toward understanding their energetic function, a concrete role for these ions has not yet been explained satisfactorily. The stimulatory effect of sodium ions on growth, methanogenesis and some membrane bound processes in methanogenic bacteria has been observed [5–10]. It was therefore assumed that Na^+ may play a role in the energetic coupling between endergonic and exergonic reactions [4]. Recently, the ATP synthesis driven by a chemical concentration gradient of Na^+ directed inwards in the presence of a permeant counterion in *Methanococcus voltae* [11] and *Methanobacterium thermoautotrophicum* has been demonstrated [12]. On the basis of these observations the existence of an electrogenic sodium ion-translocating ATPase in these organisms has been postulated [11,12]. The exact role of this electrogenic Na^+-translocating ATPase is not known but it has been suggested that under physiological conditions it might drive the exit of sodium ions from the cells of methanogenic bacteria.

The results presented here provide evidence for the existence of an inducible Na^+-translocating ATPase (synthase?) in *M. thermoautotrophicum*.

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2. MATERIALS AND METHODS

Methanobacterium thermoautotrophicum strain ΔH was cultivated as described earlier [13]. The growth medium [14] containing Na_2CO_3 instead of $NaHCO_3$ according to Schönheit et al. [15], supplemented either with 5 mM NaCl (low Na^+ medium) or 50 mM NaCl (high Na^+ medium), was used. Cells in the late logarithmic phase of growth were harvested by centrifugation, twice washed and resuspended in the appropriate anaerobic buffer (0.1 M Pipes-Tris, 5 mM $MgCl_2$; pH 6.9) as described by Al-Mahrouq et al. [12]. In some experiments a phosphate buffer (0.1 M phosphoric acid-Tris, 5 mM $MgCl_2$; pH 6.9) was used. All manipulations were performed under strictly anaerobic conditions.

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For sodium gradient-driven ATP synthesis, cells suspended in 10 ml anaerobic Pipes-Tris buffer were placed in 120 ml bottles sealed with butyl rubber stoppers, pressurized with H_2 and allowed to preincubate at 60°C in a gyratory water bath for 10 min. When indicated, monensin (10 μM) was added as an ethanolic solution and tetraphenylborate (1 mM), 2,4-dinitrophenol (0.1 mM), vanadate (1 mM), azide (10 mM) and amiloride (0.75 mM) as water solutions. The reaction was started by injecting 1 ml of 4 M NaCl into the suspension. Samples of 100 μl volume were taken at regular intervals and ATP determined according to Sprott et al. [16].

For proton gradient-driven ATP synthesis the phosphate buffer (0.1 M phosphoric acid-Tris, 5 mM $MgCl_2$; pH 6.9) was used as a reaction medium and the reaction was started by injecting 6 M HCl as described by Doddema et al. [17].

For the study of ATPase activity permeabilized cells of *M. thermoautotrophicum* were used. Cells were permeabilized with Triton X-100 in a similar way as in [18]. ATPase activity was assayed by the measurement of inorganic phosphate released from ATP [19]. The reaction mixture contained in 1 ml: 3 mM ATP, 5 mM $MgCl_2$, 0.1 M glycylglycine, pH 8.2, 50 μg Triton X-100 and 500 μg protein. After a 2 min preincubation, the reaction was started by the addition of ATP, incubated for 10 min at 65°C. A blank sample without Mg^{2+} was subtracted from every sample.

Methane formation from CO_2 and H_2 by the cell suspension was measured as described in [13] by gas chromatography on Carlo Erba Fractovap 4200 using the 2 m long steel column packed with Sepharon AE 200–300 μm. For the detection a heat-conductive detector, model 450, was used. Samples of the gaseous phase were taken from cultivation flasks by gas-tight syringes (Pierce Series A-2).

Protein was determined according to [20] using bovine serum albumin as a standard.

All chemicals used were reagent grade mostly purchased from Lachema Brno, except for monensin, dicyclohexylcarbodiimide (Serva), luciferin-luciferase preparation (Calbiochem), ATP, tetraphenylbarate (Sigma), amiloride (Léčivá Praha) and SF 6847 which was kindly donated by Professor G. Hauska from Regensburg.

3. RESULTS AND DISCUSSION

It has been known for some time now that cells of *M. thermoautotrophicum* concentrate sodium ions at various phases of growth and that the concentration in these cells can be achieved by changes of Na^+ concentration in the growth medium [21]. Correspondingly, a stimulatory effect of sodium ions on methane formation has been observed [6]. Here, we show that the rate of methane formation from $H_2 + CO^+$ by *M. thermoautotrophicum* (ΔH) under nongrowing conditions was found to be dependent on sodium ion concentrations in the growth medium on which the cells had originally been grown. When the NaCl concentration in the growth medium was low (5 mM), the formation of methane by cells suspended in a buffer containing 3 mM K_2HPO_4, 2 mM KH_2PO_4, 2 mM $MgSO_4$, pH 7.1, was very low. However, the addition of NaCl (50 mM final) to such a nongrowing cell suspension substantially increased the rate of methane formation. Cells grown in the presence of 50 mM NaCl exhibited under the same conditions a several times higher production of methane which was stimulated no further by addition of NaCl into the cell suspension. The results of a typical experiment are shown in fig.1. The finding indicates that a saturating intracellular sodium ion concentration may exist which can secure all cellular requirements for Na^+ in these cells.

The ability of cells to synthesize ATP after an imposition of Na^+ concentration gradient depended on the growth conditions too. Fig.2 shows that in whole cells of *M. thermoautotrophicum* ATP synthesis can be driven by an artificially imposed chemical gradient of sodium ions. Cells grown in the presence of a low NaCl concentration (5 mM) synthesized ATP to a small extent after imposition of a Na^+ gradient, requiring the presence of a counterion (tetraphenylborate) for the manifestation of this activity. The requirement of a counterion for Na^+-induced ATP synthesis in *M. thermoautotrophicum* has already been observed [12]. However, when cells were grown in the presence of 50 mM NaCl, they were able to synthesize higher amounts of ATP under the imposition of Na^+ concentration gradient even in the absence of the counterion and the ATP level synthesized was comparable in both cases, with or without the counterion. In this case Na^+-driven ATP synthesis has been found to be twice as high when compared with cells grown in the presence of a low NaCl concentration. Na^+ gradient-driven ATP synthesis in these experiments was completely inhibited by monensin but no inhibition was found with uncouplers (dinitrophenol, SF 6847), vanadate, azide and amiloride (not shown). This observation argues for the possibility that during ATP synthesis Na^+ movement other than via Na^+/H^+ antiporter is involved, that a proton gradient is not involved in the process and that an E_1E_2 membrane ATPase does not participate on the Na^+-driven ATP synthesis.

A chemical gradient of K^+ could not drive ATP synthesis in these cells when they were grown at a

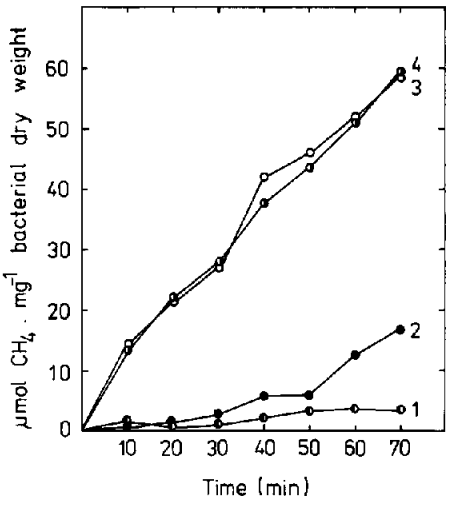

Fig.1. Effect of Na^+ on methane production in *M. thermoautotrophicum* cells grown in the presence of 5 mM (1,2) and 50 mM (3,4) NaCl. Cells were incubated in the medium according to Perski et al. [6] at 60°C in a gyratory water bath and 50 mM NaCl was added at $t = 0$ to suspensions 2 and 4.

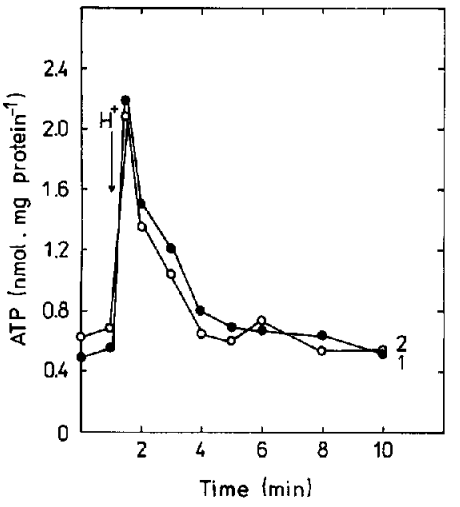

Fig.3. ΔpH-driven ATP synthesis in *M. thermoautotrophicum* cells grown in the presence of 5 mM (1) and 50 mM (2) NaCl. At $t = 1$ min, the pH value of the suspension was shifted from 6.9 to 2.9 by adding 6 M HCl.

low nor when they were grown at a high Na^+ concentration (not shown).

To compare the coupling between proton entry and ATP synthesis in *M. thermoautotrophicum* cells grown in the presence of the low and the high Na^+ concentrations we studied ATP synthesis driven by base to acid transition. Fig.3 shows ATP synthesis in these cells after the imposition of an artificial protonmotive force. It is interesting to notice that ΔpH-driven ATP synthesis is the same in both types of cells used and does not depend on the sodium ion concentration in the growth medium. It could be considered as a remarkable difference between the ΔpH-driven and ΔpNa-driven ATP synthesis in this organism.

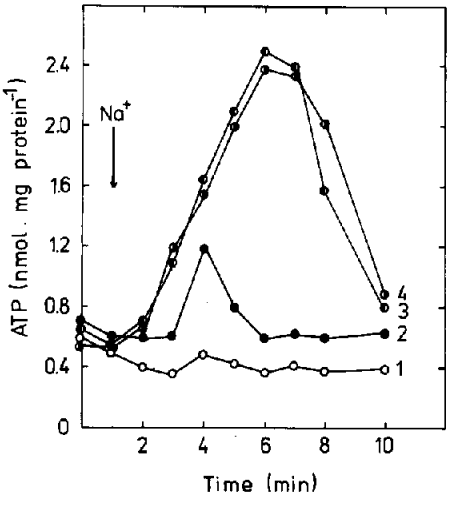

Fig.2. ΔpNa-driven ATP synthesis in *M. thermoautotrophicum* cells grown in the presence of 5 mM (1,2) and 50 mM (3,4) NaCl. 1 mM tetraphenylborate was added at the beginning of preincubation period to suspensions 2 and 4.

Excluding the opinion of Lancaster [22] it is mostly assumed that methanogenic bacteria synthesize ATP by an electron-transport phosphorylation and that ATP synthesis in these organisms is catalyzed by an ATP synthase complex (for review see [23]). Finding that Na^+ stimulates ATPase in permeabilized cells of *M. thermoautotrophicum* might also indicate a role for Na^+ in ATP synthesis and hydrolysis in the cells studied. Table 1 summarizes some features of Mg^{2+}-stimulated ATPase of permeabilized cells grown in the presence of low and high Na^+ concentrations. There is no substantial difference in the Mg^{2+}-stimulated ATPase activity of the cells grown in the presence of different Na^+ concentrations. However, ATPase activity of cells grown in the presence of 50 mM NaCl could be stimulated by Na^+ up to 30% more than that of cells grown in the presence of 5 mM NaCl. In both

Table 1

Effect of Na^+ and dicyclohexylcarbodiimide (DCCD) on ATPase activity (μmol P_i × mg protein^{-1} × min^{-1}) in cells of *M. thermoautotrophicum* grown in the presence of 5 mM and 50 mM NaCl

Additions	Cells grown in 5 mM NaCl			Cells grown in 50 mM NaCl		
	Activity	Stimulation %	Inhibition %	Activity	Stimulation %	Inhibition %
None	0.32	–	–	0.31	–	–
250 μM DCCD	0.21	–	34	0.19	–	39
50 mM NaCl	0.51	59	–	0.58	87	–
50 mM NaCl + 250 μM DCCD	0.22	–	57	0.23	–	60

cases a significant part of the ATPase activity was inhibited by dicyclohexylcarbodiimide. Unfortunately, neither DCCD nor the other inhibitors used could discriminate between H^+-ATPase and Na^+-ATPase in these cells. Despite the above-mentioned problem the entire ATPase activity of cells grown in the presence of a high sodium ion concentration is significantly more stimulated by Na^+ which correlates with the higher ability of these cells to synthesize ATP upon the imposition of a sodium ion gradient.

Based on the results obtained, it is tempting to speculate that under the described conditions either the proton translocating ATPase changes its specificity, acquiring the ability to translocate Na^+ similar to an ATPase of *Propionigenium modestum* [24], or as an alternative a novel ATPase specifically translocating Na^+ is induced complementing the work of the constitutive proton-translocating ATPase. This assumption might partly explain why methanogenesis from H_2 and CO_2 at a low Na^+ concentration requires a higher protonmotive force than at a high Na^+ concentration [25].

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