The presence of H^+ and Na^+-translocating ATPases in *Methanobacterium thermoautotrophicum* and their possible function under alkaline conditions

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Received 20 June 1995; revised version received 17 July 1995

Abstract Two ATPases with different apparent molecular masses of approx. 500 kDa and 400 kDa were identified in the EDTA extract of the cell membranes of *Methanobacterium thermoautotrophicum*. Western blotting with polyclonal antiserum reactive with β-subunit of mitochondrial ATPase from rat liver and yeast was used for further analysis of these ATPases. A strong crossreactivity with a single protein band with an apparent molecular weight of about 53 kDa (similar to β-subunit of F-type ATPase from other sources) was found in protein extracts of whole cells of *Methanobacterium thermoautotrophicum* strains ΔH and Marburg, as well as of *Methanospirillum hungatei*. This indicates the presence of F-type ATPase in methanogens. ATP synthesis driven by membrane potential which was generated by artificially-imposed ΔpH in the presence of protonophorous uncoupler and sodium ions was stimulated by bafilomycin A_1, an inhibitor of V- and A-type ATPases, as well as by harmaline, an inhibitor of Na^+/H^+ antiporter. These results indicate that cells of *Methanobacterium thermoautotrophicum* strain ΔH contain the F-type ATP synthase which is Na^+-translocating in addition to V- or A-type ATP synthase which is H^+-translocating.

Key words: Methanogens; ATP synthesis; Na^+/H^+ antiporter; Sodium ion-motive force

1. Introduction

In 1984 Skulachev [1] suggested that substitution of H^+ by Na^+ as the coupling ion might be of crucial importance in bacterial energetics, especially under alkaline conditions. This new bioenergetic paradigm has been supported by experimental observations over the last decade (for reviews, see [2–4]). The Na^+-energetic cycle composed of primary sodium ion electrochemical gradient generator(s) and consumer(s) was found in various bacterial cells in addition to the classical energetic H^+-cycle based on circulation of protons [3,5]. Na^+/H^+ antiporter serves here as an universal device for linking the H^+-cycle with Na^+-cycle [6].

Methanogens as a member of the Archae, possessing a specialized kind of metabolism, seem to belong to a group of microorganisms containing H^+- and Na^+-cycles and the Na^+/H^+ antiporter. This assumption is supported by following findings: (i) in *Methanosarcina mazei Göl*, the existence of a primary sodium pump (methyl-H_4MPT:HS-CoM methyltransferase) and an F_1F_0-ATP synthase translocating Na^+ ions have been confirmed in addition to an A_1A_0-ATP synthase using H^+ as the coupling ion [7]; (ii) in *Methanococcus voltae*, the energy transduction based on a sodium ion current has been proven [8]. (iii) In *Methanobacterium thermoautotrophicum* strain Marburg, the scheme on the involvement of Na^+- and H^+-cycles in the coupling of exergonic and endergonic reactions during methane formation has been suggested [9]. The importance of sodium ions in the bioenergetics of *Methanobacterium thermoautotrophicum* strain ΔH has also been recognized [10,11,20]; (iv) the existence of the Na^+/H^+ antiporter has been confirmed in different methanogens [8,12,13,21]. All these data strongly support the idea that H^+- and Na^+-cycles, linked by the Na^+/H^+ antiporter, coexist also in methanogens and that their mutual cooperation could be responsible for the adaptive energetic behaviour under different physiological conditions.

We report here that cells of *Methanobacterium thermoautotrophicum* strain ΔH posses two different ATPases (synthases) one of them being the F-type and the other probably the A-type ATPase. Moreover, the results presented here show that the H^+-translocating ATPase is probably the A-type and the Na^+-translocating ATPase might be of the F-type. The results also show that Na^+-translocating ATPase can work in the synthase mode under alkaline conditions.

2. Materials and methods

Methanobacterium thermoautotrophicum strain ΔH was cultivated as described earlier [14]. Growth medium containing Na_2CO_3 instead of $NaHCO_3$, supplemented with 50 mM NaCl was used. Cells in the late logarithmic phase of growth were harvested by centrifugation, washed and resuspended in the appropriate anaerobic buffer. All manipulations were performed under strict anaerobic conditions.

Experimental conditions for proton diffusion potential-driven ATP synthesis are given in the figure legends.

Measurement of Na^+/H^+ antiporter activity in whole cells was performed as follows. Anaerobically harvested cells were washed and suspended in oxygen-free HEPES buffer (20 mM HEPES-Tris, 10 mM KCl, pH 7.0) under argon atmosphere (deenergized conditions) and preincubated for 30 min at 60°C. Cell suspension (approx. 100 μg protein) was added to an anaerobic stirred cuvette containing 50 mM HEPES buffer (50 mM HEPES-Tris, 10 mM KCl, 5 mM $MgCl_2$, 2.5 μM acridine orange, pH 8.0). The change of the fluorescence intensity of Acridine orange was monitored at 60°C using a Perkin-Elmer LS 50-B spectrofluorimeter at excitation and emission wavelengths set to 493 and 530 nm, respectively.

Cell membranes and EDTA extract from these membranes were prepared as described by Roth et al. [15].

Native PAGE and ATPase activity staining were performed according to Kakinuma and Igarashi [16] with the exception that native PAGE slab gels were stained for ATPase activity at 60°C in 50 mM glycine-Tris buffer, pH 8.8 containing 5 mM ATP, 5 mM $MgCl_2$ and 0.05% (w/v) Pb-acetate.

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Abbreviations: TCS, tetrachlorosalicylanilide; $\Delta\tilde{\mu}_{Na^+}$, electrochemical potential difference of Na^+; ΔpH and ΔpNa, difference in concentrations of H^+ and Na^+, respectively

SSDI 0014-5793(95)00866-7

Standard techniques were used for SDS-PAGE [17] and Western blotting [18] of whole cell proteins from methanogens. TCA precipitates of cell homogenates were solubilized in SDS and separated in 12% gels. Separated proteins were electroblotted onto nitrocellulose membranes and decorated with anti-F_1 antiserum followed by ^{125}I-labelled protein A. Cross-reactive proteins labelled with [^{125}I]protein A were identified by autoradiography. Immunotitration of antiserum was performed with F_1 complex purified from yeast mitochondria.

Protein was quantified by the Lowry method [19] with bovine serum albumin as the standard.

All chemicals were reagent grade purity purchased mostly from Lachema Brno (CR), except for ATP (Serva), harmaline (Aldrich Chem. Co.), TCS (Kodak), bafilomycin A_1 (Sigma) and acridine orange (Int. Enzymes Ltd.).

3. Results

In a previous paper [20] we have suggested that cells of *Methanobacterium thermoautotrophicum* might contain both F- and V-types of ATPases. To test this hypothesis, we analyzed the EDTA extract prepared from the cell membranes of *Methanobacterium thermoautotrophicum* strain ΔH by native PAGE. We identified two bands with ATPase activity having apparent molecular mass approximately 500 kDa and 400 kDa (Fig. 1A). This results indicated that V- and F-types of ATPases might exist in these cells, since the observed molecular masses corresponded to molecular masses reported for these two ATPase types [30,31]. For further characterization of these ATPases, polyclonal anti-F_1 antiserum specific for β-subunit both in rat liver and yeast mitochondria was used. This antiserum gave a strong crossreactivity with a single protein band on Western blots of crude extracts of *Methanobacterium thermoautotrophicum* strains ΔH and Marburg, and *Methanospirillum hungatei*. As shown in Fig. 1B, the mobility of these bands (approx. 53 kDa) corresponded well with molecular masses reported for β-subunits from various sources (50–57 kDa). The identity of crossreacting bands as β-subunits of F-type ATPase was confirmed by the loss of crossreactivity in cell extracts of methanogens after competitive titration of the antiserum with F_1 complex isolated from yeast mitochondria (not shown). From these results it might be concluded that the methanogens tested contain the F-type ATPase.

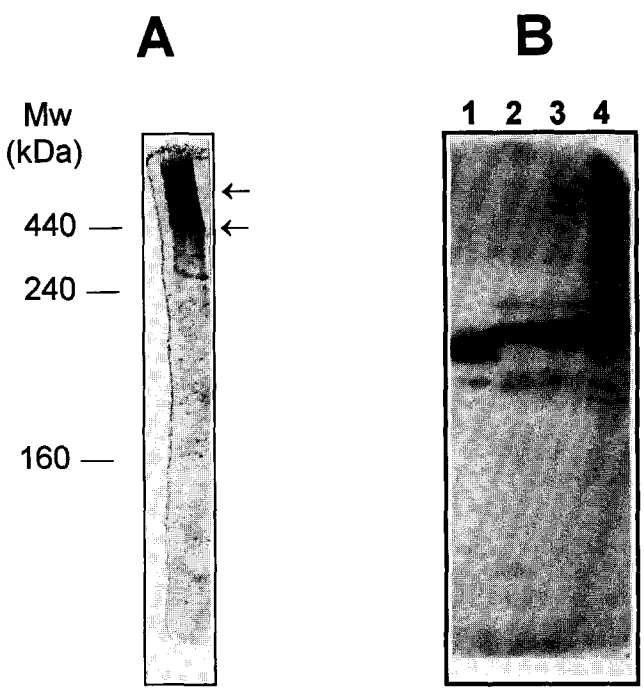

Fig. 1. Detection of ATPase in methanogens. (A) Native PAGE of EDTA extract of *Mb. thermoautotrophicum* strain ΔH. 10 μg of proteins were applied to the gel. The arrows indicate two bands with ATPase activity after ATPase activity staining. (B) Immunodetection of F_1F_0-ATPase β-subunit. Protein extracts (50 μg in lane 1, 20 μg in lanes 2–4) were separated on a 12% polyacrylamide gel and processed as described in section 2. Lane 1, isolated yeast mitochondria; lane 2, *Mb. thermoautotrophicum* strain Marburg; lane 3, *Mb. thermoautotrophicum* strain ΔH; lane 4, *Msp. hungatei*.

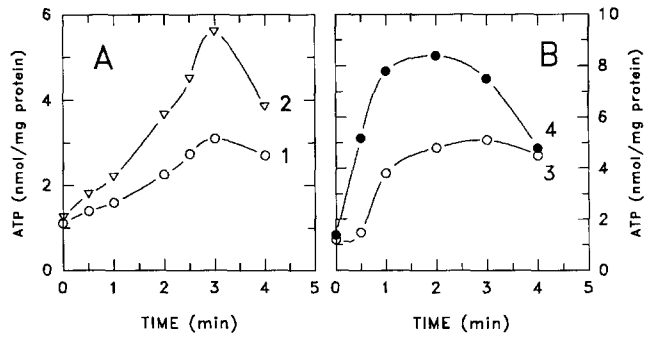

Fig. 2. Effect of bafilomycin A_1 and harmaline on proton diffusion potential-driven ATP synthesis in resting cells of *Methanobacterium thermoautotrophicum*. Cells were suspended in 50 mM MOPS-Tris buffer, pH 6.8 to a concentration of 0.93 mg protein per ml and preincubated for 5 min at 45°C (A) and 60°C (B) in the presence of 30 μM TCS. ATP synthesis was induced by the addition of 3 M KOH to final pH 9.2. The NaCl concentration of reaction mixture was 40 mM. (1) control, (2) 0.8 μM bafilomycin A_1 added 4.5 min prior to zero time (A). (3) control, (4) 300 μM harmaline added 5 min prior to zero time (B). Gas phase, Ar.

Recently, we have suggested [20] that two ATP synthases may exist in *Methanobacterium thermoautotrophicum* strain ΔH, one of these a proton-translocating and the other one a sodium ion-translocating enzyme, both having different functions under different physiological conditions. In addition, the existence of an Na^+/H^+ antiporter in these cells has been shown. The understanding of the interrelationship of Na^+-translocating ATPase (synthase), H^+-translocating ATPase (synthase) and Na^+/H^+ antiporter which is possibly playing a central role in the coupling between the circulation of sodium ions and protons could be attained by functional elimination of some of these systems with relevant inhibitors. Therefore, we studied the effect of bafilomycin A_1 (inhibitor of V-type [22] and A-type [7,23] ATPases) and harmaline (inhibitor of Na^+/H^+ antiporter [12]) on $\Delta\tilde{\mu}_{Na^+}$-driven ATP synthesis.

Fig. 2A and 2B shows that both bafilomycin A_1 and harmaline conspicuously stimulated ATP synthesis driven by proton diffusion potential induced by the acid-alkaline shift in the presence of the uncoupler (TCS) and sodium ions. The observed stimulatory effect of bafilomycin A_1 can be explained by the possibility that ATP synthetized at the expense of sodium-motive force cannot be hydrolyzed by H^+-dependent ATPase because of its inhibition by bafilomycin A_1. Moreover, this finding indicates that H^+-ATPase belongs to the A-type ATPases since bafilomycin A_1 specifically inhibits this kind of ATPase. The stimulatory effect of harmaline indicates that Na^+/H^+ antiporter competes with Na^+-translocating ATPase

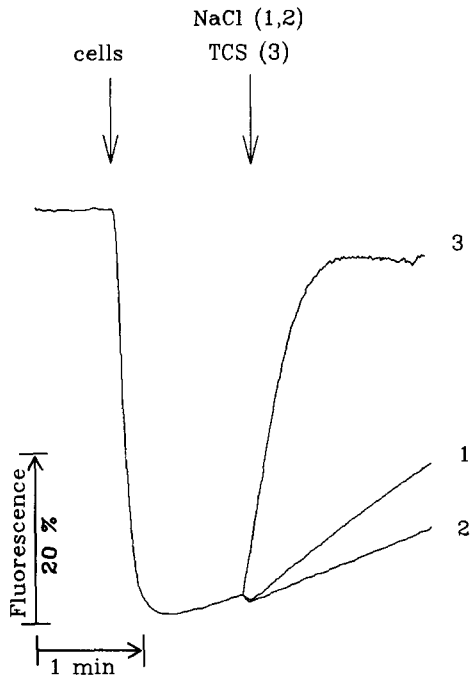

Fig. 3. Effect of harmaline and TCS on the Na^+/H^+ antiporter activity in resting cells of *Methanobacterium thermoautotrophicum*. Cells were added to an anaerobic stirred cuvette containing 50 mM HEPES buffer (50 mM HEPES-Tris, 10 mM KCl, 5 mM $MgCl_2$, pH 8.0 and 2.5 μM Acridine orange). Fluorescence intensity of Acridine orange was monitored as described in section 2. At the arrow, NaCl (50 mM final) or TCS (10 μM final) was added. (1) control, (2) 300 μM harmaline, (3) 10 μM TCS. Gas phase, Ar. Fluorescence before cells addition was assumed to be 100%.

for sodium ions. To verify that harmaline inhibits the Na^+/H^+ antiporter in these cells under alkaline conditions, we measured the effect of this inhibitor on the activity of Na^+/H^+ antiporter. As demonstrated on Fig. 3, the Na^+/H^+ antiporter activity was inhibited by 60% in the presence of 300 μM harmaline. Moreover, it is also evident that harmaline under the conditions employed, did not exhibit any uncoupling activity when compared to the effect of uncoupler TCS. Fig. 4 shows that ATP synthesis driven by methanogenic electron transport at pH 8.0 (when $\Delta\psi$ and ΔpH were oppositely directed) was very low in the presence of 5 mM NaCl (low concentration) and it was only slightly stimulated by harmaline. At the same pH but in the presence of 50 mM NaCl (high concentration), harmaline stimulated ATP synthesis by 60%. Experiments with the uncoupler TCS at pH 8.0 showed that TCS inhibited ATP synthesis at low sodium ion concentrations by 30% while at high sodium ion concentrations ATP synthesis was slightly stimulated (not shown). These results indicate that the proton-motive force might also partially participate in ATP synthesis under alkaline conditions in the presence of a low sodium ion concentration. At alkaline conditions in the presence of a high sodium ion concentration, synthesis of ATP was probably driven by sodium ion-motive force only. Growth experiments performed in the presence of 10 μM TCS have shown that the growth of these bacteria was inhibited by 100% at pH 6.8 even when 50 mM NaCl was present. On the other hand, at alkaline pH, the growth was inhibited by 100% only in the presence of low NaCl concentration. In the presence of high NaCl concentration, the growth was inhibited approx. by 20%. These findings indicate that at alkaline pH and in the presence of high NaCl concentration, Na^+-translocating ATPase works in the synthase mode.

4. Discussion

The identification of two distinct membrane-associated ATPases in *Methanobacterium thermoautotrophicum* ΔH might indicate the presence of A- and F-types of ATPases. Recently, Becher and Müller [7] have concluded from inhibitor studies that membrane vesicles of *Methanosarcina mazei Göl* contain an F_1F_0-ATP synthase (sodium ion-translocating) in addition to the A_1A_0-ATPsynthase (proton-translocating). Membrane-associated ATPases were also found in other methanogenic bacteria but their properties differed in different methanogens. It was found that ATPases of *Methanosarcina barkeri* [24] and *Methanococcus voltae* [23] belong to the V-type ATPase family, while the ATPase of *Methanolobus tindarius* [25] belongs to F_1F_0-ATPase family. These findings show that there is not a predictable distribution of ATPases in different methanogens. In immunoblotting experiments with cell proteins separated by SDS-PAGE, we detected one protein of approx. 53 kDa which cross-reacted with an anti-F_1 antiserum. Such cross-reactivity was observed not only in cells of *Methanobacterium thermoautotrophicum* strain ΔH but also in strain Marburg and in *Methanospirillum hungatei* and it could be attributed to the β-subunit by immunotitration experiments. These results indicate that the F-type ATPase can be quite frequent in methanogens. Interestingly, antisera directed against the β-subunit of *Escherichia coli* ATPase did not exert the cross-reactivity against the extracts from different methanogens [26]. The reason for these differences is not understood at the moment. We propose that our findings may demonstrate that some methanogens belonging to the group of the Archaea are more closely related to the Eucarya than to the Bacteria as it has already been suggested for other archaebacteria [27].

It can be concluded from the observed stimulatory effect of bafilomycin A_1 on Na^+-dependent ATP synthesis that these cells contain both an Na^+-dependent ATPase (which is probably F-type) and an H^+-dependent ATPase, belonging to the A-type ATPase family. The stimulatory effect of bafilomycin A_1 on Na^+-dependent ATP synthesis probably reflects the possibility that a part of ATP synthetized by Na^+-dependent ATPase could be hydrolyzed by the H^+-dependent ATPase under some conditions. Hence, the inhibition of this proton-translocating ATPase by bafilomycin A_1 could be responsible for the observed stimulation of ATP synthesis mediated by Na^+-dependent ATPase. It is not clear at present whether such an hydrolysis of ATP is physiologically relevant.

The observed stimulatory effect of harmaline on the Na^+-dependent ATP synthesis driven by the proton diffusion potential in the presence of sodium ions or by the methanogenic electron transfer could probably be explained on the basis of a competition for sodium ions between the Na^+/H^+ antiporter and the Na^+-dependent ATPase. Recently, Becher and Müller [7] have shown that inhibition of the Na^+/H^+ antiporter led to a stimulation of ATP synthesis driven by the methyl-transfer reaction (generator of sodium ion-motive force) as well as by ΔpNa. The finding that ATP synthesis driven by methanogenic electron transport at pH 8.0 in the presence of a high sodium ion concentration is insensitive to uncoupler, shows that at

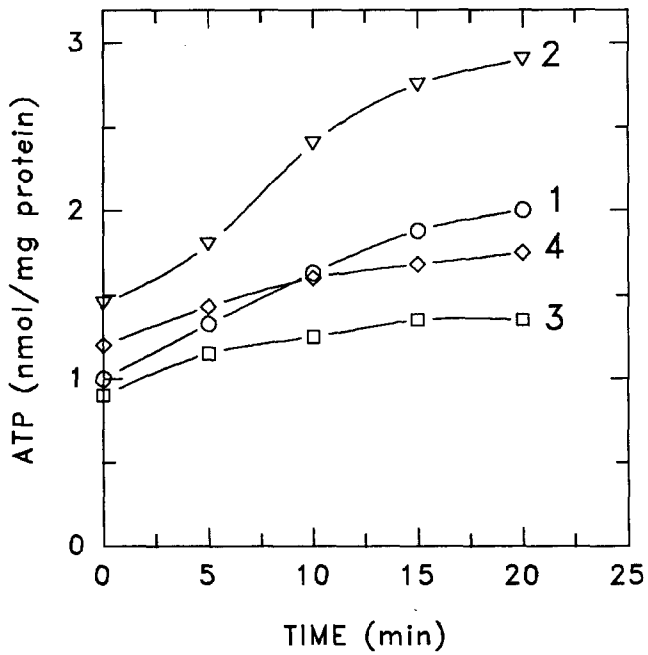

Fig. 4. Effect of harmaline on ATP synthesis driven by methanogenic electron transport in cells of *Methanobacterium thermoautotrophicum*. Cells were suspended in 50 mM HEPES-Tris buffer (50 mM HEPES-Tris, 5 mM $MgCl_2$, 50 mM NaCl(1,2) or 5 mM NaCl(3,4), pH 8.0) to a concentration of 1.0 mg protein per ml. Cells were preincubated for 5 min at 60°C in the presence of 300 μM harmaline(2,4). Gas phase, H_2/CO_2(4:1).

these conditions sodium ions can probably fully substitute for protons as the coupling ions.

All these findings support the idea that, in according with Skulachev's laws of bioenergetics [28], there exists a very close functional interrelationship among the proton-dependent ATPase, the sodium ion-dependent ATPase and the Na^+/H^+ antiporter in cells of *Methanobacterium thermoautotrophicum*. Their mutual cooperation could be responsible for the adaptive behaviour of these cells in different external conditions. With respect to the evolutionary aspects of these observations we assume that sodium energetics in these cells might reflect the observed portion of their halophilic nature. *Methanobacteriaceae*, as already been suggested, is the methanogenic group most closely related to the extreme halophilis [29]. We also speculate that magnesium ions, which occur in high concentrations in various saline biotopes (e.g. 55 mM in sea water) and which are a known inhibitor of Na^+/H^+ antiporter in *Methanobacterium thermoautotrophicum* [12] might have been the stimulatory factor which forced the cells to close the existing Na^+-cycle via the sodium ion dependent ATPase, when Na^+/H^+ antiporter was inhibited by high concentration of Mg^{2+}.

Acknowledgements: The authors gratefully acknowledge the technical assistance of Mrs. Petra Melicherová. We are also grateful to Prof. Štefan Kužela for the gift of rabit anti-F_1 antiserum. This investigation was supported by Research Grant GAV 2/1052/95 from the Slovak Academy of Sciences.

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