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Na^+-driven ATP synthesis in *Methanobacterium thermoautotrophicum* and its differentiation from H^+-driven ATP synthesis by rhodamine 6G

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Abstract

Rhodamine 6G (3 μM) effectively inhibited ΔpH-driven ATP synthesis in *Methanobacterium thermoautotrophicum* while ΔpNA-driven ATP synthesis was not affected by it. Rhodamine 6G inhibited Mg^{2+}-stimulated ATPase activity of membrane vesicles prepared from these cells but the ATPase catalytic sector detached from the membrane was insensitive to this inhibitor. Methanogenesis-driven ATP synthesis at pH 6.8 of the cells grown in the presence of 50 mM NaCl was inhibited by rhodamine 6G both in the presence of 5 mM and 50 mM NaCl. On the other hand, the methanogenesis-driven ATP synthesis at pH 8.0 of cells grown in the presence of 50 mM NaCl was slightly inhibited by rhodamine 6G in the presence of 5 mM NaCl and was not inhibited at all in the presence of 50 mM NaCl. The growth experiments have shown that cells of *Methanobacterium thermoautotrophicum* can grow under alkaline conditions even in the presence of rhodamine 6G and of high NaCl concentration when the growth media were inoculated with the cells which had been grown in the presence of 50 mM NaCl. These results indicate that sodium-motive force-driven ATP synthase in *Methanobacterium thermoautotrophicum* operates effectively at alkaline conditions and it might be the sole ATP synthesizing system when the proton motive force-supported ATP synthesis is inhibited by rhodamine 6G.

Key words: Methanogen; Sodium-motive force; ATP synthesis; Rhodamine 6G

1. Introduction

Evidence has been obtained with different bacterial species that sodium ions can under specific conditions substitute for protons as the coupling ions [1–5]. The stimulatory effect of sodium ions on growth, methane production and some membrane-bound processes in methanogenic bacteria [6–8] indicated that Na^+ can be directly involved in energy transduction of this special division of archaebacteria. In general, the electrochemical potential of Na^+ is generated directly by a primary Na^+ pumps which are independent of H^+ circulation [4]. The methyl-H_4MPT:HS-CoM methyltransferase seems to be such primary Na^+ pump at least in *Methanosarcina barkeri* strain Go1 [8,9]. As another possible Na^+ primary pump in methanogenic bacteria, the formyl-methanofuran dehydrogenase in reverse direction might be considered [8].

A set of Na^+ electrochemical potential consuming systems, including Na^+-translocating ATP synthase, is already known [4]. The Na^+-isoleucine symporter as the first $\Delta\tilde{\mu}_{Na^+}$ consuming system of methanogenic bacteria was described [10]. However, in spite of that the synthesis of ATP driven by artificially-imposed sodium ions gradient has been shown in cells of some methanogenic bacteria [11,12] the presence of Na^+-translocating ATP synthase in methanogenic bacteria is still unclear.

We report here that cells of *Methanobacterium thermoautotrophicum* possess a $\Delta\tilde{\mu}_{Na^+}$-utilizing ATP synthesizing system which can be differentiated from $\Delta\tilde{\mu}_{H^+}$-driven ATP synthesis. Moreover, the results presented here show also that Na^+ dependent ATPase might drive the ATP synthesis at alkaline conditions when H^+-translocating ATP synthase is inhibited by rhodamine 6G.

2. Materials and methods

Methanobacterium thermoautotrophicum strain ΔH was cultivated as described earlier [13]. The growth medium [14] containing Na_2CO_3 instead of $NaHCO_3$, supplemented with 50 mM NaCl (high Na^+ medium), or containing K_2CO_3 supplemented with 5 mM NaCl (low Na^+ medium) 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.

Sodium ion gradient driven and proton gradient driven ATP synthesis of the cell suspension was measured as described elsewhere [15].

Methane formation from CO_2 and H_2 by the cell suspension was measured by gas chromatography [13] on Carlo Erba Fractovap 4200 using the 2 m long steel column packed with Sepharon AE 200–300 μm. A heat-conductive detector (Model 450) for gas detection was used. Samples of the gaseous phase were taken from cultivation flasks (20 ml bottles sealed with butyl-rubber stoppers) by gas-tight syringes Pierce Series A-2.

For the measurement of ATPase activity the membrane preparation was used. The cell membrane fraction was prepared as follows: cells of *Mb. thermoautotrophicum* were harvested by centrifugation and washed once with 10 mM K-glycyl-glycine, 10 mM $MgCl_2$ (pH 8.7) anaerobic buffer solution. Then cells were resuspended in 10 mM K-glycyl-glycine, 10 mM $MgCl_2$, 0.4 M sucrose (pH 8.0) solution. A few grains of DNase were added to the cell suspension and cells were disrupted by two passages through a French press (FA 030 40K, SLM Instruments, Urbana, USA) at 70 MPa. The unbroken cells and large debris were removed by centrifugation at 25,000 × *g* at 4°C for 15 min. The cell-free supernatant was centrifuged aerobically at 120,000 × *g* at 4°C

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

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for 1 h. The resulting sediment was washed once with the buffer described above. The membrane fraction was resuspended in the same buffer (10–15 mg protein per ml) and used for the measuring of ATPase activity.

Preparation and purification of a soluble ATPase from *Mb. thermoautotrophicum* was done according to Roth et al. [16].

ATPase activity was assayed by the measurement of inorganic phosphate released from ATP [17]. The reaction mixture contained in 1 ml: 1 mM ATP, 5 mM $MgCl_2$, 10 mM K-glycyl-glycine, 0.4 M sucrose (pH 8.0) and 100–500 μg protein. After 5 min preincubation, the reaction was started by the addition of ATP and incubated for 5 min at 60°C. A blank sample without Mg^{2+} was substracted from every sample.

Protein was quantified by the Lowry method [18] with bovine serum albumin as the standard.

All chemicals were of reagent grade purity purchased mostly from Lachema Brno, except for ATP (Serva), dicyclohexylcarbodiimide (DCCD) (Serva) and rhodamine 6G (Lambda Physic).

3. Results

As the intact cells of *Methanobacterium thermoautotrophicum* are resistant to the low concentration of DCCD and as a higher concentration of it may exert some non-specific interactions, in order to discriminate between ATP synthesis driven by ΔpH and ΔpNa in *Methanobacterium thermoautotrophicum* an inhibitor rhodamine 6G has been selected. According to Higuti et al. [19] the inhibition site of rhodamine 6G in mitochondria is an F_0 subunit and probably some other components related to H^+ ejection by redox systems of the respiratory chain.

Fig. 1A shows that ATP synthesis in *Mb. thermoautotrophicum* after the imposition of an artificial ΔpH was strongly inhibited by low (3 μM) concentration of rhodamine 6G. On the other hand, the synthesis of ATP driven by the imposition of Na^+ concentration gradient was insensitive to the action of rhodamine 6G (Fig. 1B). Moreover, the ATP synthesis driven by proton diffusion potential induced by the acid–alkaline shift in the presence of an uncoupler (tetrachlorosalicylanilide) and sodium ions, was even slightly stimulated in the presence of rhodamine 6G (Fig. 2). In this case the observed stimulation can be explained by the possibility that ATP synthetized on the account of sodium-motive force can not be hydrolyzed by H^+-dependent ATPase because of its inhibition by rhodamine 6G. These results indicate that ATP synthesis in *Mb. thermoautotrophicum* can be driven by two different ionmotive forces via two enzymatic entities which are different in their response to rhodamine 6G.

To localize the inhibition site of rhodamine 6G in *Mb. thermoautotrophicum* we tested its effect on Mg^{2+}-stimulated ATPase activity of the membrane vesicles prepared from these cells. In the presence of 1.5 μM rhodamine 6G the rate of ATP hydrolysis dropped to 68.8 nmol P_i/mg protein/min which corresponds to 61.1% of the control (112.6 nmol P_i/mg protein/min). At 3 μM concentration of this inhibitor the rate of ATP hydrolysis dropped to 58.4 nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$ which corresponds to 51.8% of the control. The inhibitory effect of rhodamine 6G at 3 μM concentration was roughly comparable with DCCD inhibitory effect at 150 μM concentration. However, the isolated soluble ATPase from these vesicles was insensitive to the inhibitor. The results indicate that the inhibitory site of rhodamine 6G is on the membrane part

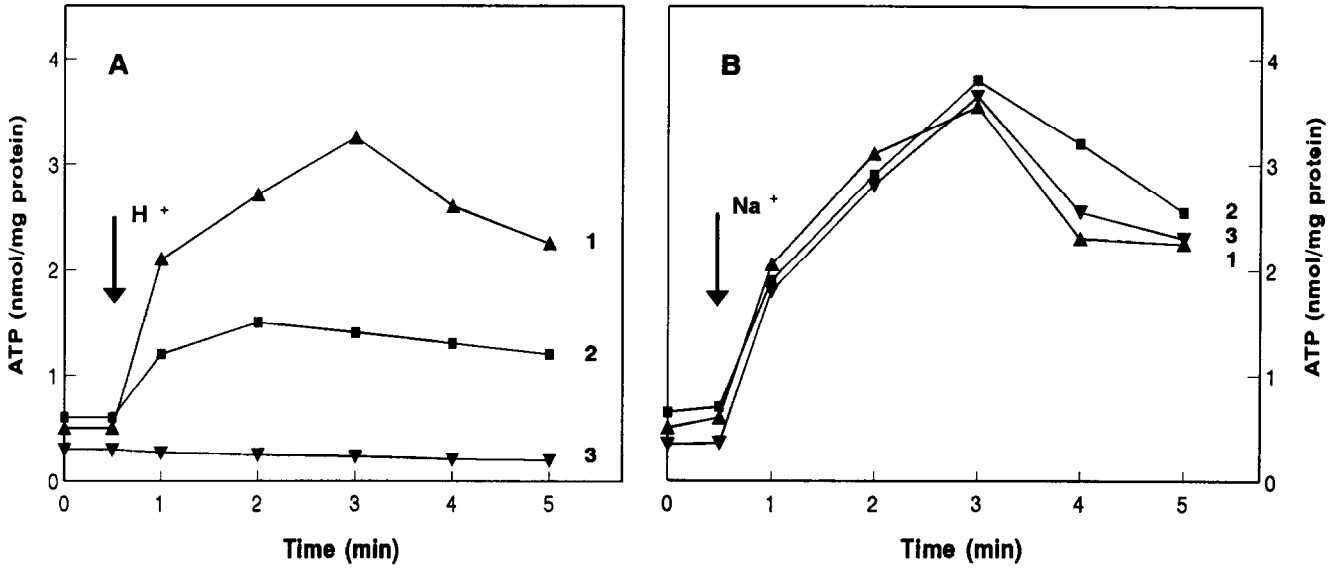

Fig. 1. Effect of rhodamine 6G on ΔpH-driven and ΔpNa-driven ATP synthesis in *Methanobacterium thermoautotrophicum*. Cells were suspended (A) in 100 mM phosphate buffer (0.1 M phosphoric acid-TRIS, 5 mM $MgCl_2$, pH 6.9), or (B) in 100 mM HEPES buffer (0.1 M HEPES-TRIS, 5 mM $MgCl_2$, pH 6.9), to a concentration of 1.13 mg protein per 1 ml. Cells were preincubated for 5 min at 60°C in the presence of rhodamine 6G. At t = 0.5 min, the pH value of the suspension was shifted from 6.9 to 3.0 by adding 6 M HCl (A) or the reaction was started by injecting 4 M NaCl to a final concentration 0.4 M (B). Gas phase, Ar. (1) control, (2) 1.5 μM rhodamine 6G, (3) 3 μM rhodamine 6G. 1 mM tetraphenylborate was added at t = 4 min of preincubation period to all suspensions.

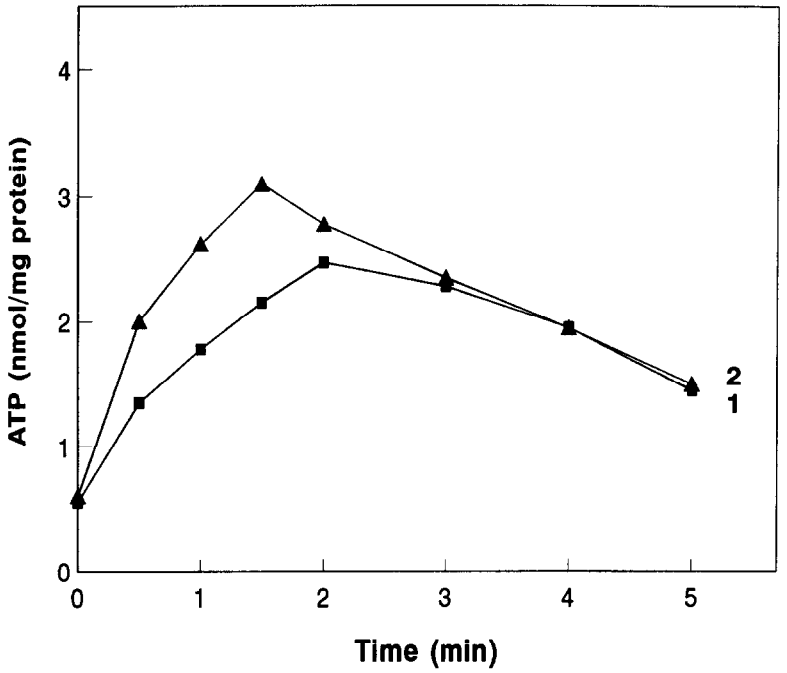

Fig. 2. Proton diffusion potential-driven ATP synthesis in resting cells of *Methanobacterium thermoautotrophicum*. Cells were suspended in 50 mM MOPS-TRIS buffer (pH 6.8) to a concentration 1.26 mg protein per 1 ml. ATP synthesis was induced by addition of 3 M KOH to a final pH of 9.2. The NaCl concentration of reaction mixture was 40 mM. 7 min prior to zero time 30 μM TCS was added. (1) control; (2) 3 μM rhodamine 6G added 5 min prior to zero time. Gas phase, Ar.

of the ATPase complex of *Mb. thermoautotrophicum*. The similar conclusion based on the inhibitory effect of rhodamine 6G was done also for isolated mitochondria [19].

Fig. 3A shows that ATP synthesis driven by methanogenic electron transport at pH 6.8 was effectively inhibited by rhodamine 6G both in the presence of low (5 mM) and high (50 mM) NaCl concentration. The methanogenesis itself at the concentration of rhodamine 6G used in those experiments was not affected (not shown). On the other hand, the same experiments done at pH 8.0, when $\Delta\Psi$ and ΔpH were oppositely directed, showed that

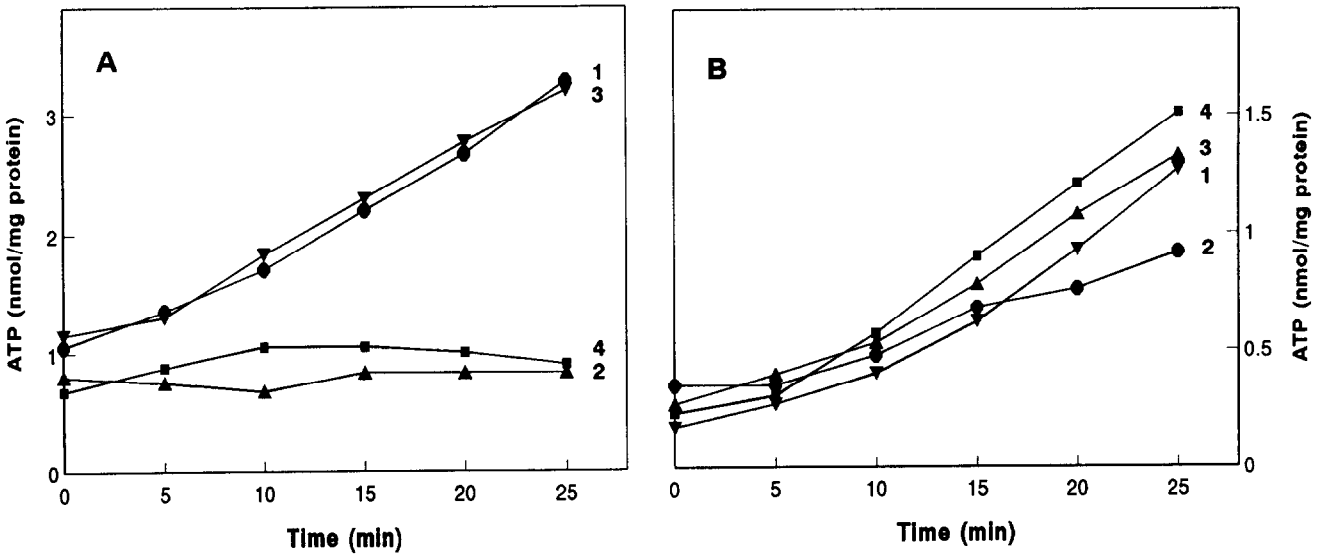

Fig. 3. Effect of rhodamine 6G on electron transfer-driven ATP synthesis in *Methanobacterium thermoautotrophicum*. Cells were suspended (A) in 50 mM HEPES buffer (50 mM HEPES-TRIS, 5 mM $MgCl_2$ with 5 mM NaCl (1,2) or 50 mM NaCl (3,4) pH 6.9), or (B) in 50 mM TRIS-buffer (50 mM TRIS-HEPES, 5 mM $MgCl_2$ with 5 mM NaCl (1,2) or 50 mM NaCl (3,4) pH 8.0), to a concentration 1.5 mg protein per 1 ml. Cells were preincubated for 5 min at 60°C in the presence of 3 μM rhodamine 6G (2,4). Gas phase, H_2/Co_2 4:1.

electron transport-driven ATP synthesis in the presence of the low NaCl concentration was inhibited by rhodamine 6G too. Such an inhibitory effect of rhodamine 6G on ATP synthesis was not observed in the presence of the high NaCl concentration (Fig. 3B). These results have shown that ATP synthesis driven by methanogenic electron transport can occur at alkaline pH in the presence of 50 mM NaCl even when the proton translocating ATP synthase is inhibited by rhodamine 6G.

The above described results have raised the question: can cells of *Mb. thermoautotrophicum* grow when proton translocating ATP synthase is inhibited by rhodamine 6G? To answer this question, further physiological experiments were conducted. Table 1 summarizes the inhibitory effect of rhodamine 6G on the growth of *Mb. thermoautotrophicum* at pH 6.8 and 8.0. In agreement with our previous results [15], the inhibitory effect of rhodamine 6G on the growth was dependent on the inoculum used. When the growth media containing rhodamine 6G were inoculated with cells originally grown in the presence of the low (5 mM) NaCl concentration, the cell growth was inhibited by 100% in all cases. However, a different picture was observed when growth media containing rhodamine 6G were inoculated with cells originally grown in the presence of the high (50 mM) NaCl concentration. In this case, the growth at pH 6.8 in the presence of the high NaCl concentration was inhibited by rhodamine 6G up to 90%, while the growth at pH 8.0 under the same conditions was inhibited only by 40%. To explain this observation we suppose the existence of the sodium cycle, in which Na^+-translocating ATPase might operate at alkaline pH as a synthase.

4. Discussion

It has been previously shown [20] that cells of *Mb. thermoautotrophicum* at pH higher than 7.5 have reversed polarity of proton-motive force, i.e. interior acid with a $\Delta\tilde{\mu}_{H^+}$ up to + 145 mV. This situation is unfavorable not only from a kinetic aspect but also from a thermodynamic aspect for using H^+ as a coupling ion [21]. Substitution of Na^+ for H^+ as a coupling ion seems to be very effective solution to this problem. The existence of Na^+- translocating ATPase which can be modulated with sodium ion concentration in the growth medium has already been described in cells of *Mb. thermoautotrophicum* [15]. In this context it should be mentioned that the requirement of a high concentration of Na^+ for induction of the *Enterococcus hirae* Na^+-ATPase and for the Na^+-motive oxidase of *Escherichia coli* was reported [22,23]. In different methanogenic bacteria the existence of a sodium cycle has been suggested [9,24,25]. Up to now it is not clear why methanogenic bacteria establish a primary sodium concentration gradient. One of the possibilities is that this concentration gradient of sodium ions is consumed by Na^+-translocating ATP synthase.

To test this hypothesis it was important to find an inhibitor which could inhibit H^+ gradient-dependent ATP synthesis and which does not exert the inhibitory effect on Na^+ gradient-dependent ATP synthesis. We found rhodamine 6G as very potent inhibitor of H^+ gradient-dependent ATP synthesis. The strong inhibitory effect of this compound was also shown at pH 6.8 when ATP synthesis was driven by methanogenic electron transport. ATP synthesis driven by methanogenic electron transport at pH 8.0 in the presence of a low Na^+ concentration was partly inhibited by rhodamine 6G which indicates that ATP synthesis driven by proton-motive force takes place also at pH 8.0. However, the amount of synthetized ATP might be too low to be sufficient to drive all energy requiring processes in *Mb. thermoautotrophicum*. This is documented also by the finding that cells of *Mb. thermoautotrophicum* do not grow under such conditions. A different picture was obtained at pH 8.0 when a high Na^+ concentration was present in the medium. Rhodamine 6G did not exert the inhibitory effect on ATP synthesis and cells were able to grow under

Table 1
Growth inhibition by rhodamine 6G at pH 6.8 and 8.0 in the presence of 50 mM and 5 mM NaCl

Additions	Growth inhibition %			
	pH 6.8		pH 8.0	
	5 mM NaCl 45 mM Chol·Cl	50 mM NaCl	5 mM NaCl 45 mM Chol·Cl	50 mM NaCl
(A) 3 μM Rhodamine 6G	100	80	100	40
(B) 3 μM Rhodamine 6G	100	100	100	100

Medium buffered to pH 6.8 and 8.0 with HEPES containing 5 mM or 50 mM NaCl was inoculated with 2×10^6 cells per ml of *Mb. thermoautotrophicum*. The growth yield (expressed in cells per ml) was evaluated after 48 h by counting the cells in a haemocyte chamber. In the case when the medium contained 5 mM NaCl, 45 mM choline chloride was added. (A) Medium inoculated with cells which had grown in the presence of 50 mM NaCl. (B) Medium inoculated with cells which had grown in the presence of 5 mM NaCl and 45 mM choline chloride.
The control cells yield after 48 h, 2×10^8 cells per ml.

such conditions even in the presence of rhodamine 6G. All these findings might indicate that cells of *Mb. thermoautotrophicum* can utilize the primary sodium ion gradient for ATP synthesis mediated by Na^+-translocating ATP synthase at least at pH 8.0 when H^+-translocating ATP synthase is inhibited by rhodamine 6G.

Based on our results presented here, it is not yet clear whether the cells of *Mb. thermoautotrophicum* contain two different ATP synthases with different function under different conditions. The stimulation of ATP synthesis driven by proton diffusion potential in the presence of sodium ions and rhodamine 6G indicates the existence of both H^+-translocating and Na^+-translocating ATP synthases in these cells. Our preliminary results have shown that the antibodies raised against the β-subunit of mitochondrial F_1 crossreacted with ATPase from *Mb. thermoautotrophicum*. This finding indicates that an F-type ATPase exists in the cells of *Mb. thermoautotrophicum*. Sumi et al. [26] and Kakinuma et al. [27] have suggested that *Ms. barkeri* and *E. hirae* contain both F and V types of ATPases. Sumi et al. [26] have also suggested that F-type in *Enterococcus hirae* is a H^+-ATPase and V-type is an Na^+-ATPase which is induced at high Na^+ concentration in the culture medium. These results might support the possibility that the cells of *Mb. thermoautotrophicum* also could contain similar F- and V-types of ATPases. Further studies on this matter are in progress.

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