Discrimination between transmembrane ion gradient-driven and electron transfer-driven ATP synthesis in the methanogenic bacteria

H.A. Al-Mahrouq, S.W. Carper[+] and J.R. Lancaster, jr*

Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322, USA

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As with *Methanococcus voltae* [(1986) FEBS Lett. 200, 177–180], ATP synthesis in *Methanobacterium thermoautotrophicum (ΔH)* can be driven by the imposition of a sodium gradient, but only in the presence of a counterion. Monensin (but not SF6847) inhibits this synthesis. Methanogenic electron transfer-driven ATP synthesis, however, is insensitive to the combination of these two ionophores. In *M. voltae*, 117 μM diethylstilbestrol effectively inhibits both membrane potential- and sodium gradient-driven ATP synthesis, but has no effect on ATP production coupled to methanogenesis. In *Mb. thermoautotrophicum (ΔH)*, a similar pattern of inhibition is exhibited by harmaline, an inhibitor of sodium-linked membrane transport systems. We conclude that ATP-driven sodium translocation and electron transfer-driven ATP synthesis are accomplished by separate entities, at least for these two only distantly related species of methanogen.

Methanogen *Bioenergetics* *Na^+ pump* *ATPase*

1. INTRODUCTION

Sodium has recently been shown to function as a coupling ion in membrane bioenergetics in several microbial systems [1]. In the methanogenic bacteria, sodium has been implicated in several cellular functions, including growth [2], methane formation [2–4], electron transfer [4], amino acid transport [5], pH homeostasis [6,7], and membrane potential-induced ATP synthesis [8,9]. We have recently presented evidence for a role for sodium as a coupling ion in *Methanococcus voltae* for internal ion and solute homeostasis (powered by an electrogenic sodium-translocating ATPase), with cellular ATP production coupled to electron transfer by a separate, direct mechanism [9–12]. The experimental evidence includes ATP synthesis driven by electrogenic movement of sodium through a membrane-bound ATPase and uncoupler-insensitive ATP synthesis coupled to electron transfer. Here we report the presence of a similar system in an only distantly related species of methanogen (*Methanobacterium thermoautotrophicum (ΔH)*) and, with the use of inhibitors, show that in neither species is this system involved in electron transfer-driven ATP synthesis.

2. MATERIALS AND METHODS

Mb. thermoautotrophicum (ΔH) (DSM 1053) was cultured in the medium described in [13]. *M. voltae* strain PS (DSM 1537) was cultured in the medium described by Whitman et al. [14]. All manipulations were performed under strictly anaerobic conditions. Actively growing cells were collected by centrifugation (10000 × *g*, 10 min), washed, and resuspended in the appropriate anaerobic buffer [Pipes buffer (0.1 M Pipes-Tris, pH 6.9; 5 mM $MgCl_2$); sucrose buffer (0.8 M sucrose, 10 mM $MgCl_2$, 10 mM Tricine, pH 8.0 (NaOH)); salt buffer (0.4 M NaCl, 10 mM $MgCl_2$,

[+] Present address: Department of Radiation Oncology, University of Arizona Health Sciences Center, Tucson, AZ 85724, USA

* To whom correspondence should be addressed

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10 mM Tricine, pH 8.0 (NaOH)]. Whenever TPB^- was used potassium was not added. For *Mb. thermoautotrophicum (ΔH)*, cells suspended in anaerobic Pipes buffer (3 ml) were placed in 120 ml sealed bottles and ionophores or lipophilic ions added as ethanolic solutions [sodium tetraphenylboron (TPB^-; 1 mM final), SF6847 (5 μM final), monensin (10 μM final), or harmaline (0.3 mM final)]. The cell suspension was pressurized with N_2/CO_2 (80:20%, v/v) and allowed to preincubate at 60°C in a gyratory water bath for 35 min. NaCl (0.2 M final concentration), H_2/CO_2 (90:20%, v/v), or valinomycin (10 μM final) was added at the times indicated in the figures. 50 μl samples were subsequently removed anaerobically for determination of cellular ATP levels by the method of Kimmich et al. [15]. For $\Delta\psi$- and methanogenesis-driven ATP synthesis in *M. voltae*, cells were treated as for *Mb. thermoautotrophicum (ΔH)* except salt buffer was used and the temperature was 38°C. Diethylstilbestrol (DES) was added in ethanol before the preincubation to a final concentration of 117 μM. For sodium gradient-driven ATP synthesis in *M. voltae*, cells suspended in anaerobic sucrose buffer were placed in test tubes inside the glove bag and allowed to incubate at 38°C in a heating block for 25 min after ethanolic additions (TPB^-, DES). NaCl was added from a 4 M stock solution to a final concentration of 0.4 M and ATP was assayed as described above. Protein was determined by the method of Markwell et al. [16]. Harmaline, valinomycin, monensin, DES and sodium tetraphenylboron were purchased from Sigma. SF6847 was a gift from P. Hinkle, Cornell University.

3. RESULTS

As with *M. voltae* [9], addition of NaCl to cells of *Mb. thermoautotrophicum (ΔH)* in the presence of TPB^- as counterion results in ATP synthesis (fig.1). The level attained is comparable to that induced by electron transfer (fig.2, vide infra). The elimination of ATP synthesis by the sodium ionophore monensin shows that the effect is due to sodium movement (as opposed to, for example, intracellular sodium appearance). The requirement for TPB^- indicates that the movement is electrogenic [9]. Sodium gradient-driven ATP synthesis is insensitive to the uncoupler SF6847 (which has been shown to be competent to catalyze electrogenic proton movement in *M. voltae* [11]), showing that the link between these two processes is direct, not involving the obligatory intermediacy of proton translocation (as has also been shown for *M. voltae* [17]). Fig.2 shows that ATP synthesis in cells of *Mb. thermoautotrophicum (ΔH)* coupled to methanogenic electron transfer is insensitive to the presence of both SF6847 and monensin. This result argues against the obligatory intermediacy of a proton or sodium gradient in this process.

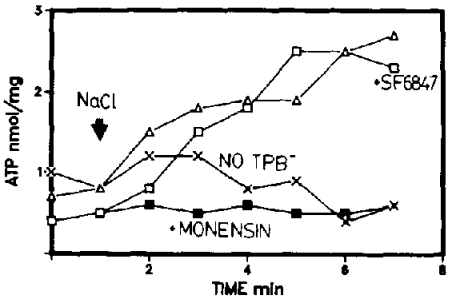

Fig.1. Δp*Na*-driven ATP synthesis in *Mb. thermoautotrophicum (ΔH)*. Cells were preincubated with the indicated additions as described in section 2. 0.4 M NaCl added at t = 1 min. (△) Plus 1 mM TPB ; (□) plus TPB^- plus 5 μM SF6847; (×) ethanol control (no TPB^-); (⊠) plus TPB^- plus 10 μM monensin.

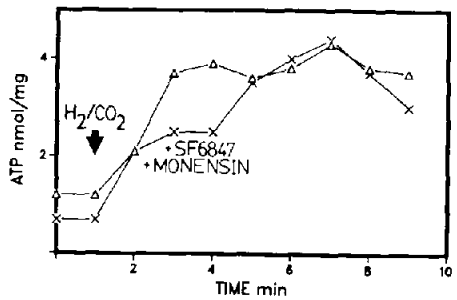

Fig.2. Effect of ionophores on electron transfer-driven ATP synthesis in *Mb. thermoautotrophicum (ΔH)*. Conditions were as for fig.1, except ATP synthesis was initiated by pressurizing the headspace gas with H_2/CO_2 (80/20%) and no TPB^- was present. (△) Ethanol control, (×) plus 5 μM SF6847 plus 10 μM monensin.

Diethylstilbestrol (DES) is an inhibitor of several plant and fungal ATPases [18,19]. As shown in fig.3, 117 μM DES effectively inhibits ATP synthesis induced by valinomycin addition (at t =

10 min) in *M. voltae*, which has been shown to result in the formation of a membrane potential, negative inside [9]. Under these conditions, however, ATP synthesis driven by methanogenic electron transfer (initiated by the subsequent addition of H_2/CO_2) is unaffected. The concentration range of DES resulting in this differential effect is fairly narrow, with higher levels resulting in inhibition of methanogenesis. This result indicates that the ATP synthesis induced by these two driving forces occurs by separate entities. The inhibition by DES of sodium gradient-driven ATP synthesis at this same concentration (fig.4) supports the assignment of Na^+ as the ion responsible for $\Delta\psi$-driven ATP synthesis [9], which is consistent with the requirement of external sodium for $\Delta\psi$-driven ATP synthesis in at least two species of methanogen [8,9].

In *Mb. thermoautotrophicum (ΔH)*, DES effects

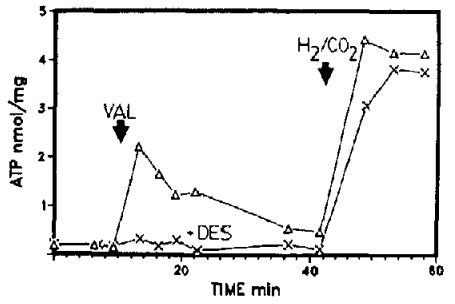

Fig.3. Effect of diethylstilbestrol (DES) on membrane potential- and electron transfer-driven ATP synthesis in *M. voltae*. Cells were treated as described in section 2. At the times indicated by the arrows, 10 μM valinomycin or H_2/CO_2 was added. (Δ) Ethanol control, (×) cells pretreated with 117 μM DES.

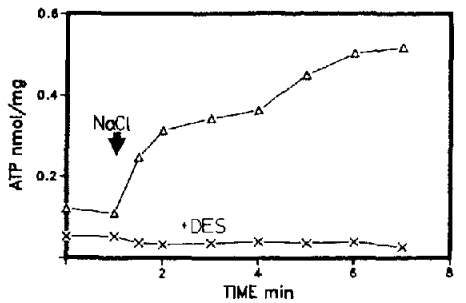

Fig.4. Effect of DES on Δp*Na*-driven ATP synthesis in *M. voltae*. Cells were preincubated with 1 mM TPB^+ as described in section 2. 0.4 M NaCl added at t = 1 min. (Δ) Control, (×) cells pretreated with 117 μM DES.

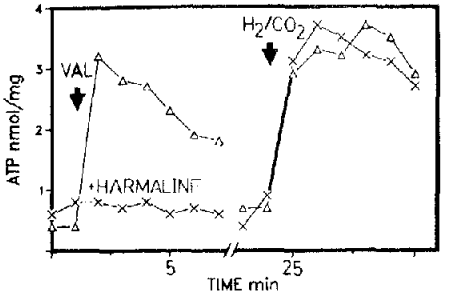

Fig.5. Effect of harmaline on membrane potential- and electron transfer-driven ATP synthesis in *Mb. thermoautotrophicum (ΔH)*. Cells were treated as described in section 2. (Δ) Ethanol control, (×) cells pretreated with 0.3 mM harmaline.

are not discriminatory for these two processes, i.e. the only observed effect is inhibition of methane formation (not shown). Schonheit and Beimborn [7] have shown that harmaline, an inhibitor of sodium-translocating systems in eukaryotic cells [20], inhibits Na^+/H^+ antiport in *Mb. thermoautotrophicum (Marburg)*. Since the inhibition has been suggested to be due to binding to a common sodium site on these different systems [20], we tested the effects of this compound on electrical potential-driven ATP synthesis in *Mb. thermoautotrophicum (ΔH)*. As shown in fig.5, 0.3 mM harmaline effectively eliminates $\Delta\psi$-driven ATP synthesis, but has no effect on ATP production coupled to electron transfer. Concentrations of up to 0.6 mM do not inhibit methanogenesis-driven ATP synthesis (not shown).

4. DISCUSSION

Within the Archaebacteria, the Methanococcaceae and the Methanobacteriaceae are only distantly related; indeed, the latter appear to be more closely related to the Halobacteriaceae than to the former [21]. It is thus perhaps not surprising that the two species in the present study differ in sensitivity to DES (the lipid compositions of the membranes of the two genera, for example, are different [22]). In both cases, however, the overall energetic scheme appears to be similar in that the system responsible for sodium translocation coupled to ATP hydrolysis is not involved in ATP synthesis driven by methanogenic electron transfer.

A puzzling feature of the Δp*Na*-driven ATP synthesis (fig.4 and [9]) is the slow rise, low level at-

tained, and surprisingly long duration of the ATP rise when compared to that driven by a $\Delta\psi$. We would expect the effect to be transient, due to the equilibration of internal and external sodium. In this regard, we note that, according to our present view of the function of this pump, the physiological direction of sodium movement is outwards so that sodium gradient-driven ATP synthesis corresponds to driving the system 'backwards'. Undoubtedly the best-characterized analogous system is the proton-translocating ATPase from the obligately anaerobic organism *Streptococcus lactis*. The physiological function of this ATPase is the same as we propose for the sodium pump of methanogens, i.e. maintenance of internal ion homeostasis as opposed to energy-coupled ATP synthesis. Indeed, Maloney and Wilson [23], in a detailed study of proton gradient-driven ATP synthesis in this organism, have reported a phenomenon essentially identical to that shown here, specifically a slow ΔpH-driven ATP synthesis to relatively low and sustained ATP levels when compared to that exhibited by an artificially driven $\Delta\psi$. That this difference is due to a requirement for counterion movement (as is also true for methanogens [9]) was shown by the stimulation of ΔpH-driven ATP synthesis in the presence of valinomycin plus potassium. If the analogy is strict, this would mean that these phenomena in methanogens are due to the slow movement of TPB^- as counterion (possibly due to the low concentration (1 mM) relative to potassium in the presence of valinomycin). Unfortunately, a major difference in these two systems is the use of Na^+ as the driving ion in methanogens, an ion which could move via valinomycin at the concentrations used for Δp*Na*-driven ATP synthesis (compared to protons); the lack of a significant rise in cellular ATP upon imposition of a Δp*Na* in the presence of valinomycin and potassium [9] supports this interpretation.

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