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A sodium-stimulated ATP synthase in the acetogenic bacterium *Acetobacterium woodii*

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Experiments with resting cells of *Acetobacterium woodii* were performed to elucidate the coupling ion used by the ATP synthase. *A. woodii* synthesized ATP in response to an artificial ΔpH, indicating the presence of a proton-translocating ATPase. On the other hand, a ΔpNa, as well as a proton diffusion potential, could serve as a driving force for ATP synthesis with the latter strictly dependent on Na^+. These results are indicative for the presence of a Na^+-translocating ATP synthase in *A. woodii*.

Acetogenic bacterium; Bioenergetics; Sodium cycle; ATP synthesis; Na^+; *Acetobacterium wooddii*

1. INTRODUCTION

Acetogenic bacteria are able to grow on and to produce acetate from a number of organic substrates as well as from H_2 + CO_2. During heterotrophic growth, ATP is synthesized in sufficient amounts by substrate-level phosphorylation. This is not the case during acetogenesis from H_2 + CO_2 were the net ATP formation by this mechanism is zero [1]. This prompts the question about additional mechanisms of ATP synthesis in these organisms. Thermodynamic considerations bring into focus the conversion of methylene-tetrahydrofolate (THF) to the methylated CO dehydrogenase which is catalyzed by the enzymes methylene-THF reductase, methyl transferase and CO dehydrogenase. The free energy change of these reactions under standard conditions is large enough to be coupled to ATP synthesis by a mechanism of electron transport phosphorylation and has therefore already been discussed by several authors as a possible site for energy conservation during acetogenesis [2].

In methanogens the corresponding sequence of reactions, the conversion of methylene-THMP to the formal redox level of methanol, has been shown to be coupled to a primary electrogenic sodium translocation across the membrane [3], thus conserving energy in the form of a primary electrochemical sodium gradient. This finding initiated a study about the role of Na^+ in the bioenergetics of acetogens. Growth as well as acetogenesis by *Acetobacterium woodii* is sodium-dependent with the reduction of methylene-THF to the formal redox level of methanol as the sodium-dependent step. Furthermore, acetogenesis is accompanied by the generation of a ΔpNa of −90 mV [4]. The energy stored in the electrochemical Na^+ gradient could then be used to drive ATP synthesis via a sodium-translocating ATP synthase. To prove this hypothesis the presence of a $\Delta\mu_{Na^+}$ utilizing ATP synthase is crucial. Therefore we examined the effect of artificial driving forces on ATP synthesis by whole cells of *A. woodii* and present evidence for the presence of a sodium-stimulated ATP synthase in this organism.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; SF6847, 3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalonitrile; ETH 2120, *N,N,N',N'*-tetracyclohexyl-1,2-phenylenedioxydiacetamide; TCS, 3,5,4',5'-tetrachlorosalicylanilide; THMP, tetrahydromethanopterin; THF, tetrahydrofolate; TRICINE, *N*-tris-[hydroxymethyl] methylglycine; ΔpH, transmembrane chemical gradient of H^+; ΔpNa, transmembrane chemical gradient of Na^+; $\Delta\Psi$, transmembrane electrical gradient; $\Delta\mu_{Na^+}$, transmembrane electrochemical gradient of Na^+.

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2. MATERIALS AND METHODS

2.1. *Growth of organisms and preparation of cell suspensions*

Acetobacterium woodii (DSM 1030) was grown under anaerobic conditions on 20 mM fructose as described [4], except that Na_2S was omitted and cysteine increased to 0.4 g/l. Cultures were harvested at the end of the exponential growth phase by centrifugation (10 300 × *g*, 15 min, 4°C) and washed twice with 20 mM imidazole/HCl buffer, pH 6.8, containing 20 mM $MgSO_4$ and 6 mM DTT. The cells were resuspended in the same buffer to a final protein concentration of 30–40 mg/ml under an atmosphere of nitrogen. This suspension was used immediately for the experiments. The protein concentration of the cell suspensions was determined by the method of Schmidt et al. [5]. All manipulations were done under strictly anaerobic conditions in an anaerobic chamber.

2.2. *Experiments with cell suspensions*

Determination of ATP synthesis by artificial driving forces was

performed in 58-ml glass bottles filled with 10 ml buffer (20 mM imidazole/HCl, pH 7.0, 20 mM $MgSO_4$ for ΔpNA-driven ATP synthesis; 20 mM imidazole/HCl, pH 7.0, 20 mM $MgSO_4$, 10 mM potassium citrate for ΔpH-driven ATP synthesis; 10 mM imidazole/HCl, pH 5.9, 20 mM $MgSO_4$, 10 mM TRICINE, 5 mM potassium citrate for proton diffusion potential-driven ATP synthesis) under an atmosphere of nitrogen and cells as given in the legends. Incubation was done at 30°C on a rotary shaker (70 rpm). ATP synthesis was induced by addition of HCl, NaCl or KOH, respectively, from anaerobic stock solutions at time points indicated in the figures. In case of inhibitor studies, cell suspensions were pre-incubated with the indicated inhibitor for 10–20 min prior to the experiment. At time points indicated for each experiment 0.4 ml were removed by syringe to determine the intracellular ATP content as described before [4,6].

All buffers used were reduced with 6 mM DTT and stored under N_2. Ionophores and inhibitors were added as ethanolic solutions; controls received the solvent only.

3. RESULTS

The coupling ion used by the ATPase was determined by applying artificial ion gradients to whole cells of *A. woodii*. Addition of HCl resulted in the transient formation of ATP (Fig. 1) which was dependent on the magnitude of the transmembrane proton gradient. ATP synthesis was inhibited by 89% by addition of the protonophore TCS (40 μM) and by 74% in the presence of 18 μM of the proton conductor SF6847. The sodium ionophore ETH 2120 had no effect on ΔpH-driven ATP synthesis excluding the possibility that the ΔpH was converted to a ΔpNa prior to ATP synthesis (data not shown). Of interest now was the effect of sodium pulses on the intracellular ATP content. Addition of NaCl resulted in the formation of ATP (Fig. 2). ATP synthesis was transient and dependent on the ΔpNa applied with a maximum ΔATP at a final Na^+ concentration of 400 mM (data not shown). NaCl could not be substituted for by KCl, indicating a specific effect of the sodium ion. The synthetic Na^+/H^+ antiporter monensin, as well as the sodium ionophore ETH 2120, completely inhibited ΔpNa-driven ATP synthesis. The extent of ATP synthesis driven by ΔpNa was only small as compared to ΔpH as the driving force but was significantly increased by addition of tetraphenylborate; this membrane-permeable anion distributes across the membrane in response to a $\Delta\Psi$ and, therefore, increases the accumulation of Na^+ by dissipating the artificial $\Delta\Psi$ which results from the Na^+ influx. These findings are in good agreement with a sodium-translocating ATP synthase.

Na^+-dependent ATP synthesis was further investigated by applying proton diffusion potentials to whole cells of *A. woodii*. Resting cells were incubated at pH 6 in the presence of the proton conductor SF6847. Alkalisation of the medium by addition of KOH results in an efflux of protons along their chemical gradient thus forming a membrane potential (inside negative). It has to be pointed out that, because of the reverse magnitudes of ΔpH and $\Delta\Psi$, the electrochemical proton gradient is zero at each time point of the experiment. Any observed endergonic reaction has then to be driven by an electrochemical ion gradient other than protons. Addition of KOH to resting cells of *A. woodii* in the presence of SF6847 resulted in ATP formation which was strictly dependent on the magnitude of the artificial $\Delta\Psi$ (Fig. 3A); similar results were obtained in the presence of TCS (data not shown). Interestingly, the maximal ATP content was reached after 60 s as compared to 5 min in the case of ΔpH as driving force. ATP synthesis was observed neither in the absence of protonophores nor in the presence of 10 μM valinomycin and 125 mM KCl. Since protons cannot be the coupling ions under these conditions it was of interest to study the

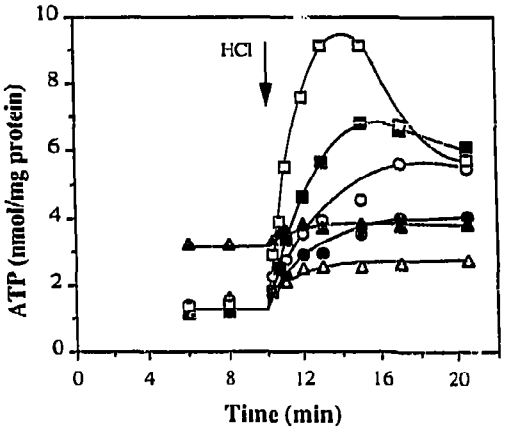

Fig. 1. ΔpH-driven ATP synthesis in resting cells of *A. woodii* incubated at pH 7.0 (protein content: 0.97 mg/ml). ATP synthesis was induced by addition of HCl to a final pH of 5.6 (△), 4.7 (●), 4.1 (○), 3.3 (■) or 2.9 (□) as indicated by the arrow. HCl was added to a final pH of 3.3 in the presence of 40 μM of TCS (▲).

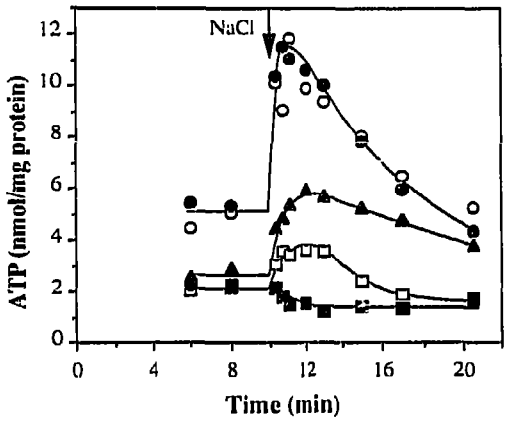

Fig. 2. ΔpNa-driven ATP synthesis in resting cells of *A. woodii* (protein content: 0.96 mg/ml). Cells were incubated in the absence (□, ■) or presence of 10 (▲), 25 (○), 50 (●) μM tetraphenylborate. One suspension was pre-incubated with 10 μM monensin (■). NaCl was added to a final concentration of 400 mM. The external Na^+-concentration at the beginning of the experiment was 0.2 mM.

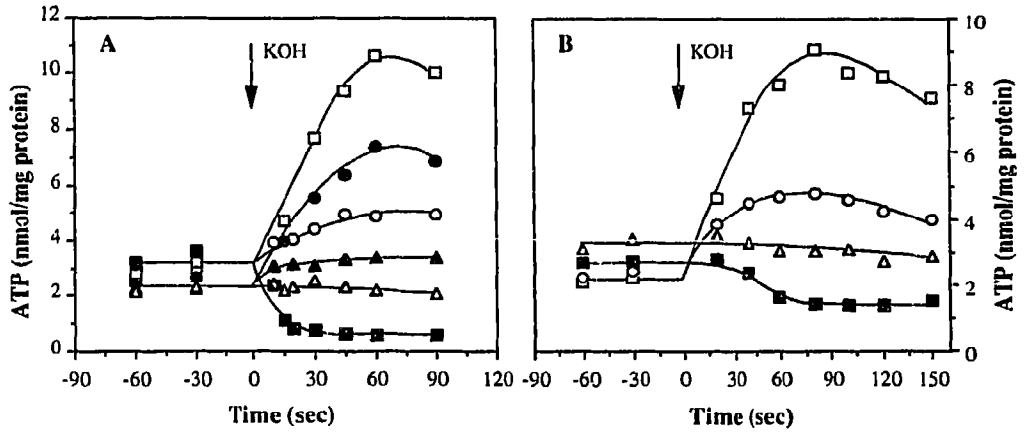

Fig. 3. Proton diffusion potential-driven ATP synthesis in resting cells of *A. woodii* incubated at pH 6.0 (protein content: 0.96 mg/ml). (A) ATP synthesis was induced by addition of KOH to a final pH of 7.8 (▲), 8.4 (⊃), 9.2 (●) or 9.8 (□). The Na^+ concentration of the reaction mixture was 30 mM. Control experiments were done in the absence of SF6847 (△) or presence of 10 μM valinomycin + 125 mM KCl (■). (B) Cell suspensions were pre-incubated in the absence (△) or presence of 3 (⊃) or 30 (□) mM NaCl or 20 μM ETH 210 (■). ATP synthesis was induced by addition of KOH to a final pH of 9.9.

effect of Na^+ present in the reaction mixture. It is apparent from Fig. 3B that the rate as well as the extent of ATP formation was strictly dependent on the sodium concentration of the buffer; ATP synthesis was inhibited by monensin (data not shown) and ETH 2120. These experiments are in accordance with the function of Na^+ as the coupling ion for ATP synthesis.

To elucidate the type of ATP synthase, inhibitor studies were performed. DCCD and venturicidin are known potent inhibitors of F_1F_0-ATPases, whereas vanadate inhibits E_1E_2-type proteins. Vanadate in concentrations up to 75 nmol/mg protein had no effect on ATP synthesis. On the other hand, DCCD (200 nmol/mg protein) inhibited proton diffusion potential- and ΔpNa-driven ATP synthesis by 94 and 81%, respectively; venturicidin inhibited similar effects. These inhibition patterns are as expected for a Na^+-translocating F_1F_0-ATPase.

4. DISCUSSION

A prerequisite for electron transport phosphorylation is the presence of membrane bound electron carriers and electron donor/acceptor systems as well as an ATP synthase. In a number of acetogens, cytochromes and menaquinones as well as iron sulfur proteins are found [7]. Furthermore, recent studies indicate that CO dehydrogenase, hydrogenase and methylene-THF reductase are membrane-bound [8] and capable of producing a $\Delta\Psi$ which in turn can drive uptake of amino acids [9–11]. An ATP synthase of the F_1F_0-type was documented in *C. thermoaceticum* [12] which resembles the F_1F_0-ATPase of *E. coli* [13].

Acetogenium kivui, *Peptostreptococcus productus* and *A. woodii* differ from other acetogens investigated so far by a dependence on Na^+ for metabolic activity [4,14,15]. In *A. woodii* the reduction of methylene-THF to the redox level of methanol is sodium-dependent and accompanied by the generation of a ΔpNa of −90 mV [4]. In analogy to methanogens this was discussed in terms of a primary mechanism of sodium translocation conserving energy in the form of an electrochemical sodium ion gradient [16]. In this paper we have presented evidence that *A. woodii* contains an ATPase which uses Na^+ as a coupling ion: transient ATP synthesis was observed with ΔpNa or a proton diffusion potential as driving force with the latter strictly dependent on Na^+. These results indicate the presence of a sodium-translocating ATPase in *A. woodii*. From the inhibitor studies it can be assumed that the sodium-stimulated ATPase of *A. woodii* is of the F_1F_0-type. An interesting finding is the fact that an artificial ΔpH (also in the presence of the sodium ionophore ETH 2120, excluding a Na^+/H^+ antiporter involvement) drives ATP synthesis as well. Therefore, the question arises whether *A. woodii* contains 2 enzymes with different ion specificities or just 1 enzyme which can use either H^+ or Na^+. Whereas the Na^+-ATPase present in *Propionigenium modestum* [17,18] is also able to pump protons in the absence of Na^+ [19], *Vibrio alginolyticus* seems to have two enzymes with different ion specificities [20–22]. This question can only be answered with purified systems, which will also be able to show more conclusively that the subunit composition of the enzyme under study is of the F_1F_0-type. The physiological function of the Na^+-ATPase in *A. woodii*, together with an as yet to be identified primary sodium pump, is to synthesize ATP and ensure net ATP formation during the operation of the Wood-Ljungdahl pathway. The elucidation of the coupling

mechanism as well as the further characterization of the sodium ATPase is now under way in our laboratory.

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