Energy transduction in the thermophilic anaerobic bacterium Clostridium fervidus is exclusively coupled to sodium ions

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ABSTRACT The thermophilic, peptidolytic, anaerobic bacterium Clostridium fervidus is unable to generate a pH gradient in the range of 5.5-8.0, which limits growth of the organism to a narrow pH range (6.3-7.7). A significant membrane potential (Δψ ≈ −60 mV) and chemical gradient of Na⁺ (−ZApNa ≈ −60 mV) are formed in the presence of metabolizable substrates. Energy-dependent Na⁺ efflux is inhibited by the Na⁺/H⁺ ionophore monensin but is stimulated by uncouplers, suggesting that the Na⁺ gradient is formed by a primary pumping mechanism rather than by secondary Na⁺/H⁺ antiport. This primary sodium pump was found to be an ATPase that has been characterized in inside-out membrane vesicles and in proteoliposomes in which solubilized ATPase was reconstituted. The enzyme is stimulated by Na⁺, resistant to vanadate, and sensitive to nitrate, which is indicative of an F/V-Type Na⁺-ATPase. In the proteoliposomes Na⁺ accumulation depends on the presence of ATP, is inhibited by the ATPase inhibitor nitrate, and is completely prevented by the ionophore monensin but is stimulated by protonophores and valinomycin. These and previous observations, which indicated that secondary amino acid transport uses solely Na⁺ as coupling ion, demonstrate that energy transduction at the membrane in C. fervidus is exclusively dependent on a Na⁺ cycle.

Clostridium fervidus is a Gram-positive, spore-forming, thermophilic anaerobe that has been isolated from a hot spring in New Zealand. The organism ferments peptides and amino acids, yielding acetate, NH₃, CO₂, and H₂ as major end products (ref. 1; G.S., unpublished). Amino acid transport in this organism is coupled exclusively to sodium ions, indicating that the electrochemical sodium ion gradient (ΔpNa⁺) plays a prominent role in the uptake of substrates essential for growth (2, 3). In accordance, growth of C. fervidus is completely inhibited by the Na⁺/H⁺ ionophore monensin and is partially inhibited by protonophores (G.S., unpublished).

In a strictly fermentative bacterium such as C. fervidus the generation of a sodium gradient is most easily envisaged by (i) a Na⁺-pumping ATPase in combination with a Na⁺/H⁺ antiporter, (ii) a membrane-bound Na⁺-pumping decarboxylase (4, 5), or (iii) a Na⁺-pumping ATPase (6). To establish how the Na⁺ gradient of C. fervidus is generated, the ion gradients across the cytoplasmic membrane have been estimated in intact cells and the Na⁺-pumping mechanism has been characterized in inside-out membrane vesicles and proteoliposomes. An F/V-Type Na⁺-ATPase is detected that functions in vivo as a Na⁺-extruding ATP hydrolyase. To our knowledge, a primary Na⁺ pump for a thermophilic eubacterium has not been reported previously. C. fervidus appears to be unique in the sense that energy transduction across the membrane is exclusively dependent on Na⁺ and that no H⁺ cycle is present.

METHODS

Growth of the Organism and Preparation of Inside-Out Membrane Vesicles. C. fervidus ATCC 43204 was grown anaerobically at pH 7.0 and 68°C in tryptone/yeast extract/glucose (TYEG) medium as described (3). Cells were harvested in the mid-exponential phase of growth (A₆₀₀ = 0.3–0.35). Inside-out membrane vesicles were prepared as described (7) with the following modifications: 3-(N-Morpholino)propanesulfonic acid (Mops) was used as a buffer, EDTA was omitted, and MgSO₄ (5 mM) and DNase and RNase (20 μg/ml each) were added prior to passage through a French press cell.

Determination of Transmembrane Ion Gradients. C. fervidus cells were harvested in the exponential phase of growth, washed once with basal medium supplemented with 5 mM NaCl and 3 mM dithiothreitol (MMND), and resuspended to an A₆₀₀ of 1.0 into 4 ml of the same medium. All operations were performed at room temperature and with the aid of an anaerobic cabinet. Cells were incubated for 10 min at 68°C with 10 mM glucose or arginine and the appropriate radioactive probes. The membrane potential (Δψ), the pH gradient (ΔpH), and the ΔpNa⁺ were estimated by the filtration and silicon oil centrifugation method (2, 8). [H³⁺]Tetraphenylphosphonium ion (TPP⁺), [³¹C]benzoic acid, [¹⁴C]methylamine, and α-amino [¹⁴C]isobutyrate (AIB) were used at final concentrations of 5 μM, 5 μM, 4 μM, and 5 μM, respectively. Samples to which 10 μM valinomycin and 1 μM nigericin were added to fully dissipate the Δψ and ΔpH served as blanks for the estimation of the gradients. In parallel experiments, the internal volume was determined from the difference in the distribution of H²O and [¹⁴C]isobutylrub. The internal volume of C. fervidus was 1.85 μl/mg of protein.

22Na⁺ Efflux. Cells of C. fervidus were handled as described above and resuspended to a final A₆₀₀ of about 13 into MMND and equilibrated with 5 mM ²²Na⁺ overnight at room temperature. ²²Na⁺ efflux was assayed by the filtration method (2).

ATPase Activity. ATP hydrolysis was determined as described (9). Measurements were performed at 45°C in 50 mM 2-(N-morpholino)ethanesulfonic acid (Mes; pH 6.0) containing 1 mM MgCl₂, 50 mM NaCl, plus 50 mM KCl for the inside-out membrane vesicles, and in 50 mM Mops (pH 7.0).

Abbreviations: ΔpNa⁺, electrochemical gradient of sodium ions (in mV); ΔpNa⁺, concentration gradient of sodium ions; ΔpH, proton motive force (in mV); Δψ, pH gradient; Z, 2.3RT/F, conversion factor to express pH or Na⁺ concentration gradients (in mV); AIB, α-aminoisobutyrate; ΔpAB, concentration gradient of AIB (in mV); Δψ, membrane potential (in mV); TPP⁺, tetrathenylphosphonium ion; S13, 5-chloro-3-tert-butyl-2'-chloro-4'-NO₂-salicylanilide; SF-6847, 3,5-di(t-tert-butyl)-4-hydroxybenzilidene malonitrile.

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containing 100 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (buffer A) plus 50 mM NaCl for the proteoliposomes.

Reconstitution of the ATPase. Inside-out membrane vesicles (4 mg of protein) were solubilized for 10 min at 45°C in 2.5 ml of buffer A containing 1% (wt/wt) Triton X-100 plus 20% (wt/wt) glycerol. The Triton X-100 extract was centrifuged for 60 min at 4°C at 48,000 × g. The supernatant, preformed liposomes, and Triton X-100 were mixed as described (10). The mixture was equilibrated for 2 h at 4°C. Two times 2 g (for 3 and 16 h) and one times 4 g (for 3 h) of Bio-Beads were added per 200 mg of Triton X-100. The mixture was centrifuged for 2 h at 4°C at 200,000 × g. The liposomes were resuspended in buffer A to about 70 mg of phospholipid per ml and stored at 4°C.

22Na⁺ Uptake in Proteoliposomes. Proteoliposomes were diluted into 1 ml of buffer A (0.1 mg of protein per ml, final concentration). 22Na⁺ was added (0.5 mM, final concentration) and allowed to equilibrate. Tris-ATP (3 mM, final concentration) was used as substrate. Samples of 100 μl were taken and assayed for 22Na⁺ uptake by the filtration method (2).

Other Methods. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard. Liposomes were prepared as described (3) in buffer A.

Materials. The following radioactively labeled materials were purchased from Amersham: [3H]TPP⁺ (92.5 GBq/mol), [14C]benzoic acid (0.81 TBq/mol), [14C]methylamine (2.07 TBq/mol), 3H₂O (37 GBq/l), [14C]sorbitol (12.3 TBq/mol), [14C]AIB (2.18 TBq/mol), and 22Na⁺ (27.2 TBq/mol).

RESULTS

Effect of pH on Growth and Energization. The effect of the initial medium pH on growth of C. fervidus is shown in Fig. 1. Growth rates and maximal optical densities sharply decreased below pH 6.3 and above pH 7.7. No growth was observed at or below pH 6.0 and at or above pH 8.0. This observation and the observations that amino acid transport is exclusively coupled to Na⁺ led us to estimate the Δψ, the ΔpNa⁺, and ΔpNa⁺, in intact cells in the presence of glucose or arginine and at various pH values (Table 1). The ΔpNa⁺ has been used to estimate the ΔpNa⁺, by assuming that the steady-state transmembrane gradient of AIB (ΔpAIB) is in thermodynamic equilibrium with the electrochemical sodium gradient. The ΔZpNa was obtained by subtracting the Δψ from the ΔpAIB. A significant ΔpH, either intracellular or extracellular, would be able to detect Na⁺ or arginine served as energy source. The Δψ assay used was found to be sufficiently sensitive to detect artificially imposed pH gradients of 0.3 unit (ΔZpH = -20 mV) (G.S., unpublished). However, under these conditions a large Δψ, ΔpNa⁺, and ΔpNa⁺ were formed, and these gradients were relatively constant in the pH range 5.5–8.0 (Table 1). No significant differences were found between the gradients estimated with the filtration or silicone oil centrifugation method. The observation that C. fervidus is unable to generate a Δψ and to regulate the cytoplasmic pH is consistent with the observed narrow pH range of growth.

Effect of Ionophores on Transmembrane Ion Gradients. The electrochemical gradient of Na⁺ in C. fervidus can be generated by two distinct mechanisms that are shown in Fig. 2. In Fig. 2A, a proton motive force (in mV) (ΔpH) is formed by the activity of a H⁺-ATPase and this ΔpH is converted into a ΔpNa⁺, by a H⁺/Na⁺ antiporter system. Fig. 2B shows a Na⁺-ATPase as the mechanism. To discriminate between these two mechanisms of ΔpNa⁺ generation, the effect of ionophores on Na⁺ efflux in intact cells was studied. Arginine was used as an energy source since it is taken up in exchange for ornithine without net movement of 22Na⁺, and intracellular ATP is formed rapidly by substrate level phosphorylation (3). Resting cells equilibrated with 22Na⁺ displayed little or no efflux of Na⁺. Upon addition of arginine 22Na⁺ was extruded (Fig. 3A), and after correction for binding, a 7- to 10-fold decrease of the internal Na⁺ concentration could be calculated, yielding a -ZpNa⁺ of -50 to -60 mV. These values are in agreement with those presented in Table 1. 22Na⁺ efflux was stimulated by the protonophore S13 but was inhibited by monensin (Fig. 3A). After energization monensin caused an immediate influx of Na⁺, whereas S13 had less effect than the ethanol control (Fig. 3B). These results are in agreement with the model of Fig. 2A: The protonophore would dissipate the proton motive force, thereby decreasing the Na⁺/H⁺ antiporter activity, which indirectly results in the dissipation of a Na⁺ gradient. The electroneutral Na⁺/H⁺ exchanger monensin would serve as a substitute for a Na⁺/H⁺ antiporter and convert a pH gradient into a sodium gradient or vice versa, depending on the magnitude and direction of these gradients. According to the model in Fig. 2B, dissipation of the Δψ by protonophores would lead to an increase of the chemical gradient of sodium due to additional pumping of Na⁺ by the

![Fig. 1. pH dependence of growth of C. fervidus. Cells were grown at 68°C in TYEG medium adjusted to various pH values with HCl or KOH.](image-url)
ATPase. Monensin would convert the Na⁺ gradient into a pH gradient. These are indeed the effects that are observed in C. fervidus. These results are also consistent with previous ones in which in membrane vesicles no Na⁺/H⁺ but only Na⁺/Na⁺ exchange activity was observed (3). Since ATP production from the metabolism of arginine is able to elicit the Na⁺ extrusion, the primary Na⁺-pump mechanism most likely is a Na⁺-ATPase.

**Effect of Monovalent Cations on ATP Hydrolysis.** Inhibitor and immunological studies indicated the presence of an F/V-type ATPase in membranes of *C. fervidus* (G.S., unpublished). This ATPase was studied in inside-out membrane vesicles and in proteoliposomes in which solubilized ATPase was reconstituted. The effect of increasing NaCl concentrations (at constant ionic strength) on ATP hydrolysis is shown in Fig. 4. The stimulation by Na⁺ ions shows Michaelis-Menten kinetics from which an apparent Km (Na⁺) of 3.2 mM can be estimated. The "zero" concentration in Fig. 4 reflects ~25 μM (contaminating) Na⁺ (data not shown). This activation of ATPase activity by Na⁺ is indicative of a Na⁺-translocating ATPase.

**Functional Reconstitution of ATPase Activity into Liposomes.** The reconstituted ATPase activity exhibits the same Na⁺ dependency as the ATPase activity in inside-out membrane vesicles (Fig. 4). Again, an apparent Km of about 3 mM can be estimated for the activation by Na⁺. The reconstituted ATPase exhibits properties identical to the ATPase activity in inside-out membrane vesicles (Table 2). Upon lowering the pH from 7.0 to 6.0 the activity is 2-fold stimulated. NO₃ inhibits the ATPase activity, SO₄ activates, whereas orthovanadate has no effect on the enzyme (Table 2). ATP hydrolysis in the proteoliposomes was stimulated by valinomycin, indicating that a Δψ is formed. Triton X-100 had a far more stimulating effect on ATP hydrolysis in the proteoliposomes than in the inside-out membrane vesicles, indicating that in proteoliposomes the ATPase is for the major part oriented with the binding side for ATP on the inside.

ATP-Driven ²²Na⁺ Uptake in Proteoliposomes. ²²Na⁺ was accumulated into the proteoliposomes upon the addition of ATP (Fig. 5). This accumulation was dependent on ATPase activity, since in the absence of ATP or in the presence of the ATPase inhibitor KNO₃ only equilibration of ²²Na⁺ was observed (Fig. 5A). The effects of ionophores and protono-
Table 2. Na\(^+\)-ATPase activity in inside-out membrane vesicles and proteoliposomes

<table>
<thead>
<tr>
<th>Addition</th>
<th>pH</th>
<th>Rate of ATP hydrolysis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inside-out vesicles</td>
<td>Without TX-100</td>
</tr>
<tr>
<td>None (control)</td>
<td>7.0</td>
<td>100</td>
</tr>
<tr>
<td>None (control)</td>
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<td>190</td>
</tr>
<tr>
<td>KNO$_3$ (50 mM)</td>
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<td>8</td>
</tr>
<tr>
<td>Na$_2$SO$_3$ (25 mM)</td>
<td>7.0</td>
<td>ND</td>
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<td>Na$_2$SO$_3$ (25 mM)</td>
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<td>400</td>
</tr>
<tr>
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<td>50</td>
</tr>
<tr>
<td>Valinomycin (4 uM)</td>
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<td>ND</td>
</tr>
<tr>
<td>Vanadate (200 uM)</td>
<td>6.0</td>
<td>104</td>
</tr>
</tbody>
</table>

One hundred percent values for ATPase activity in inside-out membrane vesicles in the absence and presence of 0.05% Triton X-100 (TX-100) and in proteoliposomes in the absence or presence of 0.1% Triton X-100 correspond to 59, 88, 38, and 657 nmol of Pi per min per mg of protein, respectively. ATPase measurements were performed at 45°C at a Na\(^+\) concentration of 50 mM. ND, not determined.

DISCUSSION

Previous studies demonstrated that all secondary transport systems in C. f ervidus use Na\(^+\) instead of H\(^+\) as coupling ions (2, 3). To use these secondary transport systems effectively, C. f ervidus requires a $\Delta$P$_{\text{Na}^+}$ generating system. All attempts to detect a secondary Na\(^+\)/H\(^+\) antiport system failed. The observations that in intact cells only a $\Delta$ψ and $\Delta$P$_{\text{Na}^+}$ can be detected between pH 5.5 and 8.0 and that the $\Delta$P$_{\text{H}^+}$ is close to zero also argue against the presence of a Na\(^+\)/H\(^+\) antiporter. The results on the Na\(^+\) efflux in intact cells and ATP-driven Na\(^+\) uptake in proteoliposomes clearly demonstrate that an F/V-type ATPase generates the $\Delta$P$_{\text{Na}^+}$. The concept of a Na\(^+\)-ATPase as the sole Na\(^+\)-translocating mechanism in C. f ervidus is supported by the observation that substrates that can function as energy sources do not function as substrates of Na\(^+\)-translocating decarboxylases but yield ATP by substrate level phosphorylation (G.S., unpublished).

Thermophilic organisms like C. f ervidus are confronted with serious energetic problems since their cytoplasmic membrane is drastically more permeable for ions, including protons, at the optimal temperature of growth than the membrane of their mesophilic counterparts. Bacillus stearothermophilus compensates for this increased H\(^+\) leakage by a very high respiratory chain H\(^+\)-translocating activity (12). Also the thermophiles PS3 (13, 14), the anaerobic Clostridium thermoaceticum, Clostridium thermoautotrophicum (15, 16), and Clostridium thermocellum (17) use both Na\(^+\) and H\(^+\) as coupling ions. These organisms are able to grow over a wider pH range than C. f ervidus but consequently are confronted with high maintenance energy requirements.

The use of a sodium instead of a proton motive force can be of important energetic advantage for C. f ervidus. Phospholipid membranes are 6–10 orders of magnitude less permeable for Na\(^+\) than for H\(^+\) (18, 19). By using Na\(^+\) as a sole coupling ion in vectorial energy-transducing processes and by avoiding H\(^+\) cycling, metabolic energy can be spared. In doing so the organism has to pay a price since it is unable to regulate the internal pH, which narrows the pH range of growth. So far, the bacterium C. f ervidus is unique by relying completely on Na\(^+\) as coupling ion for primary and secondary transport processes. Although a primary Na\(^+\) pump and a Na\(^+\)-driven ATP synthase have been reported for Propionigenium modestum (20) and Acetobacterium woodii (21), the presence or absence of a Na\(^+\)/H\(^+\) antiporter and the nature of the coupling ion in secondary solute transport processes are not known. In P. modestum the latter process is even supposed to be coupled to H\(^+\) (5).