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The F- or V-Type Na\(^+\)-ATPase of the Thermophilic Bacterium

*Clostridium fervidus*

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*Clostridium fervidus* is a thermophilic, anaerobic bacterium which uses solely Na\(^+\) as a coupling ion for energy transduction. Important features of the primary Na\(^+\) pump (ATPase) that generates the sodium motive force are presented. The advantage of using a sodium rather than a proton motive force at high temperatures becomes apparent from the effect of temperature on H\(^+\) and Na\(^+\) permeation in liposomes.

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**TABLE 1.** Effect of monovalent cations on ATP hydrolysis

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate of ATP hydrolysis (%)</th>
<th>- Triton X-100</th>
<th>+ Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>100(^a)</td>
<td>100(^a)</td>
</tr>
<tr>
<td>10 mM NaCl</td>
<td></td>
<td>226</td>
<td>156</td>
</tr>
<tr>
<td>10 mM KCl</td>
<td></td>
<td>120</td>
<td>114</td>
</tr>
<tr>
<td>50 mM NaCl</td>
<td></td>
<td>417</td>
<td>217</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td></td>
<td>144</td>
<td>129</td>
</tr>
<tr>
<td>50 mM LiCl</td>
<td></td>
<td>387</td>
<td>213</td>
</tr>
<tr>
<td>50 mM RbCl</td>
<td></td>
<td>145</td>
<td>ND(^d)</td>
</tr>
<tr>
<td>50 mM choline chloride</td>
<td></td>
<td>135</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Corresponds with 52 nmol of P\(_{\text{m}}\)/min/mg of protein.
\(^b\) Corresponds with 107 nmol of P\(_{\text{m}}\)/min/mg of protein.
\(^d\) ND, not determined.

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**TABLE 2.** Activators and inhibitors of ATP hydrolysis in inside-out membrane vesicles of *C. fervidus*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rate of ATP hydrolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Triton X-100</td>
</tr>
<tr>
<td>Control</td>
<td>100(^b)</td>
</tr>
<tr>
<td>200 (\mu)M ortho-vanadate</td>
<td>95</td>
</tr>
<tr>
<td>200 (\mu)M DCCD(^f)</td>
<td>90</td>
</tr>
<tr>
<td>2 (\mu)M EDAC(^e)</td>
<td>ND</td>
</tr>
<tr>
<td>200 (\mu)M DES(^f)</td>
<td>98</td>
</tr>
<tr>
<td>5 (\mu)M Na(_2)SO(_4)</td>
<td>97</td>
</tr>
<tr>
<td>100 (\mu)M pCMBS</td>
<td>0.5</td>
</tr>
<tr>
<td>100 (\mu)M triphenyltin</td>
<td>20</td>
</tr>
<tr>
<td>25 mM K(_2)NO(_3)</td>
<td>40</td>
</tr>
<tr>
<td>25 mM Na(_2)SO(_4)</td>
<td>ND</td>
</tr>
<tr>
<td>25 mM Na(_2)SO(_4)</td>
<td>ND</td>
</tr>
<tr>
<td>100 (\mu)M bafilomycin A(_1)</td>
<td>ND</td>
</tr>
<tr>
<td>1 mM ADP</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^b\) Corresponds with 167 nmol of P\(_{\text{m}}\)/min/mg of protein.
\(^c\) Corresponds with 117 nmol of P\(_{\text{m}}\)/min/mg of protein.
\(^d\) ND, not determined.
\(^e\) Diethylstilbestrol.

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\(\text{Na}^+\) and \(\text{Li}^+\) are not substrates for the Na\(_+-\)translocating ATPase. In the presence of 5 mM EDTA, only 10% residual activity was observed. Mg\(^2+\) could be replaced by Mn\(^2+\) (90% activity), could be substituted for only partially by Ca\(^2+\) (50% activity), but could not be substituted for by Co\(^2+\), Ni\(^2+\), or Zn\(^2+\). The simultaneous presence of Mg\(^2+\) and Ca\(^2+\) enhanced ATP hydrolysis to intermediate values (75% activity).

In the absence of Triton X-100, NaCl and LiCl stimulated ATP hydrolysis approximately fourfold, compared with KCl, RbCl, or choline chloride. In the presence of Triton X-100, the stimulating effect of NaCl and LiCl was lower but was still significantly higher than the effect of KCl (Table 1). The lower activation by NaCl and LiCl in the presence of Triton X-100 is an indication that the interaction between the F\(_o\) and F\(_i\) parts is affected by the detergent. Stringent precautions were not taken to exclude Na\(^+\) from the assay buffer, and as a result, the zero concentrations in Table 1 reflect 50 \(\mu\)M (contaminating) Na\(^+\). Altogether, the activation of ATPase activity by Na\(^+\) and Li\(^+\) is a further indication of the existence of a Na\(^+\)-translocating ATPase.

A number of classical ATPase inhibitors/activators were tested for their effect on the Na\(_+-\)translocating ATPase. A few of the most potent inhibitors/activators are listed in Table 2. It is striking that choline chloride and Mn\(^2+\) are also inhibitors, whereas K\(_2\)SO\(_4\) and Mg\(^2+\) are activators. This suggests that the thermophilic ATPase is characterized by both a Na\(_+-\) and a K\(_+\)-type transport system.
VOL.

with 115 nmol of mM Tris-ATP membrane vesicles. The inhibitory effect, inhibited by tested for their effect in absence of Triton X-100, in the presence and tested for their effect in absence of Triton X-100, in the presence (●) and absence of 50 mM NaCl (○). One hundred percent activity corresponds with 115 nmol of P/min/mg of protein.

FIG. 1. Effect of pH on the rate of ATP hydrolysis in inside-out membrane vesicles. P_i released was measured upon the addition of 2 mM Tris-ATP at 45°C in the absence of Triton X-100, in the presence (●) and absence of 50 mM NaCl (○). One hundred percent activity corresponds with 115 nmol of P/min/mg of protein.

tested for their effect on ATPase activity in the presence and absence of Triton X-100 (Table 2). ATPase activity was not inhibited by the P-type ATPase inhibitor ortho-vanadate, while bafilomycin A_1 had a small effect. F-type ATPase inhibitors, such as N,N'-dicyclohexylcarbodiimide and N_3^-, had a small inhibitory effect, whereas diethylsilibestrol inhibited ATPase activity moderately. Strikingly, the ATPase was inhibited most strongly by the V-type ATPase inhibitor NO_3^- and was strongly activated by SO_4^{2-}. SH reagents, such as triphenyltin and pCMBS, inhibited P_i release, as did the product of the reaction, ADP.

Polyclonal antibodies directed against the β subunit of the Escherichia coli F_{0}F_{1} ATP synthase showed weak cross-reactivity with membranes of C. fervidus. A single faint band with an apparent molecular mass of 55 kDa was observed (in agreement with the mass of the β subunit of an F- or V-type ATPase).

The pH optimum of the ATPase was 6.0, in both the presence and the absence of Na^+ (Fig. 1). The ATPase activity was not inhibited by ortho-vanadate, in either the absence or the presence of Na^+ and over the entire pH range tested (data not shown), which is consistent with the suggestion that a single ion-translocating ATPase is present in the membrane of C. fervidus.

ATPase hydrolysis showed a high optimum temperature and temperature stability (Fig. 2), as would be expected of an enzyme from a thermophile. The optimum temperature of ATPase activity was 68°C, whereas the activation energy was 64 kJ/mol. ATPase hydrolysis activity was enhanced in the presence of Triton X-100, but the enzyme was less temperature stable. The inactivation temperature T_i (defined as the temperature at which 50% of activity is lost within 10 min) was 67°C and 72°C in the presence and absence of Triton X-100, respectively. At 45°C the ATPase was fully stable for at least 1 h.

The ATPase activities described in this study correspond with the enzyme that has been shown to translocate Na^+ upon the addition of ATP (13). No indications were obtained for the presence of a (additional) H^+-pumping ATPase. Other bacteria also possess Na^+-ATPases or Na-translocating ATP synthases but maintain H^+ cycling at the same time (for examples, see references 3–5, 7, 11, 16). The best characterized Na^+-ATPase is that of Propionigenium modestum (7, 8). The Na^+-ATPase from C. fervidus differs from the P. modestum ATPase in several respects. (i) The C. fervidus enzyme functions as a Na^+-extruding ATPase, rather than an ATP synthase consuming the electrochemical gradient of sodium ions. (ii) The ATPase of C. fervidus is stimulated to the same extent by LiCl as by NaCl. (iii) The pH dependence of the enzyme is not influenced by the presence of Na^+. The pH optima of the Na^+-ATPase of P. modestum are 6.0 and 7.0 in the absence and presence of Na^+, respectively, which is

FIG. 2. (A) Effect of temperature on the rate of ATP hydrolysis. ATPase activity was measured in inside-out membrane vesicles at pH 6.0 in the presence of 50 mM NaCl upon the addition of 2 mM Na_2-ATP and in the absence (□) or presence (●) of 0.1% Triton X-100. One hundred percent activities represent 1.37 and 0.65 μmol of P_i/min/mg of protein with and without Triton X-100, respectively. (B) Arrhenius plots of the temperature dependency of ATP hydrolysis with (●) or without (□) Triton X-100.
indicator of the ability of the enzyme to pump H\textsuperscript{+} at low Na\textsuperscript{+} concentrations. (iv) The ATPase of \textit{C. fervidus} is inhibited by NO\textsubscript{3} and activated by SO\textsubscript{4}\textsuperscript{2-}. The latter characteristics have also been described for the V-type ATPases found in \textit{Archaean} (9, 15), \textit{Thermus thermophilus} (17), and \textit{Enterooccus hirae} (5).

\textbf{Na\textsuperscript{+} and H\textsuperscript{+} fluxes into liposomes.} The effects of increased temperature on H\textsuperscript{+} and Na\textsuperscript{+} influx were examined in order to discern whether the use of Na\textsuperscript{+} as the sole coupling ion would have some bioenergetic advantage for thermophilic fermentative organisms like \textit{C. fervidus}. Liposomes were prepared in 10 mM potassium phosphate (pH 7.0) containing 100 mM KCl, 5 mM MgSO\textsubscript{4}, and 100 \muM pyranine and were diluted 100-fold into buffer with 1 mM NaCl and 100 mM N-methylglycinechloride. H\textsuperscript{+} influx in response to a membrane potential (inside negative) was started by the addition of valinomycin (200 nM) and was measured as a change in fluorescence of internal pyranine (2). The rate of Na\textsuperscript{+} influx was estimated under the same conditions, from the uptake of \textit{22Na} (43 MBq/liter) as determined by the filtration method (12). Liposomes prepared from phospholipids extracted from \textit{C. fervidus} showed almost the same H\textsuperscript{+} permeability as liposomes prepared from \textit{E. coli} phospholipids plus egg phosphatidylcholine (data not shown). In these liposomes and under the conditions employed, the Na\textsuperscript{+} influx was approximately 7 orders of magnitude lower than was the H\textsuperscript{+} influx (Fig. 3). Upon an increase in temperature from 20 to 38\degree C, the absolute H\textsuperscript{+} influx increased from 3.6 \times 10\textsuperscript{-8} to 14.2 \times 10\textsuperscript{-8} mol/s, while the Na\textsuperscript{+} flux increased from 3.3 \times 10\textsuperscript{-15} to 23.6 \times 10\textsuperscript{-15} mol/s.

The use of a Na\textsuperscript{+} (instead of a H\textsuperscript{+}) cycle for energy transduction could be of energetic advantage, particularly for an anaerobic thermophile in which the yield of metabolic energy per molecule of substrate is much less than that in aerobic organisms. Moreover, membrane-permeable pH-gradient-dissipating weak acids and bases are produced (1, 6). At higher temperatures bacterial membranes become more H\textsuperscript{+} permeable (Fig. 3). Therefore, less energy has to be invested in maintaining a Na\textsuperscript{+} gradient than in maintaining a H\textsuperscript{+} gradient, especially at elevated temperatures. This advantage will be valid only if no H\textsuperscript{+} cycling occurs at the same time, which turns out to be the case with \textit{C. fervidus}. We propose that the exclusive use of Na\textsuperscript{+} as a coupling ion in energy transduction is an adaptation by \textit{C. fervidus} to environmental conditions, in order to minimize its bioenergetic costs.

\section*{Acknowledgments}

We thank K. H. Altendorf from the University of Osnabrück, Germany, for the gift of bafilomycin A\textsubscript{1} and of polyclonal antibodies directed against the β subunit of the \textit{E. coli} ATPase and J. Knol for performing the sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting experiments.

\section*{References}


