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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.**

Clostridium fervidus is a thermophilic anaerobe that ferments peptides and amino acids (10). Metabolic energy is obtained from processes that lead to ATP synthesis by substrate-level phosphorylation, i.e., glycolysis and degradation of amino acids, and not by decarboxylation of substrates by membrane-bound ion-pumping decarboxylases (unpublished results; 3). Amino acid transport in *C. fervidus* is coupled exclusively to sodium ions, indicating that the electrochemical gradient of sodium ions plays a pivotal role in the energy transduction of the organism (12, 14). Recent studies have indicated that energy transduction at the membrane of *C. fervidus* is exclusively dependent on Na^+ cycling (13). Strikingly, Na^+/H^+ exchange activity could not be detected in cells or membrane vesicles of this bacterium (14). The present paper elaborates further on the bioenergetics of *C. fervidus* (i) by characterizing the Na^+-translocating ATPase and (ii) by comparing the ion permeabilities of artificial phospholipid membranes.

ATPase activity in inside-out membrane vesicles. ATPase activity in inside-out membrane vesicles was assayed as described previously (12). ATP, dATP, and GTP were hydrolyzed equally rapidly, whereas the rate of UTP hydrolysis was about 30% of the rate of ATP hydrolysis. Mg^{2+} ions were required for ATP hydrolysis, with an optimum concentration of Mg^{2+} of 1 mM (100% activity). 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_0 and F_1 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 μM (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

TABLE 1. Effect of monovalent cations on ATP hydrolysis

Addition	Rate of ATP hydrolysis (%)	
	− Triton X-100	+ Triton X-100
0	100[a]	100[b]
10 mM NaCl	226	156
10 mM KCl	120	114
50 mM NaCl	417	217
50 mM KCl	144	129
50 mM LiCl	387	213
50 mM RbCl	145	ND[c]
50 mM choline chloride	135	ND

[a] Corresponds with 52 nmol of P_i/min/mg of protein.
[b] Corresponds with 107 nmol of P_i/min/mg of protein.
[c] ND, not determined.

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

Compound	Rate of ATP hydrolysis (%)[a]	
	+ Triton X-100	− Triton X-100
Control	100[b]	100[c]
200 μM *ortho*-vanadate	95	104
200 μM DCCD[d]	90	77
2 mM EDAC[e]	ND[f]	86
200 μM DES[g]	98	45
5 mM NaN_3	97	75
100 μM pCMBS	0.5	ND
100 μM triphenyltin	20	ND
25 mM K_2NO_3	40	8
25 mM Na_2SO_3	ND	400
25 mM Na_2SO_4	ND	27
100 μM bafilomycin A_1	ND	77
1 mM ADP	35	35

[a] ATPase activity was measured in the presence and absence of 0.05% Triton X-100. Data presented are corrected for side effects of ionic strength and/or $[Na^+]$.
[b] Corresponds with 167 nmol of P_i/min/mg of protein.
[c] Corresponds with 117 nmol of P_i/min/mg of protein.
[d] *N,N'*-Dicyclohexylcarbodiimide.
[e] 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide.
[f] ND, not determined.
[g] Diethylsilbestrol.

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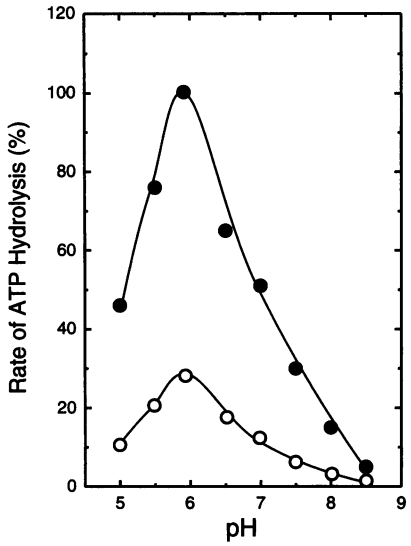

FIG. 1. Effect of pH on the rate of ATP hydrolysis in inside-out membrane vesicles. P_i released was measured upon the addition of 2 mM Tris-ATP at 45°C in the absence of Triton X-100, in the presence (●) and absence of 50 mM NaCl (○). One hundred percent activity corresponds with 115 nmol of P_i/min/mg 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 diethylsilbestrol 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_3^{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_0F_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 activity 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 as 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

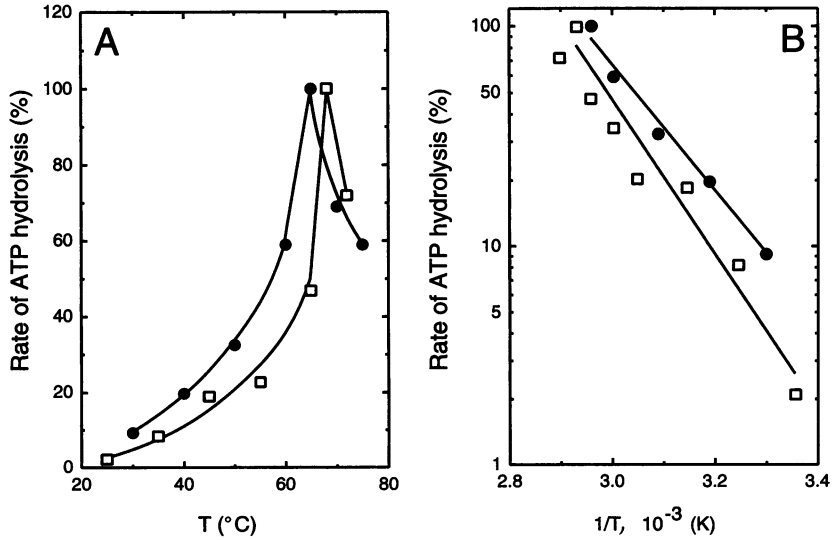

FIG. 2. (A) Effect of temperature on the rate of ATP hydrolysis. ATPase activity was measured in inside-out membrane vesicles at pH 6.0 in the presence of 50 mM NaCl upon the addition of 2 mM Na_2-ATP and in the absence (□) or presence (●) of 0.1% Triton X-100. One hundred percent activities represent 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.

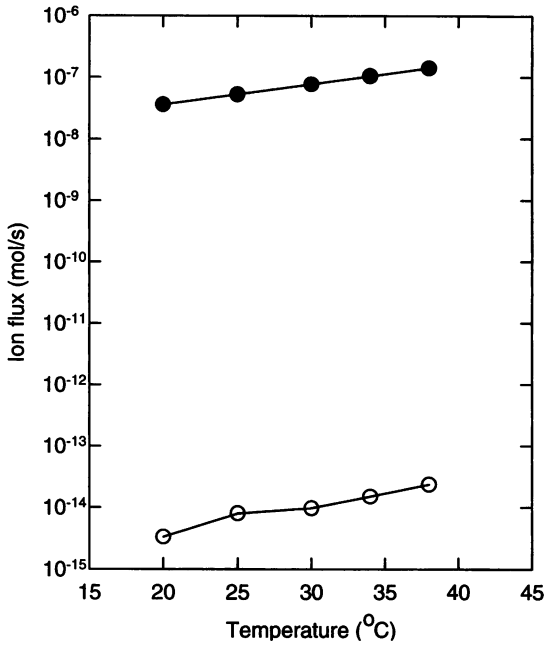

FIG. 3. Effect of temperature on the passive influx of Na^+ (○) and H^+ (●) into liposomes composed of *E. coli* phospholipids plus egg phosphatidylcholine (3/1 [wt/wt]). H^+ and Na^+ influxes were measured as described in the text; 12 μg of liposomal phospholipid was used per assay.

indicative of the ability of the enzyme to pump H^+ at low Na^+ concentrations. (iv) The ATPase of *C. fervidus* is inhibited by NO_3 and activated by SO_3^{2-}. The latter characteristics have also been described for the V-type ATPases found in *Archaea* (9, 15), *Thermus thermophilus* (17), and *Enterococcus hirae* (5).

Na^+ and H^+ fluxes into liposomes. The effects of increased temperature on H^+ and Na^+ influx were examined in order to discern whether the use of Na^+ as the sole coupling ion would have some bioenergetic advantage for thermophilic fermentative organisms like *C. fervidus*. Liposomes were prepared in 10 mM potassium phosphate (pH 7.0) containing 100 mM KCl, 5 mM $MgSO_4$, and 100 μM pyranine and were diluted 100-fold into buffer with 1 mM NaCl and 100 mM *N*-methylglucamine-chloride. H^+ 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^+ influx was estimated under the same conditions, from the uptake of $^{22}Na^+$ (43 MBq/liter) as determined by the filtration method (12). Liposomes prepared from phospholipids extracted from *C. fervidus* showed almost the same H^+ permeability as liposomes prepared from *E. coli* phospholipids plus egg phosphatidylcholine (data not shown). In these liposomes and under the conditions employed, the Na^+ influx was approximately 7 orders of magnitude lower than was the H^+ influx (Fig. 3). Upon an increase in temperature from 20 to 38°C, the absolute H^+ influx increased from 3.6×10^{-8} to 14.2×10^{-8} mol/s, while the Na^+ flux increased from 3.3×10^{-15} to 23.6×10^{-15} mol/s.

The use of a Na^+ (instead of a H^+) 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^+ permeable (Fig. 3). Therefore, less energy has to be invested in maintaining a Na^+ gradient than in maintaining a H^+ gradient, especially at elevated temperatures. This advantage will be valid only if no H^+ cycling occurs at the same time, which turns out to be the case with *C. fervidus*. We propose that the exclusive use of Na^+ as a coupling ion in energy transduction is an adaptation by *C. fervidus* to environmental conditions, in order to minimize its bioenergetic costs.

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