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An Na^+-Pumping V_1V_0-ATPase Complex in the Thermophilic Bacterium *Clostridium fervidus*

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Energy transduction in the anaerobic, thermophilic bacterium *Clostridium fervidus* relies exclusively on Na^+ as the coupling ion. The Na^+ ion gradient across the membrane is generated by a membrane-bound ATPase (G. Speelmans, B. Poolman, T. Abee, and W. N. Konings, J. Bacteriol. 176:5160–5162, 1994). The Na^+-ATPase complex was purified to homogeneity. It migrates as a single band in native polyacrylamide gel electrophoresis and catalyzes Na^+-stimulated ATPase activity. Denaturing gel electrophoresis showed that the complex consists of at least six different polypeptides with apparent molecular sizes of 66, 61, 51, 37, 26, and 17 kDa. The N-terminal sequences of the 66- and 51-kDa subunits were found to be significantly homologous to subunits A and B, respectively, of the Na^+-translocating V-type ATPase of *Enterococcus hirae*. The purified V_1V_0 protein complex was reconstituted in a mixture of *Escherichia coli* phosphatidylethanolamine and egg yolk phosphatidylcholine and shown to catalyze the uptake of Na^+ ions upon hydrolysis of ATP. Na^+ transport was completely abolished by monensin, whereas valinomycin stimulated the uptake rate. This is indicative of electrogenic sodium transport. The presence of the protonophore SF6847 had no significant effect on the uptake, indicating that Na^+ translocation is a primary event and in the cell is not accomplished by an H^+-translocating pump in combination with an Na^+-H^+ antiporter.

Membrane-bound ATPases play an important role in H^+ and Na^+ cycling in all bacteria and archaea. The enzymes couple the synthesis or hydrolysis of ATP to the translocation of H^+ or Na^+ across the cell membrane. In respiratory and phototrophic bacteria, ATPases normally function in the synthesis mode, i.e., the enzymes use the H^+ or Na^+ electrochemical gradient to drive ATP synthesis. In fermentative bacteria, the enzymes usually work in the opposite direction, i.e., an H^+ or Na^+ electrochemical gradient is generated upon hydrolysis of ATP. The ATPases can be categorized into F-type and V-type ATPases (for reviews, see references 14 and 21). F-type ATPases are commonly found in chloroplasts and mitochondria of eukaryotes and in bacteria. In contrast, V-type ATPases are more abundant in endomembranes and some plasma membranes of eukaryotes and in archaea. V-type ATPases of the latter are now referred to as A-type ATPases (7). The only two known examples of bacterial V-type ATPases are the H^+-ATPase of *Thermus thermophilus* (33) and the Na^+-ATPase of *Enterococcus hirae* (27). Of both F- and V-type ATPases, H^+- and Na^+-pumping examples have been described. The architectures of both types of ATPases are similar and consist of a soluble part (F_1, V_1, A_1) and an integral membrane part (F_0, V_0, A_0). The former contains the ATP binding sites, while the latter mediates the translocation of the ion across the membrane.

Clostridium fervidus is a strictly anaerobic and thermophilic bacterium that ferments peptides and amino acids and grows optimally at a temperature of 68°C (16) but only in a very narrow pH range (6.3 to 7.7). Studies of the energy transduction processes in *C. fervidus* have demonstrated that this bacterium does not use an H^+ cycle. An Na^+-H^+ exchanger could not be detected, and all amino acid uptake systems that have been tested use Na^+ exclusively as the coupling ion (22, 23). Furthermore, *C. fervidus* generates a large electrochemical gradient of Na^+ but not of protons. The sodium motive force is generated by an Na^+-ion pumping ATPase. Experiments with membrane vesicles showed that the hydrolysis of ATP was stimulated by the presence of Na^+ in the medium, and ATP-driven accumulation of Na^+ could be demonstrated after reconstitution of the solubilized membranes in proteoliposomes. Inhibitor studies of the ATPase activity did not conclusively reveal which type of ATPase is present in the membrane of *C. fervidus*. The ATPase activity was moderately sensitive to typical F-type inhibitors like *N*,*N*′-dicyclohexylcarbodiimide (DCCD), diethylstilbestrol (DES), and azide, while the V-type inhibitors nitrate and sulfate were the most inhibitory. In accordance with the properties of other V-type ATPases, sulfite stimulated the activity. On the other hand, the V-type inhibitor 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC) affected the enzyme only marginally (24). These studies may have been hampered by the presence of other ATPase activities in the cytoplasmic membranes.

In this paper, we describe the purification of the Na^+-ATPase of *C. fervidus* and report its subunit structure. N-terminal sequencing of the subunits and immunoanalysis confirm that the ATPase is a V-type ATPase. The purified enzyme is reconstituted in proteoliposomes, and transport studies show that the ATPase indeed is a primary sodium ion pump.

MATERIALS AND METHODS

Materials. Malachite green was obtained from Serva Feinbiochemica (Heidelberg, Germany). $^{22}Na^+$ (27.2 TBq/mol) was purchased from Amersham (Amersham, United Kingdom). All other reagents were from Sigma.

Purification of the Na^+-ATPase. *C. fervidus* ATCC 43024 was grown anaerobically at pH 7.2 and 68°C in tryptone-yeast extract-glucose medium as described previously (16) and harvested in the late log phase of growth (A_{660}, 0.5 to 0.6). Inside-out membrane vesicles were prepared as described previously (24) and stored in liquid N_2 until use. For purification of the ATPase, membranes (40 mg)

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were thawed quickly and suspended in 3 ml of 50 mM *N*-2-morpholinepropanesulfonic acid (MOPS; pH 7.0)–50 mM KCl–5 mM $MgCl_2$–5% glycerol–0.1% Triton X-100–2 mM dithiothreitol (buffer A) plus 2 mM phenylmethylsulfonyl fluoride. The membranes were solubilized by incubation for 10 min at 45°C after the addition of the detergent Triton X-100 to a final concentration of 3%. Nonsolubilized material was removed by ultracentrifugation at 350,000 × *g* for 30 min at 4°C. The supernatant was diluted with buffer A to obtain a Triton X-100 concentration of 1% and subsequently loaded onto an anion-exchange column (MonoQ HR 5/5; Pharmacia) equilibrated at room temperature with buffer A at a flow rate of 0.5 ml/min. After washing with 10 ml of buffer A, the column was eluted with a 50 to 350 mM KCl gradient in buffer A. Fractions were collected every minute, immediately stored on ice, and assayed for ATPase activity. The active fractions were pooled and, after the addition of 0.2 mg of phospholipids per ml (phosphatidylethanolamine [PE] and phosphatidylcholine [PC] at a ratio of 3:1), concentrated to 200 μl by ultrafiltration at 4°C (Microconcentrator-50; Amicon). The concentrated solution was loaded onto a gel filtration column (Superose 6; Pharmacia) equilibrated at room temperature with buffer A containing 150 mM KCl and 0.2 mg of phospholipids per ml and eluted with the same buffer at a flow rate of 0.3 ml/min. Fractions of 0.5 ml were collected on ice and assayed for ATPase activity. Active fractions were pooled and immediately reconstituted in liposomes. The protein concentration in the fractions was determined by the method of Dulley and Grieve (4), with bovine serum albumin as a standard.

Assay of ATPase activity. ATP hydrolysis was estimated from the amount of P_i released as described previously (3), with modifications. The volume of the protein samples was scaled down to 30 μl for use in microtiter plates. The ATPase activity was determined in the presence and absence of 10 mM NaCl in 50 mM *N*-2-morpholineethanesulfonic acid (MES; pH 6.0) containing 5 mM $MgCl_2$, 15 mM KCl, 5% glycerol, and 0.01% Triton X-100 (ATPase assay buffer). Samples were incubated at 45°C, after which the reaction was started by the addition of Tris-ATP (3 mM final concentration) and allowed to proceed for 10 min. Subsequently, 200 μl of the malachite green molybdate reagent kept at room temperature was added. After additional incubation for 5 min at room temperature, the reaction was stopped with 30 μl of a 34% citric acid solution. The optical densities of the samples in the microtiter plates were read at 620 nm in a Titertek multiscan spectrophotometer. For ATPase activity staining in native polyacrylamide gel electrophoresis (PAGE), the gel was incubated for 20 min at 45°C in ATPase assay buffer supplemented with 50 mM NaCl and 3 mM Tris-ATP. Liberated P_i was made visible by staining the gel for 5 min with malachite green molybdate reagent before fixation in 34% citric acid.

Electrophoresis and blotting. For native gel electrophoresis, a gradient gel (3.5 to 11%) based on a method described by Schägger and von Jagow (20) was used. The anode buffer contained 50 mM MES-HCl (pH 6); the cathode buffer contained 50 mM MES–Bis-Tris-propane (pH 6) and 0.1% Triton X-100. The samples were gently mixed 1:1 with sample buffer (1% Triton X-100, 10% glycerol, 0.1 M MES [pH 6], 0.1% bromophenol blue). The gel was run at a constant current of 25 mA for 4 h. Proteins were visualized after Western blotting (immunoblotting) with the Western-Light protein detection kit (Tropix). For N-terminal sequencing, the subunits were separated by sodium dodecyl sulfate (SDS)-PAGE of the purified complex and subsequently electroblotted onto polyvinylidene fluoride membranes (Immobilon transfer; Millipore). After staining with Coomassie brilliant blue, the protein bands were cut out of the blot and the N-terminal amino acid residues were determined with a peptide sequenator (Applied Biosystems model 470A).

Reconstitution of the ATPase. A mixture of *Escherichia coli* PE (washed with acetone-ether) and egg yolk PC at a ratio of 3:1 (wt/wt), dissolved in chloroform, was dried under vacuum and suspended to a concentration of 20 mg/ml in 50 mM MOPS (pH 6.0)–5 mM $MgCl_2$ (buffer B). The mixture was stored in liquid nitrogen until use. Freshly purified ATPase (1.4 mg) was added to 30 mg of PE-PC solubilized with 45 mg of Triton X-100 in a volume of 9 ml. The mixture was allowed to equilibrate for 30 min at room temperature. Triton X-100 was removed by adsorption to Biobeads as described previously (18). The suspension was continuously mixed by rotation at 4°C for 3 h after the addition of 15 mg of Biobeads per 1 mg of Triton X-100, and this procedure was repeated twice with 16- and 3-h incubation times. The solution was passed through glass wool to remove excessive Biobeads. The proteoliposomes were collected by centrifugation at 350,000 × *g* for 1 h at 4°C and resuspended in buffer B to a concentration of 20 mg of phospholipid per ml. After a freeze-thaw step, the liposomes were extruded by two successive passages through 400-nm and 200-nm polycarbonate filters.

Transport assays. The proteoliposomes were diluted 1:1 with buffer B (final concentration, 10 mg of phospholipids per ml) at 45°C. $^{22}Na^+$ was added (final concentration, 0.44 μM) and allowed to equilibrate. After 2 min, Tris-ATP (final concentration, 3 mM) was added. In control experiments, ATP was omitted from the reaction mixture. At given time intervals, 100-μl samples were taken, diluted into 2 ml of ice-cold KCl (0.1 M), and immediately filtered through cellulose-nitrate filters (Millipore; 0.45-μm pore size). Filters were washed once with 2 ml of cold KCl (0.1 M) and transferred to scintillation vials. Scintillation fluid was added, and the radioactivity was measured with a scintillation counter.

TABLE 1. Purification of the Na^+-ATPase of *C. fervidus*

Fraction	Amt of protein (mg)	% Activity[a]	Sp act (nmol/min/mg)	Na^+ stimulation[b]
Membranes	20.8	100	37	1.8
Solubilized membranes	12.4	118	74	1.9
Anion-exchange chromatography	6.6	58	68	4.4
Gel filtration chromatography	2.1	24	88	3.9

[a] ATPase activity in the presence of 50 mM NaCl and 0.2 mg of phospholipids per ml.
[b] Ratio of the ATPase activity in the presence and absence of 50 mM NaCl.

RESULTS

Purification of the Na^+-ATPase. Cytoplasmic membranes isolated from *C. fervidus* by French press treatment and resuspended in buffer A supplemented with 2 mM phenylmethylsulfonyl fluoride were solubilized with 3% Triton X-100 as described in Materials and Methods. The procedure solubilizes about 60% of the protein from the membranes with a twofold increase in specific ATPase activity (Table 1). The increase upon solubilization may be a combined effect of differential extraction of ATPase activity from the membranes and improved accessibility of ATPases by solubilization. The Na^+ stimulation of the ATPase activity in the membranes and the solubilized fraction remains the same, indicating no enrichment of Na^+-dependent ATPase activity. The solubilized fractions were loaded onto an anion-exchange column (MonoQ) equilibrated in buffer A, washed with the same buffer, and eluted with a linear gradient of KCl from 50 to 350 mM. ATPase activity in the fractions was measured in the presence and absence of 10 mM NaCl. A single peak of Na^+-stimulated ATPase activity eluted at 200 mM KCl (Fig. 1). The fractions containing the highest ATPase activity (fractions 39 to 46) were pooled, and a mixture of phospholipids consisting of PE and PC at a ratio of 3:1 was added to a final concentration of

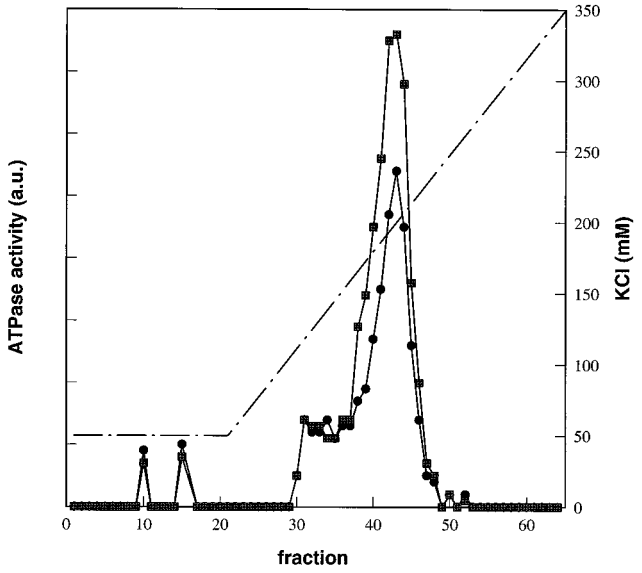

FIG. 1. Anion-exchange chromatography of solubilized membrane vesicles. The ATPase activity in arbitrary units (a.u.) of each fraction was assayed in the presence (■) and absence (●) of 50 mM NaCl. —·—, KCl gradient.

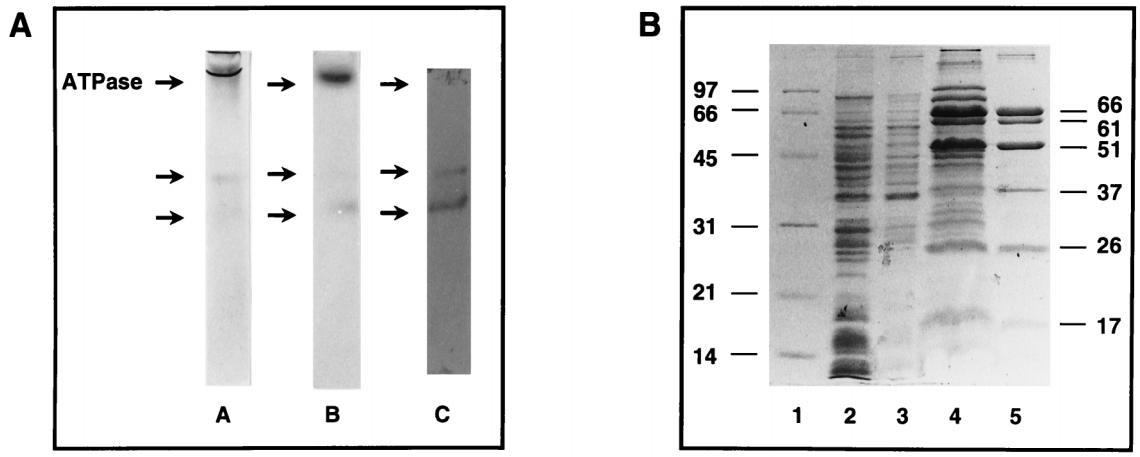

FIG. 2. Analysis of the purified ATPase by nondenaturing (A) and denaturing (B) gel electrophoresis. (A) Native PAGE. Proteins in the gel were visualized by silver staining (lane A), activity staining (lane B), and cross-reaction with a polyclonal antibody against the 60-kDa subunit B of the *S. cerevisiae* V-type ATPase (lane C). Each lane contains 30 μg of purified ATPase. The holoenzyme (ATPase) and two minor bands that run at a higher mobility are indicated by arrows. (B) SDS-PAGE. Proteins in the gel (12% polyacrylamide) were visualized by Coomassie brilliant blue R staining. Lanes: 1, molecular weight markers; 2, cytoplasmic membrane vesicles (20 μg); 3, solubilized membrane vesicles (20 μg); 4, pool of the MonoQ column (40 μg); 5, pool of the Superose 6 column (30 μg). Numbers on the left and right indicate molecular weights (in thousands).

0.2 mg/ml. The addition of phospholipids stabilized the protein and had a significant effect on the activity in the presence of Na^+. The stimulation of the ATPase activity increased from about a factor of 1.5 in the absence of phospholipids (Fig. 1) to more than a factor of 4 in the presence of added phospholipids (Table 1). The stimulation of the ATPase activity by Na^+ increased more than twofold in the anion-exchange step, showing enrichment of the Na^+-ATPase. About half of the protein is lost in this step with no increase in specific activity, indicating significant loss of Na^+-ATPase activity (Table 1). The pooled fractions were concentrated by ultrafiltration, and the Na^+-ATPase was further purified by size exclusion chromatography on a Superose 6 column. The ATPase activity eluted in the void volume (data not shown). The size exclusion chromatography step reduced the amount of protein by a factor of 3 with a small increase in specific activity, again indicating significant loss of ATPase activity. Na^+ stimulation was similar to that observed after anion-exchange chromatography.

The protein fraction thus obtained migrated as a single band on a nondenaturing polyacrylamide gel (Fig. 2A, lane A) stained with silver. Two minor proteins bands were observed at higher mobilities. The activity of the complex could be demonstrated in situ by activity staining (Fig. 2A, lane B). The gel was incubated at 45°C in the presence of ATP, after which the P_i produced was visualized by staining the gel with malachite green molybdate reagent. The two minor protein bands running at a higher mobility (Fig. 2A, lane A) both hydrolyzed ATP, suggesting that they represent dissociated states of the complex. This conclusion is substantiated by the cross-reaction of the protein bands with a polyclonal antibody raised against the B subunit of the vacuolar ATPase of *Saccharomyces cerevisiae*, a V-type ATPase (Fig. 2A, lane C). Relative to the major band, the two minor bands show a strong cross-reaction when the relative amounts of protein visible in lane A are taken into consideration. It is likely that the two minor bands are dissociated fragments of the ATPase complex that still have the ability to hydrolyze ATP. Apparently, epitopes recognized by the antibodies are not accessible in the native ATPase complex but become accessible after dissociation of the fragments.

The protein composition in the different steps of the purification procedure was analyzed by SDS-PAGE (Fig. 2B). While the major subunits of the Na^+-ATPase were not readily visible in membrane vesicles (Fig. 2B, lane 2) or solubilized membranes (lane 3), a significant purification took place in the anion-exchange step (lane 4). The gel filtration step resulted in a distinct pattern of bands (lane 5), and the single band on the nondenaturing gel (Fig. 2A, lane A) suggests that the polypeptides observed in lane 5 are all subunits of the Na^+-ATPase. A minor fraction of protein remained on top of the gel and is most likely aggregated material. The complex consists of six different subunits with molecular masses of 66, 61, 51, 37, 26, and 17 kDa. The 51-kDa subunit is recognized specifically with the polyclonal antibody raised against the 60-kDa B subunit of the V-type H^+-ATPase of *S. cerevisiae* (data not shown).

N-terminal sequencing. The six subunits of the purified Na^+-ATPase were electroblotted onto a polyvinylidene difluoride membrane after SDS-PAGE, and the N-terminal amino acid residues were determined. No sequences were obtained for the 61- and 26-kDa subunits, presumably because of blocked N termini. The amino-terminal amino acid sequences of the 37- and the 17-kDa polypeptides were determined to be MDLTYFSQSIARVKAMENKML and AKEVVKQVIDVE RQAEEIVRQAQQKANE, respectively. No homology was found with subunits of other ATPases. The N-terminal sequences of the 66- and 51-kDa subunits were also determined and compared with the N-terminal sequences of the major subunits of several V-type and F-type ATPases from archaea, bacteria, and eukaryotes (Table 2). The 30-residue-long amino-terminal sequence of the 66-kDa subunit (subunit A) of the *C. fervidus* ATPase contains 10 amino acids that are found in all three bacterial V-type ATPases. Of these residues, seven are conserved in the A subunits of all five V-type ATPases listed. The highest similarity was found with the A subunit of the Na^+-translocating V-type ATPase of *E. hirae*, with as many as 15 identical and 5 similar amino acids. The similarity with the six β subunits of the listed F-type ATPases was clearly less. The N terminus of the 51-kDa subunit (subunit B) again showed the highest similarity with the V-type subunits, with 15 of 27 amino acids identical and 9 amino acids similar to the sequence of subunit B from *E. hirae*. The sequence similarity between the B subunits of the V-type ATPases and the α

TABLE 2. N-terminal sequence alignment[a]

Subunit	Source	N-terminal sequence	Type	Reference
A	*Clostridium fervidus*	MKA---GGGGRIILVSGPLVVAEGMEHACMFDV	V	This work
	Enterococcus hirae	MQI------GKIIKVSGPLVMAENMSEASIQDM	V	27
	Thermus thermophilus	MIQ------GVIQKIAGPAVIAKGMLGARMYDI	V	28
	Sulfolobus acidocaldarius	MVSE-----GRVVRVNGPLVIADGMREAQMFEV	V	2
	Neurospora crassa	MAPQQNGAEVDGIHTGKIYSVSGPVVVAEDMIGVAMYEL	V	1
	Acetobacterium woodii	MAQNI----GKVVQVIGPVVDVKFQKDKLPKLN	F	6
	Propionigenium modestum	MENK-----GVITQIIGPVVDVTFENE-LPRIY	F	9
	Vibrio alginolyticus	MAT------GKIVQVIGAVVTVEFPQSNVPSVY	F	10
	Escherichia coli	MAT------GKIVQVIGAVVDVEFPQDAVPRVY	F	29, 30
	PS3	MTR------GRVIQVMGPVVDVKFENGHLPAIT	F	15
	Bovine mitochondria	SPSKAGAT-----TGRIVAVIGAVVDVQFDEG-LPPIL	F	31
B	*Clostridium fervidus*	M-L---REYRTIREIA-GPLMLVDGVEGAWYD	V	This work
	Enterococcus hirae	M-I---KEYRTIKEVV-GPLMAVEKVSGVKYE	V	27
	Thermus thermophilus	MDLLK-KEYTGITYIS-GPLLFVENAKDLAYG	V	28
	Sulfolobus acidocaldarius	MSLLNVREYSNISMIK-GPLIAVQGVSDAAYN	V	2
	Saccharomyces cerevisiae	MVLSDKELFAINKKAVEQGFNVKPRLNYNTNSGVN-GPLVILEKVKFPRYN	V	13
	Propionigenium modestum	MKIRPEEISGIIKTEIENYKKSLTVKTSGSVVQVGDGIAR-IYGLSNAKAG	F	9
	Vibrio alginolyticus	MQLNSTEISDLIKQRIESFEVVSEARNEGTIVSVSDGIIR-IHGLADVMQG	F	10
	Escherichia coli	MQLNSTEISELIKQRIAQFNVVSEAHNEGTIVSVSDGVIR-IHGLADCMQG	F	29, 30
	PS3	MSIRAEEISALIKQQIENYESQIQVSDVGTVIQVGTGIAR-AHGLDNVMSG	F	15
	Bovine mitochondria	EKTGTAEVSSILEERILGADTSVDLEETGRVLSIGDGIAR-VHGLRNVQAE	F	31

[a] The A (66-kDa) subunit of the *C. fervidus* ATPase was aligned with the larger subunits of V-type ATPases (termed A or α for bacterial or archael enzymes) and the β subunits of F-type ATPases. The B (51-kDa) subunit was compared with the equivalent V-type subunits (B or β) and the α subunit of F-type ATPases. Residues which are identical to the *C. fervidus* sequence are indicated in bold type.

subunits of the F-type ATPases is so poor that a meaningful alignment is not possible. In conclusion, the N-terminal sequences suggest that the 66- and 51-kDa subunits of the *C. fervidus* Na^+-ATPase correspond to the A and B subunits of V-type ATPases and are similar to the A and B subunits of the Na^+-ATPase of *E. hirae*, respectively.

Reconstitution in proteoliposomes. Optimal conditions for reconstitution of the purified Na^+-ATPase complex in liposomes were sought by mixing a freshly purified aliquot of enzyme with preformed liposomes treated with different amounts of the detergent Triton X-100 (19). The state of solubilization of the liposomes was monitored by measuring light scattering at a wavelength of 540 nm (Fig. 3A). Typically, the addition of Triton X-100 results at first in a swelling of the liposomes due to the incorporation of detergent molecules in the phospholipid bilayer. As the concentration of detergent increases, a gradual disintegration of the liposomes follows due to the formation of mixed phospholipid-detergent micelles. The purified ATPase was added to liposomes at the onset of solubilization and after complete solubilization (Fig. 3A). After incubation, the detergent was removed by treatment with polystyrene beads (Biobeads) and the proteoliposomes were

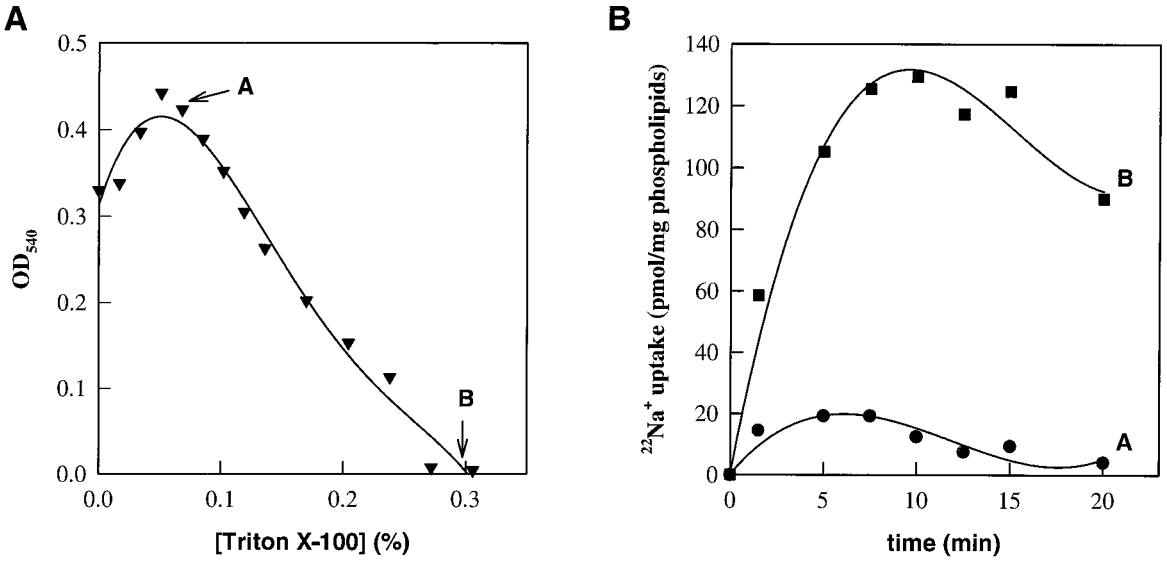

FIG. 3. Reconstitution in liposomes. (A) Preformed liposomes were titrated with increasing amounts of Triton X-100 until the optical density of the solution at 540 nm (OD_{540}) reached a stable value. (B) The uptake of $^{22}Na^+$ catalyzed by the Na^+-ATPase reconstituted in liposomes treated with the amounts of Triton X-100 indicated by arrows A (●) and B (■) in panel A is shown. The ATP concentration was 3 mM.

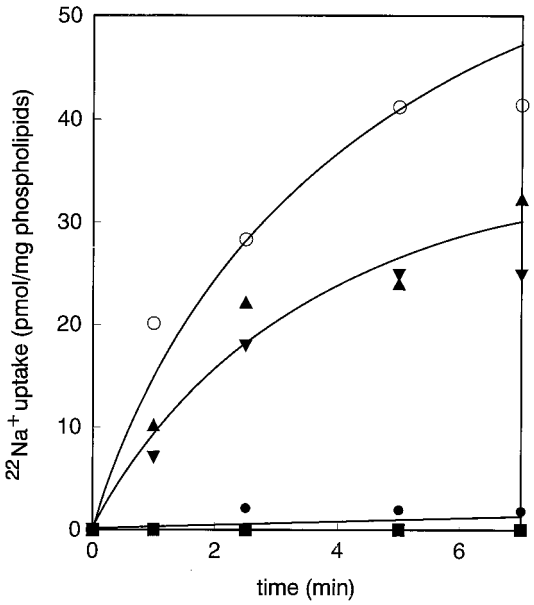

FIG. 4. Na^+ transport catalyzed by the purified ATPase of *C. fervidus* reconstituted in proteoliposomes. $^{22}Na^+$ uptake in proteoliposomes containing the purified Na^+-ATPase was measured in the presence (▼) and absence (●) of 3 mM ATP and in the presence of ATP with 1 μM valinomycin (○), 1 μM SF6847 (▲), and 1 μM monensin (■). The $^{22}Na^+$ concentration was 0.44 μM.

collected by ultracentrifugation. Figure 3B shows that proteoliposomes obtained from both procedures accumulated $^{22}Na^+$ upon the addition of ATP when assayed at 45°C, indicating that the purified Na^+-ATPase couples the hydrolysis of ATP to the transport of Na^+ ions. However, Fig. 3B shows that the proteoliposomes formed from completely solubilized liposomes have a significantly higher activity. Further studies were conducted with the purified enzyme complex reconstituted with detergent-solubilized lipids without preforming liposomes.

The ion specificity of the Na^+-ATPase was studied by examining the effect of different ionophores on the uptake of $^{22}Na^+$ into the proteoliposomes (Fig. 4). Upon addition of ATP, Na^+ ions are rapidly accumulated in the lumen of the proteoliposomes. As incubation continues, the rate of Na^+ uptake gradually declines, and after about 6 min, the internal Na^+ concentration reaches a steady state. The ATP-dependent accumulation of $^{22}Na^+$ is completely prevented by the sodium ionophore monensin. Dissipation of the membrane potential in the presence of the potassium ionophore valinomycin stimulated the uptake rate of the Na^+ ions about twofold, suggesting electrogenic Na^+ transport. The protonophore SF6847 had no obvious effect, showing that a proton gradient is not intermediate between the pump and the accumulation of Na^+ ions.

DISCUSSION

C. fervidus is a fermentative, thermophilic organism that uses the electrochemical gradient of the sodium ions as the driving force for the uptake of amino acids that are essential substrates for growth. Studies with membrane vesicles suggest that the organism uses a primary Na^+ pump to generate the transmembrane sodium gradient, and inhibition studies suggest that this ATPase might be a V-type ATPase (24). In the present study, the complete ATPase complex of *C. fervidus* was purified and reconstituted in proteoliposomes to confirm these observations. The purified, reconstituted complex drives the accumulation of Na^+ upon ATP hydrolysis in the presence of a protonophore, showing that Na^+ translocation is the primary event (Fig. 4). The molecular weight pattern of the subunits (see below) and the specific cross-reaction of the 51-kDa subunit with the polyclonal antibodies directed against the B subunit of the *S. cerevisiae* V-type ATPase provide further evidence that the enzyme belongs to the V-type ATPases. The strongest evidence is provided by the N-terminal sequences of the 66- and 51-kDa subunits (Table 2). The alignment of the amino-terminal sequences revealed a remarkably high similarity between the Na^+-ATPase from *C. fervidus* and the Na^+-translocating V-type ATPase from *E. hirae.*

Subunit structure. Known V-type ATPase holoenzymes are large complexes of 450 to 750 kDa with a variable number of subunits. All enzymes contain the A and B subunits that form the peripheral V_1 part and the proteolipid subunit of the membrane-bound V_0 part. Characteristic of the subunits of the V-type ATPases is the larger difference in size of the A and B subunits when compared to the corresponding α and β subunits of the F-type ATPases and the size of the proteolipid that is twice as big as the corresponding subunits c of the F-type ATPases (~8 kDa). The *C. fervidus* V-type Na^+-ATPase complex consists of at least six subunits with molecular sizes of 66 (subunit A), 61, 51 (subunit B), 37, 26, and 17 kDa (Fig. 2B). The 66- and 51-kDa subunits could be identified as the V_1 A and B subunits by homology of the N-terminal sequences of known V-type ATPases especially with the sequence of the Na^+-ATPase of *E. hirae* (Table 2). The *ntp* gene cluster for the V-type ATPase of *Enterococcus hirae* was sequenced and contains 11 genes, *ntpA* to *ntpK* (27). *ntpJ* was later shown to code for a component of the KtrII K^+-uptake system and not to be a subunit of the ATPase (12). The genes *ntpA* to *ntpE* and *ntpK* have been reported to correspond to proteins on SDS-PAGE with apparent molecular masses of 69 (subunit A), 52 (subunit B), 38 (subunit C), 29 (subunit D), 24 (subunit E), and 16 (subunit K) kDa, respectively (26). Therefore, besides the high degree of N-terminal sequence similarity, the major subunits A and B of the *E. hirae* and the *C. fervidus* enzymes are also similar in size. Furthermore, the sizes of subunit C and the proteolipid subunit K suggest that they correlate with the 38- and 17-kDa subunits of the *C. fervidus* ATPase. The subunit of 26 kDa could correspond to the D or E subunit. The different sizes of the A and B subunits and the presumed 17-kDa proteolipid subunit is in agreement with the characteristics of V-type ATPases and discriminates it from F-type ATPases. The subunit of 61 kDa between subunits A and B is not typical for V-type ATPases. A similar band has been described for the V-type ATPase from *Thermus thermophilus* (32). However, this band does not strictly copurify with the ATPase in *T. thermophilus.*

A V-type Na^+ ATPase. Na^+-translocating ATPases have been demonstrated in a couple of other bacteria, such as *Acetobacterium woodii* (17), *Propionigenium modestum* (11), *Vibrio* sp. strain ABE-1 (25), the bacterium *Vitreoscilla* (5), and *E. hirae* (8, 27). The latter example and the H^+-pumping V-type ATPase of *T. thermophilus* (33) were the only known bacterial V-type ATPases. At first, V-type ATPases were believed to be found only in the membranes of vacuolar vesicles of eukaryotes. The discovery of a third example of a vacuolar-type ATPase in the thermophilic, strictly anaerobic bacterium *C. fervidus* shows that this type of ATPase may be more common in the bacterial kingdom.

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REFERENCES

1. **Bowman, E. J., K. Tenney, and B. J. Bowman.** 1988. Isolation of genes encoding the Neurospora vacuolar ATPase. Analysis of vma-1 encoding the 67-kDa subunit reveals homology to other ATPases. J. Biol. Chem. **263:** 13994–14001.
2. **Denda, K., J. Konishi, T. Oshima, T. Date, and M. Yoshida.** 1988. Molecular cloning of the β-subunit of a possible non-F_1F_0 type ATPase from the acidothermophilic archaebacterium, *Sulfolobus acidocaldarius*. J. Biol. Chem. **263:**17251–17254.
3. **Driessen, A. J. M., L. Brundage, J. P. Hendrick, E. Schiebel, and W. Wickner.** 1991. Preprotein translocase of Escherichia coli: solubilization, purification and reconstitution of the integral membrane subunits SecY/E, p. 147–165. *In* A. M. Tartakoff (ed.), Methods in cell biology, vol. 34. Vectorial transport of proteins into and across membranes. Academic Press, Inc., Orlando, Fla.
4. **Dulley, J. R., and P. A. Grieve.** 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal. Biochem. **64:**136–141.
5. **Efiok, B. J. S., and D. A. Webster.** 1992. Sodium-coupled ATP synthesis in the bacterium *Vitreoscilla*. Arch. Biochem. Biophys. **292:**102–106.
6. **Forster, A., R. Daniel, and V. Mueller.** 1995. The Na(+)-translocating ATPase of Acetobacterium woodii is a F_1F_0-type enzyme as deduced from the primary structure of its beta, gamma and epsilon subunits. Biochim. Biophys. Acta **1229:**393–397.
7. **Ihara, K., T. Abe, K.-J. Sugimura, and Y. Mukohata.** 1992. Halobacterial A-ATP synthase in relation to V-ATPase. J. Exp. Biol. **172:**475–485.
8. **Kakinuma, Y., and K. Igarashi.** 1995. Electrogenic Na^+ transport by *Enterococcus hirae* Na^+-ATPase. FEBS Lett. **359:**255–258.
9. **Krumholz, L. R., U. Esser, and R. D. Simoni.** 1992. Characterization of the genes coding for the F_1F_0 subunits of the sodium-dependent ATPase of *Propionigenium modestum*. FEMS Microbiol. Lett. **70:**37–42.
10. **Krumholz, L. R., U. Esser, and R. D. Simoni.** 1989. Nucleotide sequence of the unc operon of Vibrio alginolyticus. Nucleic Acids Res. **17:**7993–7994.
11. **Laubinger, W., and P. Dimroth.** 1988. Characterization of the ATP synthase of *Propionigenium modestum* as a primary sodium pump. Biochemistry **27:** 7531–7537.
12. **Murata, T., K. Takase, I. Yamato, K. Igarashi, and Y. Kakinuma.** 1996. The ntpJ gene in the Enterococcus hirae ntp operon encodes a component of KtrII potassium transport system functionally independent of vacuolar Na^+-ATPase. J. Biol. Chem. **271:**10042–10047.
13. **Nelson, H., S. Mandiyan, and N. Nelson.** 1989. A conserved gene encoding the 57-kDa subunit of the yeast vacuolar H^+-ATPase. J. Biol. Chem. **264:** 1775–1778.
14. **Nelson, N.** 1992. The vacuolar H^+-ATPase—one of the most fundamental ion pumps in nature. J. Exp. Biol. **172:**19–27.
15. **Ohta, S., M. Yohda, M. Ishizuka, H. Hirata, T. Hamamoto, Y. Ottawara-Hamamoto, K. Matsuda, and Y. Kagawa.** 1988. Sequence and overexpression of subunits of adenosine triphosphate synthase in thermophilic bacterium PS3. Biochim. Biophys. Acta **933:**141–155.
16. **Patel, B. K. C., C. Monk, H. Littleworth, H. W. Morgan, and R. M. Daniel.** 1987. *Clostridium fervidus* sp. nov., a new chemoorganotrophic acetogenic thermophile. Int. J. Syst. Bacteriol. **37:**123–126.
17. **Reidlinger, J., and V. Mueller.** 1994. Purification of ATP synthase from *Acetobacterium woodii* and identification of a Na^+-translocating F_1F_0-type enzyme. Eur. J. Biochem. **223:**275–283.
18. **Richard, P., Y.-L. Rigaud, and P. Gräber.** 1990. Reconstitution of CF_1F_0 into liposomes using a new reconstitution procedure. Eur. J. Biochem. **193:**921–925.
19. **Rigaud, J.-L., and B. Pitard.** 1995. *In* J. R. Philippot and F. Schuber (ed.), Liposomes as tools in basic research and industry, p. 71–78. CRC Press, Inc., Boca Raton, Fla.
20. **Schägger, H., and G. von Jagow.** 1991. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. **199:**223–231.
21. **Senior, A. E.** 1990. The proton translocating ATPase of *Escherichia coli*. Annu. Rev. Biophys. Biophys. Chem. **19:**7–41.
22. **Speelmans, G., B. Poolman, and W. N. Konings.** 1993. Amino acid transport in the thermophilic anaerobe *Clostridium fervidus* is driven by an electrochemical sodium gradient. J. Bacteriol. **175:**2060–2066.
23. **Speelmans, G., B. Poolman, T. Abee, and W. N. Konings.** 1993. Energy transduction in the thermophilic anaerobic bacterium *Clostridium fervidus* is exclusively coupled to sodium ions. Proc. Natl. Acad. Sci. USA **90:**7975–7979.
24. **Speelmans, G., B. Poolman, T. Abee, and W. N. Konings.** 1994. The F- or V-type Na^+-ATPase of the thermophylic bacterium *Clostridium fervidus*. J. Bacteriol. **176:**5160–5162.
25. **Takada, Y., N. Fukunaga, and S. Sasaki.** 1991. Na^+-driven ATP synthesis of a psychrophilic marine bacterium, *Vibrio* sp. strain ABE-1. FEMS Lett. **82:**225–228.
26. **Takase, K., S. Kakinuma, L. Yamato, K. Konishi, K. Igarashi, and Y. Kakinuma.** 1994. Sequencing and characterization of the *ntp* gene cluster for vacuolar-type Na^+-translocating ATPase of *Enterococcus hirae*. J. Biol. Chem. **269:**11037–11044.
27. **Takase, K., L. Yamato, and Y. Kakinuma.** 1993. Cloning and sequencing of the genes coding for the A and B subunits of vacuolar-type Na^+-ATPase from *Enterococcus hirae*. J. Biol. Chem. **268:**11610–11616.
28. **Tsutsumi, S., K. Denda, K. Yokoyama, T. Oshima, T. Date, and M. Yoshida.** 1991. Molecular cloning of genes encoding major two subunits of a eubacterial V-type ATPase from *Thermus thermophilus*. Biochim. Biophys. Acta **1098:**13–20.
29. **Walker, J. E., M. Saraste, and N. J. Gay.** 1984. The unc operon. Nucleotide sequence, regulation and structure of ATP-synthase. Biochim. Biophys. Acta **768:**164–200.
30. **Walker, J. E., N. J. Gay, M. Saraste, and A. N. Eberle.** 1984. DNA sequence around the Escherichia coli unc operon. Completion of the sequence of a 17 kilobase segment containing asnA, oriC, unc, glmS and phoS. Biochem. J. **224:**799–815.
31. **Walker, J. E., L. Fearnly, N. J. Gay, B. W. Gibson, F. D. Northrop, S. J. Powell, M. Runswick, M. Saraste, and V. L. J. Tybulewicz.** 1985. Primary structure and subunit stoichiometry of F_1-ATPase from bovine mitochondria. J. Mol. Biol. **184:**677–701.
32. **Yokoyama, K., Y. Akabane, N. Ishii, and M. Yoshida.** 1994. Isolation of prokaryotic V_0V_1-ATPase from a thermophilic eubacterium *Thermus thermophilus*. J. Biol. Chem. **269:**12248–12253.
33. **Yokoyama, K., T. Oshima, and M. Yoshida.** 1990. *Thermus thermophilus* membrane associated ATPase. Indication of a eubacterial V-type ATPase. J. Biol. Chem. **265:**21946–21950.