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Properties of the V_0V_1 Na^+-ATPase from *Enterococcus hirae* and Its V_0 Moiety[1]

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We report here the large-scale purification of vacuolar (V_0V_1)-type Na^+-ATPase from *Enterococcus hirae* achieved using column anion-exchange and gel filtration chromatographies; 32 mg of purified enzyme comprising nine subunits, A, B, C, D, E, F, G, I, and K, was obtained from 20 liter culture. This amount is 500-fold larger than that reported in the previous paper [Murata, T., Takase, K., Yamato, I., Igarashi, K., and Kakinuma, Y. (1997) *J. Biol. Chem.* 272, 24885-24890]. The purified enzyme shows a high specific activity of ATP hydrolysis (35.7 μmol P_i released/min/mg protein). ATP-driven $^{22}Na^+$ uptake by reconstituted V_0V_1-proteoliposomes exhibited an apparent K_t value for Na^+ of 40 μM, which is near the K_m value (20 μM) for Na^+ of the ATP hydrolytic activity. Denatured gel electrophoresis revealed that six subunits, A, B, C, D, E, and F, are releasable as the V_1 subunit from the V_0V_1 complex by incubation with ethylenediaminetetraacetic acid; subunit G was not identified. The remaining V_0-liposomes containing I and K subunits catalyzed Na^+ uptake in response to potassium diffusion potential ($\Delta\psi$, inside negative); the K_t value for Na^+ of this reaction was estimated to be about 2 mM. Inhibition by *N,N'*-dicyclohexylcarbodiimide (DCCD) of the Na^+-ATPase activity and $\Delta\psi$-driven Na^+ uptake by the V_0-liposomes was prevented by the presence of Na^+, suggesting that the Na^+ binding site overlaps with the DCCD-reactive site.

Key words: *Enterococcus hirae*, Na^+ pumping properties, Na^+-translocating ATPase, vacuolar ATPase, V_0-liposomes.

Ion-motive ATPases that do not form phosphorylated intermediates are divided into two types: vacuolar V_0V_1-type ATPase (V-ATPase) and F_0F_1-ATPase (F-ATPase). V-ATPase is known as the proton pump of acidic organelles and plasma membranes in eukaryotic cells (*1-4*). V-ATPases also occur in bacteria (*5, 6*); archaebacterial proton-translocating ATPases are thought to mediate ATP synthesis (*6*). F-ATPase functions as an ATP synthase in mitochondria, chloroplasts, and oxidative bacteria (*7, 8*). Both ATPases are similar multisubunit enzymes consisting of a hydrophilic catalytic portion (V_1 and F_1, respectively) and a membrane-embedded portion (V_0 and F_0). The proteolipid of the membrane sector, which contains a DCCD-reactive acidic amino acid residue, is thought to be the pathway through which protons cross the membrane. In both cases, energy transfer between ATP hydrolysis/synthesis and proton movement calls for three catalytic sites in the catalytic moiety and multiple proton-translocating proteolipids in the membrane-embedded portion (*7-10*). Although it has been proposed that the eukaryotic V-ATPase reaction is unidirectional toward ATP hydrolysis coupled with proton pumping (*3*), there must be a common principle of energy-transduction by these ATPases. The rotation catalysis mechanism (*11*), experimentally verified for F_1-ATPase (*12-14*), is probably applicable to V-ATPase.

On the other hand, there are several notable differences between the V-ATPase and F-ATPase molecules. First, a sequence stretch (about 90 amino acid residues), not found in the sequence of the β subunit of *Escherichia coli* F-ATPase, is conserved in the N-terminal region of the V-ATPase A subunit. Second, the size of the eukaryotic V-ATPase proteolipid is generally 16-17 kDa, a size thought to have arisen by tandem duplication of the 7-8 kDa c subunit gene of F-ATPase (*3, 4, 15*). The size of the ATPase proteolipid is variable in archaebacteria; some archaea have the c subunit-like proteolipid (*6*), and the proteolipid of *Methanococcus jannaschii* ATPase is likely to be a triplication of the c subunit gene (*16*). Third, the resemblance between the amino acid sequences of V-ATPase minor subunits and those of F-ATPase minor subunits is conspicuous. For instance, from the sequence

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Abbreviations: CBB, Coomassie Brilliant Blue R-250; DCCD, *N,N'*-dicyclohexylcarbodiimide; DM, *n*-dodecyl α-D-maltoside; $\Delta\psi$, membrane potential.

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data, it remains unclear which V-ATPase subunit rotates as the γ subunit of F-ATPase rotates. Finally, the subunit composition of eukaryotic V-ATPase molecules is still unsettled. New proteolipids comparable to the V_0 subunit of V-ATPase were recently discovered in yeast (*17*) and *Caenorhabditis elegans* (*18*). In yeast, the vacuolar ATPase complex appears to contain at least 13 polypeptides, including three proteolipids (*19*). It is necessary to consider the significance of these characteristics in the molecular architecture of V-ATPase to elucidate the mechanism of eukaryotic V-ATPase.

A unique variant among V-ATPases is the enzyme from *Enterococcus hirae*, which transports Na^+ rather than H^+ under physiological conditions (*20, 21*). The *E. hirae* Na^+-translocating ATPase is encoded by a Na^+-responsive operon (designated *ntp*) consisting of eleven genes, *ntpFIKECGABDHJ* (*22-25*). The deduced amino acid sequences of the *ntp* gene products, except for *ntpH* and *ntpJ*, are very similar to those of archaebacterial ATPases and moderately similar to eukaryotic vacuolar ATPases. The *ntpJ* gene product is a component of the KtrII K^+ transport system, but not a subunit of the purified V_0V_1-ATPase (*26*). As there is no strong Shine-Dalgarno sequence upstream of the mini *ntpH* gene, we tentatively consider that *ntpH* is not an open reading frame. Recently, we purified an *E. hirae* Na^+-ATPase complex consisting of nine *ntp* gene products, *ntpA, -B, -C, -D, -E, -F, -G, -I*, and *-K*, but not *-H* and *-J*. ATP-driven electrogenic sodium ion transport was observed by the reconstituted proteoliposomes (*27*). The sodium-pumping V-ATPase of *E. hirae* thus can be used to elucidate the fundamental mechanism of V-ATPase equivalent to phylogenetically related V-ATPases. Although *E. hirae* V_0V_1 Na^+-ATPase is routinely purified by glycerol gradient centrifugation, the amount of purified enzyme obtained is limited, and the amount is crucial for investigations of the enzyme at the molecular level.

In this paper, we report the large-scale purification of *E. hirae* Na^+-ATPase, and describe some of the properties of sodium movement by the V_0V_1- and V_0-proteoliposomes.

MATERIALS AND METHODS

Strain and Culture—*E. hirae* strain 25D, a mutant defective in the production of F_0F_1, H^+-ATPase (*28*), harboring plasmid pCemtp18 was used; this plasmid contains the 13.4-kb *Xba*I-*Xba*I fragment of pKAZ171 (*24*), which extends from the promoter region of the *E. hirae* Na^+-ATPase (*ntp*) operon to the end of the *ntpJ* gene. Cells were cultured at 37°C in 20 liters of complex medium (*29*) containing 0.5 M NaCl supplemented with 10 μg/ml erythromycin, and harvested at the mid-exponential phase of growth.

Purification of V_0V_1 Na^+-ATPase—Membrane vesicles prepared as described previously (*29*) were suspended in buffer A [100 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride; pH 7.5], and stored at −80°C. Na^+-ATPase was solubilized by incubating the membrane vesicles (25 mg protein/ml) with 2.5% *n*-dodecyl α-D-maltoside (DM) (Calbiochem-Novabiochem) for 10 min at room temperature, and recovered in the supernatant after centrifugation at 150,000×*g* (60 min at 4°C). The supernatant (20 ml) was loaded onto an anion-exchange column (Bio-Scale DEAE20, Bio-Rad; column dimensions, 15×113 mm) equilibrated at room temperature with buffer B (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, and 0.05% DM; pH 7.5) containing 400 mM KCl. After washing with 40 ml of the same buffer, the column was eluted with a linear gradient of 400 to 800 mM KCl in this buffer at a flow rate of 0.5 ml/min. Eighty fractions of 3 ml each were collected. The ATPases that eluted from 660-750 mM KCl were pooled and concentrated to 2 ml by ultrafiltration (YM10 filter, Amicon, Beverly, MA) at 4°C. One milliliter of the concentrated ATPase fraction was loaded onto a gel filtration column (Superose 6HR; Pharmacia, column dimensions, 20×500 mm) equilibrated with buffer B and eluted with the same buffer at a flow rate of 0.3 ml/min. Fifty fractions of 2.5 ml each were collected. Gel filtration was repeated for the remaining 1 ml of the concentrated DEAE pool. The ATPases recovered in fractions 28 to 30 were finally pooled. In these purification steps, the Na^+-ATPase activity was measured in the presence of 25 mM NaCl, 0.05% DM, and 0.1 mg/ml dioleoylphosphatidylglycerol (Sigma Chemicals) as described previously (*27*).

Reconstitution of Proteoliposomes Containing the V_0V_1-ATPase—Purified V_0V_1-ATPase (1.5 mg of protein) in 0.5 ml of buffer B and 20 μl of 1 M $MgCl_2$ (final 2 mM) were added to a liposome suspension formed by sonicating a suspension of 300 mg phosphatidylcholine (Sigma Chemicals, Type II S) in 10 ml of buffer C (10 mM Tris-HCl, 1 mM DTT; pH 7.5) four times for 10 min with a tip type sonicator (Brason sonifier 250; micro tip, output 50). The mixture was incubated for 10 min at room temperature with occasional shaking, frozen in dry ice/acetone, and thawed at 4°C. The proteoliposomes were sonicated with a tip type sonicator twice for 10 s, and collected by centrifugation at 150,000×*g* for 60 min. The liposomes were resuspended in 10 ml of buffer D (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and 100 mM KCl; pH 7.5) and stored at −80°C until use. Approximately 10% of the purified Na^+-ATPase used was recovered as V_0V_1-liposomes by this reconstitution procedure.

Dissociation of V_1 ATPase from V_0V_1-Proteoliposomes—V_1-ATPase was dissociated from the V_0 moiety by incubation of the V_0V_1-proteoliposomes (300 mg of phospholipid) in 10 ml of buffer E [1 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, and 10% glycerol; pH 7.5] at 25°C for 2 h, and separated from the V_0-liposomes by centrifugation at 150,000×*g* for 60 min. The V_0-liposomes were suspended in the same volume of buffer E, incubated for 50 min at 25°C, and centrifuged again. The V_0-proteoliposomes were finally suspended in 10 ml of buffer C containing 10% glycerol.

Rebinding of V_1 ATPase to V_0-Proteoliposomes—The dissociated V_1-ATPase fraction was dialyzed against buffer A and concentrated to 10 mg protein/ml by ultrafiltration (YM10). In the presence of 5 mM $MgCl_2$, 0.5 mg of dissociated V_1-ATPase was incubated with 6 mg of the V_0-proteoliposome suspension for 45 min at 4°C. The reconstituted V_0V_1-proteoliposomes were subsequently collected by centrifugation (150,000×*g*, 60 min) and resuspended in 0.2 ml of buffer C containing 10% glycerol.

Measurement of Na^+-ATPase Activity in V_0V_1-Proteoliposomes—The Na^+-ATPase activity of the V_0V_1-proteoliposomes was measured by the same method used to

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measure the activity of membrane vesicles (*29*). The initial rate of the ATPase reaction was determined within 5 min, and one enzyme unit was defined as the amount of 1 μmol inorganic phosphate liberated per minute per mg phospholipid. DCCD (0.5 mM), when used, was added 5 min before the addition of ATP.

Measurement of Na^+ Transport—ATP-dependent Na^+ transport by the V_0V_1-proteoliposomes was measured by diluting the proteoliposomes 5-fold in buffer D (final 6 mg proteolipids/ml); and the assay was performed as described previously (*27*).

For the measurement of Na^+ transport driven by membrane potential, V_0-proteoliposomes or V_0V_1-liposomes reconstituted with the released V_1 moiety and V_0-liposomes in buffer C containing 10% glycerol were incubated overnight at 4°C with 200 mM KCl for loading. K^+-loaded proteoliposomes were collected by centrifugation at $150{,}000 \times g$ for 60 min and resuspended in the same buffer (300 mg of proteolipids/ml). K^+-loaded proteoliposomes were diluted 47-fold in buffer C containing 200 mM choline chloride, 2 mM ^{22}NaCl (340 kBq/mmol) and 10% glycerol. After 10 min, the reaction was started by adding 100 nM valinomycin. DCCD (0.5 mM), when used, was added 5 min before the addition of valinomycin. At intervals, 90 μl of the reaction mixture was filtered through a nitrocellulose filter (0.2 μm pore size, Toyo Roshi, Tokyo) with suction, and quickly washed twice with 4 ml of buffer D. The radioactivity trapped on the filter was measured with a γ-scintillation counter (Aloka, Tokyo). The initial rate of the transport reaction by the V_0V_1 and V_0 proteoliposomes was determined within 5 min.

Other—SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (*30*), and stained with Coomassie Brilliant Blue R-250 (CBB). Western blotting was performed as described elsewhere (*25*); spots were visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase. Rabbit antisera against various Ntp proteins (Ntp I, -C, -D, -E, -F, -K, -G) were prepared by injecting the synthetic peptide for each protein conjugated to KLH into rabbits as described previously (*27*). All antisera were purchased from Nippon Bio-Test Lab. (Tokyo) and Takara Shuzo (Kyoto). Protein was determined according to the method of Lowry *et al.* (*31*) with bovine serum albumin as the standard. ^{22}NaCl (1.36 TBq/mmol) was obtained from Dupont/NEN Research Products (America).

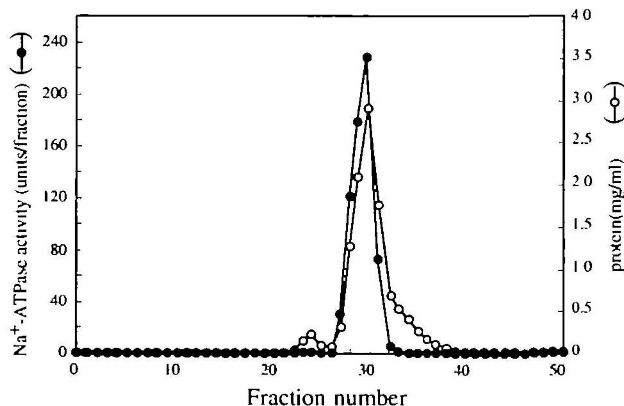

Fig. 1. **Elution profile of *E. hirae* Na^+-ATPase in Superose 6HR chromatography.** Gel filtration was performed as described in "MATERIALS AND METHODS." Na^+-ATPase activity, ●; protein concentration, ○.

RESULTS

Purification of Na^+-ATPase—*E. hirae* Na^+-ATPase was purified by column chromatographies. The final step was achieved by gel filtration on Superose 6HR (Fig. 1). We observed a single peak of Na^+-ATPase activity that coincided with a protein peak. The specific activities of the peak fractions (fraction Nos. 28, 29, and 30) were constant at 35.7 units/mg protein, which indicates a 4-fold purification from the DM extracts. More than 90% of the membrane protein (420 mg) was solubilized by DM, and about 8% of the solubilized protein was recovered in these peak fractions (Table I). More than 30% of the Na^+-ATPase solubilized was recovered, and we finally obtained 32 mg of purified enzyme retaining a specific activity 1.7-fold higher than that of samples purified by glycerol gradient centrifugation according to the previous paper (*27*). The amount of purified enzyme obtained here was more than 500-fold greater than the amount (0.6 mg) obtained by the previous method, which is enough for further biochemical analysis. The purified enzyme was stable at 4°C for at least one week, and for three months at −80°C.

The subunit composition of the pooled peak fractions (Nos. 28-30) from gel filtration was analyzed by SDS-PAGE in 12% gels (Fig. 2, lane 1). Eight polypeptides with

TABLE I. **Purification of *E. hirae* Na^+-ATPase.**

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Membrane	420	882	2.1	–
DM extract	398	3,340	8.4	100
DEAE pool	114	2,580	22.6	77
Superose 6HR	32	1,140	35.7	34

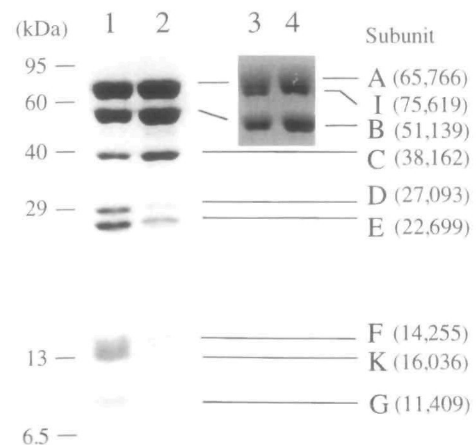

Fig. 2. **SDS-PAGE profile of purified V_0V_1 Na^+-ATPase and the V_1 moiety dissociated from V_0V_1-proteoliposomes.** The purified Na^+-ATPase (10 μg; lanes 1 and 3) and V_1 moiety (10 μg; lanes 2 and 4) were electrophoresed in a 12.5% gel (lanes 1 and 2) or 10% gel (lanes 3 and 4), and the gels were stained with CBB. The numbers in parentheses are the molecular masses of Ntp proteins calculated from the deduced amino acid sequences.

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apparent molecular masses of 69, 52, 38, 27, 24, 15, 14, and 8 kDa were observed; the 69 kDa protein band was split into two bands of 69 and 65 kDa in 10% gels (Fig. 2, lane 3). These SDS-PAGE profiles are equivalent to those of the purified enzyme in the previous paper (*27*). Western blotting using antisera raised against the catalytic moiety of this ATPase consisting of the A, B, and D subunits (*32*), and the I, C, D, E, F, K, and G subunits revealed that the 69, 65, 52, 38, 27, 24, 15, 14, and 8 kDa polypeptides correspond to the Ntp A, I, B, C, D, E, F, K and G subunits, respectively (data not shown). Densitometric analysis of the purified enzyme stained with CBB suggested that the A, I, B, C, D, E, F, K, and G subunits occur in the molar ratio 3:1-2:3:1: 1:3:1-2:3-4:1.

Na^+ Transport and Na^+-ATPase Activities of V_0V_1-Liposomes—In the previous paper, reconstitution into V_0V_1-proteoliposomes was performed with total phospholipids extracted from *E. faecalis*. Here soybean phosphatidylcholine was used for reconstitution because of its commercial availability. ATP-driven $^{22}Na^+$ uptake, which is accelerated by carbonylcyanide *m*-chlorophenylhydrazone and valinomycin, but inhibited by monensin (data not shown), was observed by the proteoliposomes. Therefore, the amount of phospholipid as well as that of purified enzyme are not crucial for reconstitution experiments of electrogenic sodium translocation by *E. hirae* V_0V_1 Na^+-ATPase.

Figure 3A shows the effect of NaCl concentration on the initial rates of ATP-driven $^{22}Na^+$ uptake by V_0V_1-liposomes. The maximum velocity (9 pmol $^{22}Na^+$ transported/min/mg lipid) by the liposomes estimated under the present conditions coincided with that of reconstituted V_0V_1-proteoliposomes prepared with *E. faecalis* phospholipids reported previously (*27*). All assay media were contaminated with 10 μM Na^+. Therefore, considering the contaminating Na^+, double reciprocal plots of the Na^+ transport reaction indicated an apparent K_t value for Na^+ of V_0V_1 Na^+-ATPase of 40 μM (Fig. 3A, inset). Under the same assay conditions, the effect of NaCl concentration on the initial rates of Na^+-ATPase activity by V_0V_1-liposomes was also examined (Fig. 3B). The rate of ATP hydrolysis of the V_0V_1-liposomes increased with Na^+ concentration until saturation was reached at 100 mM NaCl (Fig. 3B). Although 20% of the maximal ATPase activity was observed at zero Na^+ concentration, this activity can be accounted for by the presence of the contaminating Na^+. Therefore, we conclude that the ATPase reaction of V_0V_1-liposomes is tightly coupled to Na^+. Double reciprocal plots of the data suggest the presence of two K_m values for Na^+: 20 μM (high affinity) and 3 mM (low affinity) (Fig. 3B, inset). These K_m values are similar to the two K_m values for Na^+ (20 μM and 5 mM) of the purified enzyme in detergent micelles (*27*). The low K_m (high affinity) value of the ATPase activity of V_0V_1-proteoliposomes is equivalent to the K_t value of Na^+ transport. The high K_m value (5-7 mM) has also been reported for the Na^+-ATPase activity of membrane-bound ATPase (*29*). Therefore, the low affinity component of this enzyme is not necessarily an artifact of the *in vitro* reconstitution system. It is important to examine whether the Na^+ transport reaction also has the low affinity K_t value for Na^+ (K_m=5-7 mM). However, an accurate determination of the initial velocities of Na^+ transport at more than 10 mM Na^+ by V_0V_1 liposomes is not possible under the present conditions. Further evaluation in a revised assay system is required.

Dissociation and Association of V_1 and V_0 Moieties—In order to know the functional roles of each subunit in the Na^+-ATPase reaction, it is important to assign nine Na^+-ATPase subunits to the catalytic V_1 moiety and the membrane-embedded V_0 moiety. Since the 16-kDa K subunit is a proteolipid (*23*) and the deduced amino acid sequence of the 76-kDa I subunit suggests that this protein has several membrane-spanning domains in its C-terminal half, both the K and I subunits can be tentatively assigned to the V_0 subunit. Because the Na^+-ATPase activity of V_0V_1-liposomes is not stimulated by the addition of 0.1% DM, nearly all the V_0V_1-ATPase in the proteoliposomes is oriented toward the outside. Dissociation of V_1 and V_0 moieties was accomplished by incubating the V_0V_1-proteoliposomes with EDTA to chelate the Mg^{2+} essential for the direct binding of V_1 and V_0 (*33*). After EDTA treatment, the V_0-liposomal fraction showed only 6% of the Na^+-

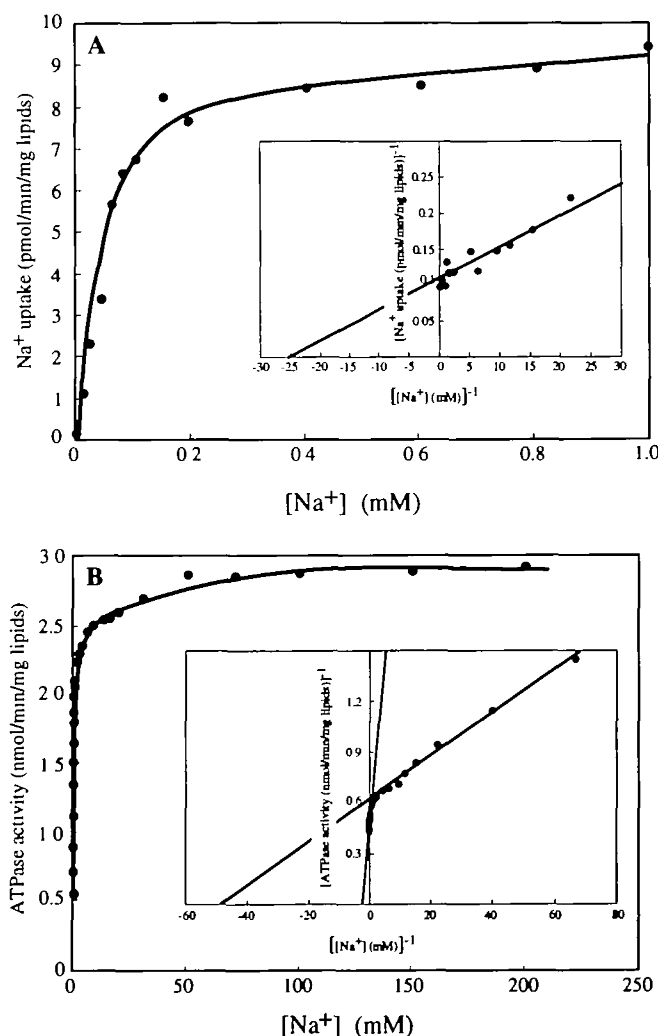

Fig. 3. **Na^+ concentration dependence of the Na^+-transport and ATPase activities of V_0V_1-liposomes.** The initial rates were determined as described in "MATERIALS AND METHODS." The insets show the double-reciprocal plots of the initial rates against Na^+ concentration. Panel A, Na^+-transport activity; Panel B, ATPase activity.

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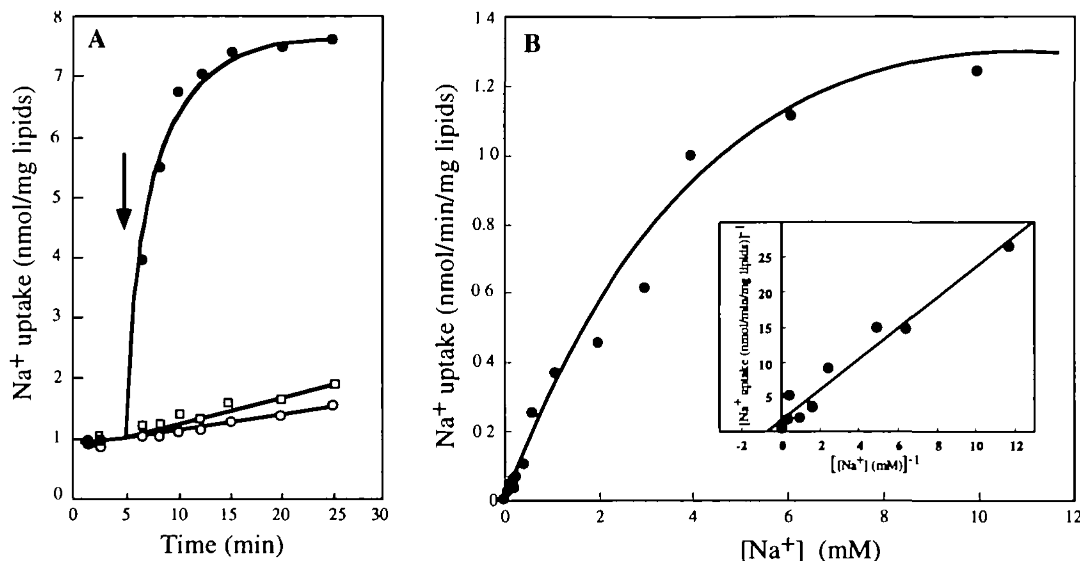

Fig. 4. **$\Delta\psi$-induced Na^+ uptake by V_0-liposomes.** Panel A: Time course of Na^+ uptake. K^+-loaded V_0-liposomes and reconstituted V_0V_1-liposomes were prepared as described in "MATERIALS AND METHODS," and $\Delta\psi$ was imposed as a K^+-diffusion potential by the addition of 100 nM valinomycin at the time shown by an arrow. Symbols: ○, without valinomycin; ●, with valinomycin; □, V_0V_1-liposomes with valinomycin. Panel B: Na^+ concentration dependence of Na^+ uptake. The initial rates of $\Delta\psi$-induced Na^+ uptake after the addition of valinomycin was determined within 5 min. The inset shows the double-reciprocal plot of the initial rates of Na^+ uptake activity vs. Na^+ concentration.

ATPase activity of untreated proteoliposomes. Importantly, incubation of the dissociated V_1 fraction with V_0-liposomes resulted in a full recovery of the V_0V_1-ATPase activity; the activities of the Na^+-ATPase (1.4 nmol/min/mg of phospholipids) and ATP-driven Na^+ transport (7 pmol/min/mg of phospholipids) by the reconstituted liposomes were similar to those of the original V_0V_1-proteoliposomes prior to EDTA treatment. EDTA treatment did not cause the inactivation of either the V_1 or V_0 moieties of Na^+-ATPase.

The V_1 moieties dissociated from the V_0 moieties were concentrated by ultrafiltration (YM10) and analyzed by SDS-PAGE (Fig. 2, lanes 2 and 4). Six polypeptides with apparent molecular masses of 69, 52, 38, 27, 24, and 15 kDa were observed. Western blotting using antisera against various Ntp subunits revealed that the 69, 52, 38, 27, 24, and 15 kDa polypeptides correspond to the A, B, C, D, E, and F subunits, respectively (data not shown). As expected, we did not observe any polypeptides cross-reacting with antisera against the I and K subunits in the dissociated V_1 fraction (data not shown). We also did not observe subunit G in this V_1 fraction. Since subunit G is small, it may be lost during concentration by ultrafiltration with YM10 (pore size; MW 10,000); the assignment of the G subunit as part of the V_1 or V_0 subunits was not confirmed. These results suggest that the A, B, C, D, E, and F subunits constitute the V_1 moiety while the V_0 moiety comprises of at least the I and K subunits.

Na^+ Transport by V_0-Liposomes—The characterization of ion movement through the F_0 part of F-ATPase was helpful for understanding the overall mechanism of the F_0F_1-ATPase reaction (*34, 35*). Proton movement through isolated V_0-liposomes has been demonstrated for *Thermus thermophilus* V-ATPase (*36*) and eukaryotic V-ATPase (*37, 38*). The effect of membrane potential (interior negative) on sodium movement by V_0-liposomes was examined (Fig. 4A). In this experiment, K^+-loaded V_0-liposomes were diluted in K^+-free buffer and then $^{22}Na^+$ movement was followed. $^{22}Na^+$ uptake into V_0-liposomes was very slow in the absence of valinomycin. When valinomycin was added to generate a membrane potential (interior negative) by potassium diffusion, rapid $^{22}Na^+$ uptake into the V_0-liposomes was observed. No $^{22}Na^+$ uptake was observed by liposomes without V_0 or in the presence of 200 mM KCl (data not shown). Thus, Na^+ movement through the *E. hirae* V_0 moiety responds to membrane potential. Membrane potential-driven $^{22}Na^+$ uptake by V_0-liposomes is blocked by the rebinding of V_1 (Fig. 4A), suggesting that $^{22}Na^+$ translocation is catalyzed by V_0 with its V_1 binding site exposed to the outside. Tight coupling of the V_1 and V_0 moieties stops the passive movement of Na^+ though the V_0 moiety. Figure 4B shows the dependence of Na^+ uptake by V_0-liposomes on the external Na^+ concentration. Double reciprocal plots of the initial velocities of $\Delta\psi$-induced Na^+ uptake by V_0-liposomes indicated a K_t value for Na^+ of 1.4 mM (Fig. 4B, inset), suggesting that the translocation of Na^+ through the V_0 pathway requires the binding of an alkaline cation to a specific site.

Sodium Ions Protect against the Inhibitory Effect of DCCD on V_0V_1-ATPase—DCCD inhibits the Na^+-ATPase activity of the purified enzyme and ATP-driven sodium ion translocation by V_0V_1-liposomes (*27*), probably by attacking a glutamic acid residue (Glu136) in the fourth membrane-spanning domain of the K proteolipid (*23*). On the other hand, we know that DCCD does not inhibit sodium ion extrusion by the Na^+-ATPase in intact cells or the Na^+-ATPase activity of membrane vesicles under certain experimental conditions where treatment with DCCD is carried out in the presence of Na^+ (*29*). The effect of DCCD

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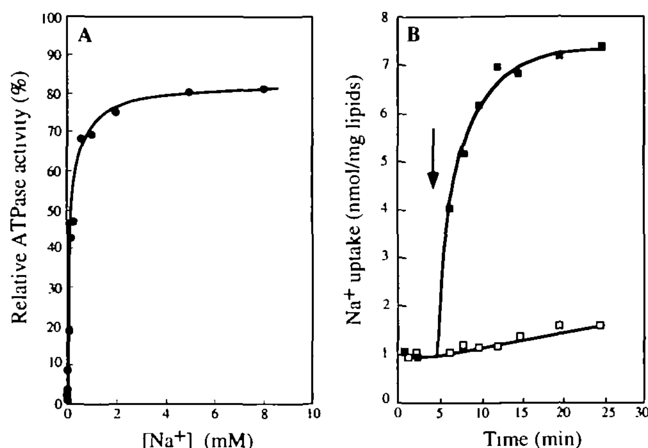

Fig. 5. **Protective effect of Na^+ on the reactivity of DCCD with purified V_0V_1 Na^+-ATPase and V_0-liposomes.** Panel A: Effect of Na^+ on the DCCD-inhibited ATPase activity of purified V_0V_1-ATPase. Purified Na^+-ATPase (3 μg/ml) was treated with 0.5 mM DCCD for 5 min in the presence of NaCl at the various concentrations indicated, and the reaction was initiated by the simultaneous addition of 5 mM ATP and 25 mM NaCl. The activity without DCCD treatment was taken as 100%. Panel B: Effect of Na^+ on the DCCD of $\Delta\psi$-induced Na^+ uptake by DCCD in V_0-liposomes. 0.5 mM DCCD was added 5 min before the addition of valinomycin (shown by the arrow) in the presence (■) or absence (□) of Na^+.

on the activities of the proteoliposomes was examined (Fig. 5). DCCD (0.5 mM) completely inhibits the Na^+-ATPase activity of the purified V_0V_1-ATPase (Fig. 5A), but the activity is retained by preincubation with NaCl before DCCD treatment. The protective effect saturates at 2 mM NaCl and is not perfect; about 80% of the original activity is recovered. Membrane potential-driven $^{22}Na^+$ movement by V_0-liposomes is inhibited by 0.5 mM DCCD in the absence of Na^+ (Fig. 5B). The inhibition by DCCD is prevented by the presence of 2 mM NaCl, suggesting that the Na^+ binding site overlaps the DCCD-reactive site.

DISCUSSION

By using column chromatographies, we could obtain more than 30 mg of purified V_0V_1 Na^+-ATPase from 20 liters of culture; the purified V_0V_1 ATPase showed a high specific activity for the Na^+-ATPase. The activity of reconstituted V_0V_1-proteoliposomes prepared with commercially available phospholipid corresponds to that of V_0V_1-proteoliposomes reconstituted with *E. faecalis* phospholipids. The initial rate of Na^+ uptake of V_0V_1-proteoliposomes is about 8 pmol/min/mg lipids, which is only 0.5% of the ATP hydrolytic activity (1.6 nmol/min/mg lipid) of liposomes measured under the same conditions. Since (i) the Na^+-ATPase activities of liposomes reconstituted with the V_1 fraction and V_0-liposomes is coincides with that of the original V_0V_1-proteoliposomes, and (ii) the ATPase activity of the V_0V_1-liposomes is not affected by 0.1% DM, it appears that nearly all of the incorporated V_0 and V_1 moieties are oriented toward the outside of the V_0V_1 complex. The discrepancy between the activities of ATP hydrolysis and Na^+ translocation may be attributed to the leakiness to Na^+ of the reconstituted liposomes.

SDS-PAGE and Western blotting experiments (data not shown) revealed that the A, B, C, D, E, and F subunits are released from the V_0 moiety by incubation of V_0V_1-proteoliposomes with EDTA. All these proteins are suggested to be hydrophilic based on their deduced amino acid sequences. The I, K, and G subunits were not detected in this V_1 fraction. We are now going to disrupt each gene in the *ntp* operon by inserting an erythromycin resistance gene cassette, and recent data suggest that the nine *ntp* genes encoding the A, B, C, D, E, F, G, I, and K subunits are indispensable for a functional Na^+-ATPase (data not shown). A functional Na^+-ATPase complex was recovered by reconstituting the released V_1 and V_0-liposomes, suggesting that these six Ntp proteins are functionally active, probably forming a V_1 multimeric complex. The subunit ratio of the dissociated V_1 fraction, estimated by densitometric analysis of the amounts of bound Coomassie dye, was slightly different from that of the purified V_0V_1 (Fig. 2). The ratio of the A, B, and C subunits (3:3:1) of the dissociated V_1 moiety is equal to the ratio of the same subunits from purified V_0V_1, but the amounts of D, E, and F subunits were lower in the dissociated V_1 fraction (Fig. 2). Some of the D, E, and F subunits may be bound to the V_0 moiety, probably associating with the stalk region between the V_1 and V_0 sectors.

It is reasonable that both the I and K subunits are not released in the V_1 fraction. However, we wonder whether NtpG is tightly associated with the V_0 moiety, since it has been reported that an NtpG-homolog protein, the F subunit of eukaryotic V-ATPases such as yeast Vma7p, behaves as the V_1 subunit (*39*). NtpG is a small (11 kDa) hydrophilic protein and may easily pass through the ultrafiltration filter (YM10). Reexamination of the assignment of the G subunit to the V_1 or V_0 subunit is now in progress.

Incubation of the *E. hirae* V_0V_1-ATPase with DCCD results in the inactivation of the enzyme in a pH-dependent manner (data not shown). The inactivation of the ATPase activity by DCCD is specifically prevented by the presence of Na^+ (Fig. 5A), suggesting that the Na^+ binding site overlaps with the DCCD-reactive site. Protective effects of Na^+ against DCCD inactivation have also been observed in the F-type Na^+-ATPases from *Propionigenium modestum* (*40*) and *Acetobacterium woodii* (*41*). It is likely that the DCCD-reactive amino acid residue in subunit K (probably Glu139) must be protonated for the reaction with carbodiimide and provides the Na^+-binding site in its deprotonated state; the dissociation of a proton from the carboxylic acid (at high pH) and the binding of Na^+ to the carboxylate thus abolish the reactivity toward DCCD. $\Delta\psi$-induced $^{22}Na^+$ uptake by V_0-liposomes is also inhibited by 0.5 mM DCCD in the absence of Na^+, but not in the presence of 2 mM Na^+ (Fig. 5B). These results suggest that Na^+ binds to the cation binding site of V_0 in the absence of DCCD regardless of whether the V_1 moiety associates with V_0, and that this Na^+ binding abolishes the reactivity of the residue toward DCCD.

The K_t value (1.4 mM) for Na^+ in the $\Delta\psi$-induced Na^+ uptake reaction of V_0-liposomes is very high compared with the K_t value (40 μM) for Na^+ in ATP-dependent Na^+ uptake reaction of V_0V_1-liposomes. These K_t values for Na^+ uptake of the V_0-liposomes and V_0V_1-liposomes do not change much, even when measured in the presence of both a potassium diffusion potential and ATP. Therefore, we assume that these K_t values are intrinsic for Na^+ binding in

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V_0- and V_0V_1-moieties. We believe that the rotation mechanism demonstrated in the operation of F_1-ATPases (*11*) can be applied to V_1-ATPase. However, the ion pumping mechanism at the F_0- or V_0-portion coupled to rotation remains poorly understood. In this report, we demonstrate that the V_0-portion of *E. hirae* shows a low affinity site for Na^+; the V_1-portion changes the affinity for Na^+ to high affinity. This finding should be relevant to the energy coupling mechanism of V-type ATPases. The purification/reconstitution system for *E. hirae* V_0V_1 Na^+-ATPase described in this paper is valuable for further extensive studies into the energy coupling mechanism.

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