Purification and Reconstitution of Na⁺-translocating Vacuolar ATPase from Enterococcus hirae*

(Received for publication, May 15, 1997, and in revised form, July 21, 1997)

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Vacuolar ATPases make up a family of proton pumps distributed widely from bacteria to higher organisms. An unusual member of this family, a sodium-translocating ATPase, has been found in the eubacterium Enterococcus hirae. We report here the purification of enterococcal Na⁺-ATPase from the plasma membrane of cells, whose ATPase content was highly amplified by expression of the cloned ntp operon that encodes this Na⁺-ATPase (ntpFIECGABDHHJ). The purified enzyme appears to consist of nine Ntp polypeptides, all the above except for the ntpH and ntpJ gene products. ATPase activity was strictly dependent on the presence of Na⁺ or Li⁺ ions and was inhibited by nitrate, N-ethylmaleimide, and the peptide antibiotic destruxin B. When the purified ATPase was reconstituted into liposomes prepared from Enterococcus faecalis phospholipids, ATP-driven Na⁺ uptake was observed; uptake was blocked by nitrate, destruxin B, and monensin, but it accelerated by carbonyl cyanide m-chlorophenylhydrazone and valinomycin. These data demonstrate that E. hirae Na⁺-ATPase is an electrogenic sodium pump of the vacuolar type. This is a promising system for research on the fundamental molecular structure and mechanism of vacuolar ATPase.

Vacuolar ATPases comprise a family of structurally and functionally related enzymes that translocate protons across organelles and plasma membranes of eukaryotic cells (1, 2). Proton movements linked to ATP hydrolysis acidify membrane-bound spaces and establish electrochemical proton potentials that serve as the driving force for diverse proton-coupled secondary transporters. Vacuolar ATPase consists of a water-soluble, catalytic V₁ moiety and a membrane-integrated V₀ portion that conducts protons (3–5); the vectorial and chemical reactions are coupled into membranes.

The V₁ portions and V₀V₁ complexes, some of which are reconstitutively active in proton pumping, have been purified from a variety of eukaryotic species, and the amino acid sequences of these components have been determined (4–6). The V₀V₁ complex has three major subunits: the A and B subunits of the V₁ portion and the 17-kDa proton-conducting proteolipid of the V₀ portion. The amino acid sequences of these subunits are highly conserved among species and are known to resemble the sequences of the β, α, and ε subunits of the FₐFₐ-ATPase (another family of proton-translocating ATPases). It has been proposed that these two ATPase families are phylogenetically related and that the 17-kDa proteolipid of the vacuolar ATPase arose by tandem duplication of the gene for the 8-kDa proteolipid of the FₐFₐ-ATPase (5–7). The amino acid sequences of other subunits of vacuolar ATPases are also well conserved among species, but the resemblance of these subunits to those of FₐFₐ-ATPases is not conspicuous. Characterization of the subunits of vacuolar ATPase is most advanced in Saccharomyces cerevisiae, based on molecular genetics (4, 8). Recently two new proteolipids, Vma11p and Vma16p, were found to be subunits of the vacuolar ATPase (9). Thus, the vacuolar ATPase complex of yeast appears to contain at least 13 polypeptides, and purification of the holo enzyme has been attempted (8, 10).

Vacuolar-type ATPases also occur in bacteria (5, 6, 11); archaeabacterial proton-translocating ATPases are thought to mediate ATP synthesis (12). An important variant among vacuolar ATPases is the enzyme from Enterococcus hirae, which transports Na⁺ rather than H⁺ under physiological conditions (13, 14). Our group has purified the catalytic V₁ moiety of this ATPase (15) and cloned the genes for the complex; the genes form an operon (designated ntp) consisting of 11 genes, ntpFIECGABDHHJ (16–18). The molecular properties of purified V₁ and the sequence analysis of these ntp genes indicate that Enterococcus Na⁺-ATPase belongs to the vacuolar ATPase family. Three major subunits of Na⁺-ATPase, NtpA, NtpB, and NtpK (16-kDa proteolipid), resemble those of the eukaryotic ones; the other genes, ntpD, ntpE, ntpF, and ntpG, encode the polypeptides that resemble the Vma8p, Vma4p, Vma10p, and Vma7p subunits of yeast vacuolar ATPase, respectively, although the sequence similarities were only moderate (23–29% identity; 40–50% similarity). The similarities of the sequences of NtpC and NtpI to those of Vma6p and Vph1p of yeast vacuolar ATPase were less prominent (15–16% identity; 36–37% similarity). However, some amino acid clusters conserved among the corresponding subunits in eukaryotic vacuolar ATPases are conserved in these sequences; in case of NtpI, the sequence around the first membrane-spanning domain of Vph1p is conserved (18). The ntpJ gene product is a component of a K⁺ transport system linked to the Na⁺-ATPase, but it is not essential for the assembly or the operation of the ATPase (19). It appears, then, that this vacuolar-type Na⁺-ATPase consists of fewer than 10 polypeptides.

We report here the purification and reconstitution of the Enterococcus Na⁺-ATPase complex as a stage in its biochemical characterization. The ATPase activity of the purified enzyme, which appears to consist of nine Ntp proteins, was inhibited by vacuolar ATPase inhibitors; ATP-driven Na⁺ uptake by reconstituted proteoliposomes was blocked by nitrate and monensin but accelerated by valinomycin. These results suggest that the
vacuolar Na\textsuperscript+-ATPase of \textit{E. hirae} is an electrogenic sodium pump.

**MATERIALS AND METHODS**

\textbf{Plasmids, Strains, and Culture—}An \textit{Enterococcus-Ascherichia coli} shuttle vector pCEm3 (4.6-kb) was constructed by ligating the 1.8-kb \textit{Bam}HI-HindIII fragment (the erythromycin resistance gene) of pJEM2 (19) with the 2.8-kb \textit{Bgl}II-HindIII fragment of pCS (20) carrying the replication origin for these bacteria; pCEm3 has single XbaI site. The 18-kb pCEmtp18 was constructed by ligation of the 13.4-kb XbaI-XhoI fragment of pKAZ171 (18), which extends from the promoter region of \textit{E. hirae} Na\textsuperscript{+}-ATPase (\textit{ntp}) operon to the end of the \textit{ntp} gene (see Fig. 2A) to the 4.6-kb XbaI cut of pCEm3. pCEmtp18 was introduced into \textit{E. hirae} strain 22D, a mutant defective in production of \textit{F. P. H}.-ATPase (14). The transformed cells were grown on a complex medium (21) containing 0.5 m NaCl supplemented with 10 \mu g/ml erythromycin. Phospholipids were extracted from a large batch of \textit{Enterococcus faecalis} cells, strain AD1001, grown on the same complex medium.

\textbf{Purification of Na\textsuperscript{+}-ATPase—}Membrane vesicles were prepared by disintegration of spheroplasts with a French press as described previously (21), suspended in buffer A (100 mM Tris-HCl, 10 mM MgSO\textsb{4}, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) containing 10% glycerol, and stored at \textdegree C. The Na\textsuperscript{+}-ATPase was solubilized by incubation of the membrane vesicles (1.5 mg/ml) with 1.5% n-dodecyl \textit{d}-maltoside (DM) (Calbiochem) for 10 min at room temperature and recovered in the supernatant after centrifugation at 22,000 \times g (30 min at 4 \textdegree C). The supernatant (0.6 ml) was applied to the top of a linear glycerol gradient from 10 to 30% (Fig. 1) in buffer A containing 0.1% DM and 0.1 mg/ml dioleoylphosphatidylglycerol (Sigma) and was centrifuged at 175,000 \times g for 12 h at 4 \textdegree C. After dividing into 18 fractions (0.6 ml each), the ATPase activity of the fractions was determined. One unit of the ATPase activity was defined as corresponding to 1 \mu mol of ATP hydrolyzed/min.

\textbf{Reconstitution of Proteoliposomes—}The phospholipids of \textit{F. eaealis} AD1001 were extracted and purified from the cell mass by silica gel chromatography, as described elsewhere (22). Proteoliposomes were produced by a freeze-thaw and dilution method (22) as follows. 25 \mu l of the purified enzyme (0.05 mg/ml) was mixed with 100 \mu l of the liposome suspension (50 mg of phospholipid/ml in buffer B (50 mM Tris-HCl, 100 mM KCl, 10 mM MgSO\textsb{4}, 1 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol, pH 7.5) and supplemented with 11.4 \mu l of 15% CHAPS. After incubation for 10 min at 4 \textdegree C, the mixture was frozen in dry ice/acetone, thawed, and sonicated for 5 s. The mixture was finally diluted 200-fold into buffer B, and the proteoliposomes were recovered by centrifugation at 150,000 \times g for 90 min. The liposomes were suspended in 0.1 ml of buffer B and stored at 4 \textdegree C.

\textbf{Determination of Na\textsuperscript{+} Uptake by Proteoliposomes—}The proteoliposomes were suspended in 2 ml of buffer B (0.6 \mu g of protein/ml); 0.1 mM \textsuperscript{22}NaCl (6,500 cpm/\mu mol) was added to the mixture and allowed to equilibrate for 30 min. The uptake reaction was started by the addition of 5 mM ATP. Inhibitors or ionophores were added at 10 min before the addition of ATP. At intervals, 90 \mu l of the reaction mixture was filtered on a nitrocellulose filter (0.2-\mu m pore size, Toyo Rishi Co. Ltd., Tokyo) with suction and washed twice quickly with 4 ml of buffer B. The radioactivity trapped on the filter was measured with a liquid scintillation counter.

\textbf{Preparation of Antiserum against NtpI, -E, -F, -K, and -J—}Rabbit antisera against various Ntp proteins were prepared by injection of a synthetic peptide for each protein conjugated to keyhole limpet hemocyanin. All antiserum were purchased by Nippon Bio-Test Lab. Inc. (Tokyo) and Takara Shuzo Co. (Kyoto). The amino acid sequences of the oligopeptides synthesized were the following: CTKTVEKYVNHKKK (near the COOH terminus of NtpI), GLIDDAOSIQ(INFL (the COOH terminus of NtpF), MMDYLITGOMMV (the NH\textsb{2} terminus of NtpK), and RAE- NYKIPESIM (near the COOH terminus of NtpJ). 500 \mu l of the purified enzyme was subjected to SDS-PAGE on 12.5% gel and electroblotted to a polyvinylidene difluoride membrane (Bio-Rad). After staining with Coomassie Brilliant Blue, the bands corresponding to the 38-, 29-, and 8-kDa subunits were cut out, and the NH\textsb{2}-terminal sequences of the polypeptides were analyzed with the HP G1005A protein sequencing system (Hewlet Packard) by Takara Shuzo Co.

\textbf{RESULTS}

\textbf{Purification of Na\textsuperscript{+}-ATPase of \textit{E. hirae}—}The efficacy of various detergents for the solubilization of Na\textsuperscript{+}-ATPase was examined first. Among the detergents tested (DM, Tween 80, octyl \textit{d}-glucoside, Brij-58, Brij-35, CHAPS, cholate, C\textsubscript{12}E\textsb{8}, C\textsubscript{12}E\textsb{8}, C\textsubscript{12}E\textsb{10}, and Triton X-100), DM was the best for this enzyme. When the membrane vesicles were incubated with 1.5% DM, the activity of Na\textsuperscript{+}-ATPase in the suspension doubled. This increase probably resulted from the disintegration of membrane structure because about half of the \textit{E. hirae} vesicles prepared with the French press are everted. Under the conditions described in this paper, more than 80% of the membrane proteins were solubilized, and nearly 100% of total Na\textsuperscript{+}-ATPase activity was recovered, suggesting that nearly all of the Na\textsuperscript{+}-ATPase was solubilized in its native form (Fig. 1, inset). Further purification was accomplished by centrifugation through a linear glycerol gradient from 10 to 30% (Fig. 1). We observed a single peak of Na\textsuperscript{+}-ATPase activity which was relatively sharp and also a peak of protein which coincided with that of ATPase activity; most of the applied protein was recovered in the upper fractions. Importantly, the specific activities of Na\textsuperscript{+}-ATPase in fractions 4, 5, 6, and 7 were constant at 21 units/mg protein, which indicates a 10-fold purification from the DM extracts. About 80% of the Na\textsuperscript{+}-ATPase solubilized with DM was recovered in these four fractions. The activity of solubilized enzyme was stable at 4 \textdegree C for at least 1 week and for at least 3 months at \textdegree C.

\textbf{Molecular Properties—}Eight polypeptides with apparent molecular masses of 69, 52, 38, 27, 24, 15, 14, and 8 kDa were observed when the purified fractions (fractions 5, 6, and 7) were analyzed by SDS-PAGE (12.5% gel; Fig. 2B, lanes 3, 4, and 5). The 69-kDa protein band split into two bands of 69 and 65 kDa on 10% gel (Fig. 2B, lane 6), but no additional bands were observed by electrophoresis with different concentrations of

\textbf{Others—}SDS-PAGE was carried out using the system of Laemmli (23) and stained with Coomassie Brilliant Blue R-250 or silver. Western blotting was performed as described elsewhere (24); spots were visualized by using goat anti-rabbit IgG conjugated to alkaline phosphatase. The Na\textsuperscript{+}-ATPase activity of the purified enzyme was determined in the presence of 25 mM NaCl as described previously (21); the reaction was started by the addition of 2 mM ATP, sometimes after a 10-min preincubation with various compounds. Protein was determined according to the method of Lowry et al. (25), with bovine serum albumin as standard.

\textbf{\textsuperscript{22}NaCl was obtained from NEN Life Science Products (1.36 TBq/mmol).}
acrylamide. The purified enzyme most probably consists of these nine polypeptides.

Determination of the NH₂-terminal sequences of the 38-kDa (MEYHELIN), 27-kDa (MKLNVPN), and 8-kDa (TYKIGVV) bands indicated that these polypeptides correspond to the ntpC, ntpD, and ntpG gene products, respectively, of the Na⁺-ATPase operon (Fig. 2A). Fig. 2C shows Western blots of the fractions obtained from glycerol gradient centrifugation (Fig. 1), with various antisera. We have purified the V₁ catalytic moiety of this enzyme previously; it has a molecular mass of about 400 kDa and consists of three subunits, A, B, and D, with a stoichiometry of 3:3:1 (14). The 69- and 52-kDa polypeptides of the enzyme purified here were assigned to the A and B subunits, by Western blotting with antisera against purified V₁, moiety or the oligopeptides for the ntp gene products I, E, K, and F. Blotting and visualization were performed as described under “Materials and Methods.”

Purification of E. hirae Na⁺-ATPase

Panel A, structure of the Na⁺-ATPase operon. The arrow indicates the transcriptional direction. Panel B, SDS-PAGE. Washed membranes of 25D/pCempt18 (15 μg; lane 1), the DM extract (15 μg; lane 2), and the peak fractions, 5, 6, and 7 (1 μg) from the glycerol gradient as shown in Fig. 1 (lanes 3, 4, and 5, respectively) were electrophoresed on 12.5% gel and stained with Coomassie Brilliant Blue R-250; fraction 6 was also electrophoresed on 10% gel (lane 6). The parentheses indicate the molecular masses of Ntp proteins calculated from the deduced amino acid sequences. Panel C, Western blotting of the glycerol gradient fractions with various antisera, raised against the purified V₁ moiety or the oligopeptides for the ntp gene products I, E, K, and F. Blotting and visualization were performed as described under “Materials and Methods.”

FIG. 2. SDS-PAGE and Western blotting of several fractions during the purification of E. hirae Na⁺-ATPase. Panel A, structure of the Na⁺-ATPase operon. The arrow indicates the transcriptional direction. Panel B, SDS-PAGE. Washed membranes of 25D/pCempt18 (15 μg; lane 1), the DM extract (15 μg; lane 2), and the peak fractions, 5, 6, and 7 (1 μg) from the glycerol gradient as shown in Fig. 1 (lanes 3, 4, and 5, respectively) were electrophoresed on 12.5% gel and stained with Coomassie Brilliant Blue R-250; fraction 6 was also electrophoresed on 10% gel (lane 6). The parentheses indicate the molecular masses of Ntp proteins calculated from the deduced amino acid sequences. Panel C, Western blotting of the glycerol gradient fractions with various antisera, raised against the purified V₁ moiety or the oligopeptides for the ntp gene products I, E, K, and F. Blotting and visualization were performed as described under “Materials and Methods.”

FIG. 3. Effect of salts and ATP concentrations on the ATPase activity of purified enzyme. Panel A, salt concentration. The activity was measured at 2 mM ATP as described under “Materials and Methods.” Under these experimental conditions, at least 5 μM Na⁺ was always present as a contaminant. The inset shows the effect of higher concentrations of salts. ○, NaCl; □, LiCl. Panel B, ATP concentration. The assays were performed in the presence of 25 mM NaCl.
The Na\textsuperscript{+}-ATPase activity of purified enzyme was determined as described under "Materials and Methods" after a 10-min incubation of the enzyme at 25 °C with the inhibitor at the concentrations indicated. 100% activity corresponds to 0.5 unit.

| Reagent                  | Concentration (mM) | Relative Na\textsuperscript{+}-ATPase activity (%) |
|--------------------------|--------------------|---------------------------------------------------|
| Control                  |                    | 100                                               |
| KNO\textsubscript{3}     | 30                 | 56                                                |
| N-Ethylmaleimide         | 0.2                | 52                                                |
| Concanamycin A           | 0.005              | 96                                                |
| Destruxin B              | 0.03               | 49                                                |
| N,N'-Dicyclohexylcarbodiimide | 0.2            | 10                                                |
| Vanadate                 | 1                  | 90                                                |
| Azide                    | 10                 | 100                                               |

Purification of E. hirae Na\textsuperscript{+}-ATPase

NtpJ protein may be the part of the Na\textsuperscript{+}-ATPase complex. We have suggested recently that the ntpJ gene product is a component of the KtrII K\textsuperscript{+} transport system, functionally independent of the Na\textsuperscript{+}-ATPase activity (19). Western blotting with antisera against the oligopeptide for the NtpJ sequence was done for the fractions from glycerol gradient centrifugation (Fig. 5). We found that the NtpJ protein, observed mainly in fractions 13 and 15 (Fig. 5B), was separable from the Na\textsuperscript{+}-ATPase complex; only the A, B, C, and K subunits were observed here by silver staining in the fractions containing the peak of Na\textsuperscript{+}-ATPase activity (Fig. 5A, fractions 5 and 7). Moreover, neither the activity nor the assembly of Na\textsuperscript{+}-ATPase was impaired in an ntpJ-disrupted strain (19). These results suggest that the NtpJ protein is not part of the E. hirae Na\textsuperscript{+}-ATPase complex.

DISCUSSION

Sodium ions regulate the induction of Na\textsuperscript{+}-ATPase in E. hirae (30). Transcription of the Na\textsuperscript{+}-ATPase operon is stimulated under conditions where the intracellular Na\textsuperscript{+} concentration is high (24). In Na\textsuperscript{-}-limited medium, on the other hand, the sodium pumping activity via this ATPase is minimal. In the background to this work, we transformed pKAZ191 (18), harboring the whole ntp operon without its 5'-promoter region, into an Na\textsuperscript{+}-ATPase-defective mutant Nak1 (31); the operon is transcribed by the promoter activity of the vector. Cells of Nak1/pKAZ191 exhibited sodium pumping activity even on low Na\textsuperscript{+} medium. We also observed that the Na\textsuperscript{+}-ATPase activity of strain Nak1 transformed with pKAZ192 (18), harboring the whole ntp operon including its promoter, was stimulated by the Na\textsuperscript{+} concentration in the medium, suggesting that active Na\textsuperscript{+}-ATPase is expressed by the Na\textsuperscript{-}-responsive ntp operon containing these ntp genes (Fig. 2A) (24).

Several aspects of the purification of Na\textsuperscript{+}-ATPase should be emphasized. First, the enzyme was induced strongly by Na\textsuperscript{+}; complex medium supplemented with 500 mM NaCl (final, 650 mM Na\textsuperscript{+}) was used. The specific activity of the Na\textsuperscript{+}-ATPase of the membrane vesicles was 0.15 unit/mg of protein. The amount of Na\textsuperscript{+}-ATPase was enhanced further by introducing the operon on a multicopy shuttle vector pCem3, although the copy number was not determined exactly; the ATP hydrolytic activity of the membranes was 0.75 unit/mg of protein. Because the organism also contains the proton-translocating F\textsubscript{o}F\textsubscript{\textgamma}{\textsubscript{\textgamma}}.
the Ntp proteins in databases indicated that nine putative subunits. The

fractionated with anti-NtpJ antiserum. Fractions (10 μl) from a glycerol gradi-

cient was used as an immunogen for the NtpJ gene product (panel B).

ATPase (33), the Na\(^{+}\)-ATPase was purified from a mutant (25D) defective in the production of the H\(^{-}\)-ATPase (14). This

The V\(_1\) moiety is easily dissociated from the V\(_0\) moiety by

27). EDTA washing of the proteoliposomes suggested that the A, B, C, D, E, and G subunits are likely to make up the V\(_0\) subunit. Densitometric analysis of purified enzyme stained with Coomassie dye suggested that the A, I, B, C, D, E, F, K, and G subunits of E. hirae Na\(^{+}\)-ATPase occurred in a molar ratio of 3:1–2:3:1:3:1–2:3–4:1. The ratios of the A, B, and D subunits were identical with those of the purified catalytic moiety of this Na\(^{+}\)-ATPase (15). Peng et al. (32) found higher molar ratio of E subunit/vacuolar ATPase complex in clathrin-coated vesicles. In this context, it is noteworthy that the ratio of E subunit/complex was reproducibly 3 by Coomassie staining in our estimation. We are now engaged in determining the exact molar ratio of Ntp subunits, especially focusing on the possibility that the E subunit may be another major subunit of the vacuolar ATPases.

The E. hirae sodium pump is the first demonstrated example of a Na\(^{+}\)-translocating ATPase having a vacuolar ATPase structure; the purified ATPase retained the capacity for elec-

trogenic Na\(^{+}\) pumping by the reconstituted proteoliposomes (Fig. 4). Although this Na\(^{+}\)-ATPase, as well as archaeobacterial ATPases, is sensitive to macrolide antibiotics such as bafilomycin A\(_1\) or concanamycin A, the molecular resemblance to the H\(^{-}\)-translocating vacuolar ATPase of many other organisms suggests that the fundamental mechanism of ion transport is equivalent in these phylogenetically related enzymes. This so-

dium-coupled E. hirae system has yielded new insights into the
catalytic mechanism of these enzymes, and it has many advantages for the investigation of the energy coupling mechanism.

Acknowledgments—We thank Dr. F. M. Harold for critical reading of this manuscript. We also thank ADVANCE Co. (Chofu, Japan) for help in culturing E. facalis at the large scale and Dr. A. Takatsuki, the Institute of Physical and Chemical Research (RIKEN), Wako, Japan, for providing destruxin B and concanamycin A.

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