Thermus thermophilus Membrane-associated ATPase

INDICATION OF A EUBACTERIAL V-TYPE ATPase*

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An ATPase with $M_\text{r}$ of 360,000 was purified from plasma membranes of a thermophilic eubacterium Thermus thermophilus, and was characterized. ATP hydrolytic activity of the purified enzyme was extremely low, 0.07 μmol of $P_i$ released mg$^{-1}$ min$^{-1}$, and it was stimulated up to 30-fold by bisulfite. The following properties of the enzyme indicate that it is not a usual F$_1$-ATPase but that it belongs to the V-type ATPase family, another class of ATPases found in membranes of archaebacteria and eukaryotic endomembranes. Among its four kinds of subunits with approximate $M_\text{r}$ values of 66,000 (α), 55,000 (β), 30,000 (γ), and 12,000 (δ), the α subunit had a similar molecular size to the catalytic subunits of the V-type ATPases but was significantly larger than the α subunit of F$_1$-ATPases. ATP hydrolytic activity was not affected by azide, an inhibitor of F$_1$-ATPases, but was inhibited by nitrate, an inhibitor of the V-type ATPase. N-terminal amino acid sequences determined for the purified α and β subunits showed much higher similarity to those of the V-type ATPases than those of F$_1$-ATPases. Thus the distribution of the V-type ATPase in the prokaryotic kingdom may not be restricted to archaebacteria.

F$_1$F$_0$-ATPases are responsible for ATP synthesis coupled with H$^+$ translocation across membranes. They are purified from a variety of sources such as mitochondria, chloroplasts, and bacteria and have remarkably common characteristics (1–8). A water-soluble moiety with ATP hydrolytic activity is easily detached from F$_{1}$F$_{0}$-ATPase. This moiety, F$_{1}$-ATPase, and bacteria and have remarkably common characteristics of cell. This notion has been challenged by the recent finding that archaebacteria, such as Halobacterium halobium (13), and Methanothermus fervidus (14), contain novel membrane ATPases which are related to but obviously distinct from F$_{1}$-ATPases. The evolution of archaebacterial ATPases (10, 12, 14). Comparison of amino acid sequences deduced from DNA sequences finally established that archaebacterial ATPases and eukaryotic vacuolar H$^+$-ATPases belong to a single genetically related ATPase family, which is now called V-type ATPase (15–18). The catalytic subunit and eukaryotic vacuolar H$^+$-ATPase $M_\text{r}$ 66–70 kDa subunits are catalytic subunits and, therefore, correspond to the β subunit of F$_1$-ATPase. All archaebacterial ATPases reported up to now belong to the V-type ATPase, while this ATPase has not been found in any euobacteria, that is prokaryote, other than archaebacteria. On the basis of these observations, the evolution of H$^+$-ATPases and the evolutionary relationship between archaebacteria and eukaryotes have been postulated (15–19). However, the possibility still remains that euobacteria from which isolation of F$_1$F$_0$-ATPases has as yet been unsuccessful may contain V-type ATPase. Here we report the isolation and characterization of an ATPase from a thermophilic eubacteirium Thermus thermophilus which most probably belongs to the V-type ATPase family.

MATERIALS AND METHODS

Preparation of Membranes—T. thermophilus strain HB8 (ATCC 27064) was grown at 75°C under strong aeriation in a medium containing 10 g of yeast extract, 10 g of polypeptone and 2 g of NaCl per liter (20). Cells (200 g) harvested at the log phase were suspended in 400 ml of 50 mM Tris-SO$_4$, pH 8.0, containing 5 mM MgCl$_2$ and disrupted by sonication. Membranes were precipitated by centrifugation at 100,000 × g for 15 min and washed twice by centrifugation with the same buffer. At an early stage of this study, a low speed centrifugation (5,000 × g, 20 min) was carried out to remove cell debris. However, since we learned later that omission of this step improved the yield of the enzyme in the next chloroform treatment by about 80%, the disrupted cell suspension was directly applied for high speed centrifugation. Sonication and centrifugation were performed at 0–4°C, and the washed membranes were stored at −85°C.

Solubilization and Purification of ATPase—Washed membranes obtained from 900 g of cells were suspended in 400 ml of 50 mM Tris-SO$_4$ buffer, pH 8.0, and 120 ml of chloroform was added. The mixture was vigorously stirred with a Waring blender for 3 min. As an alternative to the chloroform treatment, ATPase activity was also solubilized from membranes by shaking with a low ionic strength solution, 5 mM Tris-SO$_4$, pH 8.0, containing 5 mM EDTA. The homogenate was centrifuged for 60 min at 10,000 × g. Solubilization of ATPase was carried out at 4°C, and the procedures thereafter closely resemble eukaryotic vacuolar H$^+$-ATPases than F$_{1}$-ATPases. Archaeobacterial ATPases are not affected by N$_5$, a specific inhibitor of F$_{1}$-ATPase, but are inhibited by NO$_3$, an inhibitor of vacuolar H$^+$-ATPase. The molecular weight of the α subunits of archaeobacterial ATPases (64,000–66,000) is significantly larger than that of the α (and β) subunits of F$_1$-ATPases (10, 12, 14).
were performed at room temperature. The yellowish supernatant was loaded onto a DRAE-Sepharose column (10 x 200 cm) equilibrated with buffer A (50 mM Tris-SO4, pH 8.0, 0.1 mM EDTA). The column was washed successively with 300 ml of buffer A plus 0.1 M NaCl, 300 ml of buffer A plus 0.2 M NaCl, and 300 ml of buffer A plus 0.3 M NaCl.

ATPase activity was eluted at the last washing step. Solid ammonium sulfate (20 g) was added to the combined active fractions (200 ml), and the solution was loaded on to a butyl-Toyopearl column (Toyosoda, 2 x 30 cm) equilibrated with a solution containing 50 mM Tris-SO4 buffer, pH 8.0, containing 0.1 mM EDTA and 1.2 M ammonium sulfate. The column was washed with 200 ml of the same buffer, and then a decreasing linear gradient of ammonium sulfate from 1.2 to 0 M, each 200 ml, was applied. ATPase activity was eluted at about 0.1 M ammonium sulfate. The combined active fractions were concentrated to 2 ml by ultrafiltration with an Amicon XM-300 membrane. The concentrated solution was applied to a Sepharose 6B column (2 x 90 cm) which was eluted and eluted with buffer A. The active fractions were combined and stored in 50% ammonium sulfate at 4 °C until use.

**ATPase and Protein Assays—**ATPase activities were measured at 55 °C in 500 ml of a reaction mixture containing 50 mM Na-HEPES, 5 mM ATP, 5 mM MgCl2, and 300 mM Na2SO4. When one component of the assay conditions was varied to examine its effect, the remaining conditions were kept unchanged from those described above. Reactions were initiated by the addition of ATP. Released Pi was measured by the method of Fiske and Subbarow (21). One enzyme unit is defined as the amount of enzyme producing 1 pmol of Pi/min.

**N-terminal Amino Acid Sequencing of the α and β Subunits—**The solution containing 3 mg of the purified enzyme in 200 ml of buffer A was subjected to reversed phase HPLC on a Bio-Rad RP-304 column (4.6 x 250 mm) equilibrated with 0.1% (v/v) aqueous trifluoroacetic acid. The following gradient of increasing acetonitrile in 0.1% trifluoroacetic acid was applied to develop the chromatography at a flow rate of 1 ml min⁻¹: 0-4 min (0% acetonitrile), 4-5 min (0-43%), 5-35 min (43-62%), 35-36 min (62-95%), 36-40 min (95%). Eluted proteins were monitored by the absorbance at 280 nm. Fractions to be analyzed were dried by vacuum centrifugation, dissolved in 1% sodium dodecyl sulfate (SDS) and analyzed by SDS-polyacrylamide gel electrophoresis (24). Although the γ and subunits were eluted as a mixture at 18.5 min, the α and β subunits were eluted as separated peaks at 20.2 and 23.8 min, respectively. The N-terminal amino acid sequences of the α and β subunits were analyzed with an Applied Biosystems 470 gas phase sequenator.

**RESULTS**

**Purification of T. thermophilus ATPase—**The ATP-hydrolyzing activity of the membrane fraction from *T. thermophilus* was 0.09 unit/mg protein or 1,200 total units for membranes, although the yield was approximately half that of the decreasing molecular weight, and their apparent molecular weight of the (γ and β subunits was 12,000, respectively, from the mobility in the gel (Fig. 2, right panel). The purity of the ATPase was confirmed by a gel permeation HPLC and by polyacrylamide gel electrophoresis in the absence of SDS. The protein was eluted from a column as a single peak which had ATPase activity (Fig. 1) and migrated as a single protein band in a gel (Fig. 2, left panel).

**Molecular Weight and Subunit Composition—**In order to estimate molecular weight, the purified ATPase was analyzed with a gel permeation HPLC column (Tosoh G-3000 SWXL), and the column was eluted with 50 mM Tris-SO4, pH 7.2, 1 mM EDTA, and 200 mM Na2SO4 at a flow rate of 0.5 ml/min at room temperature. Fractions of 0.5 ml were collected and protein and ATPase activity of each fraction were assayed. Elution was monitored by absorbance at 280 nm (upper trace, arbitrary absorbance scale). Ferritin (FER, M, 450,000), TF, (M, 380,000), catalase (CAT, M, 240,000), and TF, β subunit (M, 50,000) were used as M standards. Each standard was analyzed separately. The position of each in the eluate is indicated by arrows in the figure.

**FIG. 1.** Gel permeation of HPLC of purified *T. thermophilus* ATPase. Three milligrams of purified *T. thermophilus* ATPase were injected onto a gel permeation HPLC column (Tosoh G-3000 SWXL) and the column was eluted with 50 mM Tris-SO4, pH 7.2, 1 mM EDTA, and 200 mM Na2SO4 at a flow rate of 0.5 ml/min at room temperature. Fractions of 0.5 ml were collected and protein and ATPase activity of each fraction were assayed. Elution was monitored by absorbance at 280 nm (upper trace, arbitrary absorbance scale).

**TABLE I**

| Fraction | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|----------|-------------------|-----------------------|-----------------------------|----------|
| Extract* | 997               | 110                   | 0.11                        | 100      |
| DIAE-Sepharose | 389        | 86                    | 0.22                        | 78       |
| Butyl-Toyopearl | 72        | 108                   | 1.5                         | 98       |
| Sepharose 6B | 58         | 99                    | 1.8                         | 90       |

* Extract obtained by chloroform treatment.

The abbreviations used are: HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; DCCD, dicyclohexylcarbodiimide; NBG, 7-chloro-4-nitrobenzofrazan; SDS, sodium dodecyl sulfate; TF, β subunit from a thermophilic bacterium PS3; HPLC, high pressure liquid chromatography.
Enzyme-coupled assay with an ATP stable at 55 °C in the presence of 200 mM Na₂SO₃ was not applicable for this enzyme, since auxiliary enzymes were not regenerating system using pyruvate kinase and lactate dehydrogenase in micromolar range concentrations of ATP.

The linearity of the Lineweaver-Burk plots can be extended down to ATP, GTP, ITP, UTP, and CTP at relative rates of 100, 55, 37, 37, and 46%, respectively. Lineweaver-Burk plots for ATP hydrolysis by the enzymes at pH 6.0, 6.5, 7.0, 8.0, 8.5, and 9.0 were 35, 35, 37, 37, and 46%, respectively. Lineweaver-Burk plots for ATPase activity at ATP concentrations lower than 200 μM was not measurable by this method. Enzyme-coupled assay with an ATP regenerating system using pyruvate kinase and lactate dehydrogenase was not applicable for this enzyme, since auxiliary enzymes were not stable at 55 °C in the presence of 200 mM Na₂SO₃. However, it was inactivated completely after a 10-min incubation at 95 °C. Unlike the usual F₁-ATPase, the purified enzyme (1 mg ml⁻¹) in 50 mM Tris-SO₃ (pH 8.0) and 1 mM EDTA was not inactivated by overnight exposure to low temperature at 4 °C.

Effects of Ions and Inhibitors—Similar to ATPases from archaea (10-12), but to a more pronounced extent, the inclusion of Na₂SO₃ or NaHCO₃ in the assay mixtures caused remarkable activation of the ATP hydrolytic activity of the T. thermophilus ATPase (Fig. 3). High concentrations of these salts were required to gain maximum activation. Since NaCl did not show such an activating effect, this activation was not due to Na⁺. Potassium, chloride, and sulfate ions were ineffective as activating ions. Since the extent of activation by Na₂SO₃ decreased with the increase of pH (more than a 50-fold activation at pH 6.5, 30-fold at pH 7.5, and 6-fold at pH 8.5, for example) a truly effective ion species should be a bisulfite, but not a sulfite. Similarly, bicarbonate, but not carbonate, might be an effective anion.

Sensitivity of the ATPase activity of the T. thermophilus ATPase to some of the specific inhibitors for other ATPases was examined (Table II). The ATPase activity of the T. thermophilus ATPase was neither inhibited by azide, an inhibitor of F₁-ATPases, nor by vanadate, an inhibitor of the ATPases that are characterized by the formation of a phosphorylated intermediate (27). Nitrate, known as an inhibitor of vacuolar H⁺-ATPases and archaebacterial ATPases (11, 28), showed a significant inhibitory effect on the activity of the T. thermophilus ATPase. It is known that covalent modification of a single glutamic acid residue of the β subunit of F₁-ATPases by dicyclohexylcarbodiimide (DCCD) results in inactivation of their ATPase activities (9). As shown in Table II, half of ATP hydrolytic activity of the T. thermophilus ATPase was inactivated by DCCD. Since TF₁ lost about 98% of its ATPase activity under the same conditions, the T. thermophilus ATPase was relatively more resistant to DCCD inactivation than TF₁. Another chemical modification reagent, 7-chloro-4-nitrobenzofuzin (NBf-Cl), which inactivates F₁-ATPase and V-type ATPase, was a potent inhibitor of the T. thermophilus ATPase. The inhibition by DCCD and NBf-Cl did not reach completion under the conditions described. However, further addition of these reagents to the enzyme increased to about 2.0 unit mg⁻¹, but the Kᵥ value was not changed. This kind of kinetic behavior, low specific activity in the absence of the activator anions, linear Lineweaver-Burk plots, and a large Kᵥ value have been reported for ATPases from archaea (10-12, 14). As expected, this enzyme was extremely heat-stable, and the maximum activity, 2.8 unit mg⁻¹, was observed at around 85 °C for a 5-min assay at 10 μg ml⁻¹ of the enzyme in the reaction mixture containing 200 mM Na₂SO₃. However, it was inactivated completely after a 10-min incubation at 95 °C. Unlike the usual F₁-ATPase, the purified enzyme (1 mg ml⁻¹) in 50 mM Tris-SO₃ (pH 8.0) and 1 mM EDTA was not inactivated by overnight exposure to low temperature at 4 °C.

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reaction mixture reinitiated inactivation. These results show that the sensitivity of the *T. thermophilus* ATPase to various inhibitors is more similar to that of V-type ATPases than to that of F$_i$-ATPases.

### N-terminal Amino Acid Sequence of $\alpha$ and $\beta$ Subunits of *T. thermophilus* ATPase

-Forty-three and eighteen amino acid residues from the N termini of the $\alpha$ and $\beta$ subunits, respectively, were determined by Edman degradation and compared with the N-terminal sequences of the catalytic and noncatalytic major subunits of several V-type ATPases and F$_i$-ATPases from bacteria, fungi, plants, and animals (Fig. 4). The $\alpha$ subunit of the *T. thermophilus* ATPase was compared with the catalytic subunits: eukaryotic vacuolar H$^+$-ATPase 67-70-kDa subunits, *S. acidocaldarius* $\alpha$ subunit, and F$_i$-ATPase $\beta$ subunit. The $\beta$ subunit of the *T. thermophilus* ATPase was compared with the noncatalytic subunits; eukaryotic vacuolar H$^+$-ATPase 57-58-kDa subunits, *S. acidocaldarius* $\beta$ subunit, and F$_i$-ATPase $\alpha$ subunit. The $\beta$ subunit of the *T. thermophilus* ATPase contained 10 amino acid residues that were identical to the catalytic subunits of all of the listed V-type ATPases. When the sequence of the $\alpha$ subunit of the *T. thermophilus* ATPase was compared with each of the V-type ATPases from *Arabidopsis thaliana*, Neurospora crassa, and *S. acidocaldarius*, as many as 44, 40, and 35%, respectively, of the amino acid residues were identical. In contrast, when the sequence was compared with the catalytic $\beta$ subunits of F$_i$-ATPases from *Escherichia coli*, tobacco chloroplasts, and bovine mitochondria, only 14, 14, and 19%, respectively, of the residues were identical. Similarly, 4 out of 18 amino acid residues in the N-terminal sequence of the $\beta$ subunit of the *T. thermophilus* ATPase were identical to the noncatalytic subunits of all of the listed V-type ATPases. Again, the sequence similarity in the region of the N termini between the $\beta$ subunit of the *T. thermophilus* ATPase and the noncatalytic $\alpha$ subunits of F$_i$-ATPases was so poor that a meaningful alignment appeared impossible. When a comparison was made between different combinations, between the *T. thermophilus* $\alpha$ subunit and the F$_i$-ATPase $\alpha$ subunits or between the *T. thermophilus* $\beta$ subunit and the F$_i$-ATPase $\beta$ subunits, no meaningful sequence similarity was found. These results strongly indicate that the *T. thermophilus* ATPase is not an F$_i$-ATPase but belongs to the V-type ATPase family.

### DISCUSSION

V-type ATPases have been found in a variety of eukaryotic endomembrane vacuolar vesicles, but in the prokaryotic kingdom, they have so far been detected only in archaeabacterial plasma membranes (17-19, 25). From this unique distribution of the V-type ATPases, it has been postulated that the vacuolar H$^+$-ATPase of eukaryotes arose by the internalization of the plasma membrane H$^+$-ATPase of an archaeabacterial-like ancestral cell (15). We solubilized and purified an ATPase from the membranes of *T. thermophilus*. This ATPase was expected to be an F$_i$-ATPase since the bacterium is an archaeobacterium. However, the properties of the *T. thermophilus* ATPase described in this report, such as resistance to azide inhibition, sensitivity to nitrate inhibition, a large $\alpha$ subunit

### TABLE II

| Reagents                  | Preincubation | Residual activity% |
|---------------------------|---------------|--------------------|
| None                      | 100           |                    |
| NH$_4$VO$_3$ (0.1 mM)     | 60            | 100                |
| Na$_2$SO$_3$ (1 mM)       | 60            | 100                |
| NaN$_3$ (20 mM)           | 40            |                    |
| DCCD (1 mM)               | 120           | 50                 |
| NBGCl (1 mM)              | 120           | 31                 |

*The control activity in the absence of inhibitors was 2.0 units/mg which was taken as 100%. This activity remained unchanged after 60 or 120 min of incubation without inhibitors.*

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**FIG. 4.** Comparison of N-terminal sequences of $\alpha$ and $\beta$ subunits of *T. thermophilus* ATPase with acatactyl (A) and noncatactyl (B) major subunits of V-type ATPases and F$_i$-ATPases. The upper four lines and lower three lines represent V-type ATPases and F$_i$-ATPases, respectively. The sequence of the 70-kDa subunit of bovine clathrin-coated vesicle H$^+$-ATPase is a sequence obtained by tryptic digestion (34). Other sequence data were taken from references: human clathrin-coated vesicle H$^+$-ATPase 58-kDa subunit (34), *A. thaliana* vacuolar H$^+$-ATPase 69-kDa subunit (35), *carrot* vacuolar H$^+$-ATPase 67-kDa subunit (36), *N. crassa* vacuolar H$^+$-ATPase 67- and 57-kDa subunits (37, 38), *S. acidocaldarius* ATPase $\alpha$ and $\beta$ subunits (17, 39), *E. coli* F$_i$ $\alpha$ and $\beta$ (40, 41), tobacco chloroplast F$_i$ $\alpha$ and $\beta$ (42, 43), and bovine mitochondrial F$_i$ $\alpha$ and $\beta$ (1).
molecular weight, and a high degree of similarity in the N-terminal amino acid sequences of the two major subunits to those of the V-ATPases, strongly indicate that it belongs to the V-type ATPase family, not to the F$_{1}$-ATPases. From an analysis of its 16 S rRNA (29-31) and from the fact that its membranes do not contain ether lipids (unique components of archaebacterial membranes (32,33)) it has been well established already that T. thermophilus is a eubacterium, not an archaebacterium. Comparison of the full sequences of the T. thermophilus ATPase subunits with other classes of ATPases and knowledge of the distribution of V-type ATPase in various eubacteria will provide clues to discovering the evolutionary reason why a eubacterium, T. thermophilus, has a V-type ATPase.

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