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# Rapid purification and characterization of $F_1$-ATPase of *Vibrio parahaemolyticus*

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**The $F_1$ portion of $H^+$-translocating ATPase was purified from membrane vesicles of *Vibrio parahaemolyticus* by a rapid procedure. The whole purification process (from culture of cells to purification of the enzyme) could be completed in 1 day. The $F_1$-ATPase consists of five subunits ($\alpha$, $\beta$, $\gamma$, $\delta$ and $\epsilon$) like $F_1$ of *Escherichia coli* and other microorganisms. The $F_1$-ATPase of *V. parahaemolyticus* showed some interesting properties. Its activity was greatly stimulated by high concentrations (about 0.5 M) of $SO_4^{2-}$, $SO_3^{2-}$ and $CH_3COO^-$, their effects decreasing in this order. Among the anions tested, $Cl^-$ and $NO_3^-$ were ineffective, or rather inhibitory, and cations had no significant effects. Ethanol (or methanol) stimulated the activity 2- to 3-fold. The activity was inhibited by 4-acetamido-4′-isothiocyanostilbene 2,2′-disulfonate (SITS) (an anion exchanger inhibitor), tetrachlorosalicylanilide (TCS) (an $H^+$ conductor), azide and *N*-ethylmaleimide. Zinc inhibited the activity only slightly, although it strongly inhibited the ATPase activity in membrane vesicles.**

## Introduction

The $H^+$-translocating ATPase is widely distributed in living organisms. $F_0F_1$-type $H^+$-translocating ATPase is present in membranes of microorganisms, mitochondria and chloroplasts. The main function of the $F_0F_1$-type ATPase is to synthesize ATP, which is essential for life. The driving force for ATP synthesis is an electrochemical potential of $H^+$ across the membrane established by electron transport chains. On the other hand, $Na^+$-translocating $F_0F_1$-type ATPase has been found in a strictly anaerobic eubacterium *Propionigenium modestum* [1]. The driving force for ATP synthesis by this system is an electrochemical potential of $Na^+$, which is established by $Na^+$-translocating decarboxylase [2]. Thus mechanisms by which membrane-mediated ATP synthesis are operated are more varied than originally thought.

*Vibrio parahaemolyticus* is a slightly halophilic marine bacterium. It requires $Na^+$ for growth, and possesses a system for $Na^+$ extrusion, a respiratory-driven $Na^+$ pump [3], like that of *V. alginolyticus* in which this $Na^+$ pump was originally found [4]. This pump establishes an electrochemical potential of $Na^+$, which is a driving force for active transport of nutrients [3] and for flagella rotation [5]. The respiratory chain of these *Vibrios* also extrudes $H^+$, establishing an electrochemical potential of $H^+$. It was not clear what type of ATP synthetase is present in *V. parahaemolyticus* or whether the electrochemical potential of $H^+$ or of $Na^+$ is the driving force for ATP synthesis. We have found that an $F_0F_1$-type $H^+$-translocating ATPase is present in the membranes of *V. parahaemolyticus*, and that this enzyme is involved in ATP synthesis [6]. We have also isolated mutants which lack the $H^+$-translocating ATPase. Our experimental results suggested the presence of another ATP synthetase involved in oxidative phosphorylation in *V. parahaemolyticus*. It seemed important to characterize the ATP synthetases of this organism.

Previously, we reported some properties of a membrane-bound ATPase of *V. parahaemolyticus* [7]. Since no ATPase activity was observed in the mutants de-

Abbreviations: $F_1$, extrinsic and catalytic portion of the $H^+$-translocating ATPase; $F_0$, intrinsic portion of the $H^+$-translocating ATPase; Mops, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-glycine; Ches, 2-(cyclohexylamino)ethanesulfonic acid; NEM, *N*-ethylmaleimide; DCCD, dicyclohexylcarbodiimide; TCS, tetrachlorosalicylanilide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate.

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scribed above, the ATPase we characterized using membrane vesicles seems to be $F_0F_1$. Consistent with this idea, the membrane-bound ATPase activity was inhibited by DCCD and by azide. We tried to purify the $F_1$-ATPase of *V. parahaemolyticus* to characterize it. During the course of this study, we found that the $F_1$-ATPase of this organism is very unstable, and is difficult to purify in an active form. Therefore, we tried to establish a procedure for purification of the $F_1$-ATPase in an active form. Here we report a procedure for rapid purification of the $F_1$-ATPase of *V. parahaemolyticus* and some properties of this enzyme.

## Materials and Methods

Cells of *V. parahaemolyticus* AQ3334 were grown aerobically in medium S [8] supplemented with 0.5% polypeptone at 37 °C, and harvested in the late exponential phase of growth. The cells were washed twice with 10 mM Mops-Tris (pH 7.5), 0.3 M choline chloride, 25 mM $MgSO_4$, and suspended in the same buffer containing 0.5 mM PMSF. Membrane vesicles were prepared with a French press [8], and washed twice with the above buffer. When necessary, the preparations of membrane vesicles were supplemented with 50% glycerol and stored at −80 °C until use.

$F_1$-ATPase was purified as follows. Membrane vesicles (about 20 mg protein) were incubated in 45 ml of 3 mM Tricine-Tris buffer (pH 8.0) containing 1 mM 2-mercaptoethanol, 5 mM $MgSO_4$ and 10% glycerol at 20 °C for 30 min with gentle stirring, and then centrifuged at 100000 × *g* for 60 min. The washed vesicles were incubated in 30 ml of 3 mM Tricine-Tris buffer (pH 8.0) containing 1 mM 2-mercaptoethanol, 1 mM $MgSO_4$, 0.5 mM EDTA and 10% glycerol at 20 °C for 30 min with gentle stirring, and then centrifuged at 100000 × *g* for 60 min. Then $MgSO_4$ and PMSF were added to the supernatant at final concentrations of 10 mM and 0.1 mM, respectively. The supernatant was then applied to a Mono Q column (HR5/5, Pharmacia), pre-equilibrated with buffer consisting of 50 mM Tris-HCl (pH 7.0), 1 mM 2-mercaptoethanol, 10 mM $MgSO_4$, 0.5 mM EDTA, 0.5 mM PMSF, 1 mM benzamidine and 10% glycerol. The column was washed with 4 ml of the same buffer, and then developed with 23 ml of a linear gradient of 150 to 350 mM NaCl in the same buffer at a flow rate of 1 ml/min, using a Pharmacia FPLC system. The absorbance of the eluate at 280 nm was monitored. Fractions (1 ml) were collected, and assayed for ATPase activity.

The standard reaction mixture (0.6 ml) for assay of ATPase contained 20 mM Ches-Tris buffer (pH 8.6), 4 mM $MgSO_4$, 0.5 M $Na_2SO_4$, 2% ethanol and 4 mM ATP. Reaction mixtures were incubated at 37 °C for 15 min, and then the inorganic phosphate released was determined colorimetrically [9]. One unit of activity is defined as that releasing 1 μmol of inorganic phosphate per min. For determination of the effects of DCCD and NEM, purified ATPase was incubated with these compounds at 37 °C for 30 min, and then activity was measured.

SDS-PAGE was performed as described by Laemmli [10].

Protein contents were determined by the method of Schaffner and Weissmann [11].

## Results

*Purification of $F_1$-ATPase*

For purification of the $F_1$ portion of the $H^+$-translocating ATPase of *V. parahaemolyticus*, we first tried to follow the procedure for purification of $F_1$ of *E. coli*, in which $F_1$ is released by washing membrane vesicles (everted) with buffer containing EDTA at low salt concentration [12]. $F_1$ was released from membranes of *V. parahaemolyticus* by this procedure, but we could not detect ATPase activity in the supernatant, although we detected the α and β subunits. Thus $F_1$ seemed to dissociate into subunits on this treatment. In fact, when we applied the supernatant to a DEAE-Sepharose column, we detected α and β in different fractions. Thus it seemed necessary to prevent the dissociation of $F_1$ released from membranes. Addition of 1 mM $MgSO_4$ to the buffer prevented the dissociation, and resulted in release of $F_1$ from the membranes in an active form, although in low yield. The preceding step of washing the membranes with buffer containing 5 mM $MgSO_4$ at room temperature was effective for removing loosely bound proteins. When the washing and releasing steps were done at low temperature, considerable loss of ATPase activity was observed, even in the presence of $Mg^{2+}$.

We added 10 mM $MgSO_4$ and 0.1 mM PMSF to the solubilized fraction to stabilize the $F_1$-ATPase, and then applied this solubilized fraction to a Mono Q column. The column was then washed with buffer and eluted with a linear gradient of NaCl (150 to 350 mM). The ATPase activity was eluted in one peak fraction with protein at about 260 mM NaCl (Fig. 1). Most of the protein applied to the column was eluted with 1 M NaCl (data not shown).

This fraction of ATPase gave a single band on regular PAGE (data not shown). On SDS-PAGE, the ATPase fraction gave five bands, which seemed to correspond to subunits α, β, γ, δ and ε (Fig. 2). The masses of these subunits were 55, 51, 31, 20 and 14.5 kDa, respectively. Judging from their mobilities, most of the subunits (except δ) seemed to have larger masses than the corresponding subunits of $F_1$ of *E. coli*. We did not observe any other band, so this ATPase seemed to be highly purified. We measured the density of each protein band with a densitometer. The stoichiometry of the subunits,

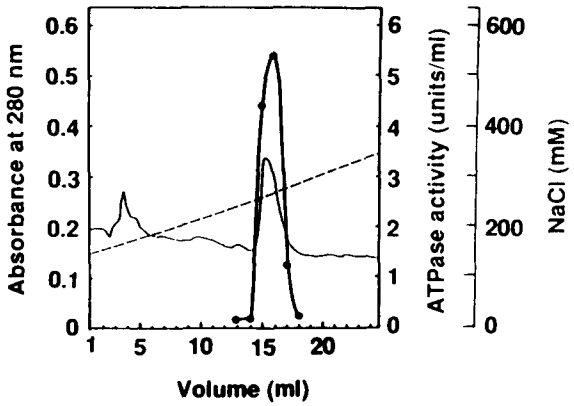


Fig. 1. Elution profile of the $F_1$-ATPase on Mono Q column chromatography. The $F_1$-ATPase was purified in an FPLC system (Pharmacia) with a Mono Q anion-exchange column. The EDTA extract was applied to the column, and proteins were eluted with a linear gradient of 150 to 350 mM NaCl (- - - - -). Protein concentration was monitored at 280 nm (———). Fractions (1 ml) were collected, and their ATPase activity was assayed (●).

calculated from their densities and masses was about $\alpha:\beta:\gamma:\delta:\epsilon = 2:3:1:1:1$. The reasons for this apparently unique stoichiometry will be discussed later.

Table I summarizes the purification. The specific activity of the purified ATPase was about 20 units/mg protein and the yield was about 20% from washed membrane vesicles. The whole purification procedure could be completed in 12 h starting from cell cultures and in 5 h starting from membrane vesicles.

TABLE I

*Purification of the $F_1$-ATPase*

| Purification step | Total activity (units) | Protein (mg) | Specific activity (units/mg protein) | Recovery (%) | Purification (Fold) |
|---|---|---|---|---|---|
| Washed membrane vesicles | 44.1 | 17.7 | 2.5 | 100 | 1 |
| EDTA extract | 17.4 | 1.8 | 9.7 | 39 | 3.6 |
| Mono Q peak fraction | 9.9 | 0.5 | 19.8 | 22 | 7.6 |

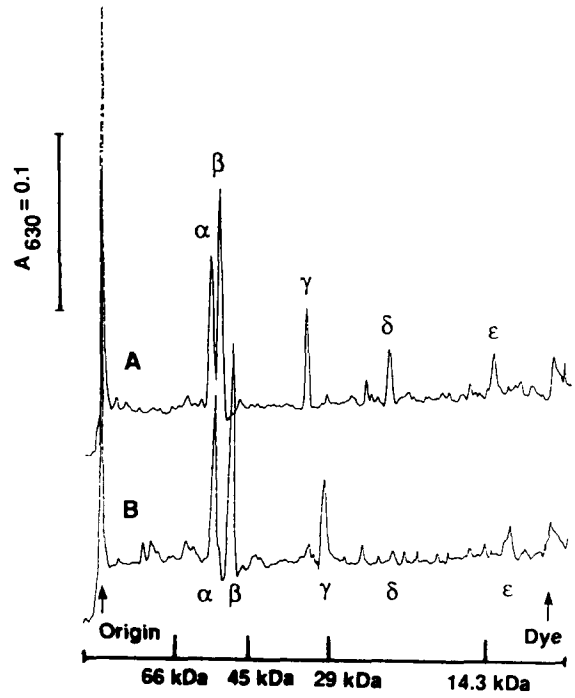


Fig. 2. Comparison of $F_1$-ATPase subunits from *V. parahaemolyticus* and *E. coli* on SDS-PAGE. Purified $F_1$-ATPases were analyzed by SDS-PAGE. The gel was stained with Coomassie brilliant blue, and scanned with a densitometer at 630 nm. The molecular mass markers were bovine serum albumin (66 kDa). ovalubumin (45 kDa), carbonic anhydrase (29 kDa) and lysozyme (14.3 kDa). (A) $F_1$ subunits of *V. parahaemolyticus*; (B) $F_1$ subunits of *E. coli*.

*Properties of $F_1$-ATPase*

The ATPase activity was dependent on $Mg^{2+}$. Activity was maximal with 4 mM $Mg^{2+}$ or more and did not decrease at higher concentrations of $Mg^{2+}$ (10 to 20 mM) (data not shown). Among the divalent cations tested, $Co^{2+}$ and $Mn^{2+}$ elicited some activity (30 to 40% of that with $Mg^{2+}$), and $Ca^{2+}$ elicited slight activity (about 20% of that with $Mg^{2+}$), but $Zn^{2+}$, $Ni^{2+}$ and $Fe^{2+}$ had no effect.

The ATPase was greatly stimulated by $Na_2SO_4$, which induced maximum activity (about 7-fold stimulation) at about 0.5 M (Fig. 3). Similar stimulation was observed with $K_2SO_4$, and less stimulation with $Li_2SO_4$ or $(NH_4)_2SO$ (data not shown). The ATPase activity was also stimulated to lesser extents by $Na_2SO_3$ and $NaOCOCH_3$, but not by NaCl and $NaNO_3$, which were rather inhibitory. Thus it seems that the $F_1$-ATPase has preference for anions, their effectiveness being in the order $SO_4^{2-} > SO_3^{2-} > CH_3COO^-$.

The ATPase activity was stimulated 2- to 3-fold by about 2% ethanol, and similar stimulation was observed with about 6% methanol (Fig. 4). This stimulation by alcohol was not observed with membrane-bound ATPase (data not shown).

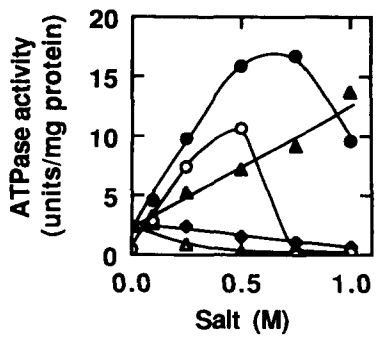


Fig. 3. Effects of salts on $F_1$-ATPase activity. The ATPase activity of the purified $F_1$ was measured in the presence of salts to test the effects of anions. Symbols: $Na_2SO_4$ (●), $Na_2SO_3$ (○), $NaOCOCH_3$ (▲), $NaNO_3$ (△), NaCl (◆).

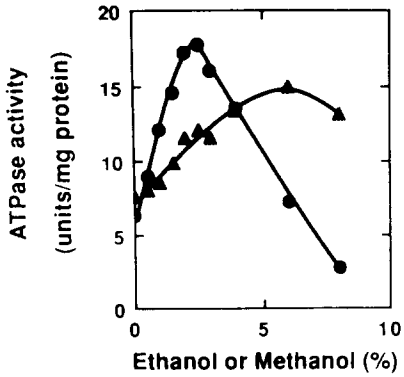


Fig. 4. Stimulation of $F_1$-ATPase activity by alcohol. Various concentrations of ethanol (●) or methanol (▲) were added to the assay mixtures, and ATPase activity was measured.

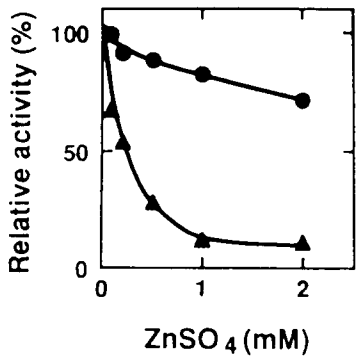


Fig. 5. Effects of $Zn^{2+}$ on purified $F_1$-ATPase and membrane-bound ATPase. The ATPase activities of the purified $F_1$ (●) and of membrane vesicles (▲) were measured in the presence of the indicated concentrations of $ZnSO_4$. The control values for $F_1$-ATPase activity and membrane-bound ATPase were 16.7 and 1.4 units/mg protein, respectively.

The optimum pH for ATPase activity in the presence of 0.5 M $Na_2SO_4$ and 2% ethanol was about 8.6 (data not shown).

We tested the effects on the $F_1$ of *V. parahaemolyticus* of chemicals that are known to inhibit ATPases. NEM and azide were considerably inhibitory (Table II), and vanadate, DCCD and $F^-$ were slightly inhibitory. Previously we reported that TCS, an $H^+$ conductor, but not CCCP, also an $H^+$ conductor, strongly inhibited the membrane-bound ATPase of *V. parahaemolyticus* [7]. Similar strong inhibitions by these compounds were observed with purified $F_1$. SITS, an inhibitor of anion-exchanger of erythrocytes [13], was the strongest inhibitor of the chemicals tested. Although $Zn^{2+}$ strongly inhibited the membrane-bound ATPase [7], it inhibited the purified $F_1$-ATPase only slightly (Fig. 5).

The $F_1$-ATPase of *V. parahaemolyticus* hydrolyzed ATP and ITP effectively, and GTP to a lesser extent (about 80% of the hydrolysis of ATP) (data not shown), but did not hydrolyze CTP, UTP, ADP or AMP.

TABLE II

*Effects of various compounds on $F_1$-ATPase*

| Compounds | ATPase activity | |
|---|---|---|
| | units/mg protein | % |
| Expt. 1 [a] | | |
| Control | 12.1 | 100 |
| NEM (1 mM) | 3.1 | 26 |
| DCCD (100 μM) | 9.9 | 82 |
| Expt. 2 [b] | | |
| Control | 16.7 | 100 |
| SITS (25 μM) | 0.9 | 5 |
| TCS (25 μM) | 4.9 | 29 |
| $NaN_3$ (1 mM) | 4.9 | 29 |
| $NaVO_3$ (0.3 mM) | 11.7 | 70 |
| NaF (1 mM) | 13.9 | 83 |

[a] Enzyme samples were preincubated in the absence or presence of DCCD or NEM at 37 °C for 30 min before the reaction.

[b] Enzyme samples were preincubated in the absence or presence of SITS, TCS, $NaN_3$, $NaVO_3$ or NaF at 37 °C for 3 min before the reaction.

## Discussion

We have developed a rapid method for purification of the $F_1$-ATPase of *V. parahaemolyticus*, and we have characterized the $F_1$-ATPase.

Some properties of the $F_1$-ATPase of *V. parahaemolyticus*, such as its subunit composition, substrate specificity and sensitivity to azide, were similar to those of the well-characterized $F_1$ of *E. coli*. However, the $F_1$-ATPase of *V. parahaemolyticus* showed some interesting properties that were different from those of *E. coli* $F_1$. First, it was greatly stimulated by $SO_4^{2-}$, $SO_3^{2-}$ or $CH_3COO^-$, $SO_4^{2-}$ causing the most stimulation. The $F_1$-ATPase of the thermophilic bacterium PS3 is also stimulated by $SO_3^{2-}$ [14], but shows no significant preference for ionic species, a property called halophilicity [14]. Nitrate and $Cl^-$ were rather inhibitory to *V. parahaemolyticus* ATPase in the absence of $SO_4^{2-}$, but not in the presence of $SO_4^{2-}$. Previously we reported some stimulation of the ATPase activity in membrane vesicles by $Cl^-$ [7]. Perhaps this activity was due to membrane-bound $Cl^-$-stimulated 5′-nucleotidase. It is interesting that $Cl^-$ and $NO_3^-$, but not $SO_4^{2-}$, $SO_3^{2-}$ or $CH_3COO^-$, stimulated the 5′-nucleotidase of *V. parahaemolyticus*. These anions do not activate the $F_1$-ATPase of *E. coli*, but activate the ATPase of archaebacteria and V-type ATPase of plants. The ATPase of *Halobacterium halobium* is activated by $SO_4^{2-}$ and $SO_3^{2-}$ [15], and that of *Sulfolobus acidocaldarius* is activated either by $SO_4^{2-}$ (under acidic conditions) or by $SO_3^{2-}$ [16]. In *Halobacterium saccharovorum*, $Cl^-$ activates the ATPase, but $SO_4^{2-}$ does not [17].

Second, the $F_1$-ATPase of *V. parahaemolyticus* was stimulated 2- to 3-fold by ethanol or methanol. This characteristic is also similar to that of $F_1$ of PS3 [14].

Ethanol or methanol did not significantly stimulate the ATPase activity in membrane vesicles (data not shown). Thus it is likely that a site(s) for the activation of alcohol was exposed as a result of release of $F_1$ from membranes.

Third, although $Mg^{2+}$ was necessary for activity, the optimal ATP : $Mg^{2+}$ ratio was not necessarily 2. With *E. coli* $F_1$ the optimum ATP : $Mg^{2+}$ ratio is reported to be 2, excess $Mg^{2+}$ reducing the ATPase activity [19]. With *V. parahaemolyticus* $F_1$, however, we observed a plateau level of activity at $Mg^{2+}$ concentrations of 4 mM or more (up to 20 mM) in the presence of 4 mM ATP. Furthermore, $Mg^{2+}$ could not be replaced by $Ca^{2+}$, unlike in the case of *E. coli* $F_1$ [19].

Fourth, NEM which inhibits ATPase of archaebacteria and V-type ATPase, but not *E. coli* $F_1$-ATPase, inhibited the $F_1$-ATPase of *V. parahaemolyticus*.

Fifth, SITS or TCS strongly inhibited the $F_1$-ATPase activity of *V. parahaemolyticus*, although these compounds do not inhibit $F_1$-ATPase of *E. coli*.

It is noteworthy that, although $Zn^{2+}$ strongly inhibited the ATPase activity in membrane vesicles [7], it inhibited purified $F_1$-ATPase only slightly. Similar effects were observed with DCCD, an inhibitor of the $F_0$ portion. DCCD is inhibitory by blocking the $H^+$ pathway of $F_0$. With $Zn^{2+}$, however, inhibition of $H^+$ flow through $F_0$ was not observed by measurement of fluorescence quenching (data not shown). Thus, the mechanism of inhibition of the $H^+$-translocating ATPase by $Zn^{2+}$ is different from that by DCCD.

The apparent stoichiometry of $\alpha : \beta : \gamma : \delta : \epsilon$ in the purified $F_1$ was about 2 : 3 : 1 : 1 : 1. The value for $\alpha$ was probably underestimated because the $\alpha$ subunit seems to be fairly unstable. When we did not add PMSF to buffers or when the purification took longer, we sometimes observed a fainter band of the $\alpha$ subunit than of the $\beta$ subunit on SDS-PAGE. We also observed much lower specific activity with these $F_1$-ATPase samples. Thus, the $\alpha$ subunit seems to be easily cleaved by proteinase(s), and the actual stoichiometry is probably 3 : 3 : 1 : 1 : 1, as in *E. coli* $F_1$.

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