Properties of Adenosine Triphosphate-Hydrolyzing Enzymes in Membrane Vesicles of Vibrio parahaemolyticus

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The properties and roles of adenosine triphosphate (ATP)-hydrolyzing enzymes in the membrane of Vibrio parahaemolyticus were investigated. At least two (perhaps three) types of ATP-hydrolyzing enzyme was present in everted membrane vesicles. The adenosine triphosphatase (ATPase) activity of one of the enzymes was sensitive to dicyclohexylcarbodiimide and the activity was lost when the membranes were washed with buffer containing ethylenediaminetetraacetic acid. This enzyme seems to be an H⁺-translocating ATPase. The other ATP-hydrolyzing activity was due to a tightly bound enzyme that could not be removed from the membranes by the washing. This enzyme required higher concentrations of Mg²⁺ (10 to 20 mM) and Cl⁻ (100 mM) for maximal activity. Zn²⁺ strongly inhibited the activity. The substrate specificity of this enzyme activity showed that it was a 5'-nucleotidase.

Keywords—membrane-bound ATPase, V. parahaemolyticus; H⁺-ATPase; 5'-nucleotidase; Na⁺-ATPase

Membrane-bound adenosine triphosphatases (ATPases) play important roles in energy transduction and cation transport. The H⁺-translocating ATPase is involved in adenosine triphosphate (ATP) synthesis and hydrolysis, both of which are coupled to H⁺ translocation across the membrane.¹ The H⁺-translocating ATPase is believed to be the most fundamental type of ion-translocating ATPase, and to be ubiquitous in nature.² A K⁺-translocating ATPase has been found in Escherichia coli membranes,³ and an Na⁺-translocating (perhaps Na⁺, K⁺-translocating)-ATPase in Streptococcus faecalis.⁴ Recently, a K⁺-ATPase, which has been called ktrA, has also been found in S. faecalis.⁵ Thus there may be other as yet unidentified cation(or anion)-translocating ATPase in microbial cell membranes.

We are interested in ion transport in Vibrio parahaemolyticus, which is a major cause of food poisoning in Japan. Although the pathogenicity of this organism is well understood,⁶ the physiological and biochemical properties have not been analyzed well. This organism requires about 0.2 M NaCl for maximal growth. We have reported that the respiratory chain of this organism extrudes Na⁺,⁷ like that of V. alginolyticus.⁸ Influx of Na⁺ down the electrochemical potential is utilized to drive transport of nutrients in several species of Vibrio.⁷,⁹,¹⁰ Furthermore, influx of Na⁺ is also utilized for ATP synthesis¹¹ and flagella rotation in V. alginolyticus.¹² Thus, the significance of Na⁺ circulation in membrane energy transduction in Vibrio has been partly clarified. However, the significance of Cl⁻ in membrane processes or energy transduction in V. parahaemolyticus is not well understood. There are many ion-translocating ATPase in biological membranes, and in this work we examined the characteristics of the ATP-hydrolyzing activities in the membrane fraction of V. para-

haemolyticus in order to obtain information on the roles of the enzymes.
Bacterium and Growth—*V. parahaemolyticus* AQ3334 cells were grown aerobically at 37°C in medium S consisting of 50 mM Tris-HCl (pH 7.8), 25 mM MgSO₄, 10 mM KCl, 1 mM CaCl₂, 0.33 mM K₂HPO₄, 0.01 mM FeSO₄, 10 mM (NH₄)₂SO₄ and 0.2 mM NaCl, supplemented with 0.5% polypeptone.

Preparation of Membrane Vesicles—Cells were harvested in the late-exponential phase of growth, washed twice with 0.3 M 4-morpholinepropanesulfonic acid (Mops)–KOH (pH 7.5) containing 5 mM MgSO₄ and suspended in the same buffer. Vesicles were prepared in a French press, washed twice with the same buffer and stored at −80°C. Ethylenediaminetetraacetic acid (EDTA)-treated membrane vesicles were prepared by washing vesicles from the French press with 3 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine)–Tris (pH 8.0) containing 0.5 mM EDTA and 1 mM 2-mercaptoethanol.

Assay of ATP-Hydrolyzing Activities—The standard reaction mixture (0.6 ml) contained 20 mM Tricine–Tris (pH 8.0), 2 mM (or 20 mM) MgSO₄, 0.2 M (or 0.1 M) NaCl and membrane vesicles (about 5 μg protein). Additions were made where indicated. After the addition of 4 mM ATP or other nucleotides, the reaction mixture was incubated at 37°C for 5 min. The inorganic phosphate released was determined colorimetrically. One unit of activity is defined as the amount releasing 1 μmol of inorganic phosphate per min at 37°C.

Protein Determination—Protein concentration was determined by a published procedure.

Results

Membrane-Bound ATP-Hydrolyzing Activities

The ATP-hydrolyzing activity (ATPase activity) in membrane vesicles was greatly enhanced by Mg²⁺, which was maximally effective at 10 to 20 mM (Fig. 1). Some of this activity was probably due to the H⁺-translocating ATPase. Since the optimum ratio of ATP to Mg²⁺ is 2 for the H⁺-translocating ATPase of *E. coli*, ATP hydrolysis by the H⁺-translocating ATPase is probably maximum at about 2 mM Mg²⁺, since the concentration of ATP in the assay mixture was 4 mM (ATP:Mg²⁺ = 2:1). If this is the case, then the ATPase activity observed at higher concentrations of Mg²⁺ should be due to some other enzyme(s) than the H⁺-translocating ATPase. This notion was confirmed by the following experiments. When membrane vesicles (everted) were washed with buffer containing EDTA, which is an easy way to remove the F₁ portion (catalytic portion) of the H⁺-translocating ATPase of *E. coli*, the ATPase activity observed at 2 mM Mg²⁺ was greatly reduced, but most of the ATPase activity observed at 10 to 20 mM Mg²⁺ was retained. This implies that the ATPase activity observed at higher Mg²⁺ concentrations was due to an enzyme(s) that was tightly bound to the membranes. We were able to solubilize the ATP-hydrolyzing enzyme(s) with a

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Fig. 1. Effect of Mg²⁺ on ATP-Hydrolyzing Activity in Membrane Vesicles

Assays were performed as described in the text. Various concentrations of Mg²⁺ were added to assay mixtures containing 0.2 mM NaCl and either untreated membrane vesicles (●) or EDTA-treated membrane vesicles (▲).

Fig. 2. Effect of DCCD on ATP-Hydrolyzing Activity

Various concentrations of DCCD were added to assay mixtures containing membrane vesicles (untreated) and either 2 mM (▲) or 20 mM Mg²⁺ (●). The ATP-hydrolyzing activities of uninhibited vesicles (100%) were 0.084 and 0.86 unit/mg of protein at 2 mM and 20 mM Mg²⁺, respectively.
non-ionic detergent, heptylthioglucoside (unpublished observation). Thus, the enzyme(s) seems to be an integral membrane protein.

We found previously that \( H^+ \)-translocation in membrane vesicles of \( V. \) parahaemolyticus is elicited by ATP hydrolysis.\(^7\) Such \( H^+ \)-translocation was maximum at 2 mM \( Mg^{2+} \), and was not observed in EDTA-washed membranes (data not shown). Thus, the \( F_1 \) portion of the \( H^+ \)-translocating ATPase seems to be removed from the membranes by washing them with buffer containing EDTA. In fact, two protein bands that seemed to correspond to the \( \alpha \) and \( \beta \) subunits of \( F_1 \) were observed when the supernatant fraction of the EDTA-wash was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

To distinguish the two ATPase activities, we tested the effect of dicyclohexylcarbodiimide (DCCD), a commonly used inhibitor of the \( H^+ \)-translocating ATPase, on the two activities. As expected, DCCD inhibited the activity measured at 2 mM \( Mg^{2+} \), but had little effect on that measured at 20 mM \( Mg^{2+} \) (Fig. 2). Azide, which is a potent inhibitor of \( F_1 \) of \( E. \) coli, did not inhibit the ATPase activity at either 2 mM \( Mg^{2+} \) or 20 mM \( Mg^{2+} \) (data not shown).

The pH optimum for the ATP-hydrolyzing activity was about 8.0 with either 2 mM \( Mg^{2+} \) or 20 mM \( Mg^{2+} \) (data not shown).

**Effects of Monovalent Cations and Anions and Divalent Cations**

We tested the effects of mono- and divalent cations and anions at both 2 mM and 20 mM \( Mg^{2+} \) (Table I). At 2 mM \( Mg^{2+} \), \( Na^+ \), \( NH_4^+ \), \( Li^+ \) and \( K^+ \) increased the ATP-hydrolyzing activity about 10 times, the strengths of their effects being in that order. We also tested the effects of anions; \( Cl^- \), \( SO_4^{2-} \) and \( CH_3COO^- \) enhanced the activity, while \( NO_3^- \) was less effective. Thus a certain level of ionic strength, but not of particular ions, seems to be necessary for the activity of the \( H^+ \)-translocating ATPase. It should be pointed out, however, that we observed significantly higher activity of ATP hydrolysis with \( Na^+ \) than with other monovalent cations.

At 20 mM \( Mg^{2+} \), \( Cl^- \) and \( NO_3^- \) (\( Cl^- > NO_3^- \)) greatly stimulated the ATP-hydrolyzing activity, but \( SO_4^{2-} \) and \( CH_3COO^- \) had little effect. Since \( V. \) parahaemolyticus lives under \( Cl^- \)-rich conditions, \( Cl^- \) would be the natural activator of the ATP-hydrolyzing enzyme.

We then tested the effect of \( Cl^- \) concentration on the ATP-hydrolyzing activity at 20 mM \( Mg^{2+} \) (Fig. 3). With increasing concentrations of \( Cl^- \) up to 100 mM, we observed higher activity. No further stimulation of ATP hydrolysis by \( Cl^- \) was observed at higher concentrations.

<table>
<thead>
<tr>
<th>Salt</th>
<th>ATP-hydrolyzing activity (units/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>At 2 mM MgSO(_4)</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.04</td>
</tr>
<tr>
<td>KCl</td>
<td>0.43</td>
</tr>
<tr>
<td>LiCl</td>
<td>0.22</td>
</tr>
<tr>
<td>NH(_4)Cl</td>
<td>0.33</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>0.40</td>
</tr>
<tr>
<td>NaOOCOCH(_3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Hydrolysis of ATP was measured with membrane vesicles (untreated). Salts were added to assay mixtures at 0.2 M with 2 mM MgSO\(_4\) or at 0.1 M with 20 mM MgSO\(_4\) to obtain maximum activities.

**Fig. 3.** Effect of \( Cl^- \) Concentration on ATP-Hydrolyzing Activity

Hydrolysis of ATP was measured in the presence of membrane vesicles (untreated), 20 mM MgSO\(_4\) and various concentrations of \( Cl^- \). Total concentration of NaCl plus Na\(_2\)SO\(_4\) in assay mixtures was 0.2 M.
Among the divalent cations tested, Mg$^{2+}$, Ca$^{2+}$ and Co$^{2+}$ stimulated the ATP-hydrolyzing activity to almost the same extent at 2 mm (Table II). On the other hand, Mg$^{2+}$ caused great stimulation, Ca$^{2+}$ and Mn$^{2+}$ considerable stimulation, and Co$^{2+}$ slight stimulation at concentrations of 20 mm.

The ATP-hydrolyzing activity in membrane vesicles (untreated) was measured in the presence of 20 mm MgSO$_4$, 0.1 M NaCl and various concentrations of ZnCl$_2$.

**Table III. Substrate Specificity of Membrane-Bound 5'-Nucleotidase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolyzing activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1.93</td>
</tr>
<tr>
<td>GTP</td>
<td>1.43</td>
</tr>
<tr>
<td>CTP</td>
<td>0.80</td>
</tr>
<tr>
<td>UTP</td>
<td>1.27</td>
</tr>
<tr>
<td>ADP</td>
<td>1.74</td>
</tr>
<tr>
<td>AMP</td>
<td>1.71</td>
</tr>
<tr>
<td>3'-CMP</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Hydrolysis of nucleotides was measured with membrane vesicles (untreated) in the presence of 20 mm MgSO$_4$ and 0.1 M NaCl. All nucleotides except cytidine-3' monophosphate (3'-CMP) were 5'-nucleotides.

Substrate Specificity

Since the ATP-hydrolyzing activity observed at 10 to 20 mm Mg$^{2+}$ was stimulated by Cl$^-$, this activity seemed to be that of anion-stimulated 5'-nucleotidase which has been reported in *V. alginolyticus*. Very similar membrane-bound 5'-nucleotidase has been reported in *V. costicola*. Thus, we tested the substrate specificity of the enzyme. With 20 mm Mg$^{2+}$, ATP, guanosine triphosphate (GTP), uridine triphosphate (UTP) and cytidine triphosphate (CTP) (ATP > GTP > UTP > CTP) were all hydrolyzed, and adenosine di-
phosphate (ADP) and adenosine monophosphate (AMP) were also hydrolyzed (Table III). Although all the 5'-nucleotides tested were hydrolyzed, 3'-nucleotide was not. Therefore, we concluded that the ATP-hydrolyzing activity observed at 10 to 20 mM Mg\(^{2+}\) was mostly due to anion (Cl\(^{-}\))-stimulated 5'-nucleotidase.

**Discussion**

The respiratory chain of *Vibrio* extrudes both H\(^{+}\) and Na\(^{+}\). We reported previously that H\(^{+}\)-translocating ATPase is present in cell membranes of *V. parahaemolyticus*. Thus, it seemed that respiration-linked ATP synthesis catalyzed by the H\(^{+}\)-translocating ATPase occurs in this organism too. As described in this paper, the properties of this ATPase were similar to those of the H\(^{+}\)-translocating ATPase of *E. coli*; namely, the enzyme required Mg\(^{2+}\), and was inhibited by DCCD, and the activity was lost from the membranes when the membranes were washed with buffer containing EDTA.

We thought that there might be an Na\(^{+}\)-translocating ATPase in addition to the H\(^{+}\)-translocating ATPase in the membranes of *V. parahaemolyticus*, and that it might synthesize ATP by using the electrochemical potential of Na\(^{+}\) established by the respiratory chain or the Na\(^{+}/H^{+}\) antiporter, which is able to convert an electrochemical potential of H\(^{+}\) to that of Na\(^{+}\). If such an Na\(^{+}\)-translocating ATPase is present in membranes of this organism, the membrane-bound ATPase activity should be stimulated by Na\(^{+}\). In fact, we detected some membrane-bound ATPase activity at 2 mM Mg\(^{2+}\), that was stimulated by Na\(^{+}\). Although it showed rather broad ion specificity, the ATP-hydrolyzing activity was obviously higher in the presence of Na\(^{+}\) than of other monovalent cations (Table I, at 2 mM Mg\(^{2+}\)). Its activity was lower in the presence of NaNO\(_{3}\), suggesting that NO\(_{3}^{-}\) was inhibitory. Since the H\(^{+}\)-translocating ATPase functions well at 2 mM Mg\(^{2+}\), most of the ATP-hydrolyzing activity observed at this Mg\(^{2+}\) concentration seems to be due to this enzyme. However, the fact that DCCD inhibited only 50–60% of the ATPase activity at 2 mM Mg\(^{2+}\) suggests the presence of some other ATPase(s). Very recently, Dibrov et al. reported the presence of “Na\(^{+}\)-ATP-synthase” in *V. alginolyticus*. They observed Na\(^{+}\)-driven ATP synthesis. However, the biochemical properties of the “Na\(^{+}\)-ATP-synthase” are not known. Thus, the presence of Na\(^{+}\)-ATPase in *V. parahaemolyticus* seems to be likely. However, it is difficult to characterize such an ATPase even if it is present, because *V. parahaemolyticus* membranes have very strong ATP-hydrolyzing activity due to Cl\(^{-}\)-stimulated 5'-nucleotidase. Therefore, we are now trying to isolate mutants defective in Cl\(^{-}\)-stimulated 5'-nucleotidase. We are also testing whether or not ATP is synthesized when an artificial electrochemical potential due to Na\(^{+}\) is imposed across the cell membrane of *V. parahaemolyticus*.

We observed the 5'-nucleotidase activity in both everted membrane vesicles and whole cells of *V. parahaemolyticus* (manuscript in preparation). It has been reported in *V. costicola* that 5'-nucleotidase is measurable with whole cells. The outwardly-oriented 5'-nucleotidase and the inwardly-oriented 5'-nucleotidase seem to be the same enzyme, because they showed very similar properties, Cl\(^{-}\)-dependency, Mg\(^{2+}\)-dependency, substrate specificity and effect of inhibitors. This suggests that the substrate could reach the catalytic site of the enzyme from either side of the membrane, because it is unreasonable to suppose that ATP, a substrate of the enzyme, can penetrate through the cell membrane of this organism. We found previously that membranes prepared by the French press method are everted vesicles. It is unlikely that they are a mixture of everted and right-side-out membrane vesicles, because their ATP-hydrolyzing activity was not increased when they were made permeable by toluene treatment (data not shown). Thus, ATP and other 5'-nucleotides may be hydrolyzed by the 5'-nucleotidase even at the inner surface of the cytoplasmic membrane, although if so, there must
be a proper control mechanism to prevent waste of 5'-nucleotides. The outwardly-oriented 5'-nucleotidase activity seems to be involved in utilization of extracellular ATP and other nucleotides. This enzyme cleaves 5'-nucleotides to nucleosides, and then nucleosides would be taken up by the cells. It should be pointed out that 5'-nucleotides have been reported to be present in sea water, although at low level.

Why is the Cl- stimulated 5'-nucleotidase an integral membrane protein? What is the role of inwardly-oriented 5'-nucleotidase activity? One possibility is that this 5'-nucleotidase functions as a Cl- pump which utilizes intracellular 5'-nucleotides, perhaps ATP. Another possibility is an involvement of this enzyme in metabolism of 5'-nucleotides on the cytoplasmic side. We characterized the 5'-nucleotidase in V. parahaemolyticus to obtain insight into the role of Cl- in membrane processes in this microorganism. Transport of Cl- may take place as a result of hydrolysis of 5'-nucleotides, especially ATP, because many ion-translocating ATPases are known in biological membranes, and these ATPases are stimulated by the ions transported. Studies are required on Cl- transport as well as Na+ transport in Vibrio, because this organism requires NaCl for growth. Our preliminary results indicated that V. parahaemolyticus has a Cl- transport system(s) (unpublished observation). We are now investigating the relationship between Cl- transport and the Cl- stimulated 5'-nucleotidase.

The ATP-hydrolyzing activity of membranes (specific activity expressed as units/mg protein) of V. parahaemolyticus varied from membrane preparation to preparation. This suggests the presence of several types of ATPase with different properties (stability, activation or inhibition by various factors, inducibility and so on). It is important to characterize these membrane ATPases of V. parahaemolyticus to get insight into their roles and into the membrane energetics of this organism.

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References