**F_0F_1-ATPase from Vibrio alginolyticus**

Subunit composition and proton pumping activity

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An F_0F_1-ATPase was isolated from the membranes of the marine bacterium Vibrio alginolyticus. Homology between the subunits of the F_0-complexes from E. coli and V. alginolyticus was found using antibodies against subunits α, β, and c of the E. coli F_0,F_1-ATPase. The F_0F_1-complex from V. alginolyticus was reconstituted into proteoliposomes, which were competent in ATP-dependent proton uptake. This process was inhibited by triphenyltin, DCCD, and venturicidin. Na⁺ did not affect proton translocation.

F_0F_1-ATPase: H⁺ pump; Proteoliposome: Vibrio alginolyticus

1. INTRODUCTION

Recently we have reported some characteristics and structural properties of the membrane ATPase from Vibrio alginolyticus which was shown to belong to the F₀F₁-type [1]. ATP-dependent Na⁺ translocation was observed in the subcellular vesicles from V. alginolyticus. Na⁺ transport was reported to be stimulated by protonophores and inhibited by DCCD [2] and triphenyltin, the hydrophobic inhibitors of the F₀F₁-type ATPases. A protonophore-resistant ATP synthesis driven by respiration of artificial Na⁺ gradient was found in intact cells of V. alginolyticus [3]. These phenomena could be accounted for by the activity of reversible Na⁺ translocating ATPase similar to the Propionigenium modestum Na⁺-ATPase. The latter is (i) of the F₀F₁-type, (ii) shows homology with the V. alginolyticus ATPase in the c subunit sequence (identity of 8 of the 9 C-terminal residues; among the next 9 residues 4 are identical and 4 are conservative replacements; the DCCD-reacting dicarboxylic amino acid is located at 25th position from the C-end and (iii) differs in the carboxy-terminal c subunit sequence from other bacterial ATPases [4].

In this study we have undertaken the purification of the F₀F₁-ATPase from the membranes of Vibrio alginolyticus and its reconstitution into the proteoliposomes.

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Abbreviations: DCCD, N,N'-dicyclohexylcarbodiimide; FCCP, carbonylcyanide-4-trifluoromethoxyphenylhydrazone; DTT, dithiothreitol

2. MATERIALS AND METHODS

Vibrio alginolyticus was grown aerobically in a medium containing 0.5 M NaCl, 100 mM succinate, 10 mM KCl, 5 mM MgSO₄, 15 mM (NH₄)₂SO₄, 2 mM KH₂PO₄, 10 mM Tris-HCl, pH 8.5. 0.1% yeast extract. Subcellular vesicles were prepared as described elsewhere [5].

2.1. Purification of the F₀F₁-ATPase

The subcellular vesicles were suspended in a medium containing 10 mM Tris-ME5, pH 8.0, 10 mM MgSO₄, 0.5% Triton X-100 (Buffer TMT) (protein concentration, 7-10 mg·mL⁻¹) and 1 mM phenylmethylsulfonylfluoride was added. The mixture was stirred at 0°C for 40 min and then centrifuged at 50 000 × g for 40 min. 50% polyethylene glycol 6000 solution (w/w) was added to the supernatant dropwise while stirring to a final concentration of 11%. The mixture was stirred at 0°C for 2 h. Precipitated protein was removed by centrifugation (25 000 × g for 10 min). Then polyethylene glycol 6000 was added once more to a final concentration of 15%. After incubating the mixture for 1 h at 0°C the precipitated proteins were collected by centrifugation. The pellet was dissolved in 2-4 mL TMT buffer supplemented with 0.1 M phenylmethylsulfonylfluoride and 1 mM DTT (TMTI buffer), and insoluble material was removed by centrifugation (50 000 × g, 10 min). The clear solution was applied to a column with Toyo-Pearl HW-60 (2.6 x 80 cm) equilibrated with TMTI buffer. Proteins were eluted with the same buffer at a flow rate of 70 ml/h. Fractions containing ATPase activity were combined and concentrated by adding polyethylene glycol 6000 to the final concentration of 14%. The precipitate was dissolved in a small amount of the TMT buffer, containing 0.05% instead of 0.5% Triton X-100. The protein solution was divided into aliquots and stored in liquid nitrogen.

Electrophoresis was performed essentially as described by Laemmli [6]. Concentration of polyacrylamide in the separating gel was either 14%, or a linear gradient 12-20% was used. The gels were stained with Coomassie brilliant blue R-250.

Electrotransfer to the nitrocellulose membranes was performed by semi-dry method in a blotting buffer containing 150 mM glycine, 20 mM Tris, 0.02% SDS, 20% methanol, pH 8.3, on NovaBlot LKB apparatus.

Immunodetection was carried out essentially as described by Baisier [7]. Polyclonal antibodies against the subunits α,β and c of E. coli were used.
coli FoF1-ATPase were kindly supplied by Dr. K. Altendorf (Osnabrück University, Germany). Peroxidase conjugated secondary goat anti-rabbit antibodies from BioRad were used. Peroxidase-sensitive staining was performed with BioRad HRP reagent [8].

2.2. Reconstitution of the purified FoF1-ATPase into the proteoliposomes

Asolectin was suspended in buffer containing 10 mM Tricine-KOH, pH 8.0, 2.5 mM MgSO4, 0.05 mM EDTA, 1 mM dithiothreitol, and either 0.25 M K2SO4 or 0.25 M Na2SO4, the lipid concentration being equal to 15 mg/ml, and sonicated under nitrogen on ice 3 times for 30 s. Purified ATPase was added to the liposome suspension at a ratio of 1 mg protein per 20-30 mg lipids, incubated for 15 min at room temperature, frozen in liquid nitrogen and thawed in an ice-water bath. Proteoliposomes were collected by centrifugation at 220 000 × g for 1 h.

2.3. Proton transport measurements

The proteoliposomes were suspended in a medium containing 10 mM Tricine-KOH, pH 8.0, 0.3 μM valinomycin, 1 μM acridine orange, 2 mM ATP, and either 0.25 M Na2SO4 or 0.25 M K2SO4. When the effect of Na+ concentration was investigated Tris salt of ATP was used. The reaction was initiated by adding 5 mM MgSO4.

ATPase activity was measured by monitoring the NADH oxidation in the presence of an ATP-regenerating system with pyruvate kinase and lactate dehydrogenase [9].

The protein concentration was determined by Lowry [10].

2.4. Materials

Tris, MES, ATP, Tricine, asolectin were from Sigma, nitrocellulose membranes from Millipore, other chemicals were of analytical grade.

3. RESULTS AND DISCUSSION

FoF1-ATPase of Vibrio alginolyticus was purified by a rapid method comprising Triton X-100 extraction of the membranes, polyethylene glycol 6000 fractionation and gel filtration on Toyopearl HW-60 (Table I). This procedure yielded an enzyme preparation with a specific activity of 2.5 μmol min/mg protein. The specific ATPase activity increased on reconstitution into the proteoliposomes up to 5 μmol min/mg protein (approx. 20-fold purification).

Purified ATPase of V. alginolyticus had a typical for FoF1-complex subunit composition. Subunits with molecular masses of 58, 55, 38, 25, 23, 17 and 14 kDa were found in these preparations and also a 77 kDa contaminant was present (Fig. 1). According to our previous paper [1] 58, 55, 38 and 23 kDa subunits belong to F1-complex (α, β, γ and δ subunits, respectively).

Western blotting of the electrophoretogram of the purified V. alginolyticus FoF1-ATPase (Fig. 2) revealed pronounced cross-reactivity (i) of the antibodies against subunit b of E. coli ATPase with the 17 kDa subunit and (ii) of the antibodies against E. coli subunit c with a low molecular mass subunit which was not visualized by Coomassie staining (cf. [12]). No cross-reactivity with any subunit of the V. alginolyticus FoF1-ATPase was observed when antibody against E. coli subunit a was used.

The purified FoF1-complex was reconstituted with phospholipids to form proteoliposomes which hydrolyzed ATP, the hydrolysis being stimulated twofold by

### Table 1

**Purification of V. alginolyticus FoF1 ATPase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific ATPase activity (U/mg)</th>
<th>Protein (mg)</th>
<th>Yield of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranes</td>
<td>0.25</td>
<td>600</td>
<td>100</td>
</tr>
<tr>
<td>Triton X-100 extraction</td>
<td>0.73</td>
<td>308</td>
<td>146</td>
</tr>
<tr>
<td>Polyethylene glycol 6000</td>
<td></td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Fractionation</td>
<td>1.6</td>
<td>24</td>
<td>40</td>
</tr>
<tr>
<td>Cell filtration</td>
<td>2.5</td>
<td></td>
<td></td>
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</tbody>
</table>

The ATPase activity is expressed in μmol ADP produced per minute.
Fig. 2. Western blotting of F_0F_1-complex of *V. alginolyticus* with antibodies against subunits a (a), b (b) and c (c) of *E. coli* F_0F_1-ATPase. All the antibodies used in the experiments were tested for a positive control with F_0 from *E. coli*. Positions of the markers of known molecular mass are shown on the right.

protonophore FCCP. ATP-dependent proton uptake by the proteoliposomes could be revealed with a fluorescent dye acridine orange (Fig. 3). Proton uptake was totally abolished by a low concentration of FCCP. The F_0 inhibitors venturicidin, triphenyltin and DCCD prevented proton transport by the proteoliposomes.

Laubinger and Dimroth [11] have found that addition of 1 mM NaCl to the proteoliposomes containing purified F_0F_1-type ATPase of *Propionigenium modestum* abolished proton uptake presumably due to the competition of Na^+ and H^+ for the ion channel of the ATPase. We have compared proteoliposomes loaded with 0.5 M Na^+ with the proteoliposomes where K^+ substituted for Na^+ (Fig. 3e,f). The proton translocation measurements were carried out in medium containing the appropriate concentration of the respective cation. In the presence of 0.5 M Na^+ proton transport was at least as active as in the absence of Na^+ , i.e. in the K^+-medium. No inhibition of proton uptake was observed when Na^+ (50 mM) was added to the incubating mixture when K^+-loaded proteoliposomes in K^+ medium were used in the experiment. Thus, no competition of protons and sodium ions could be observed when measuring ATP-dependent proton uptake by the proteoliposomes containing F_0F_1-ATPase from *V. alginolyticus*.

Recently Krumholz et al. [12] have undertaken cloning of the ATPase gene operon from *V. alginolyticus* into an *E. coli* plasmid. The enzyme encoded by this operon was expressed in *E. coli* cells and purified. The authors failed to find the ATP-dependent Na^+ uptake by the proteoliposomes containing this enzyme, though the latter were active in proton translocation. It should be noted also that the *V. alginolyticus* DNA fragment used by Krumholz et al. for cloning and expression of the ATPase in *E. coli* contained only structural genes but not gene I of an unknown function also present in the unc operon of *E. coli*. 

![Fig. 3](image-url)
Therefore, purification of the enzyme directly from the *V. alginolyticus* cells presents a more direct approach to the ion-translocating function of the *V. alginolyticus* ATPase. The very fact that, in contrast to the *P. modestum* ATPase, Na⁺ ions had no measurable effect on the ATP-dependent H⁺ uptake by the *V. alginolyticus* ATPase proteoliposomes, seems to exclude the explanation of Krumholz’s data by possible artifacts of the cloning of the ATPase operon in *E. coli*. As we already mentioned elsewhere [13], the principal difference between *V. alginolyticus* and *P. modestum* energetics consists in that the former employs both H⁺ and Na⁺ cycles whereas the latter only the Na⁺ cycle. Thus H⁺-ATPase activity of the F₀F₁-type ATPase from *P. modestum* may reveal itself under unnatural conditions (no Na⁺ in the medium). The *V. alginolyticus* H⁺-ATPase seems to be a natural activity which may be involved in the H⁺-coupled oxidative phosphorylation. As to Na⁺-coupled oxidative phosphorylation also being present in *V. alginolyticus* [3], it may be catalyzed by the same F₀F₁-complex as the H⁺-coupled one or, alternatively, by another ATP-synthase. The latter possibility seems, however, to be ruled out by the observations of Krumholz et al. who have found only one ATPase operon in the *V. alginolyticus* genome [14]. The amino acid sequences of the N-terminal regions of the major subunits of the purified F₁-complex from *V. alginolyticus* were identical to those predicted by the gene sequence [1]. Maybe the in vitro or in vivo conditions used in this study were unfavourable for Na⁺ to compete with H⁺ for the F₀ channel. Another possibility is that an additional component responsible for switching over from H⁺ to Na⁺ was lost during the purification of the ATPase. These questions are now under investigation in our group.

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REFERENCES