Volume 284, number 2, 273–276 FEBS 09853 June 1991

ADONIS 001457939100563M

F_0F_1-ATPase from *Vibrio alginolyticus*

Subunit composition and proton pumping activity

O.Yu. Dmitriev[1], I.A. Krasnoselskaya[1], S. Papa[2] and V.P. Skulachev[1]

[1]*A.N. Belozersky Laboratory for Molecular Biology and Bioorganic Chemistry, Moscow State University, 119899, Moscow, USSR* and [2]*Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy*

Received 22 April 1991

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 *a*, *b* and *c* of the *E. coli* F_0F_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 F_0F_1-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 F_0F_1-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 F_0F_1-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 F_0F_1-ATPase from the membranes of *Vibrio alginolyticus* and its reconstitution into the proteoliposomes.

Correspondence address: O.Yu. Dmitriev, A.N. Belozersky Laboratory for Molecular Biology and Bioorganic Chemistry, Moscow State University, 119899, Moscow, USSR

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide-4-trifluormethoxyphenylhydrazone; DTT, dithiotreitol

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 $MgSO_4$, 15 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 10 mM Tris-HCl, pH 8.5, 0.1% yeast extract. Subcellular vesicles were prepared as described elsewhere [5].

2.1. *Purification of the F_0F_1-ATPase*

The subcellular vesicles were suspended in a medium containing 10 mM Tris-MES, pH 8.0, 10 mM $MgSO_4$, 0.5% Triton X-100 (Buffer TMT) (protein concentration, 7–10 $mg \cdot ml^{-1}$) 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 mM phenylmethylsulfonylfluoride and 1 mM DTT (TMT1 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 × 80 cm) equilibrated with TMT1 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 Batteiger [7]. Polyclonal antibodies against the subunits *a,b* and *c* of *E.*

coli F_0F_1-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 F_0F_1-ATPase into the proteoliposomes*

Asolectin was suspended in buffer containing 10 mM Tricine-KOH, pH 8.0, 2.5 mM $MgSO_4$, 0.05 mM EDTA, 1 mM dithiothreitol, and either 0.25 M K_2SO_4 or 0.25 M Na_2SO_4, 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 Na_2SO_4 or 0.25 M K_2SO_4. When the effect of Na^+ concentration was investigated Tris salt of ATP was used. The reaction was initiated by adding 5 mM $MgSO_4$.

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

F_0F_1-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 Toyo-Pearl 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 F_0F_1-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 F_1-complex (α, β, γ and δ subunits, respectively).

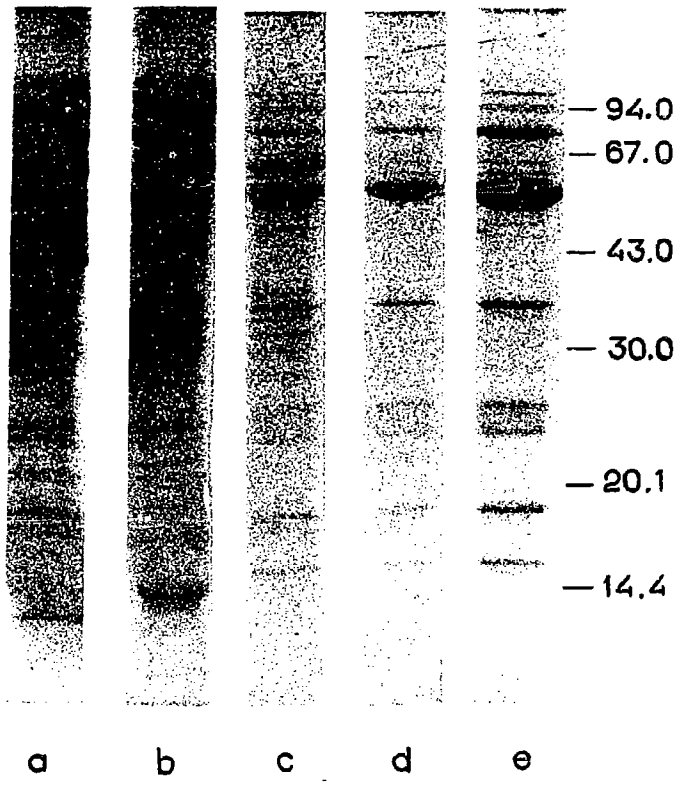

Fig. 1. SDS-electrophoresis of the preparations of membrane vesicles (a), Triton X-100 extract (b), partially purified ATPase after PEG 6000 fractionation (c), purified ATPase (d,e). The amount of protein per lane was: a, 30 μg; b, 15 μg; c, 2 μg; d, 1.3 μg; e, 6 μg. Positions of the markers of known molecular mass are shown on the right.

Western blotting of the electrophoretogram of the purified *V. alginolyticus* F_0F_1-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* F_0F_1-ATPase was observed when antibody against *E. coli* subunit *a* was used.

The purified F_0F_1-complex was reconstituted with phospholipids to form proteoliposomes which hydrolyzed ATP, the hydrolysis being stimulated twofold by

Table I

Purification of *V. alginolyticus* F_0F_1-ATPase

	Specific ATPase activity (U/mg)	Protein (mg)	Yield of activity (%)
Membranes	0.25	600	100
Triton X-100 extraction	0.73	308	146
Polyethylene glycol 6000 fractionation	1.6	39	41
Gel filtration	2.5	24	40

The ATPase activity is expressed in μmol ADP produced per minute.

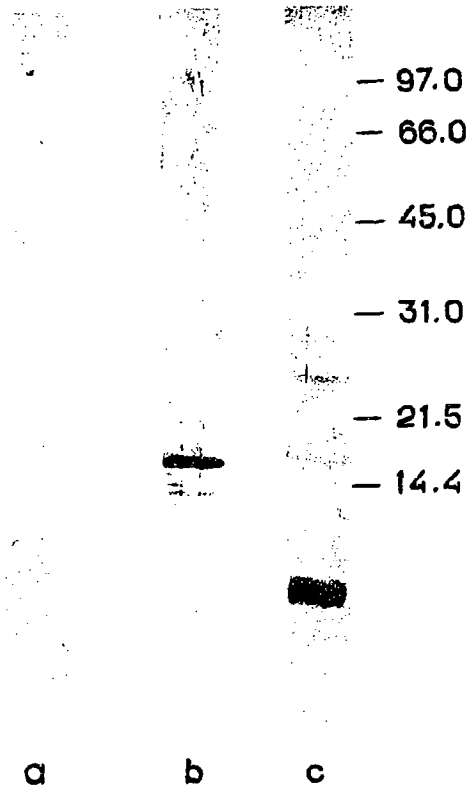

Fig. 2. Western blotting of F_0F_1-complex of *V. alginolyticus* with antibodies against subunits *a* (a), *b* (b) and *c* (c) of *E. coli* F_0F_1-ATPase. All the antibodies used in the experiments were tested for a positive control with F_0 from *E. coli*. Positions of the markers of known molecular 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*.

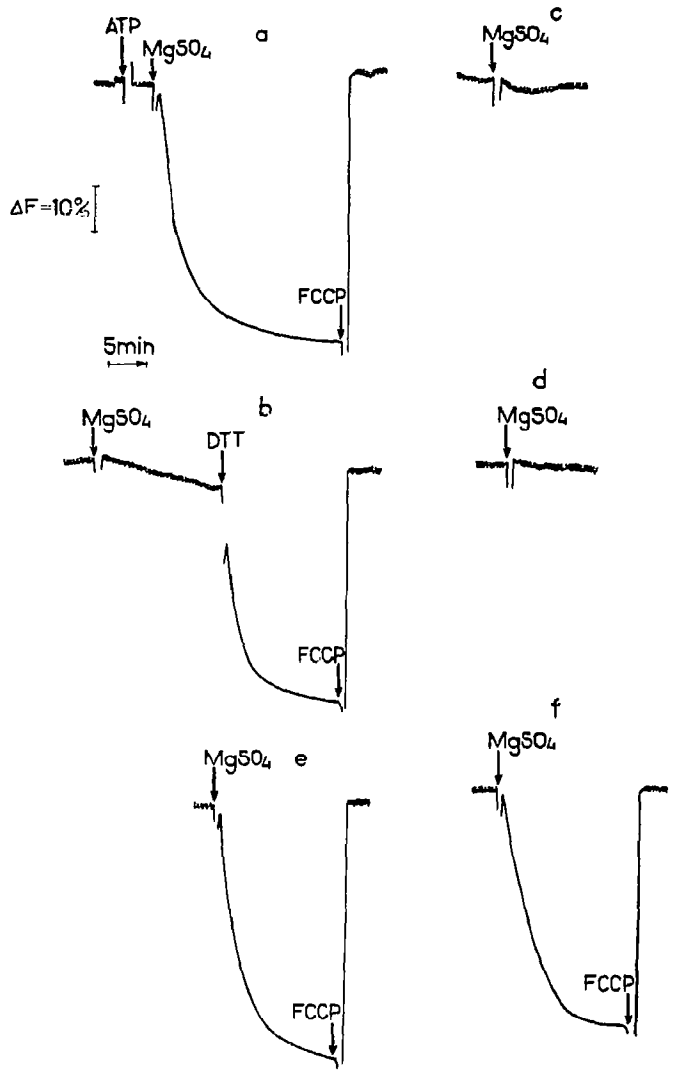

Fig. 3. Mg-ATP induced fluorescence quenching of Acridine orange in the proteoliposomes containing F_0F_1-ATPase. Additions (5 mM $MgSO_4$, 0.5 μM FCCP, 0.2 mM DTT) indicated by the arrows. a,b,c,d,f, proteoliposomes were preloaded with 0.25 M K_2SO_4 and the registration medium contained 0.25 M K_2SO_4 (cf. section 2). a,e,f, no inhibitors added before $MgSO_4$; b, 5 μM triphenyltin; c, 50 μM DCCD, d, venturicidin, 5 $mg \cdot ml^{-1}$, e, proteoliposomes were preloaded with 0.25 M Na_2SO_4 and 0.25 M Na_2SO_4 subsituted for K_2SO_4 in the incubation medium. Data of two experiments (a–d and e,f) are shown.

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*.

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 F_0F_1-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 F_0F_1-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 F_1-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 F_0 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.

Acknowledgements: We are grateful to Ms Anna Katomina (Institute of Microbiology, Moscow), Prof. Giuseppe Miragliotta (Institute of Microbiology, Bari) and Ms Laura Niccolini (Istituto Superiore di Sanità, Rome) who carried out cultivation of the bacteria and to Dr Karlheinz Altendorf (Osnabrück University) for the gift of antibodies which were used in this work.

REFERENCES

[1] Dmitriev, O.Yu., Grinkevich, V.A. and Skulachev, V.P. (1989) FEBS Lett. 258, 219–222.
[2] Dibrov, P.A., Skulachev, V.P., Sokolov, M.V. and Verkhovskaya, M.L. (1988) FEBS Lett. 233, 355–358.
[3] Dibrov, P.A., Kostyrko, V.A., Lazarova, R.L., Skulachev, V.P. and Smirnova, I.A. (1986) Biochim. Biophys. Acta 250, 449–457.
[4] Ludwig, W., Kain, G., Laubinger, W., Dimroth, P., Hoppe, J. and Schleifer, K.H. (1990) Eur. J. Biochem. 193, 395–399.
[5] Dmitriev, O.Yu. and Chernyak, B.V. (1988) FEMS Microbiol. Lett. 56, 79–82.
[6] Laemmli, U.K. (1970) Nature 227, 680–685.
[7] Batteiger, B., Newhall, W.J. and Jones, R.B. (1982) J. Immunol. Methods 42, 187–197.
[8] BioRad Immun-Blot (GAR-HRP) Assay Kit (1984)
[9] Scharschmidt, B.F., Keeffe, E.B., Blunkenship, N.H. and Ockner, D.K. (1979) J. Lab. Clin. Med. 93, 2375-2382.
[10] Lowry, O.,H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[11] Laubinger, W. and Dimroth, P. (1989) Biochemistry 28, 7194–7198.
[12] Krumholz, L.R., Esser, U. and Simoni, R.D. (1990) J. Bacteriol. 172, 6809–6817.
[13] Capozza, G., Dmitriev, O.Yu., Krasnoselskaya, I.A., Papa, S. and Skulachev, V.P., FEBS Lett., in press.
[14] Krumholz, L.R., Esser, U. and Simoni, R.D. (1989) Nucleic Acids Res. 16, 7993–7994.