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The F_1-ATPase of *Vibrio alginolyticus*

Purification and N-terminal sequence of major subunits

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The F_1-type ATPase has been isolated from membrane preparations of marine alkalotolerant bacterium, *Vibrio alginolyticus* The enzyme was found to consist of two major subunits of 55 and 58 kDa and at least two minor components (38 and 23 kDa) Amino acid sequences of N-terminal regions of the major subunits revealed close homology with those of *E coli* H^+-ATPase and of *Propionigenium modestum* Na^+-ATPase

Marine bacterium, ATPase, Sequence, (*Vibrio alginolyticus*)

1. INTRODUCTION

The bioenergetics of marine alkalotolerant bacterium, *Vibrio alginolyticus*, has been thoroughly studied in our laboratory. The $\Delta\bar{\mu}Na^+$-dependent ATP synthesis in the cells of *V. alginolyticus* and ATPase-linked Na^+-uptake by inverted membrane vesicles have been demonstrated [1,2]. These data suggest that Na^+-driven ATP-synthase is inherent in this microorganism. Recently we described an ATPase in the membrane preparations from *V. alginolyticus* [3]. This enzyme was inhibited by DCCD, NBD-Cl, sodium azide and diethylstilbestrol and was resistant to vanadate. Such pattern of inhibitor sensitivity is characteristic of F_0F_1-ATPases. Here we report purification of the F_1 component of *V. alginolyticus* ATPase and N-terminal sequence of its major subunits.

2. MATERIALS AND METHODS

2.1. *Cell cultivation and isolation of the membrane fraction*

V. alginolyticus, strain 138-2, was a gift of H Tokuda (Chiba University, Japan) Cells were cultivated and subbacterial vesicles were prepared as described elsewhere [3]

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-hydroxypropanesulfonic acid); DCCD, *N,N'*-dicyclohexylcarbodiimide; PMSF, phenylmethylsulfonylfluoride, NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; $\Delta\bar{\mu}Na^+$, electrochemical potential of Na^+-ions

2 2 *Analytic procedures*

ATPase was assayed either by liberation of inorganic phosphate by the method of Lin and Morales [4] or by ADP production in an enzyme-coupled regenerating system with pyruvate kinase and lactate dehydrogenase [5] at 28°C. The assay medium routinely contained 100 mM Tris-Mes, pH 7 6, 7 mM $MgSO_4$ and 2 mM ATP

2 3 *ATPase extraction*

Membranes were diluted 30-fold with the medium, containing 3 mM EDTA, 5 mM ATP, 10 mM Tris-Mes, pH 8.0, 1 mM PMSF, incubated for 30 min at room temperature and then pelleted by centrifugation at $50000 \times g$ for 1 h. Supernatant was used for the ATPase purification

2 4 *Chromatography techniques*

The extract containing solubilized ATPase was applied to DEAE-Sephacel column (2 8 × 3 cm) in the buffer containing 10 mM Tris-Mes, pH 8.0, 5 mM ATP, 20% ethyleneglycol, 0 1 mM PMSF (buffer A) The protein was eluted with this buffer and supplemented with NaCl (linear gradient 0–0.5 M) Fractions, containing ATPase activity were mixed and 50 μl of 5 M NaCl per ml were added This protein solution was applied to phenyl-Sepharose column (1 × 20 cm) pre-equilibrated with buffer A, containing 0 5 M NaCl Column was washed with 2 vols of this solution and then protein was eluted first with a decreasing gradient of NaCl in buffer A (0 4–0 M) and then with buffer A alone Fractions containing ATPase activity were mixed and concentrated on a small DEAE-Sephacel column Gel filtration was performed on a Toyo-pearl HW-60 column (2.8 × 100 cm) equilibrated with buffer A

SDS-electrophoresis was performed according to Laemmli [6] with 14% separating gel.

2.5. *Electroblotting*

After electrophoresis the gel was soaked in transfer buffer (20 mM $Na_2B_4O_7$, pH 9 5) for 5 min to reduce the amount of Tris and glycine PVDF membrane was rinsed with methanol and soaked in transfer buffer for 5 min The gel was sandwiched between PVDF membrane and several sheets of blotting paper and was assembled into a blotting apparatus (Midget Multiblot 205' (LKB, Sweden) and then electroeluted for 2 h at 300 mA in transfer buffer The PVDF membrane was soaked in deionized water for 3-5 min, stained with 0 1%

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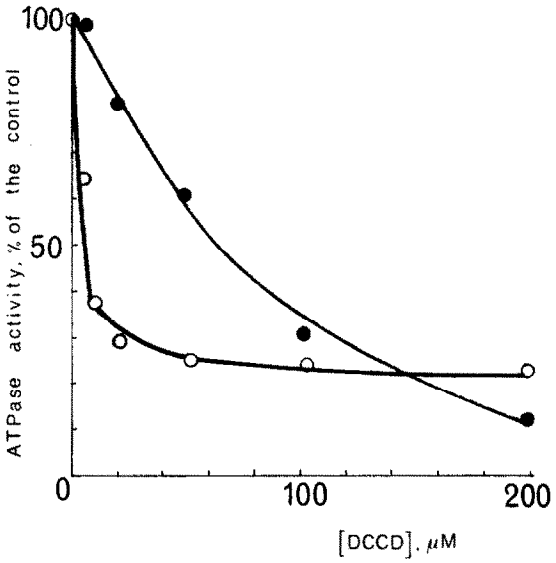

Fig.1. DCCD inhibition of the membrane-bound (○) and solubilized (●) ATPase. Prepartions were incubated with DCCD for 30 min at 20°C. Protein concentration 0.1 mg/ml. Activity without DCCD was 0.20 μmol/mg protein for the membranes and 0.37 μmol/mg protein for EDTA extract (solubilized ATPase).

Coomassie blue R-250 in 50% methanol for 5–7 min, then destained in 50% methanol/10% acetic acid at room temperature and air-dried. The membrane was stored at −30°C.

2.6. *Sequence analysis*

The protein band (80 pmol) electroblotted onto PVDF membrane and stained with Coomassie blue was cut out with a clean razor, centered on the Teflon seal and placed in the cartridge block of the sequenator. Proteins were sequenced on Applied Biosystems model 477A sequenator equipped with on-line PTH-analysis model 120 HPLC apparatus (Applied Biosystems). The PTH derivatives were separated by reverse-phase HPLC over a Microsil C-18 column (Macherey-Nagel).

Table 1

Inhibitor sensitivity of the membrane-bound and solubilized ATPase

Inhibitor	Concentration (μM)	Percent of inhibition	
		membrane ATPase	solubilized ATPase
DCCD	10	63	<5
	200	75	88
NBD-chloride	100	77	65
DES	20	59	<5
Sodium azide	1	75	93

Table 2

Purification of the solubilized ATPase

	Specific activity (μmol/min mg prot.)	Yield of activity (%)	Protein (mg)
Membranes	0.19	100	60
EDTA extract	0.42	97	23
DEAE-Sephacel	4.3	47	1.3
Phenyl-Sepharose	7.2	17	0.3

2.7. *Chemicals and materials*

Tris, Mes, Hepes and ATP were from Sigma (USA), DCCD from Serva (FRG), DEAE-Sephacel and phenyl-Sepharose from Pharmacia (Sweden), Toyo-pearl from Toyo-Soda (Japan). PVDF membranes (Immobilon Transfer), 0.4 μm pore size were obtained from Millipore (UK). Sequencing grade reagents, glass and Teflon fiber discs and Polybrene were purchased from Applied Biosystems (USA). Other chemicals were of analytical grade.

3. RESULTS

The ATPase activity of the EDTA extract was found to be sensitive to NBD-Cl and azide, which are known to interact with F_1 component of F_0F_1-ATPases. Diethylstilbestrol had no inhibitory effect on solubilized ATPase. DCCD inhibited both the membrane-bound and solubilized ATPases, though in the latter case much higher inhibitor concentrations were necessary (fig.1, table 1). In another experiment the vesicles were treated

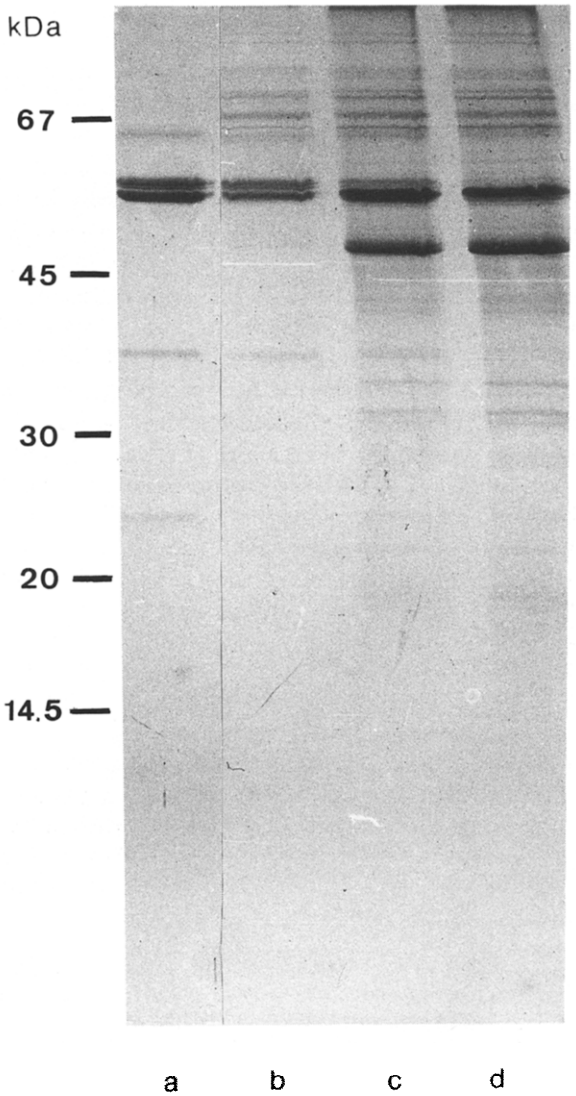

Fig.2. SDS-electrophoresis of the purified ATPase (hydrophobic chromatography) (a), partly purified ATPase (ion exchange chromatography) (b), EDTA extract (c), membranes (d). 30 μg protein was applied to each lane.

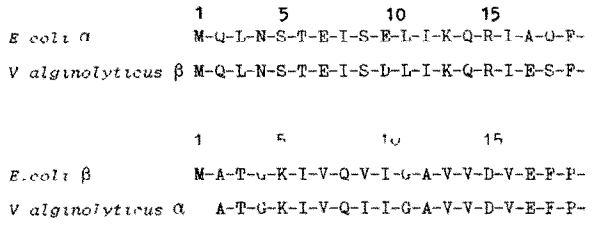

Fig 3 The N-terminal sequences of *E coli* ATPase [11] and *V alginolyticus* solubilized ATPase In the case of *V alginolyticus* ATPase α and β designations correspond to the electrophoretic mobility of the subunits rather than to their structural characteristics or functional role

with DCCD (100 μM, 10 nmol/mg protein). As a result their ATPase activity decreased to 36% of the control. Then the membranes were diluted 30-fold with EDTA-containing buffer (as described in section 2). After the dilution, DCCD concentration decreased to 3 μM. Specific ATPase activity of this extract proved to be 89% of the activity in the control extract. These data indicate that DCCD-inhibited ATPase is reactivated after its dissociation from the membrane.

We have used the two-step procedure to purify the solubilized ATPase from the EDTA extract. The extract was fractionated by ion exchange chromatography on DEAE-Sephacel and then by hydrophobic interaction chromatography on phenyl-Sepharose. Finally we obtained an ATPase preparation with a specific activity of 7.2 μmol ATP/mg protein. This preparation was shown to consist of two major polypeptides of 58 and 55 kDa (α- and β-subunits) and two minor components of 38 and 23 kDa (γ- and δ-) as revealed by SDS-disc-electrophoresis (fig.2a). A faint band at approximately the 65 kDa position is most likely to represent an impurity because we have not observed any noticeable correlation between its content and specific ATPase activity.

The molecular mass of the solubilized ATPase was estimated by gel filtration on Toyo-pearl HW-60 to be approximately 360 kDa. This value suggests a composition of $\alpha_3\beta_3\gamma\delta$ type.

We have determined the N-terminal sequence of the α- and β-subunits (fig.3). A high degree of homology with the major subunits of *E. coli* ATPase has been revealed. However, a cross-like relationship was observed: the α-subunit of *V. alginolyticus* is homologous to the β-subunit of *E. coli* and vice versa.

4. DISCUSSION

The results of inhibitor studies of *V. alginolyticus* ATPase can be explained assuming that, like other F_0F_1-ATPases, it consists of two components: i.e. (i) the integral membrane one, which is a target for hydrophobic inhibitors such as DCCD at low concentrations and diethylstilbestrol; and (ii) the peripheral soluble one, which possesses catalytic activity and is inhibited by NBD-Cl, sodium azide and DCCD at higher concentrations.

The solubilized ATPase was purified 35-fold from the EDTA extract of the membranes. Its polypeptide composition and molecular mass appear to be similar to the known F_1-ATPases, though we failed to observe the ϵ-subunit. Densitography of the gels has also shown that the γ- and δ-content in solubilized ATPase was considerably lower than $\alpha_3\beta_3\gamma\delta\epsilon$ structure requires. This may be a result of partial dissociation of the minor subunits in the course of the enzyme purification.

The N-terminal sequence of the major subunits revealed the striking homology with *E. coli* ATPase, though it was the heavier subunit of *V. alginolyticus* ATPase that was homologous to β- of *E. coli* and vice versa. This somewhat intriguing result is probably accounted for by the fact that, in the case of *V. alginolyticus* ATPase, as distinct from most of the other F_0-F_1-ATPases, the α-subunit is of higher electrophoretic mobility than β-. Such relationships were also reported for *Bacillus subtilis* [7], *Micrococcus luteus* and *Lactobacillus casei* [8]. This does not necessary imply that the α-subunit has a lower molecular mass than the β-, because molecular mass values of certain proteins, estimated by SDS-electrophoresis, sometimes appear to be slightly different from the true ones.

Thus the *V. alginolyticus* ATPase seems to be of the F_0F_1-type like H^+-ATPase of *E. coli* and the sodium-transporting ATPase of *P. modestum* [9].

It should be stressed that the *P. modestum* Na^+-ATPase is DCCD-sensitive and transports H^+, when Na^+ is absent. Its β-subunit shows 70% homology with the *E. coli* β-subunit. At the 9th position there is an isoleucine in *P. modestum* instead of the valine in *E. coli* [10]. In the *V. alginolyticus* α-subunit this position is occupied by isoleucine, like in the *P. modestum* enzyme. In this respect other bacteria (*Rps. blastica, Rh. rubrum*, thermophilic bacterium PS3, *Synechococcus sp.* strain 6301 and *Flavobacterium ferrugineum*), as well as beef heart mitochondria proved to be similar to *E. coli*, whereas chloroplasts resembled *P. modestum* and *V. alginolyticus* [10–12]. Other amino acid residues up to 19th position in *E. coli* β-subunit and *V. alginolyticus* α-subunit proved to be identical (fig.3).

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