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THE PURIFICATION AND SUBUNIT STRUCTURE OF A MEMBRANE-BOUND ATPase FROM THE ARCHAEBACTERIUM HALOBACTERIUM SACCHAROVORUM

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Summary: A membrane-bound ATPase from Halobacterium saccharovorum was solubilized using sodium dexoycholate and Zwittergent 3-10 and purified by hydrophobic and ammonium sulfate-mediated chromatography. The enzyme, which had a molecular mass of 350 kDa, was composed of two major (87 and 60 kDa) and two minor (29 kDa and 20 kDa) subunits. The halobacterial ATPases appear to be unlike any other ATPase described to date.

Light- and respiration-driven DCCD-sensitive ATP synthesis has been reported in whole cells from Halobacterium halobium [1,2], as has ATP hydrolysis (i.e., ATPase activity) in cell-free extracts [3,4,5]. However, the operation of an F_0F_1-ATP synthase in the Halobacteria, or indeed in any Archaebacterium [6], has yet to be convincingly demonstrated.

Halobacterium saccharovorum contains a cold-sensitive ATPase (5) which is inhibited by DCCD [7]. Inhibition is accompanied by the covalent binding of the inhibitor to a 60-kDa polypeptide [8]. The enzyme appeared to consist of several subunits although subunits <60 kDa were not always detected, and when present occurred in variable amounts so that their relationship to the ATPase was uncertain (unpublished data). We recently observed that cold inactivation was accompanied by the dissociation of the enzyme into subunits. By taking

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Abbreviations: TN buffer (50 mM Tris. HCl-4 M NaCl pH 8 buffer); TNM buffer (TN buffer made 10 mM with respect to $MgCl_2$); SDS-PAGE (sodium dodecyl-sulfate polyacrylamide gel electrophoresis).

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advantage of this process, we have deduced that the ATPase is composed of four subunits.

MATERIALS AND METHODS

Membranes were prepared from H. saccharovorum as previously described [7] except that the final wash was omitted. Unless otherwise stated, all operations were carried out at room temperature (ca. 22°C) and all buffers were adjusted to pH 8.0 using a low-sodium-error combination electrode.

The membrane fraction was adjusted to a protein concentration of 20 mg/ml with 4 M NaCl-50 mM Tris.Cl buffer (TN buffer) and made 0.04% with respect to octyl-β-D-glucopyranoside. The detergent-treated membranes were passed through a Sepharose 6B column (90 × 2.5 cm) previously equilibrated with TN buffer which was 10 mM with respect to $MgCl_2$ (TNM buffer). Five-milliliter fractions were collected at a flow rate of 20 ml/hr. Those fractions appearing in the void volume were combined and centrifuged for 90 min at 49K rpm in a Beckman 60Ti rotor. The pellet, which contained about 75% of the original ATPase activity, was diluted to a protein concentration of 17 mg/ml using TN buffer and made 0.1% and 0.08% with respect to Zwittergent 3-10 and sodium deoxycholate. The membrane-detergent mixture was immediately applied to a second Sepharose 6B column (90 × 2.5 cm) which was equilibrated with TNM buffer. The column was washed with TNM buffer at a flow rate of 20 ml/hr and 5-ml fractions were collected. Those fractions which contained ATPase activity, which eluted at a Ve/Vo of 1.8, were combined and designated the 6B fraction. ATPase activity was adsorbed on Phenyl Sepharose by passing the Sepharose fraction through a column containing 5 ml of the gel equilibrated in TN buffer. The gel was washed sequentially with the following buffers: 17 ml of 2.5 M NaCl-100 mM $MgCl_2$-50 mM Tris.Cl and 20 ml of 1.25 M NaCl-100 mM $MgCl_2$-50 mM Tris.Cl. ATPase activity was eluted by the latter buffer. The ATPase-containing fractions were combined (designated the Phenyl Sepharose fraction), diluted 1:2.5 with 4 M $(NH_4)_2SO_4$-50 mM Tris.Cl buffer, and applied to a column which contained 2 ml Bio-Gel DEAE A equilibrated in 2.5 M $(NH_4)_2SO_4$-50 mM Tris.Cl buffer. The gel was washed with 20 ml of 2.0 M $(NH_4)_2SO_4$-50 mM Tris.Cl buffer followed by 30 ml of 1.85 M $(NH_4)_2SO_4$-3.3 M NaCl-50 mM Tris (pH 7.4) buffer. ATPase activity was eluted by the last buffer. The enzyme was diluted 1:3 with 4 M $(NH_4)_2SO_4$-50 mM Tris.Cl buffer and passed through a column which contained 2 ml DEAE Bio-Gel A equilibrated in 2.5 M $(NH_4)_2SO_4$-50 mM Tris.Cl buffer. The gel was washed with 6 ml of 2.0 M $(NH_4)_2SO_4$-50 mM Tris.Cl buffer followed by 5-ml TN buffer. The enzyme was eluted by the latter buffer. ATPase activity was determined in the presence of 0.01% Triton X-100 with MgATP as the substrate [7]. Protein was determined from the absorbance at 215 and 225 nm [9].

Sepharose 6B and Phenyl Sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); DEAE Bio-Gel A and molecular weight markers for gel filtration were obtained from Bio-Rad Laboratories (Richmond, Calif.). The SDS-molecular weight markers and equine disodium ATP (crystalline grade) were obtained from the Sigma Chemical Company (St. Louis, Mo.). Octyl-β-D-glucopyranoside and Zwittergent 3-10 were purchased from CalBiochem-Behring (San Diego, Calif.). The sodium deoxycholate was a product of the Eastman Kodak Company (Rochester, N.Y.). Analytical gel filtration was carried out with a Superose-12 column in conjunction with a Pharmacia FPLC System.

RESULTS AND DISCUSSION

Purification was facilitated by the use of two discrete detergent steps to extract the enzyme. In the first octyl-β-glucopyranoside) extracted about

Table 1. Purification of ATPase Activity

Fraction	Total Activity (U)[1]	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purif.
Membrane	26	500	0.05	100	1
Sepharose	11	15	0.73	42	14
Phenyl Sepharose	5	3	1.7	19	34
DEAE	3.5	1	3.5	13	70

1. +Δ μmols phosphate/min.

54% of the membrane protein while leaving about 75% of the ATPase activity still associated with the membranes. In the second, 7% of the remaining protein and essentially all of the remaining ATPase activity was extracted, although only 60% of that activity was recovered following gel filtration. After the second DEAE step, the enzyme (the DEAE fraction) catalyzed the production of 3.5 μmols of P_i/min/mg protein. This represented a 70-fold purification with a 13% recovery of ATPase activity (Table 1). The enzyme had a molecular mass of 350 kDa as determined by gel filtration. Two major subunits (87 kDa and 60 kDa) were detected as were a number of minor components following SDS-PAGE according to the method of Weber and Osborn [10].

ATPase activity was unstable at low temperatures [5]. The following combination of gel filtration and electrophoretic experiments demonstrated that this was caused by the dissociation of the native enzyme. Gel filtration through Superose 12 revealed that the DEAE fraction contained three components (Fig. 1a). The major component (designated A) eluted at a volume corresponding to a molecular mass of 350 kDa and was associated with ATPase activity. The two other components (B and C) eluted at volumes corresponding to 176 kDa and 65 kDa; neither exhibited any ATPase activity. When the DEAE fraction was incubated at -13°C for 19 hr, approximately 95% of the initial ATPase activity disappeared. This was associated with a decrease in the amount of A and a corresponding increase in the amount of C (Fig. 1b). The amount of B did not appreciably change. Incubation of the cold-inactivated enzyme for 24 hr at room temperature neither restored enzyme activity nor resulted in a change in the chromatographic pattern shown in Fig. 1b (data not shown).

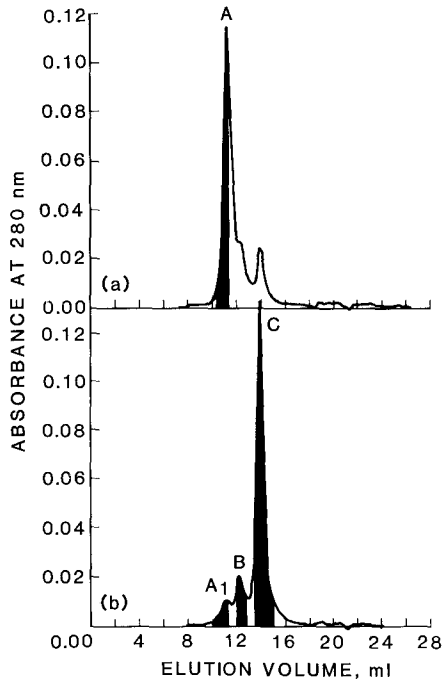

Fig. 1. Gel permeation chromatography of the second DEAE fraction.

Chromatography was carried out at room temperature (ca. 22°C) with an HR 10/30 Superose-12 column operated at 1.6 MPa and a flow rate of 0.3 ml/min. The eluant was 4 M NaCl-10 mM Tris.Cl pH 8.0 buffer. The column was calibrated using molecular weight markers made up in the same buffer. When calibration was carried out using 0.2 M NaCl-10 mM Tris.Cl pH 8.0 buffer, there was no significant effect on the elution volume of the standards. (a) The DEAE fraction (0.19 mg protein) which had been maintained at room temperature since preparation; (b) the DEAE fraction (1.9 mg protein/ml 4 M NaCl-10 mM Tris.Cl-50 mM $(NH_4)_2SO_4$ pH 8.0 buffer) was incubated at -13°C for 19 hr after which a sample containing 0.19 mg of protein was applied to the column. The shaded areas represent the fractions within the peaks which were combined and taken for subsequent SDS-PAGE (see Fig. 2).

The peaks in Fig. 1 were examined by SDS-PAGE [13]. As shown in Fig. 2, Peak A (Lane 2), was composed of the two major components (87 kDa and 60 kDa) together with three less-intensely-staining bands (49, 29, and 20 kDa). Electrophoresis of peak B (Lane 4) gave a complex pattern of polypeptides, of which none were related to those associated with component A. Peak A_1 (Fig. 1b), the material remaining after cold inactivation, was enriched with respect to the 49-kDa material whereas the 87- and 60-kDa components were depleted (lane 5 compared to lane 2). Thus, the 49-kDa component was not an ATPase constituent but was associated with material which migrated with the ATPase. Peak C (lane 3) was composed of four components: the 87 and 60 kDa

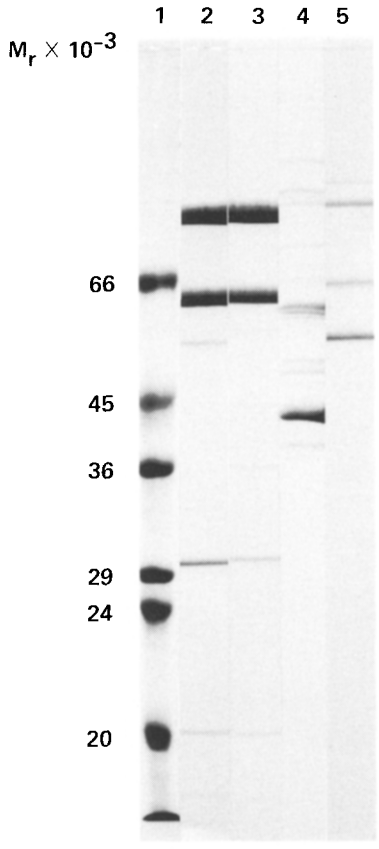

Fig. 2. Sodium dodecylsulfate polyacrylamide gel electrophoresis fractions obtained by gel permeation.

Electrophoresis was carried out according to the method of Laemmli [12] with 11% acrylamide gels using various Superose fractions (see Fig. 1 (a) and (b)). The bands were located with Coomasie Brilliant Blue R250. Lane 1, the molecular mass standards (treated in an identical manner as the gel permeation samples): bovine serumn albumin (66 kDa), ovalbumin (45 kDa), 3-phosphoglyceraldehyde dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa); lane 2, peak A (18 µg protein); lane 3, peak C (18 µg protein); lane 4, peak B (13 µg protein); lane 5, peak A_1 (5 µg protein).

polypeptides, which we designate as subunits I and II, respectively, and the 29 and 20 kDa, which we designate as III and IV. The presence of the latter components was unexpected since the Superose 12 column resolved standards over the range of 20 to 80 kDa. These results could obtain if cold dissociation resulted in three entities: subunits I and II, and a third, a dimer composed of the two low-molecular-weight subunits. It was this dimer which eluted with subunits I and II during gel filtration. Subunits III and IV were generated from the dimer prior to electrophoresis during SDS denaturation. Subunits I

and II stained with about equal intensity with Coomassie blue. Since the ATPase from H. saccharovorum is composed of four subunits, the subunit stoichiometry could be (I_2.II_2.III.IV). The molecular mass of such a complex would be 343 kDa, which is in good agreement with the value of 350 kDa obtained by gel filtration. Recently, Mukohata et al. [4] reported that the DCCD-sensitive ATPase from H. halobium was composed of two polypeptides (87 kDa and 64 kDa) with a 2:2 stoichiometry. This ATPase is probably similar to the one described by Konishi and Murakami [11], who found that the inhibition of ATP synthesis in H. halobium correlated with the binding of DCCD to a 45-62 kDa polypeptide [11]. These observations suggest the halobacterial DCCD-reactive subunits may be identical. While the function of these ATPases has not been demonstrated, the structure of the enzyme from H. saccharovorum makes it unlike any previously described ATPase. It has been suggested that the Halobacteria possess a proton-translocating ATPase unlike the ubiquitous F_0F_1-ATP Synthase [4]. These observations are consistent with this conclusion.

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