PURIFICATION OF AN *N,N'*-DICYCLOHEXYLCARBODIIMIDE-SENSITIVE ATPase FROM *ESCHERICHIA COLI*

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1. Introduction

The BF_0F_1 complex catalyzes the reversible transport of H^+ through the cytoplasmic membrane of bacteria, resulting in the formation of a protonmotive force during ATP hydrolysis or the formation of ATP during oxidative phosphorylation [1,2]. The complex has been purified to homogeneity in the thermophilic bacterium PS3 and shown to contain the 5 subunits of the F_1 and 3 subunits thought to form the F_0 [3]. It was shown [4] that only 2 subunits of 13 500 and 5400 daltons are necessary for F_0 function. The third, a 19 000 daltons polypeptide, is either a contaminant or has an undetermined function. The isolation of a DCCD-sensitive ATPase from the membrane of *E. coli* was reported [5]. We have been unable to repeat that procedure, but have developed another purification procedure which results in the isolation of highly purified BF_0F_1. A portion of this material was the subject of a preliminary report [6].

2. Materials and methods

Everted membrane vesicles were produced from stationary phase cultures of *E. coli* K12 strain 7 by lysis with a French press at 20 000 p.s.i., as in [7]. Solubilization of the BF_0F_1 was performed by suspension of membrane vesicles to 20 mg protein/ml in a solution containing 10 mM Tris–HCl, 140 mM KCl, 0.5 mM dithiothreitol, 10% (v/v) glycerol and 40 mg/ml sodium deoxycholate (pH 8.0). The 2:1 ratio of deoxycholate:protein was found to be critical for efficient extraction. The solution was incubated for 30 min at 4°C followed by centrifugation at 100 000 × *g* for 1 h to remove residual membranes. The supernatant solution was placed onto the top of a 10–40% sucrose gradient (2.5 ml supernatant/tube) in 10 mM Tris–HCl (pH 7.2), 1 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.5% Triton X-100 and centrifuged for 40 h at 25 000 rev./min in a SW 27 Beckman rotor. Fractions (1.75 ml) were collected. The ATPase-containing fractions were pooled and applied to a DEAE-cellulose column (1.6 × 7 cm). The column was eluted with a linear gradient of 0.01–0.5 M Tris–HCl (pH 7.4) containing 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% (v/v) glycerol and 0.5% Triton X-100. The ATPase activity eluted at 0.22 M Tris. Active fractions were diluted 15-fold and applied to a second DEAE-cellulose column. The column was washed with 10 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, 0.5 mM Dithiothreitol, and 10% (v/v) glycerol until the A_{280} was < 0.05. ATPase activity was then eluted with a small volume of the same buffer containing 0.5 M Tris–HCl and dialyzed against the same Triton-free 10 mM Tris–HCl buffer.

Assays for ATPase activity and DCCD sensitivity were performed as in [7]. Fractions from the sucrose gradient and DEAE columns were assayed by the spot plate method in [7]. Protein was determined by a modification of the Lowry method [8]. Dehydrogenase activities were estimated from the reduction of 2,6-dichloroindophenol, measured at 620 nm, as in [9]. SDS–urea acrylamide gradient gel electrophoresis was performed as in [10].

Purified BF_1 from *E. coli* ML 308 was the generous gift of Drs Leon Heppel and Jeffrey Smith of Cornell University. All chemicals were reagent grade and obtained from commercial sources.

3. Results

Attempts to extract and purify the DCCD-sensitive ATPase by the method in [5] were unsuccessful:

(1) Extraction with 0.2% deoxycholate was not sufficient to extract the BF_0F_1 when large amounts of membranes were used;

(2) The activity was unstable when left in deoxycholate such that little activity remained after centrifugation in a sucrose gradient containing deoxycholate.

Extraction with a fixed ratio of deoxycholate to protein, on the other hand, permitted solubilization of large amounts of membranes. The deoxycholate extract would often gel within several hours, so that it was necessary to apply the extract to the sucrose gradient as soon as possible. Centrifugation into a sucrose gradient containing Triton X-100 was extremely effective in removing the bulk of the membrane proteins (fig.1). The activity remained stable in Triton X-100 at 4°C for several weeks.

Fractionation of the sucrose gradient fractions on DEAE-cellulose in the presence of Triton X-100 was successful when a gradient of Tris–HCl was used for elution. When NaCl or KCl was used to elute, the complex was unstable, losing activity within several hours. The bulk of the Triton X-100 could be removed by binding the enzyme to a DEAE column and eluting with Triton-free buffers. The purified enzyme appeared soluble even in the absence of detergents, although the presence of bound Triton cannot be excluded. A summary of the purification is shown in table 1.

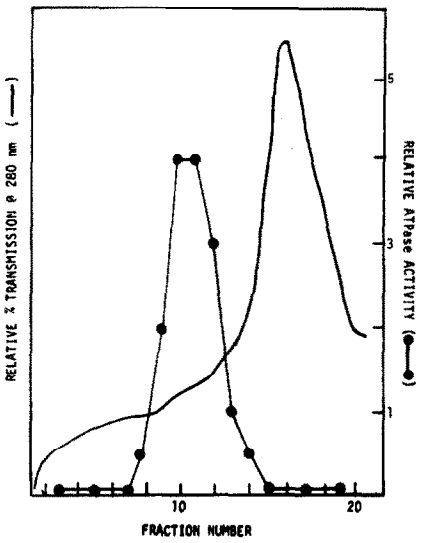

Fig.1. Fractionation of the BF_0F_1 by centrifugation in a sucrose gradient. A portion of a deoxycholate extract (2.5 ml) was placed on the top of a 10–40% (w/v) sucrose gradient containing 0.5% Triton X-100 and fractionated as in section 2. Relative % transmission at 280 nm (——) was measured using an LKB flow cell blanked against 0.5% Triton X-100. Relative ATPase activity (●——●) was measured by the spot plate assay.

The purified complex had no detectable D-lactate or α-glycerophosphate dehydrogenase activities. NADH dehydrogenase activity was observed, but only 1% of the total NADH dehydrogenase activity was found in the final purified preparation. A difference spectrum of the purified complex was flat, ruling out

Table 1
Summary of purification of BF_0F_1

Step	Protein (mg)	ATPase activity[a]		
		Spec. act. (units/mg)	Total act. (units)	Yield (%)
1. Membrane vesicles	139	3.3	458	100
2. Deoxycholate extract	100	4.9	490	106
3. Sucrose gradient	10.5	28.7	301	66
4. 1st DEAE column	3.5	45.0	157	34
5. 2nd DEAE column	1.9	80.0	152	33

[a] One unit of ATP activity is the liberation of 1 μmol P_i from ATP

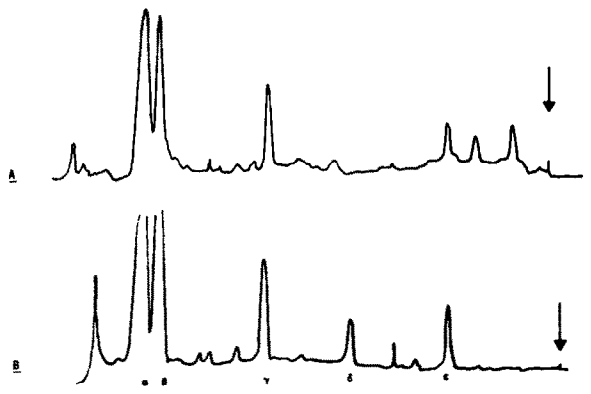

Fig.2. Polyacrylamide gel electrophoresis of purified BF_0F_1. Electrophoresis was performed in the SDS–polyacrylamide system described [10] with a gradient of acrylamide from 8–12.5% (w/v) and a gradient of urea from 32–40% (w/v). The slab was stained with Coomassie blue, and the intensity of staining estimated with a microdensitometer (Joyce, Loebl and Co.). (A) 25 μg purified BF_0F_1. (B) 20 μg BF_1 from *E. coli* ML308. Arrows indicate position of bromophenol blue dye front.

cytochrome contamination. Upon SDS–polyacrylamide gel electrophoresis (fig.2), 6 major bands were observed, 4 of which corresponded with the α, β, γ, and ε subunits of the F_1 [11]. Two bands of greater mobility were observed, corresponding to mol. wt 10 000 and 8300 daltons, respectively. No polypeptide with a mobility corresponding to the δ subunit was observed in the BF_0F_1 preparation. A sample of purified F_1 generously provided by Drs Leon Heppel and Jeffrey Smith had that subunit (fig.2). Unlike soluble BF_1, the purified ATPase complex was both stable in the cold and sensitive to DCCD (fig.3).

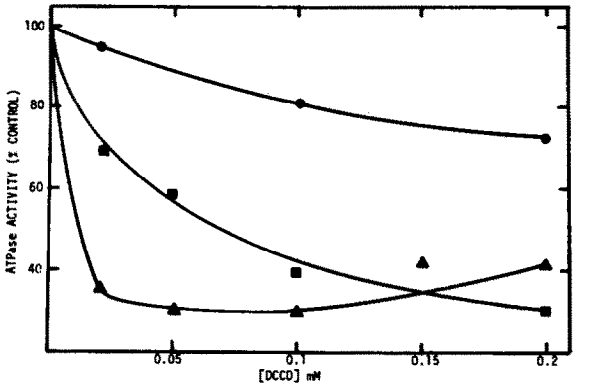

Fig.3. DCCD sensitivity of ATPase activities. ATPase activity in the presence of varying amounts of DCCD was measured as in section 2. (▲——▲) Membrane vesicles. (■——■) Purified BF_0F_1. (●——●) Crude soluble BF_1.

4. Discussion

In this communication we have reported a procedure for purification of a DCCD-sensitive ATPase from the membrane of *E. coli*. The complex is composed of the F_1 plus two additional polypeptides of 10 000 and 8300 daltons. These two polypeptides would presumably form the F_0 portion of the complex. In that respect this preparation is similar to the complex from PS3, for which it was shown [4] that only two polypeptides of mol. wt 13 500 and 5400 daltons are necessary for F_0 function [4]. The 5400 daltons polypeptide has been shown to be the DCCD-binding component of the F_0 [4]. A DCCD-binding component with mol. wt 8400 daltons was purified from *E. coli* [12]. The preparation reported [5] also has a component of about 9000 daltons, but the other additional polypeptide in that preparation was 29 000 daltons.

SDS gels of our purified preparation do not show the δ subunit of the F_1. We have found similarly that our purified F_1 preparations show little or no δ on SDS gels, even though those preparations are able to bind in a functional manner to F_0 on the membrane [7]. We cannot explain the lack of δ on the gels.

The purified complex has properties expected of an F_0F_1 complex. It is stable to storage in the cold, while the F_1 is cold labile. It is sensitive to DCCD, while the F_1 is DCCD insensitive. Reconstitution experiments to insert the complex into artificial liposomes to produce H^+-translocating activity have thus far been unsuccessful, and current efforts are designed to examine this question.

Acknowledgements

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