PURIFICATION AND PROPERTIES OF ADENOSINE TRIPHOSPHATASE FROM *CHROMATIUM VINOSUM* CHROMATOPHORES

Amira GEPSHTEIN and Chanoch CARMELI

Department of Biochemistry, The George S. Wise Center for Life Sciences, Tel-Aviv University, Tel-Aviv

and

Nathan NELSON

Department of Biology, Technion, Israel Institute of Technology, Haifa, Israel

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1. Introduction

Successful attempts have been made to separate coupling factor from chromatophores of several photosynthetic bacteria [1–5]. Highly-purified coupling factor which contained five subunits was obtained only from *Rh. rubrum* [6]. We have reported [7] on the properties of a partially-purified coupling factor from *Chromatium* chromatophores. It is the purpose of this work to present a procedure for the preparation of a highly purified ATPase from *Chromatium* and to describe its properties.

2. Materials and methods

2.1. *Isolation of chromatophores*

Chromatium strain D bacteria were grown anaerobically in the light on the medium [8] supplemented with 0.2% malate for 4 days at 32°C. The harvested bacteria were washed in 0.1 M tricine–NaOH, pH 7.8 and stored under nitrogen at −20°C. The cells were ground with alumina in 0.1 M tricine–NaOH, pH 7.8, then centrifuged at 12 000 × *g* for 15 min to remove debris. The chromatophores were sedimented by centrifugation at 144 000 × *g* for 1 h, resuspended in a small volume of a solution containing 0.1 M tricine–NaOH, pH 7.8 and glycerol (1:1, v:v), and were kept at −20°C. Bacteriochlorophyll content was determined by measuring the $A_{850\ nm}$ using the extinction coefficient determined [9].

Abbreviations: SDS, sodium dodecyl sulfate; BChl, bacteriochlorophyll; tricine, *N*-tris(hydroxymethyl)methylglycine

2.2. *Assay of photophosphorylation*

Photophosphorylation was assayed in a reaction mixture containing 33 mM tricine–NaOH, pH 7.8, 3.3 mM $K_2H^{32}PO_4$, pH 7.8 (containing 10^6 cpm), 8 mM $MgCl_2$, 3.3 mM ADP, 0.1 mM phenazine methosulfate and chromatophores containing 50 μg bacteriochlorophyll in total vol. 1.5 ml. The reaction was started by illumination at an intensity of 10^6 erg.cm^{-2}.S^{-1} for 5 min at 22°C and was stopped by addition of cold trichloroacetic acid to a final concentration of 3%. ^{32}[P]ATP formation was measured according to [10].

2.3. *Assay of ATPase activity*

ATPase activity was assayed in a reaction mixture containing: 33 mM tricine–NaOH, pH 7.8, 8 mM $CaCl_2$, 4 mM ^{32}[P]ATP, 10–50 μg coupling factor protein and 10–25 μg trypsin in total vol. 1.5 ml. The reaction was carried out in the dark by 10–20 min at 37°C and was stopped by addition of cold trichloroacetic acid to a final concentration of 3%. $^{32}P_i$ released was measured according to [10].

2.4. *SDS–gel electrophoresis*

Dissociation into subunits was performed by an

overnight treatment with 2% SDS, 2% mercaptoethanol in 10% sucrose. Subunit composition was analyzed by SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gel in the presence of 0.1% SDS as described [11].

2.5. *Preparation of antibody*

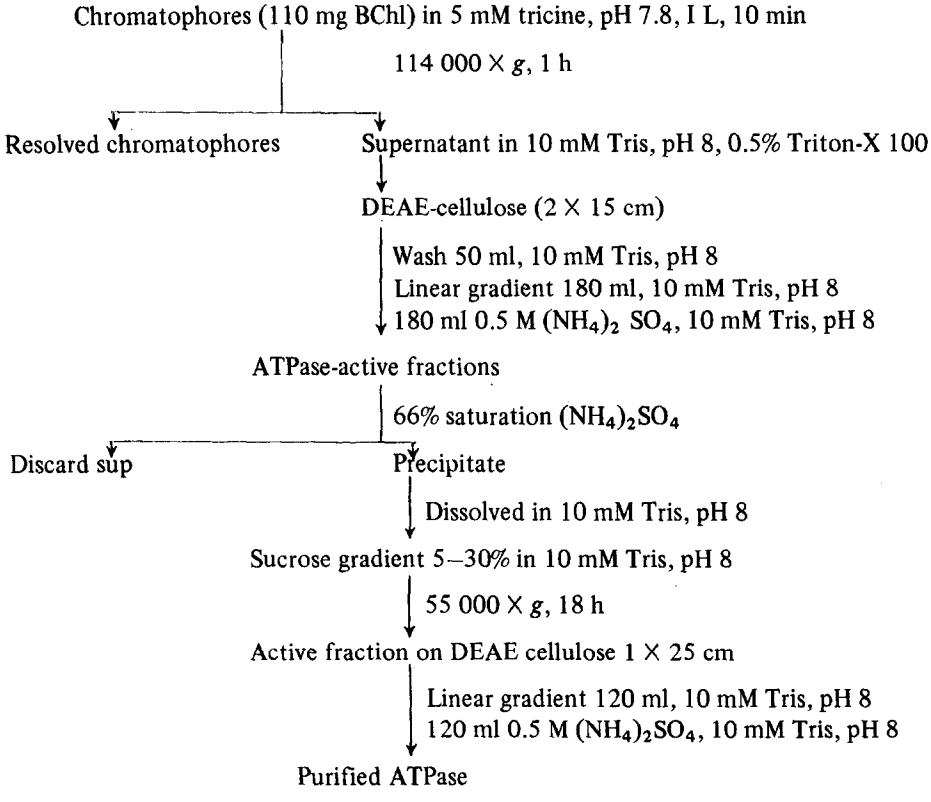
Antibody against the purified ATPase was prepared by injecting rabbits with 100 μg protein at a time as described [12]. The antibody showed a single line of precipitation without spear on an Ouchterlony plate [13].

3. Results and discussion

3.1. *Purification of ATPase*

The purification of ATPase involved extraction of the protein from chromatophores, two separations on DEAE-cellulose columns, ammonium sulfate precipitation and a sucrose gradient. The main steps are summarized in a flow sheet (scheme 1). For extraction of the crude protein chromatophores containing 100 mg bacteriochlorophyll were incubated in a medium containing 5 mM tricine NaOH, pH 7.8 for 10 min at room temperature, then sedimented by centrifugation at 144 000 × *g* for 1 h at 8°C. The clear supernatant was removed from the pellet and solutions of 1 M Tris–Cl, pH 8 and 20% Triton X-100 were added to give final concentrations of 10 mM and 0.5%, respectively. The crude ATPase obtained was applied to two DEAE cellulose DE-11 columns (2 × 15 cm) equilibrated with 10 mM Tris–Cl, pH 8 and kept at room temperature. The two columns were washed with 50 ml of the same buffer and the ATPase was eluted with 320 ml linear gradients of 0–0.5 M $(NH_4)_2SO_4$ in 10 mM Tris–Cl, pH 8. A flow rate of 5 ml/min was maintained and fractions of 5 ml were

Scheme 1
Flow sheet for purification of ATPase from chromatophores



collected. Fractions were assayed for ATPase activity as described under section 2. Protein concentration was determined by the modification of the Lowry method developed [14]. Fractions containing ATPase activity were pooled together and the enzyme was precipitated by adding 2 vol. saturated ammonium sulfate bringing the solution to 66% saturation. After incubation overnight at 4°C the ammonium sulfate suspension was centrifuged at 10 000 × *g* for 10 min and the pellet was dissolved in 2.5 ml 10 mM Tris–HCl, pH 8. The soluble enzyme was then layered on the top of a linear gradient made of 5–30% sucrose in 10 mM Tris–Cl, pH 8 and centrifuged at 15°C for 18 h at 28 000 rev/min in Spinco SW-40 rotor. The tubes were then punctured at the bottom and 0.4 ml fractions were collected. Fractions showing ATPase activity were pooled together for further purification.

Active fractions obtained from the sucrose gradient were applied to a DEAE-cellulose DE-11 column (1 × 25 cm) and the enzyme was eluted with 240 ml linear gradient of 0–0.5 M $(NH_4)_2SO_4$ in 10 mM Tris–HCl, pH 8. A 3 ml/min flow rate was maintained and 3 ml fractions collected. The active fractions were stored as a suspension in a solution of 66% saturation of ammonium sulfate.

The results of the purification are summarized in table 1. As can be seen from these data there was a 24-fold increase in the specific activity of ATPase during the purification of the enzyme. However, only 6% of the initial activity was left at the end of the purification. It was found that Triton had to be present before the application of the crude coupling factor to the first DEAE-cellulose column probably in order to dissociate hydrophobically associated proteins. In order to obtain a pure enzyme only the peak fraction of the first column had to be collected. Consequently a great part of active protein was lost.

The purity of the enzyme is indicated not only from the great increase in specific activity but also from its analysis on SDS–gel electrophoresis. In fig.1 a scan of a stained gel electrophoresis of crude coupling factor is presented. Among the multiple peaks of various proteins it is possible to see the location of the five subunits which construct the coupling factor protein. In the purified ATPase mainly four peaks are seen (fig.2). Only a small amount of the δ

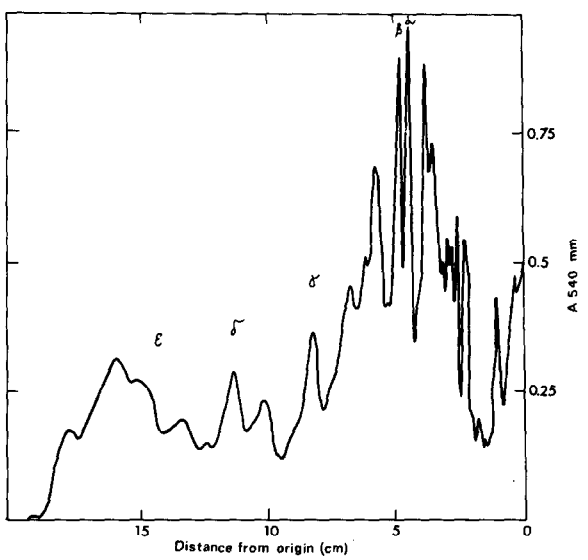


Fig.1. Scan of gel electrophoresis of crude coupling factor. Crude coupling factor, 60 μg, was applied and developed on SDS gel electrophoresis as described in section 2. The stained gel was scanned in a spectrophotometer at 540 nm.

Table 1
Purification of ATPase

Purification steps	Total protein (mg)	Specific activity (μmol P_i × mg protein^{-1} × h^{-1})	Total activity (μmol P_i × h^{-1})
Crude coupling factor	412	22.1	9105
DEAE-cellulose	36.3	99.6[a]	3025
Sucrose gradient	3.5	152.3	533
DEAE-cellulose	1.2	535	572

[a] The activity of peak fractions

Chromatophores containing 110 mg bacteriochlorophyll were used for extraction of crude coupling factor in this experiment. The various purification steps are described in section 3 and the assay procedure in section 2

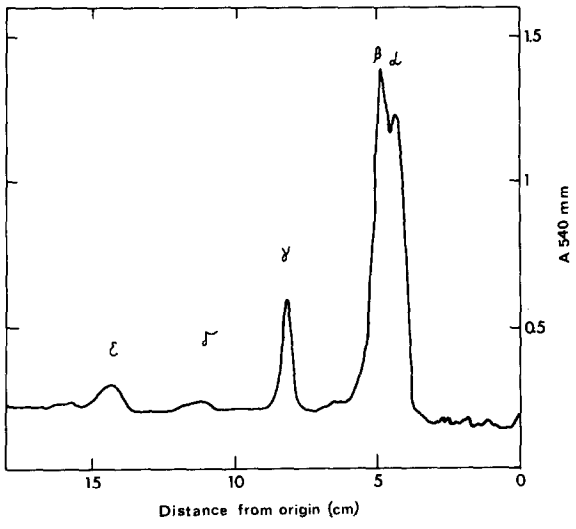


Fig.2. Scan of gel electrophoresis of purified ATPase. Purified ATPase, 26 μg, was applied and developed on SDS–gel electrophoresis as described under fig.1.

subunits remains in the purified enzyme. Although the purified enzyme catalyzed high rates of ATPase activity it did not restore phosphorylation to the resolved chromatophores from which the coupling factor was previously removed (not shown). The coupling activity of chloroplasts and *E. coli* ATPases was shown to be dependent on the presence of the δ subunits in the preparations [15]. In the crude coupling factor a peak of protein is seen where δ subunit should be located. Indeed the crude coupling factor fully restored phosphorylation to resolved chromatophores [16].

3.1. *Properties of the purified coupling factor*

Because of the low yield of the purified ATPase it could be suspected that the activity is not necessarily due to the coupling factor which catalyses ATP formation during photophosphorylation. However, the monospecific antibody prepared against the purified ATPase inhibited ATPase activity in the crude and in the purified coupling factor (table 2). It also inhibited

Table 2
Effect of antibody against the coupling factor on ATPase activity

	Phosphorylation (μmol P_i × mg $BChl^{-1}$ × h^{-1})	ATPase (μmol P_i × mg $protein^{-1}$ × h^{-1})
Chromatophores	136.4	–
Chromatophores + 1 μl antibody	115.9	–
Chromatophores + 5 μl antibody	61.1	–
Chromatophores + 10 μl antibody	12.4	–
Crude coupling factor	–	39.5
Crude coupling factor + 5 μl antibody	–	11.2
Crude coupling factor + 10 μl antibody	–	9.6
Purified coupling factor	–	205.4
Purified coupling factor + 10 μl antibody	–	0

When indicated antibody against the purified coupling factor was added to the assay mixture which contained either chromatophores when photophosphorylation was measured or with 50 μg/1.5 ml of crude or purified coupling factor when ATPase activity was measured. Assay conditions were as described in section 2

Table 3
Effect of dicyclohexylcarbodiimide on ATPase activity in purified coupling factor

Dicyclohexyl-carbodiimide (μM)	ATPase activity (μmol P_i × mg protein^{-1} × h^{-1})
–	61.2
0.7	60.0
3.3	32.4
6.6	11.8
33.0	0

Dicyclohexylcarbodiimide was preincubated with trypsin activated coupling factor for 20 h at 24°C. ATPase activity was assayed as indicated in section 2

photophosphorylation (table 2) and ATPase activity (not shown) catalyzed by the chromatophores. It seems therefore that the same enzyme catalyzed all these reactions.

A unique feature [7] has to do with the fact that dicyclohexylcarbodiimide inhibited not only phosphorylation and ATPase activity in the chromatophores but also ATPase activity in the soluble crude and partially-purified coupling factor. It was thought that these fractions could contain a subunit which binds the inhibitor and therefore induces inhibition of the activity. However, the purified enzyme is still sensitive to the inhibitor (table 3) although no additional subunit is apparent on the stained gel electrophoresis of the enzyme. It is still possible that a subunit which binds the inhibitor is attached to the enzyme but failed to be seen by the methods used in these experiments. Alternatively dicyclohexylcarbodiimide might act directly on the catalytic part of the enzyme.

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