Chloroplast ATPase in Acetabularia acutabulum: Purification and Characterization of
Chloroplast F_{1}-ATPase

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Received August 27, 1993

ATPases were isolated from chloroplasts of the unicellular marine alga Acetabularia acutabulum. Two preparations of ATPase, a chloroplast-enriched fraction and an $\alpha\beta\gamma$-complex were compared. The $\alpha\beta\gamma$-complex was released into an EDTA solution and purified by anion-exchange chromatography, hydrophobic chromatography, and gel permeation chromatography. The subunit composition of this enzyme appeared to be $52-53(\alpha), 51(\beta),$ and $40(\gamma)$kDa from SDS-PAGE. ATPase activity was enriched about 260-fold to a specific activity of approximate 4.1 U mg protein$^{-1}$. The catalytic properties of the $\alpha\beta\gamma$-complex were as follows: pH optimum at 7.5; substrate specificity, ATP $>$ GTP $>$ UTP $=$ CTP ($K_m$ for ATP 0.2 mm); divalent cation requirement, $\text{Mg}^{2+} = \text{Mn}^{2+} = \text{Co}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Ca}^{2+}$; ATPase activity was inhibited by monovalent anions ($\text{NO}_3^-,\text{SCN}^-$), while monovalent cations had neither inhibitory nor stimulatory effects. Orthovanadate had no inhibitory effect on the enzyme activity of $\alpha\beta\gamma$-complex. Azide was the most effective inhibitor of the $\alpha\beta\gamma$-complex. N-Terminal amino acid sequences of the $\alpha$ and $\beta$ subunits were not obtained and appeared to be blocked. The $\gamma$ subunit gave a sequence of AGKEMKDXIGSVXNTKLI, which showed 60% similarity to the $\gamma$ subunits of spinach and Chlamydomonas reinhardtii CF$_1$-ATPase and EF$_1$-ATPase.

ATPases play an important role in energy conversion both in prokaryotes and eukaryotes. They have been well studied biochemically and molecular biologically in connection with their cation-translocating activities. They are classified into three categories: P, V, and F types.1) Archaeabacterial ATPase has been reported to be closely related to the V type and not related to the F type.2) Mukohata et al. proposed that ATPases in archaeabacteria should be given an independent classification. A type in the ATPase family. Evolutionary aspects of the ATPase family are a current topic in the field. One hypothesis is that common progenitors may have three different progenitors of ATPase (V, A, and F types).3)–7) Acetabularia acutabulum, a unicellular marine alga, belongs to Dasyaceae, and is one of the most ancient eukaryotes. ATPases in this organism are, of course, of great interest. We have already isolated and/or characterized a $\text{Cl}^{-}$-translocating ATPase8) and a vacuolar ATPase from this organism,10) both of which showed unique properties. Chloroplast-bound ATPase has also been isolated from A. acutabulum in an inactive state,9) but the preparations (peaks II and III in ref. 11) were mostly contaminated by Rubisco through protein chemical experiments.11)

Isolation of a chloroplast-enriched fraction and partial purification of chloroplast F$_{1}$-ATPase complex ($\alpha\beta\gamma$) in an active state are described in this paper. Several catalytic properties of the respective preparation are also presented.

Materials and Methods

Materials. Most of the chemicals were the same as described in our previous reports.9) The FPLC-system, a Phenyl Superose HR5/5 column and a Superose 12 column from Pharmacia (Uppsala, Sweden) were used for isolation of chloroplast F$_{1}$-complex.

The N-terminal sequencing of the isolated subunits of F$_{1}$-complex was performed on a Model 477A protein/peptide sequencer and an on-line PTH analyzer. Model 120A-01 (Applied Bio-Systems Co., Foster City, U.S.A.) after electroblotting onto siliconized glass-fiber sheets.12) Antibodies against the $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$ subunits of spinach chloroplast F$_{1}$-ATPase were prepared as described in our previous paper.13)

Preparation of chloroplast-enriched fractions. Isolation of chloroplast-enriched fractions was carried out according to the method of Bidwell et al.13) which was established for A. acutabulum. Class I chloroplast fraction was stored at −20 C and used for experiments.

Purification of the chloroplast ATPase F$_{1}$-complex (z$\alpha\beta\gamma$). Axenic cells (3 to 5 cm in length 90 g wet weight) were cut into small pieces and extracted with the homogenization buffer (pH 7.0) without EGTA (150 mmol $\times$ three times) under vigorous stirring at room temperature for 5 min. The extract was filtered through four layers of cheesecloth and the filtrate was centrifuged at 5000 $\times$ g for 2 min. Three more steps were in principle carried out according to the method of Bender et al.14) The precipitate were suspended in 50 ml of 10 mm NaCl and centrifuged at 12,000 $\times$ g for 10 min. The washing procedure with 10 mm NaCl was repeated twice. The washed precipitates were suspended in 190 ml of 0.75 mm EDTA (1 ml for 0.1 mg chlorophael), the pH was adjusted to 7.8 with 0.1 n NaOH, and the mixture was stirred at room temperature for 10 min. After centrifugation at 12,000 $\times$ g for 10 min, the precipitate was reextracted with 0.75 mm EDTA in the same manner. The supernatants were combined, added to 10 ml of a DEAE-cellulose previously equilibrated in 50 mm Tris HCl (pH 7.8), 2 mm EDTA, 50 mm PMSF, and

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Abbreviations: FITC, fluorescein isothiocyanate; DCCD, N,N'-dicyclohexylcarbodiimide; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; NEM, N-ethylmaleimide; DES, diethylsilanolate; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; F$_{1}$, catalytic portion of ATP synthase; CF$_{1}$, F$_{1}$ from chloroplast; EF$_{1}$, F$_{1}$ from Escherichia coli; TF$_{1}$, F$_{1}$ from thermophilic bacterium PS3.
1 mM ATP, and stirred at room temperature for 20 min. The suspension was filtered through a Miracloth (60 µm) and the cellulose resin was washed three times with 50 ml of the above equilibration buffer batch-wise. The washed cellulose resin was packed into a column (2.4 cm × 5 cm). Elution of proteins was conducted with a buffer containing 50 mM Tris-HCl, pH 7.8, 0.2 M NaCl, 2 mM EDTA, 50 µM PMSF, and 1 mM ATP. Fractions with ATPase activity were pooled (ca. 40 ml) and concentrated by ultrafiltration. The concentrate (ca. 6 ml) was brought to 50% saturation with ammonium sulfate and kept at 4°C overnight. The precipitates were collected by centrifugation at 10,000 × g for 20 min, then dissolved in an equilibration buffer for Phenyl Superose (see below). The sample solution (ca. 600 µl) was applied to a Phenyl Superose column (1 ml bed-volume) previously equilibrated with a buffer consisting of 25 mM Pipes-Tris (pH 7.0), 1.2 M (NH₄)₂SO₄, 0.25 M sorbitol, 6 mM MgSO₄, 1 mM EGTA, 2 mM DTT, 50 µM PMSF, and 1 mM ATP. Proteins were eluted by a linear decrease of (NH₄)₂SO₄ in the buffer from 1.2 to 0 M. The active fractions were pooled, concentrated, and applied to a Superose 12 column previously equilibrated with a buffer consisting of 25 mM Hepes-NaOH (pH 6.5), 50 mM Na₂SO₄, 0.25 M sorbitol, 6 mM MgSO₄, 1 mM EGTA, 2 mM DTT, 25 µM PMSF, and 1 mM ATP. The final preparation was concentrated, stored at −70°C and used for further experiments.

Assay and analytical procedures. ATPase activity was measured as described previously, 90 except that the pH of the Pipes-Tris buffer was 7.5. methanol was added with a final concentration of 20% (v/v), and incubation was performed at 37°C. One unit of enzyme activity was defined as micromole Pi liberated per minute. Protein determination, analytical SDS-PAGE, western blotting, and reactions with antibodies were all carried out as described previously. 8,10

Results

Purification of chloroplast ATPase αβγ-complex

One ATPase-containing fraction was obtained through DEAE-cellulose, Phenyl Superose, and Superose 12 chromatography. The elution profile of the ATPase activity on Phenyl Superose chromatography is shown in Fig. 1. The activity was eluted from the Phenyl Superose column at 0 M (NH₄)₂SO₄. The major contaminant protein, Rubisco, was eluted at about 0.3 M (NH₄)₂SO₄. As summarized in Table I, the ATPase was about 260-fold enriched in 13% yield, and had a specific activity of 4.1 U·mg protein⁻¹.

Figure 2a shows SDS-polyacrylamide gel electrophoretograms of the crude chloroplast fraction, thylakoid mem-

![Fig. 1. Elution Profile on Phenyl Superose Chromatography.](image)

The sample solution after ammonium sulfate precipitation was applied to a Phenyl Superose column. Each fraction was assayed for ATPase activity (●) and protein was monitored by its absorption at 290 nm (○○○). The solid line indicates the linear salt gradient of (NH₄)₂SO₄ used for chromatography and the arrow shows the start of the linear gradient from 1.2 to 0 M (NH₄)₂SO₄.

![Fig. 2. SDS-Polyacrylamide Gel Electrophoretograms (Coomassie Stain) and Immunoblot of the Superose 12 Fraction and Thylakoid Membrane.](image)
a. Coomassie stain (12.5% gel): Lane A, crude chloroplast fraction (15 µg protein); lane B, thylakoid membrane after washing with 10 mM NaCl (3 µg protein); lane C, 10 mM NaCl fraction (5 µg protein). Lane D, concentrate after a DEAE-cellulose column (7 µg protein); lane E, concentrate after a Phenyl Superose column (5 µg protein); lane F, concentrate after Superose 12 column (2 µg protein); lane G, spin fertilizer complex (2 µg protein).
b. Immunoblots of the chloroplast-enriched fraction, corresponds to lane A, with antibodies against the α (lane 1) and β (lane 2) subunits of spinach CF₁-complex.
c. Immunoblots of the CF₁-complex from Acetabularia (lanes 3, 5, and 7) and spinach (lanes 4, 6, and 8). SDS-polyacrylamide gel electrophoresis was done in 10% gels, and immunostaining was done with the antibodies against the x (lanes 3 and 4), β (lanes 5 and 6), and γ (lanes 7 and 8) subunits of spinach CF₁.
Table II. Catalytic Properties of the Chloroplast ATPase \( \alpha/\beta \)-Complex (% Activity)

Substrate specificity, inhibitor effects, divalent cation requirements, and monovalent cation and anion effects were tested for the chloroplast ATPase in an assay mixture containing 25 mM Pipes-Tris buffer, 0.25 M sorbitol, 3 mM each of MgSO\(_4\) and ATP-Na, and 20% methanol for \( \alpha/\beta \)-complex. Except for the pH dependence, the buffer was pH 7.5 for \( \alpha/\beta \)-complex. For the inhibitor studies, the preincubation of each inhibitor with the enzyme was carried out at 37°C for 5 min, and the reaction was started by addition of ATP. Results are the mean of duplicate measurements.

<table>
<thead>
<tr>
<th>Substrate specificity (%)</th>
<th>Chloroplast ATPase ( \alpha/\beta )-complex</th>
<th>Chloroplast ATPase ( \alpha/\beta )-complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (3 mM)</td>
<td>100</td>
<td>None</td>
</tr>
<tr>
<td>GTP (3 mM)</td>
<td>67</td>
<td>Mg(^{2+}) (3 mM) 100</td>
</tr>
<tr>
<td>ITP (3 mM)</td>
<td>75</td>
<td>Mn(^{2+}) (3 mM) 103</td>
</tr>
<tr>
<td>UTP (3 mM)</td>
<td>17</td>
<td>Ca(^{2+}) (3 mM) 10</td>
</tr>
<tr>
<td>CTP (3 mM)</td>
<td>2</td>
<td>Zn(^{2+}) (3 mM) 53</td>
</tr>
<tr>
<td>ADP (3 mM)</td>
<td>1</td>
<td>Ni(^{2+}) (3 mM) 31</td>
</tr>
<tr>
<td>AMP (3 mM)</td>
<td>1</td>
<td>Cu(^{2+}) (3 mM) 115</td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate (3 mM)</td>
<td>1</td>
<td>Effect of monovalent ions</td>
</tr>
<tr>
<td>Inhibitors</td>
<td>None</td>
<td>Li(^+) (10 mM) 111</td>
</tr>
<tr>
<td>Orthovanadate (100 (\mu)M)</td>
<td>102 (114)(^a)</td>
<td>K(^+) (10 mM) 93</td>
</tr>
<tr>
<td>Azide (100 (\mu)M)</td>
<td>8 (59)(^b)</td>
<td>Na(^+) (10 mM) 101</td>
</tr>
<tr>
<td>DCCD (100 (\mu)M)</td>
<td>110 (76)(^b)</td>
<td>NH(_4)(^+) (10 mM) 83</td>
</tr>
<tr>
<td>DES (100 (\mu)M)</td>
<td>85 (75)(^b)</td>
<td>Choline (10 mM) 100</td>
</tr>
<tr>
<td>FITC (100 (\mu)M)</td>
<td>81 (79)(^b)</td>
<td>Effect of anion requirement</td>
</tr>
<tr>
<td>NB-D-Cl (100 (\mu)M)</td>
<td>89 (36)(^b)</td>
<td>Cl(^-) (10 mM) 70</td>
</tr>
<tr>
<td>NEM (100 (\mu)M)</td>
<td>117 (80)(^b)</td>
<td>Br(^-) (10 mM) 87</td>
</tr>
</tbody>
</table>

\(^a\) Not detectable \(^b\) ATPase activity in the chloroplast-enriched fraction.

brane after washing with 10 mM NaCl, concentrates after a DEAE-cellulose column, after a Phenyl Superose column, and after a Superose 12 column. The final preparation shows the four major polypeptides with molecular masses of 53, 52, 51, and 40 kDa (lane F in Fig. 2a). Cross-reactivities of the Superose 12 fraction were also tested using the antisera against the respective \( \alpha \), \( \beta \), \( \gamma \), \( \delta \), and \( \epsilon \) subunits of spinach chloroplast F\(_1\)-ATPase, and the results are shown in Fig. 2c. Clear cross-reactions with the anti-\( \alpha \), -\( \beta \), and -\( \gamma \) sera were observed, but the reactions with the anti-\( \delta \) and anti-\( \epsilon \) sera were not observed (data not shown). Both data supported that the Superose 12 fraction mainly consisted of the \( \alpha \), \( \beta \), and \( \gamma \) subunits of the chloroplast F\(_1\)-ATPase. Both the 53 and 52 kDa polypeptides reacted with the anti-\( \alpha \) serum.

Catalytic properties of the chloroplast-enriched fraction and \( \alpha/\beta \)-complex as ATPase

The chloroplast-enriched fraction and \( \alpha/\beta \)-complex were characterized as ATPase, and the results are summarized in Table II.

(A) pH optimum. The pH profiles of enzyme activity in the two preparations are shown in Fig. 3. The pH optima of the ATPase activities in the chloroplast-enriched fraction was around 9.0, while the pH optimum of the isolated \( \alpha/\beta \)-complex around 7.5.

(B) Substrate specificity and inhibitors. As summarized in Table II, ATP was the best substrate for the isolated \( \alpha/\beta \)-complex. The \( K_m \) of the \( \alpha/\beta \)-complex was 0.2 mM.

Table II also lists a number of compounds tested as inhibitors for the ATPase activity. The two preparations showed different susceptibilities to the inhibitors, except for orthovanadate and FITC. Azide, a typical inhibitor of F type ATPase, inhibited the activity in the isolated \( \alpha/\beta \)-complex and in the chloroplast-enriched fraction (see Fig. 4).

(C) Cation and anion requirement. Several divalent cations were tested for the isolated complexes as replacement of Mg\(^{2+}\), and the results are also listed in Table II. In the absence of Mg\(^{2+}\), the ATPase had almost no activity in both preparations. Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), and Ni\(^{2+}\) could substitute for Mg\(^{2+}\) in the isolated \( \alpha/\beta \)-complex. A number of monovalent cations were tested and shown to have no

Fig. 3. pH Profiles of ATPase Activity in the Chloroplast-enriched Fraction and the Isolated \( \alpha/\beta \)-Complex.

An aliquot (2.8 μg protein for the chloroplast-enriched fraction and 0.14 μg for the \( \alpha/\beta \)-complex) was assayed for ATPase activity at various pH values. Each value was the mean of duplicate determinations.

- ○. ATPase activity in the chloroplast-enriched fraction; ---. ATPase activity in the isolated \( \alpha/\beta \)-complex.

Fig. 4. Effects of Azide on ATPase Activity in the Chloroplast-enriched Fraction and the Isolated \( \alpha/\beta \)-Complex.

ATPase activity was assayed for the two preparations in the presence of various amounts of azide. Twenty percent methanol was added to the reaction mixture of the isolated \( \alpha/\beta \)-complex. The values were from duplicate determinations.

- ○. ATPase activity in the chloroplast-enriched fraction; ---. ATPase activity in the isolated \( \alpha/\beta \)-complex.

\( \alpha/\beta \)-complex around 7.5.
Fig. 5. Effects of Methanol and Ethanol on ATPase Activity in the Isolated $\alpha$-Complex.

An aliquot (0.14 mg for the $\alpha$-complex) was assayed for ATPase activity in the presence of the indicated concentrations of methanol and ethanol. Each value was the mean of duplicate determinations.

- ATPase activity in the isolated $\alpha$-complex in the presence of methanol; O--O, ATPase activity in the isolated $\alpha$-complex in the presence of ethanol.

significant stimulatory effects on the ATPase activity (Table II). The effects of monovalent anions tested are also summarized in the table. NO$_3^-$ and SCN$^-$ inhibited the activity in the two preparations. ID$_{50}$ concentrations of NO$_3^-$ and SCN$^-$ were 15 mM and 0.2 mM, respectively, for the isolated $\alpha$-complex.

(D) Effects of methanol and ethanol on the ATPase activities in the two preparations. The enzyme activity in the isolated $\alpha$-complex was stimulated by addition of ethanol as shown in Fig. 5, while the activity in the chloroplast-enriched fraction was not affected by methanol (data not shown). Ethanol also stimulated the activity in the isolated $\alpha$-complex (see also Fig. 5). Without addition of methanol or ethanol the isolated $\alpha$-complex had almost no ATPase activity. Thus the ATPase activity was assayed in the presence of 20% methanol for the $\alpha$-complex.

N-Terminal amino acid sequences of the $\alpha$, $\beta$, and $\gamma$ subunits of the Superose 12 fraction

Amino acid sequences of the $\alpha$ and $\beta$ subunits were not obtained and appeared to be blocked. The $\gamma$ subunit gave the sequence of AGLKEMKDXISVXNNTKI, which showed 60% similarity to the $\gamma$ subunit of spinach and Chlamydomonas reinhardtii CF$_1$-ATPase and EF$_1$-ATPase.

Discussion

Chloroplast ATPase in an active state was isolated and characterized in this work. The method of Binder et al. (14) for the preparation of CF$_1$-ATPase from spinach was modified for isolation of CF$_1$-ATPase from *Acetabularia*. The use of Phenyl Superose chromatography was very effective to remove residual Rubisco, a major contaminant protein on isolation of CF$_1$-ATPase in general. Sato et al. (15) reported that the 55 kDa polypeptide released from spinach thylakoid membrane with 1 M LiCl was not the $\beta$ subunit of chloroplast F$_1$, but the Rubisco large subunit. And they also reported that the Rubisco large subunit and the CF$_1$ $\beta$ subunit were practically indistinguishable from each other in SDS-PAGE. Therefore, this hydrophobic chromatography was a very effective step for removal of Rubisco in the case of *Acetabularia*, and might be also effective in general.

The subunit composition of the F$_1$-portion appeared to be 52 - 53($\alpha$), 51($\beta$), and 40($\gamma$) kDa from SDS-PAGE. The $\delta$ and $\varepsilon$ subunits were not detected in the isolated preparation by immunoreactivities. The $\varepsilon$ subunit of CF$_1$-ATPase in general was demonstrated to be an inhibitory subunit for ATP-hydrolyzing activity. A polypeptide in the final preparation visible below the $\varepsilon$-subunit of spinach CF$_1$-ATPase might be the Rubisco small subunit. The N-terminal amino acid sequence of the putative $\gamma$ subunit also supported that the ATPase complex originated from chloroplasts. The 52 kDa polypeptide between $\alpha$ (53 kDa) and $\beta$ (51 kDa) subunit appeared to be a polypeptide that had decomposed from the $\alpha$ subunit. This 52 kDa polypeptide cross-reacted with anti-$\varepsilon$ serum, whereas the Rubisco large subunit of *Acetabularia* showed no cross-reactions with the anti $\alpha$ and the anti $\beta$-serum (data not shown). Similar results were observed in the cross-reactivities of the TF$_1$$\beta$ subunit antibody with the spinach Rubisco and $\beta$ subunit of spinach CF$_1$-ATPase, i.e., the spinach Rubisco had no cross-reaction with the TF$_1$$\beta$ subunit antibody, while the $\beta$ subunit of spinach CF$_1$-ATPase had a cross-reaction with the TF$_1$$\beta$ subunit antibody. (15)

The catalytic properties of the two preparations presented here were different, especially the pH optimum and sensitivity to some inhibitors. An acidic shift of the pH optimum occurred during purification. This phenomenon has been reported for plasmalemma ATPase in *Dunaliella*. (16) Differences in susceptibilities to the inhibitors might be attributable to the pH shift. Concentration-dependent inhibition with azide was observed for the activities in the chloroplast-enriched fraction and for the isolated $\alpha$-complex. The $\alpha$-complex of the thermophilic bacterium *PS3 F$_1$-ATPase* is known as a catalytic core of TF$_1$-ATPase. The ATPase activity in the $\alpha$-complex was not inhibited by azide, whereas the activity in the $z_3\beta_3\gamma$ complex was inhibited by azide. (17,18)

Immunoreactivities of the CF$_1$-ATPase were tested with several antibodies against the subunits of F type and V type ATPases. The $\alpha$ and $\beta$ subunits of *Acetabularia* CF$_1$-ATPase showed almost the same immunoreactivity as the subunits of spinach CF$_1$-ATPase. It differs from the results observed for *Chlamydomonas reinhardtii* and *Dunaliella bardawil*, which belong to Dasyycladaceae like *Acetabularia acetabulum*. (19,20)

As mentioned in the introduction, evolutionary aspects of the ATPase family are the most current topics. Cloning of the genes encoding the $\alpha$ and $\beta$ subunits of the chloroplast ATPase has now been performed. Preliminary results have already been reported by Ikeda et al. (21) Three F type $\beta$-like genes, the CI$^-$$\alpha$, putative CF$_1$$\beta$, and mitochondrial F$_1$$\beta$, were cloned and sequenced. Two F type $\alpha$-like genes, the CI$^-$$\alpha$ and CF$_1$$\alpha$, were also isolated (unpublished data). Judged from the deduced amino acid sequences, CF$_1$-ATPase showed higher similarities to those of higher plants, while the CI$^-$$\alpha$-translocating ATPase to those of *Chlamydomonas*. Catalytic properties of the CI$^-$$\alpha$-translocating ATPase and CF$_1$-ATPase $\alpha$-$\beta$-complex were also different with respect to pH optimum and sensitivity to orthovanadate (see ref. 8). N-terminal sequences of the $\alpha$
and β subunit of CF₁-ATPase appeared to be blocked, while those of the a and b subunit of the Cl⁻-ATPase were obtained. For the mitochondrial F₁-ATPase, we have not tried purification or further isolation. Coexistence of mitochondrial F₁-ATPase complex is possible in the CF₁-ATPase αβγ-complex. In Acetabularia cells, however, most of the population of organelles were chloroplasts.

Cloning of the whole genes encoding three F type ATPases in Acetabularia will give more information on similarities and differences in their primary structures, and experiments on this line are now in progress.

**Acknowledgments.** We are grateful to Professor Dr. D. Oesterhelt for supporting the experiments and for discussions, and to Dr. J. Shiozawa for improvements in English style and for discussions, both of the Max-Planck-Institute of Biochemistry, Munich, FRG.

**References**