Dye-lingand chromatographic purification of intact multisubunit membrane protein complexes: application to the chloroplast H⁺-FₒF₁-ATP synthase

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n-Dodecyl-β-D-maltoside was used as a detergent to solubilize the ammonium sulphate precipitate of chloroplast FₒF₁-ATP synthase, which was purified further by dye-lingand chromatography. Upon reconstitution of the purified protein complex into phosphatidylcholine/phosphatidic acid liposomes, ATP synthesis, driven by an artificial AχH/Δφ, was observed. The highest activity was achieved with ATP synthase solubilized in n-dodecyl-β-D-maltoside followed by chromatography with Red 120 dye. The optimal dye for purification with CHAPS was Green 5. All known subunits were present in the monodisperse proton-translocating ATP synthase preparation obtained from chloroplasts.

Key words: CHAPS, detergent, dodecylmaltoside.

INTRODUCTION

Synthesis of ATP by the ATP synthase from chloroplasts (CFₒF₁) is energized by an electrochemical proton gradient across the thylakoid membrane [1]. This multisubunit protein complex consists of two portions, i.e. the soluble, catalytic F₁ part, composed of the subunits α,β,γδε [2] and the membrane-embedded Fₒ part, composed of subunits I, II, III, and IV [3]. The enzyme synthesizes about 400 ATP molecules/s in its natural environment [4]. The structure of the F₁ part of bovine mitochondrial ATP synthase was determined, in part (αβγδεγ), by Abrahams et al. [5]. To clarify the structure and function of FₒF₁ ATP synthases, a high-resolution structure of the functional intact holoenzyme is essential. A prerequisite for successful two-dimensional or three-dimensional crystallization is a highly pure and monodisperse protein sample as starting material. This has been achieved already for mitochondrial ATP synthase [6]. However, in the case of ATP synthase from chloroplasts, no adequate purification procedure yielding sufficient quantities exists. CFₒF₁, after purification by rate-zonal centrifugation [3], still contains Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39) and other contaminants. After employing a purification procedure in the presence of the detergent Triton X-100 [7] only inactive ATP synthase was obtained [7a].

Therefore, some other detergent has to be employed in order to sustain the stability of CFₒF₁ during chromatographic purification and to obtain catalytically active material suitable for crystallization or functional studies. In the case of mitochondrial ATP synthase, various detergents have been used for chromatographic purification and to obtain catalytically active material suitable for crystallization or functional studies. In the case of mitochondrial ATP synthase, various detergents have been used for chromatographic purification and to obtain catalytically active material suitable for crystallization or functional studies. Therefore, some other detergent has to be employed in order to sustain the stability of CFₒF₁ during chromatographic purification and to obtain catalytically active material suitable for crystallization or functional studies.

MATERIALS AND METHODS

Materials

DDM and CHAPS were obtained from Calbiochem (La Jolla, CA, U.S.A.). The packed dye-lingand resins (Reactive Blue 4, Reactive Blue 72, Green 5, Green 19, Red 120, Yellow 3, Yellow 86, Brown 10 and Cibacon Blue 3GA) were purchased from Sigma Chemicals (Steinheim, Germany). HiTrap desalting column (5 ml) was obtained from Pharmacia (Uppsala, Sweden). Egg yolk phosphatidylcholine was prepared according to Singleton et al. [10], and phosphatidic acid from egg yolk was a gift from Lipoid (Ludwigshafen, Germany). Luciferin/luciferase reagent was purchased from BioOrbit (Turku, Finland). Dicyclohexylcarbodiimide was obtained from Fluka (Buchs, Switzerland). Centricon 100 concentrators were purchased from Millipore (Bedford, MA, U.S.A.).

Protein analysis

Proteins were precipitated by deoxycholic acid/trichloroacetic acid according to Bensadoun and Weinstein [11]. The pellet was resuspended in 5% (w/v) SDS solution. Protein concentration was determined by the Hartree [12] modification of the Lowry method [13].

SDS/PAGE was performed according to Laemmli [14], on 14% or 15% acrylamide gels. Protein bands were revealed by silver staining [15]. Protein samples were incubated at room temperature in SDS sample buffer before electrophoretic separation, in order to maintain the integrity of the 100-kDa supramolecular complex IIIₒ of CFₒ [3].

Reconstitution of CFₒF₁

Liposomes (16 mg lipid/ml) were prepared from phosphatidylcholine/phosphatidic acid (9:1, w/w) using the reverse-phase method [16]. The liposome suspension, in liposome buffer

Abbreviations used: CFₒF₁, proton translocating ATP synthase from chloroplasts; DDM, n-dodecyl-β-D-maltoside; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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(20 mM Tricine, 20 mM succinate, 0.6 mM KCl, 80 mM NaCl, adjusted to pH 8.0 with NaOH [17]), was then passed through two polycarbonate filters (Millipore) with pore diameters of 0.4 μm and 0.2 μm, respectively, in order to obtain liposomes with a definite diameter and narrow size distribution [18]. Before reconstitution of CF$_5$F$_1$ into phosphatidylcholine/phosphatidic acid liposomes, according to Richard et al. [19], MgCl$_2$ was added to a final concentration of 5 mM.

**Activity of ATP synthesis**

For the activity assay, approx. two ATP synthase molecules/liposome were reconstituted. To determine ATP synthesis activity, an established procedure [17] was modified as described elsewhere [7a]. Energization of CF$_5$F$_1$ was achieved by applying a pH difference of 3.3 and a K$^+$/valinomycin diffusion potential of 115 mV across the proteoliposome membrane. The ATP synthesized was measured continuously with a luciferin/luciferase assay. Dicyclohexylcarbodiimide inhibition was performed as described previously [7a].

**Isolation of CF$_5$F$_1$**

CF$_5$F$_1$ was isolated from spinach chloroplasts [3,20]. The thylakoid membranes from fresh spinach were solubilized in a mixture of the detergents n-octyl-β-D-glucopyranoside (60 mM) and sodium cholate (23 mM). Thereafter, the ATP synthase was partially separated from lipids and contaminating proteins by fractionated precipitation with ammonium sulphate and rate-zonal centrifugation. The centrifugation medium contained 12 mM CHAPS [21]. Alternatively, 8 mM DDM was used as detergent. The protein solution was layered on a sucrose step gradient (12.75%, 18.75%, 24.25%, 30%, 40% sucrose, w/v). After centrifugation at 125 500 g and 16 h for DDM, and at 118 000 g and 14 h for CHAPS (Beckman VTi 50 rotor), the ATP synthase activity was found in the 30% and 40% sucrose gradients.

**Chromatographic purification with dye-ligand resins**

Before chromatographic purification in DDM, ATP synthase in DDM was desalted by passage through a HiTrap desalting column at 20 °C with equilibration buffer [20 mM Tris/HCl, pH 8.0, 20%, (w/v) glycerol, 5 mM MgSO$_4$, and 4 mM DDM or 8 mM CHAPS as appropriate] as eluent. A volume of 1 ml of the crude ATP synthase sample (only 50–60% of which was CF$_5$F$_1$, see the Results section) from the sucrose density gradient centrifugation (∼4 mg protein/ml) mixed with 0.5 ml of equilibration buffer was injected into the column. The flow rate was approx. 2 ml/min and the first peak, containing CF$_5$F$_1$, was collected. Before chromatographic purification in CHAPS, 4 ml of CF$_5$F$_1$ in CHAPS was dialysed twice against 100 ml of equilibration buffer for 2 h at 4 °C.

CF$_5$F$_1$ in equilibration buffer was applied to the various dye-ligand columns, which had been equilibrated with the equilibration buffer. Approx. 1 mg of crude protein mixture was applied to each column (2.5 ml resin volume) and chromatography was carried out at 4 °C with 6 column vols. of equilibration buffer. Fractions (1 ml) were collected and the absorbance at 280 nm was monitored. Thereafter, the column was washed with 12 ml of elution buffer (equilibration buffer including 1.5 M NaCl) and further fractions (1 ml) were collected. Fractions of the equilibration or elution step containing CF$_5$F$_1$ were pooled and concentrated (Centricon 100 concentrators).

The minor impurities could be removed by negative chromatography (with respect to CF$_5$F$_1$) with almost all dyes, but only if new columns were used. When columns were used twice or more, impurities were seen on the silver-stained gels between the γ and the δ bands of CF$_5$. Only the Red 120 dye column gave consistent purification results after several uses.

**Electron microscopy**

Electron micrographs were taken with a Philips CM 12 microscope operated at 120 kV. Specimens were stained with 1% (w/v) uranyl acetate. The samples were diluted 1:20 with water and applied immediately on to the glow-discharged copper grid (400 mesh, carbon coated).

**RESULTS**

**Isolation of CF$_5$F$_1$**

The ammonium sulphate precipitate of the ATP synthase preparation was solubilized in centrifugation medium containing 12 mM CHAPS or 8 mM DDM as detergent. CF$_5$F$_1$ in the sucrose-density gradient sedimented faster in the presence of CHAPS than in the presence of DDM. This required adjustment of the centrifugation parameters in the presence of CHAPS to 14 h at 118 000 g (instead of 16 h at 12 550 g for DDM), to avoid pelleting of the protein during rate-zonal centrifugation. For DDM, the optimal detergent concentration was found to be 8 mM; at this concentration the oligomer of subunit III was best preserved, as judged by SDS/PAGE.

After rate-zonal centrifugation, CF$_5$F$_1$ was found in the 30% and 40% sucrose steps. This pre-purified isolate still contained large amounts of Rubisco and other minor, unidentified protein contaminants, together about 40–50% of the total protein (Figure 1, lanes 2 and 6). The results of typical preparations with CHAPS and DDM are summarized in Table 1. ATP synthase samples, after rate-zonal centrifugation, contained mostly linear aggregates of ATP synthase, as demonstrated by electron microscopy (Figure 2A). This aggregation occurred via hydrophobic contacts between CF$_5$. To adjust the buffer composition before dye-ligand chromatography, the centrifugation medium was exchanged with equilibration buffer. If the buffer contained CHAPS, this was done by dialysis. In the case of DDM, gel filtration using a HiTrap desalting column was performed.

**Chromatographic purification of CF$_5$F$_1$**

Dye-ligand chromatography in the presence of CHAPS

Nine different dye resins were tested. In the presence of CHAPS, most of CF$_5$F$_1$ did not bind to any column and was found in the flow-through fractions. All subunits of CF$_5$F$_1$ were identified in the flow-through fractions by their corresponding molecular masses with SDS/PAGE. Most of the oligomer of the subunit III remained intact (sharp 100 kDa band in lane 4 of Figure 1).

The large subunit and small subunit of the main impurity, Rubisco (Figure 1, lane 6), and many minor impurities, but not CF$_5$F$_1$, bound strongly to the Green 19 and Green 5 (lane 4) columns. As a result, highly pure CF$_5$F$_1$ was obtained (Figure 1, lane 4) in the flow-through fractions.

In summary, in the presence of CHAPS optimal purification of intact CF$_5$F$_1$ was obtained by negative chromatography with the columns Green 5 and Green 19.

Dye-ligand chromatography in the presence of DDM

CF$_5$F$_1$ in DDM bound only to Green 19 and Cibacron Blue 3G, each of which bound more than 40% of the applied ATP.
Dye-ligand chromatography of chloroplast H+-ATP synthase

Figure 1 SDS/PAGE of selected fractions from dye-ligand chromatography

CFOF₁, after rate-zonal centrifugation, still contained the large subunit (LSU) and small subunit (SSU) of Rubisco, and minor amounts of other contaminating proteins (lanes 2 and 6). The α and ε subunits of ATP synthase stained very weakly with silver staining; Coomassie Blue stained the bands more strongly (results not shown). Lane 1, low-molecular-mass standards (kDa); lane 2, CFOF₁ in DDM before dye-ligand chromatography; lane 3, CFOF₁ in DDM after purification with Red 120; lane 4, CFOF₁ in CHAPS after purification with Green 5; lane 5, low-molecular-mass standards (kDa); lane 6, CFOF₁ in CHAPS before dye-ligand chromatography. The subunits, α, β, γ, δ, ε, III₁, IV, I, II and III₂ are shown on the right.

Table 1 Purification of CFOF₁

The ATP synthesis activity of reconstituted CFOF₁, energized by an artificial ΔpH/Δψ jump, was measured. CFOF₁, after rate-zonal centrifugation, represents the ATP synthase-containing fractions of the sucrose-step gradient (see the Materials and methods section), which also contain 40–50% protein contaminants. In samples purified by dye-ligand chromatography and marked with an asterisk, the specific ATP synthesis activities (mole ATP/mole CFOF₁ per s) were: Green 5, 16.8; Blue 72, 28.5 and Red 120, 34.5.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>ATP synthase activity (µmol ATP/min per mg of protein)</th>
<th>Total protein (%)</th>
<th>Activity (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilized thylakoid membrane</td>
<td>2.7</td>
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<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
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<td>30</td>
<td>100</td>
<td>3.1</td>
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<tr>
<td>Purification with CHAPS</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate-zonal centrifugation</td>
<td>15.5</td>
<td>4.0</td>
<td>100</td>
<td>5.7</td>
</tr>
<tr>
<td>Chromatography with Green 5</td>
<td>18.0*</td>
<td>4.0</td>
<td>100</td>
<td>6.7</td>
</tr>
<tr>
<td>Purification with DDM</td>
<td></td>
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<tr>
<td>Rate-zonal centrifugation</td>
<td>19.0</td>
<td>5.0</td>
<td>100</td>
<td>7.0</td>
</tr>
<tr>
<td>Chromatography with Blue 72</td>
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<td>2.0</td>
<td>100</td>
<td>11.3</td>
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<tr>
<td>Chromatography with Red 120</td>
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<td>100</td>
<td>13.7</td>
</tr>
</tbody>
</table>

The predominant impurity, Rubisco (Figure 1, lane 2), bound strongly to the Red 120 (lane 3), Green 5, Blue 72, Brown 10 and Cibacron Blue 3GA resins. All other minor impurities could be separated from ATP synthase by binding to the Green 5, Red 120, Brown 10 and Blue 72 dyes. As a result, highly pure CFOF₁ was obtained in the flow-through volume, and most of the subunit III oligomer remained intact.

For prolonged storage of the purified ATP synthase, NaN₃ was already included in the chromatographic buffers. The addition of azide did not effect the result of the chromatography.

Electron micrographs of negatively stained specimens of ATP synthase purified by dye-ligand chromatography showed mostly single CFOF₁ particles, and no aggregated forms were seen. As an example, a micrograph of Blue 72 purified ATP synthase is revealed in Figure 2(B). It illustrates that ATP synthase purified by dye-ligand chromatography is monodisperse.

In summary, in the presence of DDM optimal purification of intact CFOF₁ was obtained by negative chromatography with Green 5, Red 120, Brown 10 and Blue 72 dye-ligand resins.

DISCUSSION

The present study demonstrates that dye-ligand chromatography can be successfully employed for the purification of large multisubunit membrane proteins. As an example of a heteromeric, multisubunit membrane-protein complex, we were able to purify CFOF₁ using dye-ligand chromatography as an additional purification step to established procedures.

In the CFOF₁ isolation procedure described in the present paper, the previously used detergents Triton X-100 [3] or CHAPS [21] were replaced, in the centrifugation medium, with DDM. The crude CFOF₁ obtained (in CHAPS or DDM), however, contained more impurities (particularly Rubisco) than described previously with the detergents Triton X-100 [3] or octyl β-D-thioglucoside [21]. Subsequent chromatography with dye ligands, described in the present work, yielded purer chloroplast F₀F₁ than shown previously. In contrast to the sucrose density-gradient centrifugation step [3], no substantial amounts of protective phospholipids were necessary for the chromatography procedure.

Figure 2 Electron micrograph of negatively stained samples of purified ATP synthase

(A) Sample (0.3 mg protein/ml) purified by rate-zonal centrifugation, in the presence of DDM, showing aggregates of ATP synthase. Arrows indicate the globular F₁ and the linear aggregate of the F₀ part of ATP synthase. (B) Sample (0.5 mg protein/ml in DDM) purified by dye-ligand chromatography (Blue 72 resin) showing monodisperse ATP synthase. Arrows indicate the globular F₁ and the F₀ part of ATP synthase. The scale bars represent 50 nm.
As shown by electron microscopy, samples purified by dye-ligand chromatography were monodisperse (Figure 2B). In contrast, specimens from the rate-zonal centrifugation step contained predominantly aggregates of ATP synthase, even at a lower protein concentration than the chromatographed CF$_{10}F_1$. These aggregates have been observed previously [22].

CF$_{10}F_1$ was purified efficiently in the presence of CHAPS or DDM but, interestingly, the chromatographic separation was different for the two detergents. Strong binding of Rubisco was observed with the dyes Green 5, Red 120 and Blue 72 (depending on the detergent present). Optimal purification was achieved with Green 5 in the presence of CHAPS, and with Red 120 and Blue 72 in the case of DDM.

In contrast to the purification of mitochondrial ATP synthase in the presence of DDM under conditions where the enzyme did not bind to the column [6,23], the purification of the chloroplast enzyme with Yellow 3 chromatography was poor. Therefore the biochemical differences between mitochondrial and chloroplast ATP synthase demanded a new screening for the optimal purification conditions.

With CHAPS, only a slight increase of specific ATP synthesis activity was observed after purification, whereas it was significantly higher with DDM. The activity of the purified ATP synthase after dye-ligand chromatography with Red 120 in the presence of DDM is in good agreement with the value of crude CF$_{10}F_1$ (40 molecules of ATP/CF$_{10}F_1$ per s) reported by Richard et al. [19], when Triton X-100 was present during rate-zonal centrifugation.

The present study has shown that, by employing dye-ligand chromatography, the impurities remaining in CF$_{10}F_1$, prepurified by rate-zonal centrifugation, could be removed in a single step. The samples obtained meet the requirements for further functional and structural studies; they are highly pure, intact and active. Large amounts of monodisperse CF$_{10}F_1$ (as required for crystallization trials) can be obtained in the presence of detergents.

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