Dye-ligand chromatographic purification of intact multisubunit membrane protein complexes: application to the chloroplast H^+-F_oF_1-ATP synthase

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n-Dodecyl-β-D-maltoside was used as a detergent to solubilize the ammonium sulphate precipitate of chloroplast F_oF_1-ATP synthase, which was purified further by dye-ligand chromatography. Upon reconstitution of the purified protein complex into phosphatidylcholine/phosphatidic acid liposomes, ATP synthesis, driven by an artificial $\Delta pH/\Delta\psi$, 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 (CF_oF_1) 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 F_1 part, composed of the subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ [2] and the membrane-embedded F_o part, composed of subunits I, II, III_x and IV [3]. The enzyme synthesizes about 400 ATP molecules/s in its natural environment [4]. The structure of the F_1 part of bovine mitochondrial ATP synthase was determined, in part ($\alpha_3\beta_3\gamma$), by Abrahams et al. [5]. To clarify the structure and function of F_oF_1 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. CF_oF_1, 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 CF_oF_1 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 [6,8], and *n*-dodecyl-β-D-maltoside (DDM) was found to be an appropriate detergent for crystallization trials [9].

We have demonstrated that, by dye-ligand chromatography, structurally and functionally intact CF_oF_1 with high purity can be isolated in the presence of the detergents DDM or CHAPS.

MATERIALS AND METHODS

Materials

DDM and CHAPS were obtained from Calbiochem (La Jolla, CA, U.S.A.). The packed dye-ligand resins (Reactive Blue 4, Reactive Blue 72, Green 5, Green 19, Red 120, Yellow 3, Yellow 86, Brown 10 and Cibacron 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 III_x of CF_o [3].

Reconstitution of CF_oF_1

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: CF_oF_1, 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_oF_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_oF_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_0F_1

CF_oF_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 125500 ***g*** and 16 h for DDM, and at 118000 ***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_oF_1, see the Results section) from the sucrose density gradient centrifugation ($\approx$ 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_oF_1, was collected. Before chromatographic purification in CHAPS, 4 ml of CF_oF_1 in CHAPS was dialysed twice against 100 ml of equilibration buffer for 2 h at 4 °C.

CF_oF_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_oF_1 were pooled and concentrated (Centricon 100 concentrators).

The minor impurities could be removed by negative chromatography (with respect to CF_oF_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_1. 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_0F_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_oF_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 118000 ***g*** (instead of 16 h at 12550 ***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_oF_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_o.

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_0F_1

Dye-ligand chromatography in the presence of CHAPS

Nine different dye resins were tested. In the presence of CHAPS, most of CF_oF_1 did not bind to any column and was found in the flow-through fractions. All subunits of CF_oF_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_oF_1, bound strongly to the Green 19 and Green 5 (lane 4) columns. As a result, highly pure CF_oF_1 was obtained (Figure 1, lane 4) in the flow-through fractions.

In summary, in the presence of CHAPS optimal purification of intact CF_oF_1 was obtained by negative chromatography with the columns Green 5 and Green 19.

Dye-ligand chromatography in the presence of DDM

CF_oF_1 in DDM bound only to Green 19 and Cibacron Blue 3GA, each of which bound more than 40 % of the applied ATP

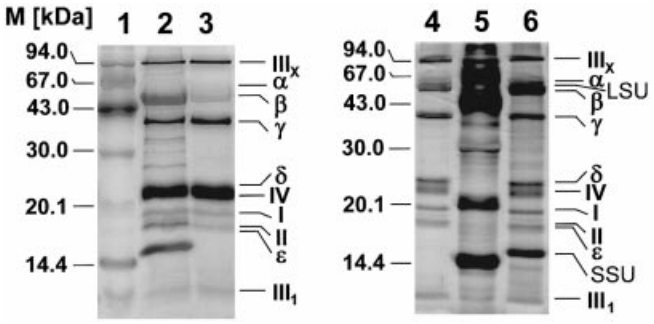

Figure 1 SDS/PAGE of selected fractions from dye-ligand chromatography

CF_0F_1, 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, CF_0F_1 in DDM before dye-ligand chromatography; lane 3, CF_0F_1 in DDM after purification with Red 120; lane 4, CF_0F_1 in CHAPS after purification with Green 5; lane 5, low-molecular-mass standards (kDa); lane 6, CF_0F_1 in CHAPS before dye-ligand chromatography. The subunits, α, β, γ, δ, ϵ, III_x, IV, I, II and III_1 are shown on the right.

Table 1 Purification of CF_0F_1

The ATP synthesis activity of reconstituted CF_0F_1, energized by an artificial $\Delta pH/\Delta\psi$ jump, was measured. CF_0F_1, 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 CF_0F_1 per s) were: Green 5, 16.8; Blue 72, 28.5 and Red 120, 34.5.

Purification step	ATP synthase activity (μmol ATP/min per mg of protein)	Total protein (%)	Activity (%)	Purification (fold)
Solubilized thylakoid membrane	2.7	100	100	1
Ammonium sulphate precipitation	8.3	30	91	3.1
Purification with CHAPS				
Rate-zonal centrifugation	15.5	4.0	23	5.7
Chromatography with Green 5	18.0*	1.5	10	6.7
Purification with DDM				
Rate-zonal centrifugation	19.0	5.0	35	7.0
Chromatography with Blue 72	30.5*	2.0	22	11.3
Chromatography with Red 120	37.0*	1.4	19	13.7

synthase. 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 CF_oF_1 was obtained in the flow-through volume, and most of the subunit III oligomer remained intact.

For prolonged storage of the purified ATP synthase, NaN_3 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 CF_oF_1 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 CF_oF_1 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 CF_oF_1 using dye-ligand chromatography as an additional purification step to established procedures.

In the CF_oF_1 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 CF_oF_1 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 F_oF_1 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.

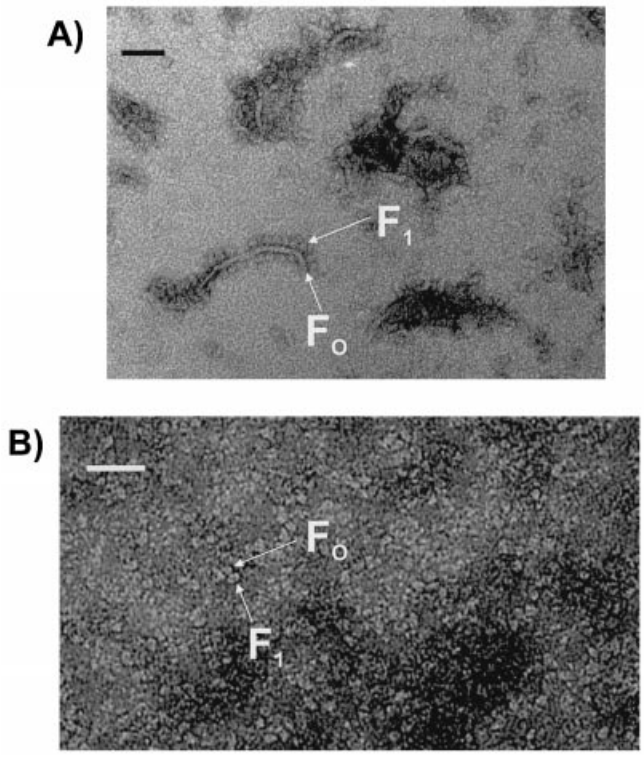

Figure 2 Electron micrograph of negatively stained samples of purified ATP synthase

(**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 F_1 and the linear aggregate of the F_0 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 F_1 and the F_0 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_oF_1. These aggregates have been observed previously [22].

CF_oF_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 withYellow 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_oF_1 (40 molecules of ATP/CF_oF_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_oF_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_oF_1 (as required for crystallization trials) can be obtained in the presence of detergents.

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