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Dye removal, catalytic activity and 2D crystallization of chloroplast H^+-ATP synthase purified by blue native electrophoresis

Ansgar Poetsch [a], Dirk Neff [a,1], Holger Seelert [a], Hermann Schägger [b], Norbert A. Dencher [a,*]

[a] *Institut für Biochemie, Abteilung Physikalische Biochemie, Technische Universität Darmstadt, Petersenstr. 22, D-64287 Darmstadt, Germany*

[b] *Zentrum der Biologischen Chemie, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, D-60590 Frankfurt, Germany*

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Abstract

The proton-ATP synthase of thylakoid membranes from spinach chloroplasts (CF_OF_1) and its subcomplexes CF_O and CF_1 were isolated by blue native electrophoresis (BN-PAGE) [Neff, D. and Dencher, N.A. (1999) Biochem. Biophys. Res. Commun. 259, 569–575] and subsequently electroeluted from the gel. A method was developed to remove most of the dye Coomassie G-250 (CBG) using gel filtration, a prerequisite for many biophysical investigations. The dye was removed from the electroeluted CF_OF_1, CF_O or CF_1 and exchanged with the detergent CHAPS. ATP hydrolysis activity of CF_1 and ATP synthesis activity of reconstituted CF_OF_1 were determined before and after dye removal. The secondary structure of CF_O was studied by CD spectroscopy in the presence and the absence of the dye. CBG neither abolishes the catalytic activity of the isolated CF_OF_1 and CF_1 nor affects the subunit composition and the high α-helical content of CF_O. In crystallization attempts, 2D arrays of CF_OF_1 and of CF_O before and after dye removal were obtained. In the aggregates of CF_O, circular structures with a mean diameter of 6.7 nm were observed. Our results indicate that the combination of BN-PAGE and dye removal by gel filtration is a suitable approach to obtain catalytically active protein complexes for further functional and structural characterization.

Keywords: Membrane protein; BN-PAGE; Proton-ATP synthase; Coomassie Blue; Detergent CHAPS

1. Introduction

Study of the structure–function relationship requires pure and intact proteins. Two- and three-dimensional (2D and 3D) crystallization, prerequisites for structural determination by electron, neutron and X-ray crystallography, respectively, can only be achieved with highly purified and homogeneous pro-

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; CBG, Coomassie brilliant Blue G-250; CF_OF_1, proton translocating ATP synthase from chloroplasts; CF_O, membrane integral proton conducting subcomplex of CF_OF_1; CF_1, hydrophilic catalytic subcomplex of CF_OF_1; CHAPS, 3-[3(cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCCD, dicyclohexylcarbodiimide; DDM, *n*-dodecyl-β-D-maltoside; EPA, phosphatidic acid from egg yolk; EPC, phosphatidylcholine from egg yolk; Rubisco, ribulose-1,5-bisphosphate-carboxylase/oxygenase; Triton X-100, *t*-octylphenoxypolyethoxyethanol

* Corresponding author. Fax.: +49-6151-164171; E-mail: dencher@pop.tu-darmstadt.de

[1] Present address: Forschungszentrum Jülich GmbH, IBI-2, D-52425 Jülich, Germany.

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tein samples as starting material. For membrane proteins, especially for multisubunit membrane protein complexes such as ATP synthases, respiratory complexes, and photosynthetic reaction centers, protein purification is usually a very cumbersome task, sometimes even without success. Promising approaches, such as the His-tag technology, can only be applied for specific expression systems and organisms. In the case of spinach chloroplast ATP synthase (CF_OF_1), for example, genetic manipulations required for the His-tag purification are not established to date. Previously, it was demonstrated that blue native polyacrylamide gel electrophoresis (BN-PAGE) can be the method of choice to successfully isolate protein complexes in high purity and sufficient yield in a one-step procedure from membranes [1,2]. Depending on the conditions chosen during electrophoresis, isolation of either intact multisubunit holoenzymes [3,4] or their subcomplexes in native state is feasible [3]. BN-PAGE combines high separation power, speed, and easiness of SDS-PAGE, but avoids the denaturing properties of the harsh surfactant SDS. The negatively charged blue dye Coomassie brilliant Blue G-250 employed (CBG, inset of Fig. 4) mimics a mild detergent. By binding to the protein surface, the protein becomes negatively charged and its aggregation is reduced. In this way, hydrophobic membrane proteins are converted into water soluble proteins, by conserving the native structure. Protein bands are visible directly during electrophoresis, i.e. subsequent staining of the gel is not necessary.

Astonishing, in spite of the many apparent advantages of BN-PAGE, this technique is not as widely applied for isolation and purification of native membrane proteins. One obvious reason is the possible interference of the blue colored dye CBG, still present in the protein sample upon electrophoresis and subsequent electroelution, with spectroscopic investigations (e.g., determination of protein concentration or secondary structure). Even more hampering is the general opinion that the isolated proteins, although structurally preserved by CBG, are functionally affected or even inactivated by the dye (there are, however, at least two reports [1,5] describing preserved activity), preventing further functional characterization. Furthermore, it is often assumed that CBG inhibits or at least interferes with protein crystallization.

We have developed a procedure to remove most of the dye from the electroeluted protein sample and to replace it by a mild detergent. Applying this method to the chloroplast ATP synthase, we could demonstrate that the purified protein complex is not only structurally intact but also functionally active, even in the presence of the dye. The dye does not interfere with the catalytic activity of the chloroplast ATP synthase, neither with ATP hydrolysis nor synthesis, and this might be true for other membrane proteins too. The purified intact CF_OF_1 complex as well as the isolated membrane integral subcomplex CF_O are amenable to 2D crystallization, which is modulated by the presence and absence, respectively, of CBG. With the advent of the dye removal procedure and the results gathered from its application, BN-PAGE has matured as a powerful and generally applicable method for the isolation and subsequent biophysical characterization of multisubunit membrane protein complexes in their native state. The 2D crystals and arrays of purified CF_OF_1 and CF_O, respectively, obtained from BN-PAGE, will facilitate the elucidation of the structure and mechanism of the chloroplast proton-ATP synthase.

2. Materials and methods

2.1. Isolation of crude CF_OF_1

CF_OF_1 was isolated from spinach chloroplasts as previously described [3,6,7]. The thylakoid membranes from fresh *Spinacia oleracea* 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 sulfate and rate-zonal centrifugation. The centrifugation medium contained 0.2% (w/v) *t*-octylphenoxypolyethoxyethanol (Triton X-100).

2.2. BN-PAGE and electroelution of protein complexes

For further purification of CF_OF_1 and the isolation of CF_1 and CF_O, BN-PAGE and electroelution were carried out according to [3]. 4.5 mg CF_OF_1 in

600–700 μl centrifugation medium were applied to a preparative BN-PA-gel (14×16×0.3 cm). CF_OF_1 was electrophoretically purified by using a cathode buffer with a concentration of 0.002% CBG (Serva Blue G). Dissociation of the holoenzyme into the subcomplexes CF_O and CF_1 during electrophoresis was achieved with a cathode buffer containing 0.02% CBG. After 4–5 h, protein bands of interest were cut out, disintegrated, and transferred into a H-shaped electroelution chamber, as described [1,2]. Electroeluted protein concentrates at the dialysis membrane (2 kDa cutoff value) and is suspended in the supernatant electroelution buffer, containing 7.5 mM Bis–Tris, 25 mM Tricine, pH 7.0.

2.3. Removal of CBG

The electroeluted protein band containing either CF_OF_1, CF_1, or CF_O from a preparative BN-PA-gel in a volume of up to 350 μl electroelution buffer was subjected to gel filtration on a column (d=1.0 cm, h=42 cm) packed with Sephacryl S-100 HR (Pharmacia). The sample was chromatographed at a flow rate of 10 ml/h with a buffer containing 25 mM Tricine, 7.5 mM Bis–Tris, 8 mM 3-[3(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 20% (w/v) glycerol, 0.02% (w/v) NaN_3, pH 7.0 at 4°C. For the chromatography of electroeluted CF_OF_1 or CF_1 5 mM $MgCl_2$ was added to the buffer to stabilize the ATP synthase. Chromatography was performed by a P-500 FPLC pump (Pharmacia) and the absorbance at 280 nm and 570 nm monitored simultaneously by a VWM 2141 UV–Vis detector (Pharmacia).

2.4. Protein analysis

Protein concentration was determined by the Hartree [8] modification of the Lowry method [9]. Alternatively, proteins were separated by SDS-PAGE [10] and the protein concentration was determined densitometrically after staining the gels with Coomassie R-250 [11]. For this purpose, the protein concentration of CF_OF_1 after zonal centrifugation was determined according to Lowry–Hartree. This sample was used as calibration standard for the gel electrophoresis. The gel lanes were scanned and the protein concentration determined using the software Quantiscan 1.25 (BioSoft).

2.5. Reconstitution of CF_OF_1

Large unilamellar liposomes (16 mg lipid/ml) in liposome buffer [12] were prepared by reverse phase evaporation [13] from phosphatidylcholine (EPC)/phosphatidic acid from egg yolk (EPA) 9:1 (w/w) as previously described [14]. Prior to reconstitution of 2 CF_OF_1 (86 nM) per liposome [15], 5 mM $MgCl_2$ was added to the liposome suspension.

2.6. ATP synthesis

ATP synthesis activity of reconstituted CF_OF_1 was determined by a slightly modified procedure [12] of Fischer et al. [14]. The proteoliposomes were energized by a fast generation of a pH/Ψ gradient and the synthesis of ATP was monitored continuously by a luciferin/luciferase assay. Dicyclohexylcarbodiimide (DCCD) inhibition of CF_OF_1 [16] was performed by adding 2 μl of 5 mM DCCD in methanol to 200 μl of the proteoliposomes in reconstitution buffer. This mixture was incubated for 7 h at 4°C.

2.7. ATP hydrolysis

ATP hydrolysis activity [17] of CF_1 was stimulated by incubation with methanol at 37°C [18]. The amount of liberated phosphate was measured spectroscopically at 740 nm [19].

2.8. 2D crystallization attempts and electron microscopy

For the crystallization of CF_OF_1 in the presence or absence (after gel filtration) of CBG after electroelution an existing procedure [20] using Bio-Beads to remove the detergent was modified: the detergent Triton X-100 was replaced with *n*-dodecyl-β-D-maltoside and phospholipase was not employed to partially deplete the added soybean lecithin lipids. Electron micrographs of the specimens obtained 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

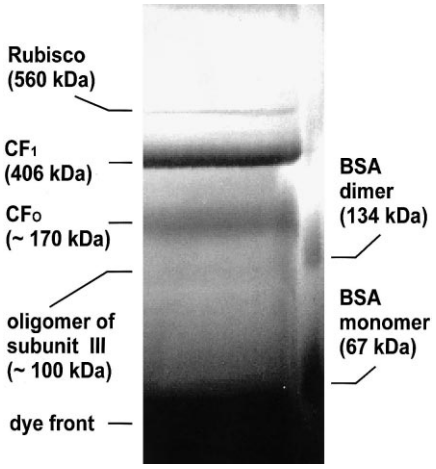

Fig. 1. Preparative BN-PAGE of CF_OF_1 after rate-zonal centrifugation, solubilized with Triton X-100. A CBG concentration of 0.02% (w/v) in the cathode buffer was applied to dissociate the ATP synthase in CF_1 and CF_O during the electrophoresis. Left lane: electrophoresis of CF_OF_1 (about 1/5 of the total width is shown). Right lane: electrophoresis of BSA used as molecular mass standard. The molecular masses were calculated from the amino acid sequence. According to SDS-PAGE after electroelution of the separated bands, the protein bands were identified as Rubisco, CF_1, CF_O and III_x, respectively.

and applied immediately onto the glow discharged copper grid (400 mesh, carbon coated).

2.9. CD spectroscopy

CD spectra of CF_O were recorded with a Jasco J-720 CD spectrometer at 4°C and a cuvette of 0.1 mm pathlength. CF_O after electroelution was diluted with an equal volume of water and CF_O after electroelution and gel filtration was diluted threefold with water just before recording the spectrum. The algorithm of Chang et al. [21] was used to analyze the secondary structure.

3. Results

3.1. BN-PAGE

To remove additional impurities (especially ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco)) from CF_OF_1 or to separate CF_O and CF_1, solubilized (Triton X-100) CF_OF_1 after rate-zonal centrifugation was applied to BN-PAGE. If the cathode buffer contained 0.002% CBG, it was possible to obtain intact and highly pure (as judged by SDS-PAGE, data not shown) CF_OF_1 in accordance with [3]. If the cathode buffer contained a 10-fold higher concentration of CBG, i.e., 0.02%, dissociation of the holoenzyme into CF_1 and CF_O was observed [3]. The corresponding BN-PAGE (Fig. 1) shows from top to bottom a narrow band (Rubisco), a very intense band (CF_1), a broad band of medium intensity (CF_O) and a narrow band of low intensity (III_X). Analysis by SDS-PAGE (data not shown) revealed that CF_1, but not CF_OF_1, had lost some of its subunit δ, but was better preserved than in a previous study [3]. Surely, the intactness of the isolated CF_1 can be improved by adequate stabilizing factors, since intact CF_OF_1 is obtained by this approach. The broad band of CF_O was divided into two halves and the respective electroeluate subjected to SDS-PAGE (Fig. 2). The upper and lower half contained intact CF_O, i.e., the subunits I, II, IV and the complex III_X were detected. In the upper half CF_O was obtained in high purity (Fig. 2, lane 4), only a faint stain of other proteins was observed in the range between 67 and 43 kDa, whereas the lower half (Fig. 2, lane 3) contained an intensely stained impurity with a molecular mass of 43 kDa. The isolated CF_O was more pure than previously reported [3], which is most likely due to differences in the protein

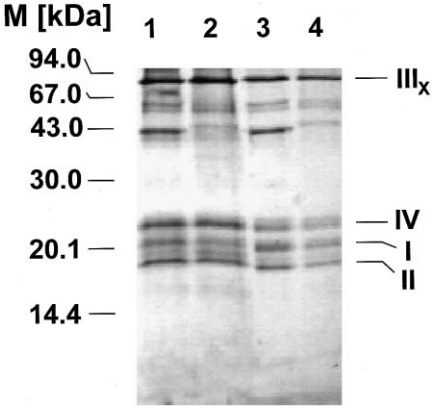

Fig. 2. SDS-PAGE of CF_O after electroelution and additional gel filtration to remove CBG. The CF_O band from the preparative BN-PAGE was cut in two halves and each half was separately electroeluted and chromatographed. Lane 1: CF_O after gel filtration (lower half), lane 2: 6.3 μg CF_O after gel filtration (upper half), lane 3: 3.8 μg CF_O after electroelution (lower half), lane 4: 2.5 μg CF_O after electroelution (upper half). Molecular masses of protein standards are indicated on the left side.

composition of the applied crude CF_OF_1 after rate-zonal centrifugation. Depending on the CF_OF_1 fraction applied, the BN-PAGE (Fig. 1) showed a faint blue band of the supramolecular complex of the subunit III, which was identified after analysis of the electroeluate by SDS-PAGE (data not shown). Most of the complex III_X may originate already from the applied solubilized CF_OF_1, which could explain the varying amount of III-oligomer. Electron microscopy of CF_OF_1 after rate-zonal centrifugation revealed that the protein complex exists to some extent in the form of string-like aggregates connected by the CF_O part (photograph not shown). In some sites of the strings, the lollypop-like CF_1 part was missing, which is an indication for the presence of the CF_O or III_X subcomplex.

Protein yields of the electroeluted protein complexes were determined by comparing the band intensities of the stained SDS-PA gel lanes. The relative yields of electroelution were 35% for CF_O, 34% for CF_OF_1 and 30% for CF_1. The yields were about threefold higher than previously reported [3]. Perhaps the larger amount of protein applied for BN-PAGE was responsible for an increase in protein yield, up to 5.5 mg/ml could be handled.

3.2. Removal of CBG

It was observed that during SDS-PAGE, the Coomassie dye at the protein surface was completely replaced with the detergent SDS. However, an obvious consequence of this process is the loss of protein functionality and native structure. To preserve the protein structure and functionality during dye removal, CBG was replaced with the zwitterionic detergent CHAPS during gel filtration. The electroeluted protein band from BN-PAGE, containing either CF_OF_1, CF_1, CF_O, or III_X was injected on the column. Soon after the sample had entered the gel, separation into a leading slightly blue band and a lagging intensive blue band became visible. During chromatography the intensity of the leading band decreased. The elution profile of the gel filtration with CF_OF_1 reflected by the absorption at 280 and 570 nm is depicted in Fig. 3. Absorption at 280 nm is caused by aromatic amino acid residues and/or the dye CBG, whereas the absorption at 570 nm is caused exclusively by CBG. Two well separated

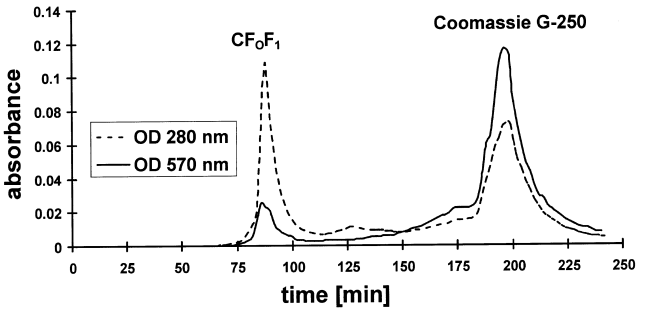

Fig. 3. Chromatogram of CBG removal from electroeluted CF_OF_1, purified by BN-PAGE. Gel filtration with Sephacryl S-100 in the presence of the detergent CHAPS was performed and the absorbance at 280 nm (protein and dye) and 570 nm (dye) recorded. The first peak corresponds to CF_OF_1 and the second to free dye.

peaks with an elution time of 82 min and 198 min, respectively, can be distinguished. For CF_O, the two peaks occurred at 80 min and 200 min, respectively. This indicates that the separation was independent of the protein complex applied. Eluted fractions corresponding to the first peak with a low absorption at 570 nm and the second peak with a high absorption at 570 nm were subjected to SDS-PAGE. Regardless of the applied sample, CF_OF_1, CF_1, CF_O or III_X, protein was only detected in fractions from the first peak. These results prove that by employing gel filtration most of the CBG could be separated from the electroeluted proteins. Apparently, mixed micelles of CHAPS and CBG, and micelles of protein, CBG and CHAPS are formed. The chromatographed proteins (72–571 kDa) cannot enter the pores of the Sephacryl gel matrix in contrast to the much smaller mixed CHAPS and CBG micelles (the mass of a CHAPS micelle is about 6 kDa). Most of the CBG dye in the electroeluate does not seem to be bound to the proteins or is easily exchangeable by CHAPS, explaining the fast separation into two bands at the beginning of the chromatography. Part of the tighter bound CBG was removed slowly in the gel filtration process. For quantification of the CBG removal the areas under the two peaks (Fig. 3) at 82 and 198 min corresponding to the absorption at 570 nm were determined in the chromatogram. For CF_1, CF_O and CF_OF_1 about 90% of the dye was found in the second peak. In order to judge, how much CBG molecules still remained bound to the proteins, dye concentration (by absorbance at 570 nm,

$\varepsilon = 4.05 \times 10^4$ M^{-1} cm^{-1}) and protein concentration (by Lowry–Hartree) were measured for one CF_O sample. It was calculated that between 1 and 2 dye molecules still remained bound to the CF_O complex of approx. 170 kDa. No disintegration of the protein complexes, even not of III_X, due to gel filtration occurred. As an example, this is illustrated by SDS-PAGE for CF_O. All the subunits III_X, I, II, and IV of CF_O are visualized by the Coomassie stain for the sample before and after gel filtration (Fig. 2, lanes 4 and 2). In addition, no significant difference in the relative stain intensities of the subunits was observed.

If CHAPS in the buffer used for gel filtration was exchanged with 0.2% (w/v) *n*-dodecyl-β-D-maltoside (DDM), only one absorbance peak at both wavelengths, corresponding to protein and dye was observed. Obviously owing to the bigger size of the DDM micelles, separation between protein/detergent micelles and dye/detergent micelles could not be achieved. However, in crystallization attempts (see below) of electroeluted CF_OF_1 in the presence of DDM and soybean lecithin, CBG was removed from the protein solution and bound to the Bio-Beads.

The relative yields (Lowry–Hartree assay) after dye removal in respect to the protein amount applied for BN-PAGE were 29% for CF_1, 19% for CF_OF_1 and 54% for CF_O. The calculated yield for the membrane integral CF_O complex seems questionable, since the yield is lower for CF_OF_1 or CF_1 and lower for electroeluted CF_O before gel filtration. It is well known that the Lowry–Hartree assay reports incorrect values for membrane proteins [22], which could explain the overestimation in the case of CF_O.

It has been reported that CF_1 after dissociation of the subunit δ is less stable than the intact complex $\alpha_3\beta_3\gamma\delta\varepsilon$. $CF_1(-\delta)$ inactivates fast in the cold [23] and precipitates fast in the presence of detergents [17]. Dissociation of CF_1 into its subunits occurs in the cold, because the hydrophobic forces between the subunits are weakened [24]. In our samples, even after a week of storage at 4°C, no precipitation could be observed with the electroeluted $CF_1/CF_1(-\delta)$ mixture from BN-PAGE. Upon exchange of the dye CBG by the detergent CHAPS with gel filtration, the $CF_1/CF_1(-\delta)$ became less stable. Occasionally, precipitates appeared within 24 h, irrespective of a storage at 4°C or at room temperature (RT). Because it seemed possible that the detergent CHAPS further destabilized $CF_1/CF_1(-\delta)$, 600 μl of the sample after gel filtration was dialyzed overnight against 1 l of a buffer containing 5 mM Tris–HCl pH 7.8, 100 mM NaCl, 5 mM $MgCl_2$ at RT before the activity assay. But this step showed no significant enhancement of the stability of the $CF_1/CF_1(-\delta)$ mixture. It is worth mentioning, that CF_OF_1 upon BN-PAGE and electroelution has not lost the δ subunit.

3.3. Reconstitution and activity of CF_OF_1

ATP synthesis activity of electroeluted CF_OF_1 after BN-PAGE and after subsequent removal of CBG was compared with the protein fraction from rate-zonal centrifugation, which was subjected to BN-PAGE. All CF_OF_1 fractions were active in ATP synthesis (Table 1). However, the activity dropped nearly 50% due to BN-PAGE and electroelution, but did not decrease further, when CBG was removed by gel filtration. Aggregation of the protein at the dialysis membrane of the anode buffer chamber during electroelution may be responsible for the drop of ATP synthesis activity after BN-PAGE. The inhibitor DCCD significantly reduced the activity of all CF_OF_1 fractions. Activity of CF_OF_1 from rate-zonal centrifugation and after CBG removal was reduced by about 75% upon DCCD treatment, whereas the activity of electroeluted CF_OF_1 from BN-PAGE was reduced by about 60%. It can be concluded, that CBG itself is not an inhibitor of CF_OF_1 and the removal of CBG and replacement by

Table 1
Enzyme activity (μmol ATP/(min × mg protein)) of protein fractions from different isolation/purification steps

	ATP synthesis activity	ATP synthesis activity+DCCD	ATP hydrolysis activity
CF_OF_1 rate-zonal	27	7.3	
CF_OF_1 BN-PAGE	15	5.8	
CF_OF_1-CBG	14	3.8	
CF_1 BN-PAGE			8.3
CF_1-CBG			3.7

CF_OF_1 rate-zonal: after rate-zonal centrifugation only; CF_OF_1 BN-PAGE, CF_1 BN-PAGE: after BN-PAGE and electroelution; CF_OF_1-CBG, CF_1-CBG: after BN-PAGE, electroelution and gel filtration.

CHAPS does not reduce the catalytic activity of CF_OF_1.

The question was addressed, whether the dye CBG is partly or fully integrated into the lipid bilayer during reconstitution or remains in the aqueous environment. Proteoliposomes were sedimented by centrifugation and the pellet as well as the supernatant examined for the presence of the dye. Since exclusively the liposome pellet had a blue color, it can be concluded that the dye was totally inserted into the lipid bilayer or remained bound to the reconstituted protein complex. It is also possible that the dye does not intercalate into the bilayer but binds to the surface of the liposomes. This seems less likely, since both the liposome surface and CBG are net negatively charged. Although the inserted dye apparently did not negatively influence the rate of ATP synthesis of CF_OF_1, there are situations, where a preceding removal of the dye is necessary. We have observed for instance that CBG raises the proton permeability of the EPC/EPA liposomes.

3.4. Activity of CF_1

During BN-PAGE at high dye concentration (0.02% CBG) CF_OF_1 fragmented into CF_1/$CF_1(-\delta)$ and CF_O. CF_1 after electroelution and additional removal of CBG was active in ATP hydrolysis (Table 1). The activity decreased by about 55% after gel filtration. Partial denaturation of the unstable [23] $CF_1(-\delta)$ could be a reason for the reduction of activity.

3.5. Secondary structure of CF_O

At first it was examined, if the study of electroeluted proteins from BN-PAGE by CD spectroscopy is hampered by the dye CBG. In the presence of 0.06% (w/v) CBG in 12.5 mM Tricine, 3.75 mM Bis–Tris, pH 7.0, CD measurements can be performed between 180 and 260 nm, the wavelength region which is used to study the peptide backbone conformation of proteins. The extended wavelength region which can be measured in the presence of CBG shows that CBG does not hamper CD spectroscopy to study the secondary structure. On the contrary it represents a suitable compound which can mimic detergents in order to make membrane proteins accessible for spectroscopic investigation. The CD spectrum of the membrane integral CF_O complex is depicted in Fig. 4. A secondary structure of about 80% α-helix and 20% β-turn was calculated. The lack of structural knowledge of the $(C)F_O$ complex makes it impossible to decide, whether the structure of the electroeluted CF_O is identical with the native protein structure. So far only the 3D structure of the monomeric and oligomeric subunit c from *Escherichia coli* and yeast mitochondrial H^+-ATP synthase, respectively [25,26] and the secondary structure of the *Propionigenium modestum* Na^+-ATP synthase [27] have been determined, which are homologues to the subunit III of CF_OF_1. The subunits c, contributing about 2/3 to the mass of the F_O complex, were mainly α-helical except for small loop regions [25–27]. The secondary structure of the electroeluted CF_O is in qualitative agreement with these results. To examine possible changes in protein structure resulting from the exchange of CBG with CHAPS, a CD spectrum of CF_O after removal of CBG was recorded (Fig. 4). The almost identical shape of the CD spectra of CF_O before and after removal of CBG leads to the conclusion that the protein conformation was preserved when CBG was replaced by CHAPS.

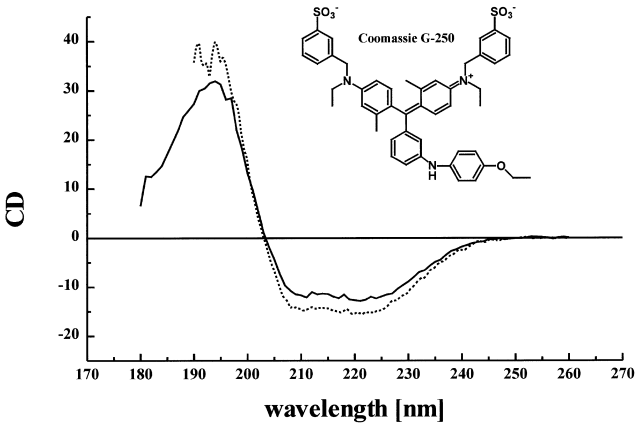

Fig. 4. CD spectrum of electroeluted CF_O (continuous line) and of the same sample after removal of CBG (inset) by gel filtration (dotted line). The average of four recordings is shown.

3.6. 2D crystallization

To our knowledge to date no reports exist on whether proteins obtained by BN-PAGE are suitable

for crystallization. Before crystallization trials were initiated, we have characterized the electroeluted proteins by electron microscopy. Electroeluted CF_O, and to a lesser extent CF_OF_1 and CF_1, consisted of aggregates, which are presumably formed when the protein concentrates at the surface of the dialysis membrane during electroelution. If this was prevented, the occurrence of aggregates was suppressed in case of CF_1. Already the cathode buffer itself with 0.02% (w/v) for BN-PAGE [3] contained aggregates, which consisted most likely of CBG. The aggregates of CF_O had a bushy morphology, which could be clearly distinguished from the dye aggregates in the cathode buffer and from CF_O aggregates in the buffer for rate-zonal centrifugation, which have a string-like appearance [28]. It seems that the aggregates of electroeluted CF_O are formed by hydrophobic contacts between the protein and the dye CBG, because they disappeared when the detergent Triton X-100 was added to a final concentration of 0.03% (w/v).

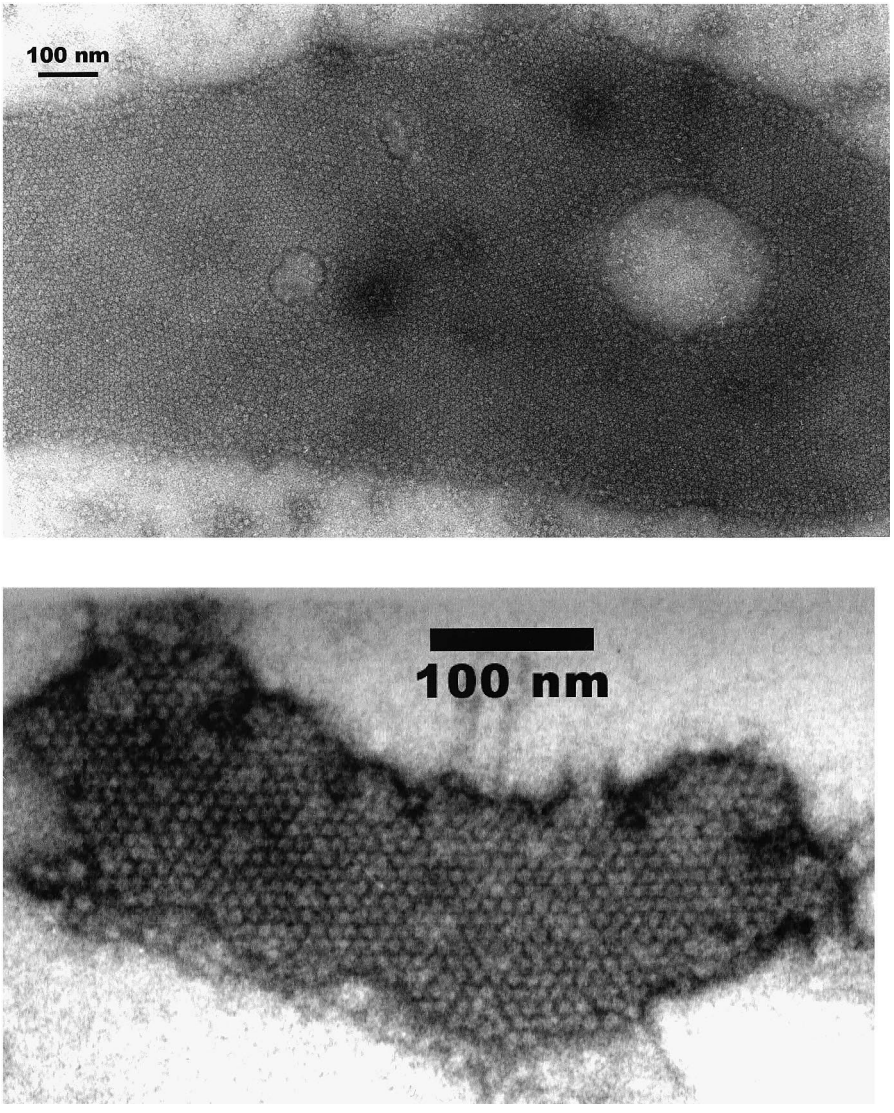

Fig. 5. Electron micrographs of negatively stained samples (0.3 mg protein/ml) of 2D crystallization trials with CF_OF_1. The scale bar represents 100 nm. (upper panel) 2D array obtained from ATP synthase sample after electroelution and before removing the Coomassie dye. The knob-like structures characteristic for F_1 are densely packed. (lower panel) Ordered 2D array obtained in 2D crystallization trials with CF_OF_1 after gel filtration.

In crystallization attempts, the best ordered arrays of CF_OF_1 were obtained, if initially the detergent DDM was used instead of Triton X-100. ATP synthase samples from BN-PAGE before and after removal of CBG were used as starting material for 2D crystallization. After addition of lipids and detergent DDM, the latter was removed by Bio-Beads according to Lacapère et al. [29]. After adding Bio-Beads to the samples, not only detergent was removed but also the Coomassie dye resulting in blue colored beads. After removal of the majority of detergent in crystallization experiments with CF_OF_1 a colorless precipitate was observed independent of prior CBG removal by gel filtration. The 2D arrays obtained were stained with uranyl-acetate and visualized by electron microscopy (Fig. 5). The surface of the arrays is composed of knobs. Because of their size (about 10 nm) the knobs are considered as the hydrophilic F_1 part of CF_OF_1 which is protruding from the membrane [20,29,30]. CF_O of the ATP synthase is membrane embedded and not visible. 2D crystallization trials with electroeluted CF_OF_1 samples from BN-PAGE resulted in much larger aggregates (Fig. 5, upper panel) (about 1 μm) compared with samples after additional removal of CBG by gel filtration. On the other hand CF_OF_1 in the smaller aggregates (about 0.2 μm) appear to be better ordered (Fig. 5, lower panel).

2D crystallization trials with CF_O resulted in 'strings' (1D aggregates) [28] or in liposomes with occasionally 2D protein arrays. The liposome consisted of many circular objects (Fig. 6) with central dimples. These rings are formed by the oligomer of the subunit III. SDS-PAGE revealed that the aggregates were composed of III_X and IV. Apparently, the subunits I and II were lost partly during formation of the aggregates. We measured a mean diameter of 6.7 ± 1.4 nm for the circular structures. This size is in good agreement with the diameter of 5.9–7.4 nm for the subunit III_X oligomer [7,31] and monomeric CF_O [30], determined by electron and atomic force microscopy, respectively.

Even though most dye was attached to the Bio-Beads, the samples obtained in CF_O crystallization trials were still colored blue if the dye was not removed by gel filtration before crystallization. Obviously, the dye does not prevent the formation of 2D protein aggregates and crystals.

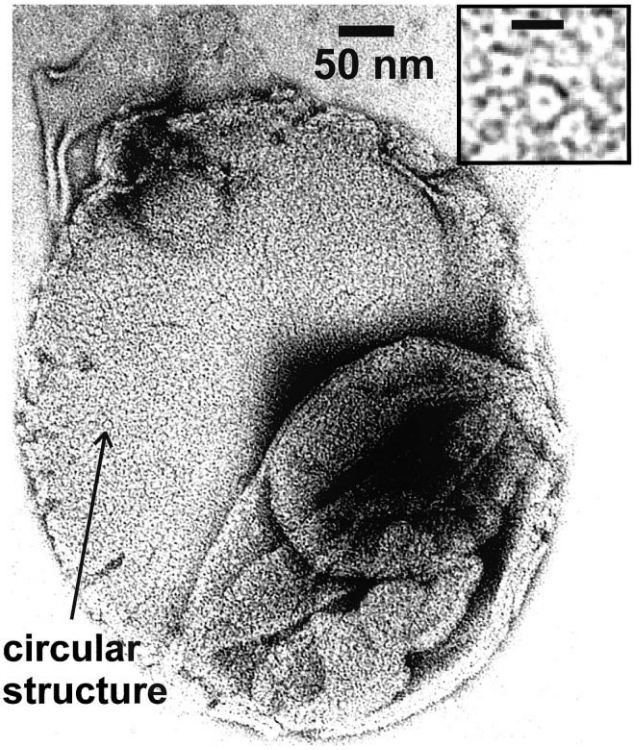

Fig. 6. Electron micrograph of negatively stained samples obtained by 2D crystallization trials with CF_O. The protein array displays circular objects, III_x and IV, with a mean diameter of 6.7 nm and the scale bar represents 50 nm. The inset shows a magnification of some circular objects (scale bar in the inset represents 10 nm).

4. Discussion

The capability of BN-PAGE to resolve complex mixtures of proteins has already made this method a powerful tool in protein analysis. It is especially useful in the analysis of membrane proteins, for instance the separation of membrane protein complexes from mitochondria [32], chloroplasts [33] and microsomes [34] was achieved. Furthermore, BN-PAGE was applied to monitor the protein import into mitochondria [35]. However, many researchers are still sceptical, if the proteins are separated in their native, active form by BN-PAGE, although enzymatic activity of mitochondrial protein complexes has already been demonstrated in the BN-gel [5] and after electroelution [1]. Our results show that by employing BN-PAGE, purification of catalytically active ATP synthase from chloroplast (CF_OF_1) can be achieved. If required, active CF_1 and CF_O can be isolated, too.

Mixtures of membrane proteins [32,33] can be easily separated by BN-PAGE. In addition, subcomplexes [3,4,36] can be obtained, which are otherwise difficult to isolate and purify. Therefore, it is tempting to use preparative BN-PAGE as a biochemical method to obtain membrane proteins for further structural and functional studies. The physicochemical properties of CBG make the addition of detergents unnecessary and the yield of milligrams of protein (up to 5.5 mg/ml) is sufficient to carry out 2D or 3D crystallization or various spectroscopic techniques like CD, IR or NMR. In this study, we could demonstrate that CBG is a suitable alternative for, e.g., detergents in order to make membrane proteins accessible for the determination of protein secondary structure with CD spectroscopy. In crystallization trials, we were able to produce 2D aggregates and crystals with electroeluted CF_OF_1 and CF_O. Although proteins in the CF_OF_1 arrays show optical diffraction, further optimization of crystallization conditions is still required. Protein aggregates were only formed when all or at least most of the dye or detergent was removed from the protein surface by binding to the Bio-Beads during crystallization. This is in line with the fact that the net-negatively charged CBG (Fig. 4) was originally introduced to prevent protein aggregation during BN-PAGE. The 2D aggregates of CF_O (Fig. 6) obtained revealed a circular structure which size is in agreement with data published for *E. coli* F_O [37], CF_O [30] and the oligomer of subunit III [7,31], respectively. With AFM, the topography of the subcomplex III_X of CF_O in 2D arrays has been visualized and cylindrical structures with outer diameters of 5.9 and 7.4 nm imaged. 14 subunits III per oligomer were determined [31]. For the first time, CF_O is now available in pure form and milligram quantities. Since diffracting domains of CF_O can be obtained, these samples are promising candidates for structural studies.

Depending on the wavelength region, CBG can interfere in spectroscopic measurements, like CD, IR or UV–Vis. Especially colorimetric protein assays can be affected. For this reason it has been already attempted to remove the dye after BN-PAGE [1]. The authors were able to remove free dye only, if CBG was omitted in the cathode buffer for BN-PAGE before completion of the electrophoresis. But protein bound dye could not be removed and protein precipitation could not always be avoided. By employing gel filtration, we succeeded to remove most CBG from the electroeluted protein and simultaneously replace it with the detergent CHAPS in order to keep the membrane proteins in solution. Separation of the dye from the proteins was achieved due to large differences in the size of protein/detergent and dye/detergent micelles. The general principle of this procedure should be applicable to other membrane proteins, too. For proteins unstable in CHAPS, *n*-octyl-β-glucopyranoside or dimethylamine-*N*-oxides may be alternative detergents because of their small micelles. Dye removal had no negative effect on the activity of CF_OF_1 and the secondary structure of CF_O. At least for CF_OF_1, the removal of CBG by gel filtration improves the formation of ordered 2D protein aggregates.

In conclusion, we have demonstrated that BN-PAGE in combination with the newly introduced procedure for CBG removal is a rather suitable method to isolate protein complexes in catalytically active form for further biophysical studies. It has been shown for the first time, that proteins from BN-PAGE can be studied by CD spectroscopy and can be aligned into extended 2D arrays under appropriate crystallization conditions, a prerequisite for structural studies by electron crystallography. A fast and relatively easy approach is now available to isolate and investigate fragile membrane protein complexes, which are difficult to isolate with other, for example chromatographic, techniques.

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