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Self-assembly of ATP synthase subunit c rings

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Abstract Subunit c of the H^+ transporting ATP synthase is an essential part of its membrane domain that participates in transmembrane proton conduction. The annular architecture of the subunit c from different species has been previously reported. However, little is known about the type of interactions that affect the formation of c-rings in the ATPase complex. Here we report that subunit c over-expressed in *Escherichia coli* and purified in non-ionic detergent solutions self-assembles into annular structures in the absence of other subunits of the complex. The results suggest that the ability of subunit c to form rings is determined by its primary structure. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: F-ATPase; Subunit c; Oligomerisation; Analytical Ultracentrifugation; Electron microscopy

1. Introduction

In eubacteria, chloroplasts and mitochondria the formation of ATP from ADP and phosphate by ATP synthases uses energy from the transmembrane proton electrochemical gradient generated by oxidative metabolism or photosynthesis [1,2]. The enzyme is composed of two major domains, F_1 and F_0. The catalytic F_1 domain, is an extrinsic membrane sector, consisting of five different subunits present in the ratio $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$. The α and β subunits alternate around a central α-helical structure in the subunit γ [3,4]. Rotation of the γ subunit with respect to the $\alpha_3\beta_3$ subcomplex during catalysis has been directly observed [5,6]. There is strong evidence [7,8] to support a mechanism in which the central stalk of the soluble F_1 domain, together with the oligomeric c-ring in the membrane domain, rotates as an ensemble, coupling ion movement with ATP hydrolysis/synthesis.

The F_0 membrane domain in its simplest form (in *Escherichia coli*) consists of three different polypeptides in the experimentally determined ratio of $a_1b_2c_{10-11}$ [9]. *E. coli* subunit c has been traditionally purified in organic solvents [10] and the structure of the monomer has been elucidated by nuclear magnetic resonance (NMR) [11,12]. Preliminary evidence obtained by immunoelectron [13] and scanning force microscopy [14] of *E. coli* F_0 suggested that subunit c arranges into ring structures. Structural data from yeast [15], chloroplasts [16] and sodium-driven bacterial [17] ATPases indicate that subunit c forms rings with 10, 14 and 11 monomers per complex, respectively. This came much as a surprise as it has been widely anticipated that the number would be fixed between species and likely to be 12 as suggested by cross-linking and genetic experiments in *E. coli* [18]. More recent cross-linking studies [19] concluded that the preferred stoichiometry for the *E. coli* rotor is 10.

The role of other subunits of F_1F_0 ATPase in the formation of the c-rings is unknown. Here, we report that recombinant subunit c forms rings after expression in *E. coli* and purification in mild detergents in the absence of any other subunits of the complex. The results suggests that the ability of subunit c to form rings is determined by its amino acid sequence.

2. Materials and methods

2.1. Analytical methods

Protein concentrations were determined by the BCA method (Pierce Chemicals, Rockford, IL, USA) using bovine serum albumin as standard, and by amino acid analysis (for determination of partial specific volume) on a Beckman 7300 Amino Acid Analyzer after hydrolysis in 6 M HCl for 18 h at 110°C. Proteins were analysed by SDS–PAGE in 12–22% gels. Amino-terminal sequences were determined with the aid of an Applied Biosystems Procise model 494 protein sequencer. Protein masses were determined by electro-spray ionization mass spectrometry (e.s.i.-m.s.) in a Perkin Elmer-Sciex API III^+ triple quadrupole instrument.

2.2. Bacterial expression

Subunit c of *E. coli* F-ATP synthase was cloned and transformed into the mutant host strains *E. coli* C41(DE3) and C43(DE3) [20] as described previously [21]. Bacteria were grown at 37°C in 2×TY medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.4) to an optical density of 0.6 at 600 nm. Then isopropyl-2-D-thiogalactoside was added to a final concentration of 0.7 mM, and the cells were grown for a further period at either 37 or 25°C. Bacteria were disrupted at 4°C by passing the suspension twice through a French pressure cell. Unbroken cells and debris were removed by low-speed centrifugation (2000×*g*, 10 min). Membranes containing subunit c were obtained by high-speed centrifugation (100 000×*g*, 1 h) of the supernatants and resuspended in TEP buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA and 0.001% (w/v) phenylmethylsulfonyl fluoride) at protein concentrations of 25–30 mg/ml, frozen in liquid nitrogen and stored at −20°C.

2.3. Protein purification

Membrane pellets were thawed and diluted to a protein concentration of 6 mg/ml in TEP buffer containing 2% LDAO (*N*,*N*-dimethyldodecylamine-*N*-oxide) (Fluka, Dorset, UK). The suspension was agitated gently for 1 h at 4°C, centrifuged (100 000×*g*, 1 h) and the supernatant was applied to an hydroxylapatite HR (Calbiochem Novabiochem Ltd., Nottingham, UK) column packed in TEP buffer and equilibrated with 0.05% LDAO. The column was washed with the equilibration buffer and subunit c-containing fractions were collected in the flowthrough. The pooled fractions were concentrated and applied to a HiLoad 26/60 Sephacryl S-300 HR (Amersam Pharmacia

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Biotech, Uppsala, Sweden) column equilibrated in a buffer containing 20 mM Tris–HCl, pH 8.0, 100 mM sodium chloride, 0.1% LDAO, 5% glycerol and 0.02% sodium azide. Fractions containing subunit c were pooled and concentrated (5 mg/ml). Aliquots (200 μl) were loaded into a Sephacryl S-300 HR 10/30 column equilibrated in the same buffer containing either 0.05% LDAO or 1% OG (*n*-octyl-β-D-glucopyranoside; Anatrace, Maumee, OH, USA) and eluted at a flow rate of 0.3 ml/min.

For e.s.i.-m.s. determinations, subunit c purified in detergent solutions was treated with chloroform–methanol followed by diethyl–ether precipitation as previously described [10].

2.4. Analytical ultracentrifugation

Sedimentation equilibrium experiments were performed with a Beckman Optima XL-A centrifuge, using an An-60Ti rotor, at either 9000 or 20 000 rev/min at 20°C. Samples were loaded at different concentrations into the cells (long radial path) and scans taken at 230 nm. Initial over-speeding, for 6 h at 150% of the equilibrium speed, was carried out to reduce the time to attain equilibrium. The apparent weight average molecular mass ($\bar{M}_{w,app}$) was calculated with the equation:

$$\bar{M}_{w,app} = \frac{d \ln c_r}{d\, r^2} \frac{2RT}{(1-\phi'\rho)_0 \omega^2};$$

where c_r is the concentration at radius, R the gas constant, T the temperature, ϕ' the apparent partial specific volume and ρ_0 the solvent density.

Densities of the solvent and solution were measured with a DMA60/DMA602 precision density meter (Anton Paar, Graz, Austria). The apparent partial specific volume for the protein was then calculated from the equation:

$$\left(\frac{\partial \rho}{\partial c_2}\right)_\mu = (1-\phi'\rho_0);$$

with the protein concentration (c_2) measured by amino acid analysis. Using these values for ϕ' and ρ_0, the molecular mass obtained will be that for the protein alone, without any direct contribution from bound detergent.

2.5. Electron microscopy and image analysis

Subunit c purified in LDAO was dialysed against 1% OG in the gel filtration buffer. Protein solutions (5 μl, 0.12 mg/ml) were applied to freshly glow-discharged carbon-coated grids and stained with 1% (w/v) uranyl acetate. Images were recorded at a 50 000× nominal magnification on Kodak SO 163 negatives in a Philips EM208S electron microscope operating at 80 kV. Negatives were scanned at a resolution of 7 μm/pixel into a computer with a Zeiss-SCAI scanner and demagnified (×4) by linear interpolation, giving a final pixel size of 5.6 Å. Images were screened by eye and 1097 particles were selected using XIMDISP, which is part of the MRC image processing suite [22]. Images were normalised, low-pass filtered and rotationally averaged using the SPIDER image processing software [23]. The resulting average was used as a reference for translational and rotational alignment and classification by multivariate statistical analysis. This process was iteratively repeated and convergence was achieved after five rounds of alignment.

3. Results

3.1. Expression of E. coli subunit c and purification in detergent solutions

High levels of over-production of subunit c (20–50 mg of subunit c per liter of culture) were obtained both in *E. coli* strain C41(DE3) and C43(DE3) as described previously [20,21]. Despite the high levels of expression, recombinant subunit c did not form inclusion bodies but was targeted

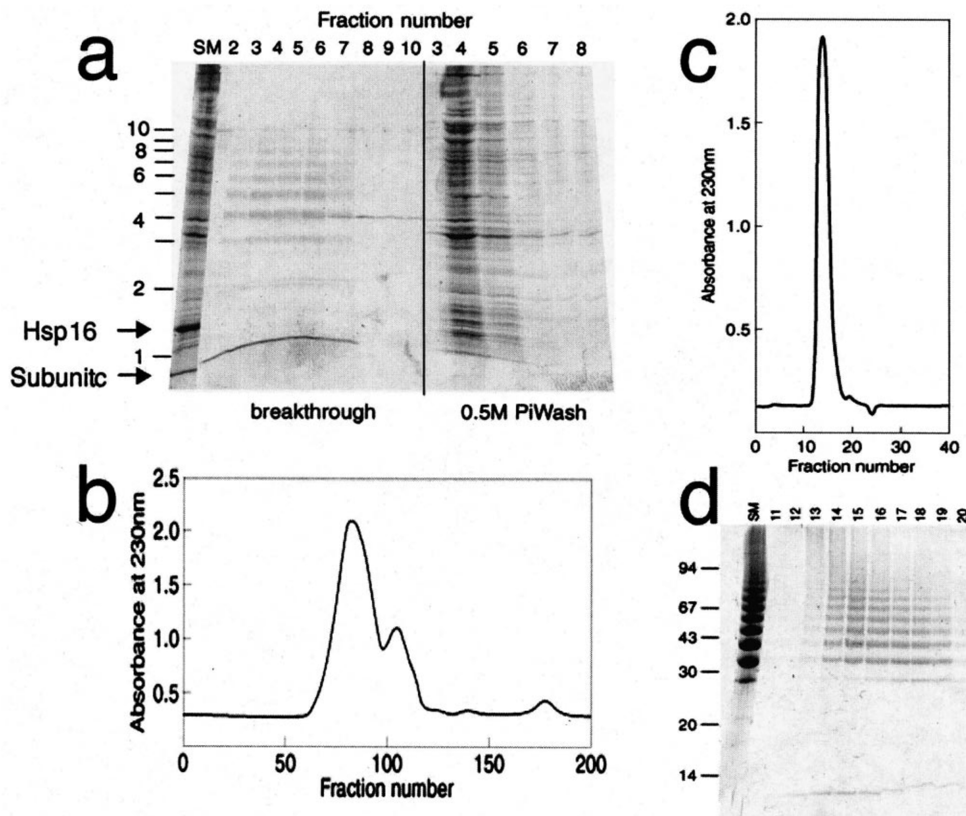

Fig. 1. Purification of subunit c of *E. coli* F_1F_0 ATPase. (a) Membranes (6 mg/ml) were solubilised in 2% LDAO (SM) and loaded to an hydroxylapatite column equilibrated in 0.05% LDAO. Molecular mass is expressed in terms of monomers (8285 Da). (b) Breakthrough fractions were pooled, concentrated and loaded to a Sephacryl S-300 HR xk 26/60 column equilibrated in 0.1% LDAO. Fractions (2 ml) from the main peak (fraction numbers 70–90) were pooled, concentrated (SM) and then (c) loaded to a Sephacryl S-300 HR 10/30 column equilibrated in 0.05% LDAO. Fractions (0.9 ml) were collected and analyzed by SDS–PAGE (d) (SM; starting material)

into the membranes. Interestingly, the over-production of subunit c was accompanied by over-expression of the heat shock protein Hsp 16 (identified by N-terminal sequencing) which also targeted to the membrane fraction (see Fig. 1a)

A variety of detergents were investigated for their ability to solubilize subunit c from the membrane fractions. Highly pure subunit c was obtained in the flowthrough of hydroxylapatite columns only when membranes were solubilized with LDAO (Fig. 1a). The pattern of ladder formation observed on the polyacrylamide gels is dependent on the protein concentration and may be disrupted by incubation of samples in 3 M trichloroacetic acid adjusted to neutral pH (data not shown). Minor impurities and excess of detergent micelles were removed by chromatography on a Sephacryl S-300 gel-filtration column (Fig. 1b). Analytical gel-filtration experiments in a S-300 HR 10/30 column (Fig. 1c,d) showed that subunit c purified in LDAO eluted from the column as a single, symmetric peak, which is an indication of the homogenity of the sample.

Protein purity was examined by amino acid analysis, N-terminal sequencing and e.s.i.-m.s. Two abundant peaks with MH^+ average masses of 8284.83 and 8257.01 were identified. The calculated mass of the *E. coli* ATPase subunit c is 8285.19. Therefore, the two observed masses may correspond to the formylated and deformylated forms of the protein.

3.2. Analytical ultracentrifugation

Plots of the apparent weight average molecular mass against concentration for the subunit c, in 0.05% LDAO and in 1% OG, at different initial loading concentrations are shown in Fig. 2, with the molecular mass expressed in terms of monomers (8285 Da) and the concentration as molarity of total monomer. In each case, but particularly in 1% OG, the protein is aggregating well beyond dimer and, as it is not possible to formulate a reliable model, no attempt was made to fit the optical density against radius data. Rather, the results were treated qualitatively as giving an overall picture of the aggregation states under these conditions.

In 0.05% LDAO, the data from different loading concentrations overlie each other closely, showing that the aggregation is reversible and up to four or five subunits (over this concentration range). The picture is completely different in 1% OG, where the curves at different loading concentrations are clearly separated, showing that the aggregation is effectively irreversible (on the time scale of the experiment, i.e. days). The aggregation is now to much higher orders but, interestingly, the data are still only compatible with a limit to the size under these conditions, as the curves are tending to a plateau, with an average value around 25–30 monomers, rather than increasing indefinitely. The value of this plateau cannot be taken as indicating the size of the largest aggregate present; for example a 4:1 (on a number basis) mixture of 10-mer and 40-mer would have a weight average size of 25-mer. Other models are also compatible with the data but without other evidence of the sizes of aggregates present it is not possible to draw any valid conclusions about the highest aggregation state under these conditions.

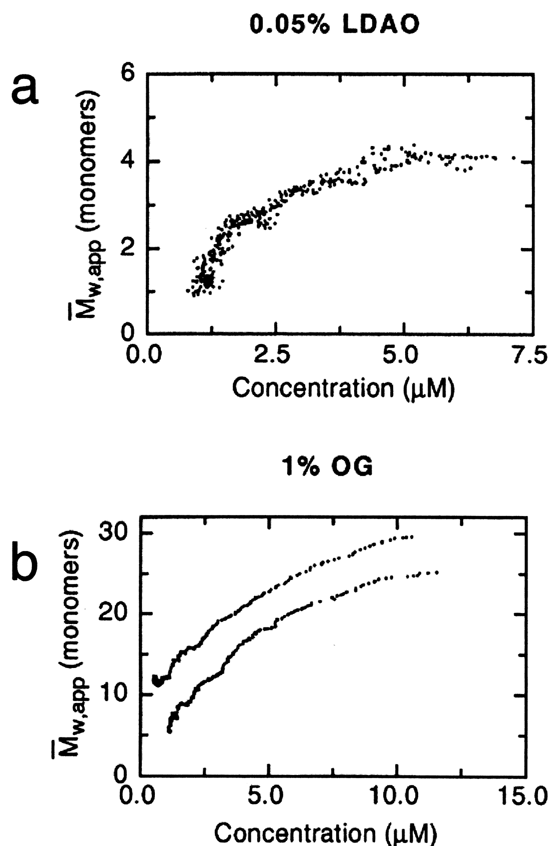

Fig. 2. Equilibrium sedimentation analysis of subunit c purified in 0.05% LDAO (a) and in 1% OG (b). Molecular mass is expressed in terms of monomers (8285 Da) and the concentration as molarity of total monomer.

3.3. Electron microscopy and image analysis

Subunit c purified in 0.1% LDAO and dialysed extensively against a buffer containing 1% OG revealed the presence on the electron micrographs of more than one species, mainly small single rings and larger objects interpreted as tetramers of rings (Fig. 3a). Assuming an equal adsorption of both types of particles on the electron microscope grid, the ratio tetramers to single rings is about 1:4, which is consistent with the data obtained in the analytical ultracentrifugation experiments.

The small rings (1097 particles) were selected and aligned (Fig. 3b). Multivariate statistical analysis, classification and average of similar clusters was iterated until the number of classes was reduced to seven. One of these classes, consisting of 319 particles and representative of a perpendicular view to the plane of the membrane, is shown in Fig. 3c. The dimensions of the ring are approximately 105 Å diameter on the outside and 23 Å on the inside. If it is assumed that the detergent substitutes totally or partially for the lipid environment of the c-rings, given a length of detergent of approximately 20 Å, then the outer diameter of the subunit c ring would be about 65 Å which is consistent with reported values for subunit c rings from different species [15–17,24].

4. Discussion

Recombinant ATP synthase subunit c from *E. coli* purified in mild detergent solutions self-assembles into annular structures in the absence of any other subunits of the ATPase complex. The results suggest that the ability of recombinant subunit c to form rings is specified by its amino acid sequence.

Previous observations of c-rings in other species [15–17] were obtained after partial or total disruption of the entire

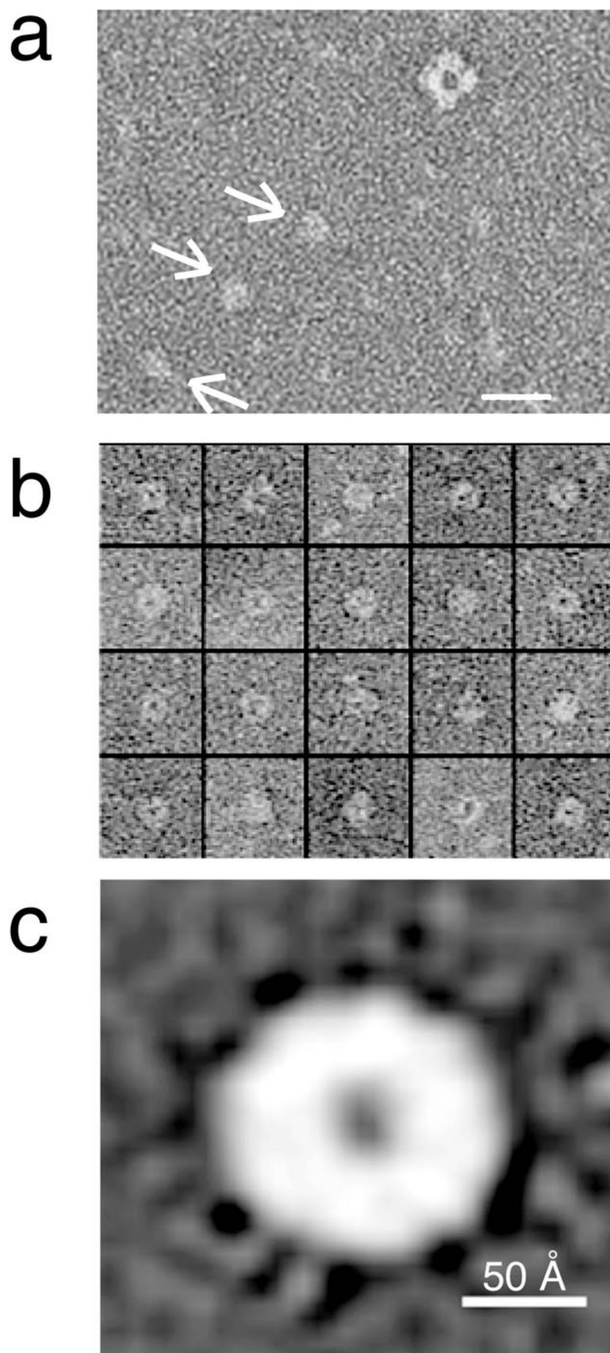

Fig. 3. Electron microscopy and image analysis of subunit c in detergent solutions. (a) Subunit c purified in LDAO and dialysed in 1% OG buffer was stained with 1% uranyl acetate and examined by electron microscopy. A mixture of small rings (white arrows) and tetramers of rings in a ratio 4:1 were detected on the grids (scale bar: 25 nm). (b) Small rings (1097) were selected and aligned using SPIDER. After correspondence analysis, classification and average of similar classes the number of classes was reduced to seven. (c) A class (319 particles) representative of a top view of the c-rings is shown in (scale bar: 5 nm).

complex into stable c oligomers. It is unclear whether other subunits in F_1F_0 ATPase could affect the size and shape of the rings. Evidence obtained by AFM studies of *Ilyobacter tartaricus* c-rings indicate that the diameter of the ring is determined by interactions between neighbouring subunits and their shape [24]. The interactions of the c-ring with the central stalk in F_1 may be very strong as suggested by cross-linking experiments [25–27] and structural studies [15]. Interactions of subunit c with the subunits b and a in the F_0 domain [28–29] may also affect the formation of c-rings.

Recent studies suggest that the central plug in the reconstituted undecameric c cylinder consists of phospholipids [30], which can be removed without affecting the stability of the cylinder. This could explain the extreme stability of these c-rings, which are resistant to boiling in SDS. Subunit c from the sodium-driven bacteria *Propionigenium modestum* is also stable in SDS at high temperatures but it can not be purified in its oligomeric form after over-expression in *E. coli* [31]. It is worth noting that recombinant *P. modestum* subunit c purified in SDS as a monomer has been suggested to retain the structural domains present in the oligomer as indicated by circular dicroism and NMR studies [32].

E. coli subunit c purified in organic solvents as a monomer [10] can be reconstituted into liposomes in an active form in the presence of subunits a and b [33], suggesting that the NMR structure of the monomer [11,12] may be similar to that one present in the oligomer. It is very likely that the detergents used in the purification of recombinant *E. coli* subunit c reported here will also preserve the secondary structural elements that contribute to form an active complex in the native enzyme.

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