The central plug in the reconstituted undecameric c cylinder of a bacterial ATP synthase consists of phospholipids

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Abstract The isolated rotor cylinder of the ATP synthase from *Ilyobacter tartaricus* was reconstituted into two-dimensional crystalline arrays. Atomic force microscopy imaging indicated a central cavity on one side of the rotor and a central plug protruding from the other side. Upon incubation with phospholipase C, the plug disappeared, but the appearance of the surrounding c subunit oligomer was not affected. This indicates that the plug consists of phospholipids. As the detergent-purified c cylinder is completely devoid of phospholipids, these are incorporated into the central hole from one side of the cylinder during the reconstitution procedure.

Key words: ATP synthase; Subunit c ring; Phospholipid; Atomic force microscopy; *Ilyobacter tartaricus*

1. Introduction

ATP synthases utilize a transmembrane H^+ or Na^+ potential to drive ATP formation by a rotary catalytic mechanism. Bacterial enzymes are typically composed of eight types of subunits with a biologically unique stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ for the F_1 sector at the periphery of the membrane and $a_1b_2c_{10-11}$ for the transmembrane F_0 sector. Recent evidence supports an ATP synthesis mechanism by which cation movement via subunit a and the oligomeric c subunit cylinder induces the rotation of this cylinder [1–3]. This rotation is connected to that of the central stalk subunits ε and γ, which is essential for ATP formation at the catalytic β subunits [4–6].

High-resolution structural information is available on the $\alpha_3\beta_3\gamma\varepsilon c_{10}$ subcomplex from yeast [7], but the structure of the peripheral stalk consisting of the a subunit and the two b subunits still awaits elucidation. It came much as a surprise that the number of c subunits in the c rotor is not fixed but varies among species. Structural work has identified c_{10}, c_{11} or c_{14} stoichiometries for the rotor rings from yeast [7], *Ilyobacter tartaricus* [8] and chloroplasts [9], respectively, and based on recent cross-linking studies it was concluded that c_{10} is the preferred stoichiometry for the *Escherichia coli* rotor [10].

The stoichiometry of the oligomer is apparently determined by intrinsic properties of the c monomers because shape and diameter of the cylinder did not change if one or several monomeric units were missing [11]. One intriguing property of the undecameric c oligomer from *I. tartaricus* reconstituted into two-dimensional (2D) arrays was the appearance of a central hole on one side and a central plug on the other side of the cylinder [8]. Here, we report that the central plug consists of phospholipids, which can be removed without affecting the stability of the cylinder.

2. Materials and methods

2.1. Purification and crystallization of the subunit c oligomer

I. tartaricus cells were grown on tartrate minimal medium at 30°C for 18 h [12]. Cells were harvested and the ATP synthase (25 U/mg protein) was purified by fractionated precipitation with polyethylene glycol as described [13]. The c oligomer was isolated from 10 mg heat-disrupted ATP synthase by sucrose density gradient centrifugation and gel filtration chromatography in a yield of 0.5 mg. 2D crystallization was performed at low lipid-to-protein ratio as described [8] to yield 2D crystals of 0.5–1 μm.

2.2. Phospholipase treatment of 2D crystals

Crystals of reconstituted subunit c oligomer in 100 mM Tris–HCl, pH 7.2 (0.18 mg/ml protein) [8] were mixed with 2.5 units phospholipase C (type V, *Bacillus cinereus*, Sigma, St. Louis, MO, USA). After 2 h incubation at 37°C samples were analyzed by atomic force microscopy (AFM).

2.3. AFM

The samples were diluted to a concentration of ~10 μg/ml in 300 mM KCl, 10 mM Tris–HCl, pH 7.8 and adsorbed to freshly cleaved mica. Contact mode AFM topographs were recorded in the same buffer, at room temperature, at a stylus loading force of < 100 pN and a line frequency of typically 4–6 Hz. The AFM used was a Nanoscope III (Digital Instruments, Santa Barbara, CA, USA) equipped with a J-scanner (scan size 120 μm) and a fluid cell. Cantilevers (Olympus, Tokyo, Japan) had oxide-sharpened Si_3N_4 tips and a spring constant of 0.09 N/m. No differences between topographs recorded simultaneously in trace and in retrace direction were observed, indicating that the scanning process did not influence the appearance of the biological sample.

2.4. Phosphate determination

Phospholipids were determined as phosphate concentrations according to [14]. Protein solutions of 0.05 ml were mixed with 0.05 ml of 10% $Mg(NO_3)_2{\cdot}6H_2O$ in ethanol and evaporated to dryness over a flame. 0.3 ml of 1 M HCl was added and the tubes were heated in a boiling water bath for 15 min to hydrolyze any pyrophosphate formed in the ashing procedure. After addition of 0.7 ml of ascorbic acid–molybdate mixture [15] and incubation at 45°C for 20 min, all probes were measured at 820 nm. The calibration curve was generated with 5–100 nmol potassium phosphate buffer, pH 8.0. An absorbance of 0.13 was obtained from 5 nmol of phosphate.

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Abbreviations: AFM, atomic force microscopy; SDS, sodium dodecyl sulfate

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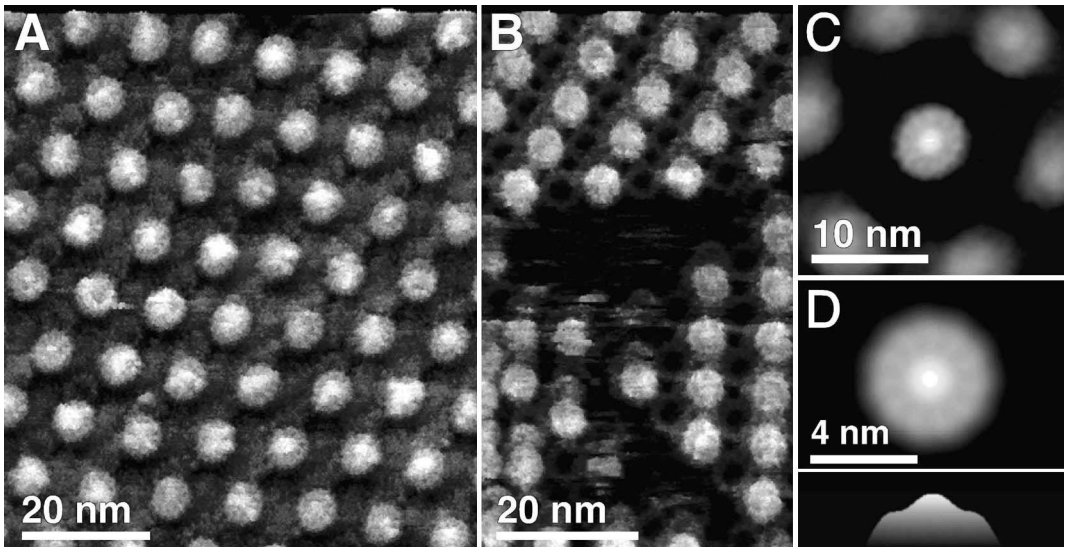

Fig. 1. AFM topographs of reconstituted c_{11} oligomers from ATP synthase of *I. tartaricus*. A: Oligomers assembled in a 'pseudo'-hexagonal 2D crystal [8]. B: Occasionally, oligomers were observed to assemble into a rectangular 2D crystal. Both crystalline areas are organized in a head-to-tail packing. The higher ends of the c_{11} oligomers are well resolved, whereas the AFM stylus cannot effectively penetrate to image the lower ends. C: Average of the 220 higher protruding oligomers as in A and B, showing a left-handed vorticity and a central plug. D: 11-fold symmetrized average of C. Bottom of D: height section through the oligomeric center. The outer diameter of the oligomers was 5.4 ± 0.3 nm. These values are slightly larger than those determined by electron microscopy (5.0 ± 0.2 nm) due to the AFM tip geometry. Topographs were recorded in 300 mM KCl, 10 mM Tris–HCl, pH 7.8 at room temperature.

2.5. Thin-layer chromatography (TLC)

Lipid-containing samples were applied to silica gel plates (10×10 cm) together with standard lipids (Sigma). After developing in $CHCl_3$/MeOH/H_2O 65:25:4 the plate was dried, sprayed with primulin dye solution, and lipid spots were visualized by UV light [16].

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Schägger and Jagow [17] with separating gels containing 13.6% acrylamide–bisacrylamide (Acrylamide/Bis solution, 37.5:1.0, Bio-Rad, Hercules, CA, USA). Silver staining was performed as described [18].

3. Results

AFM analysis of 2D crystals of the c_{11} oligomers of the ATP synthase of *I. tartaricus* showed densely packed individual rotors protruding from the lipid bilayer (Fig. 1). Whereas most oligomers were assembled in a 'pseudo'-hexagonal crystal (Fig. 1A), about 5% were found to consist of a rectangular 2D arrangement (Fig. 1B). Alternately orientated subunit c oligomers were found, which differed in their protrusion from the membrane surface. Since the higher ends of the rings

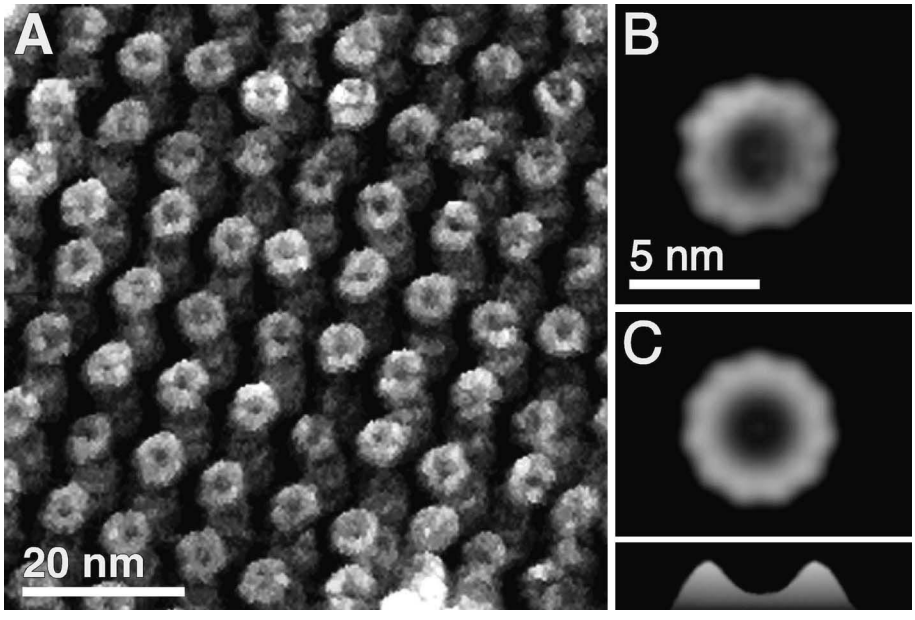

Fig. 2. Topograph of reconstituted c_{11} oligomers treated with phospholipase C. A: The central plug of the oligomers was removed. Interestingly, all oligomers of the digested samples were assembled into a rectangular assembly. B: Average of 170 oligomers as observed in A, showing 11 subunits arranged into a ring-like structure and a central depression. C: 11-fold symmetrized average of B showing the left-handed vorticity of the oligomer. Bottom of C: height section through the oligomeric center. After digestion, the outer diameter of the oligomers (5.4 ± 0.3 nm) remained unaffected. Topographs were recorded in 300 mM KCl, 10 mM Tris–HCl, pH 7.8 at room temperature.

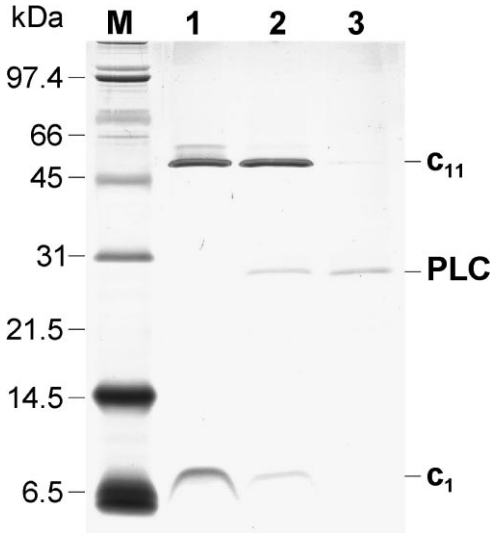

Fig. 3. Subunit c oligomer is unaffected by phospholipase C treatment. 2D crystals of c_{11} from *I. tartaricus* (1 μg protein, lane 1), phospholipase C-treated 2D crystals of c_{11} after incubation with phospholipase C (PLC; 0.5 μg protein, lane 2) and 0.5 μg of phospholipase C (lane 3) were applied to 13.4% SDS–PAGE [17] and protein bands were visualized by silver staining [18]. Protein standards were applied to lane M. The molecular masses of these proteins are shown.

protrude 1.0 ± 0.2 nm above the lower ends, the AFM stylus cannot resolve the subunits of the lower rings.

A central plug exhibiting a diameter of 1.7 ± 0.3 nm and protruding by 0.7 ± 0.3 nm above the surrounding oligomeric ring was visible on top of all the higher protruding rings. The dimensions of these protrusions were extracted from averaged topographs of 220 c oligomers (Fig. 1C). In order to identify the composition of the central plug, a sample with 2D crystals of subunit c cylinders was incubated with phospholipase C. Subsequent analysis by AFM showed an almost complete removal of the central protrusions surrounded by the subunit c oligomers (Fig. 2). Instead, a central hole became apparent, similar to the image of the molecule from its opposite side. We conclude therefore that the central plug consists of phospholipids that became degraded during the incubation with phospholipase C. Coherent with the average of the plugged oligomer (Fig. 1D), that of the digested oligomer (Fig. 2C) showed a left-handed vorticity. Thus, it can be excluded that the unplugged oligomer could potentially represent the other oligomeric surface exhibiting a right-handed vorticity [8]. The 2D crystals uniformly exhibited rectangular arrangements, indicating that digestion of phospholipids shifts the 'pseudo'-hexagonal into a rectangular crystal lattice. This finding suggests that the crystalline packing of the oligomers is mainly determined by the lipid-mediated interactions between individual oligomers. Fig. 3 shows a comparison by SDS–PAGE of crystalline samples of the c oligomer before and after incubation with phospholipase C. The mobility of the c oligomer was not changed by this treatment, which is consistent with the view that the oligomeric status of the ring was not dependent on the presence of phospholipids in its central cavity. Furthermore, the isolated c_{11} oligomer which does not contain phospholipids (see below) has the same mobility on SDS–PAGE as the two crystalline samples analyzed in Fig. 3 (not shown). Therefore, the same species is apparently migrating in SDS–PAGE, implying that the phospholipid plug is removed during SDS treatment.

It has been observed that the c oligomer from the ATP synthase of *I. tartaricus* is extremely stable, resisting boiling in SDS for at least 5 min [13]. To investigate whether protein–protein interactions are exclusively responsible for this stability or whether this depends on an interaction with phospholipids, the phospholipid content of the ATP synthase and of isolated c_{11} samples was determined. Based on organic phosphate analysis, about 300 mol phospholipids were present per mol of the purified ATP synthase. No phospholipids were found, however, in the isolated c_{11} samples. With the amounts analyzed (0.5 mg; 5.2 nmol) we would have been able to detect less than 0.5 mol of phospholipid per mol of c_{11}. The lipid content of isolated c_{11} was also analyzed by TLC and again negative results were obtained (Fig. 4). Hence, our isolated c_{11} preparations do not contain any bound (phospho)-lipids. The stability of the c_{11} oligomer is therefore entirely due to tight protein–protein interactions. We conclude further that the phospholipid plug observed in the central hole on one surface of the c_{11} cylinder is inserted into this location during the reconstitution procedure.

4. Discussion

The data presented here do not make it possible to draw unequivocal conclusions about the presence or absence of a phospholipid plug in the central hole of the c_{11} cylinder within the ATP synthase incorporated into the biological membrane. The association with phospholipids during the reconstitution procedure merely indicates a strong affinity of the c_{11} cylinder for hydrophobic molecules to its central cavity. From a physiological point of view, such an affinity seems to be highly desirable. It is obvious that a cell would not survive with a water-filled hole in the middle of the c_{11} cylinder because this would inevitably lead to the collapse of the membrane poten-

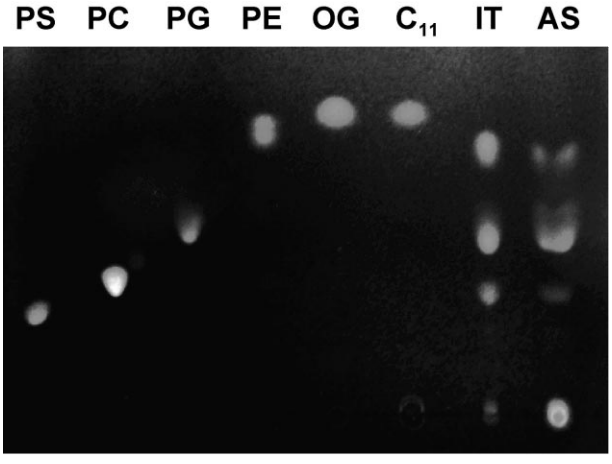

Fig. 4. TLC of purified ATP synthase and subunit c oligomer. Samples were applied to a silica gel plate and developed in $CHCl_3$/MeOH/H_2O 65:25:4. The lipid standards (2 μg each) L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylcholine (PC), L-α-phosphatidyl-DL-glycerol (PG), L-α-phosphatidylethanolamine (PE) and 20 μg β-octylglucoside (OG) are indicated. Whereas no lipids could be detected with 2 μg purified c_{11} in 2% β-octylglucoside-containing buffer (c_{11}), the chloroform/methanol extracts of 1 μg *I. tartaricus* cells (IT) and of 20 μg ATP synthase from *I. tartaricus* (AS) showed similar distribution patterns of lipid types.

tial. Hence, there must be a seal within the c_{11} cylinder, which prevents ions from penetrating through the hole across the entire bilayer. Such a seal could be provided by the phospholipid plug seen in AFM of reconstituted c_{11} or by another hydrophobic compound. Future examinations with the entire ATP synthase are necessary to determine whether these compounds are lipids, proteins or a combination of both. In this context it is interesting to note that in the X-ray structure of F_1 with the adhering c_{10} moiety from yeast an elongated mass has been identified within the c_{10} cylinder, which was suggested to consist of phospholipids [7].

Biochemical data of the ATP synthase from *Propionigenium modestum* and NMR studies of its subunit c monomer support a free accessibility of the ion-binding site of subunit c from the cytoplasm [2,19]. According to these observations an ion-binding site located near the membrane boundary was proposed [20]. Consequently, the higher protruding rings as observed by AFM (Figs. 1 and 2) might represent the cytoplasmically exposed region of the protein complex. However, lipid insertion from the cytoplasmic side would prevent the binding of γ and ε to the hydrophilic loops of the c subunits, and therefore the assignment of the higher and lower rings in the 2D crystals to the periplasmic and cytoplasmic sides in the native environment still remains to be elucidated.

References

[1] Kaim, G. and Dimroth, P. (1999) EMBO J. 18, 4118–4127.
[2] Kaim, G., Matthey, U. and Dimroth, P. (1998) EMBO J. 17, 688–695.
[3] Jiang, W. and Fillingame, R.H. (1998) Proc. Natl. Acad. Sci. USA 95, 6607–6612.
[4] Pänke, O., Gumbiowski, K., Junge, W. and Engelbrecht, S. (2000) FEBS Lett. 472, 34–38.
[5] Sambongi, Y. et al. (1999) Science 286, 1722–1724.
[6] Tsunoda, S.P., Aggeler, R., Noji, H., Kinosita Jr., K., Yoshida, M. and Capaldi, R.A. (2000) FEBS Lett. 470, 1–5.
[7] Stock, D., Leslie, A.G.W. and Walker, J.E. (1999) Science 286, 1700–1705.
[8] Stahlberg, H., Müller, D.J., Suda, K., Fotiadis, D., Engel, A., Meier, T., Matthey, U. and Dimroth, P. (2001) EMBO Rep. 2, 229–233.
[9] Seelert, H., Poetsch, A., Dencher, N.A., Engel, A., Stahlberg, H. and Müller, D.J. (2000) Nature 405, 418–419.
[10] Jiang, W., Hermolin, J. and Fillingame, R.H. (2001) Proc. Natl. Acad. Sci. USA 98, 4966–4971.
[11] Müller, D.J., Dencher, N.A., Meier, T., Dimroth, P., Suda, K., Stahlberg, H., Engel, A., Seelert, H. and Matthey, U. (2001) FEBS Lett. 504, 219–222.
[12] Schink, B. (1984) Arch. Microbiol. 139, 409–414.
[13] Neumann, S., Matthey, U., Kaim, G. and Dimroth, P. (1998) J. Bacteriol. 180, 3312–3316.
[14] Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775.
[15] Chen Jr., P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758.
[16] White, T., Bursten, S., Federighi, D., Lewis, R.A. and Nudelman, E. (1998) Anal. Biochem. 258, 109–117.
[17] Schägger, H. and Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[18] Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.
[19] Kaim, G. and Dimroth, P. (1998) EMBO J. 17, 5887–5895.
[20] Dimroth, P., Matthey, U. and Kaim, G. (2000) Biochim. Biophys. Acta 1459, 506–513.