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Two-dimensional crystallization of intact F-ATP synthase isolated from bovine heart mitochondria

Mitochondrial F-ATP synthase produces the majority of ATP for cellular functions requiring free energy. The structural basis for proton motive force-driven rotational catalysis of ATP formation in the holoenzyme remains to be determined. Here, the purification and two-dimensional crystallization of bovine heart mitochondrial F-ATP synthase are reported. Two-dimensional crystals of up to 1 μm in size were grown by dialysis-mediated detergent removal from a mixture of decylmaltoside-solubilized 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and F-ATP synthase against a detergent-free buffer. A projection map calculated from an electron micrograph of a negatively stained two-dimensional crystal revealed unit-cell parameters of $a = 185.0$, $b = 170.3$ Å, $\gamma = 92.5^\circ$.

1. Introduction

Mitochondrial F-ATP synthase provides the majority of ATP to eukaryotic cells through exploitation of Mitchell's proton motive force produced by the respiratory chain (Mitchell, 1961). In ATP synthesis mode, the proton motive force provides power for rotation of a proton-translocating rotor ring in the membrane-stemming F_o domain of F-ATP synthase. Rotation of the rotor ring is mechanically coupled *via* a central stalk to the catalytic water-soluble F_1 domain, where consecutive changes are induced in the nucleotide-binding affinity of the catalytic $\alpha_3\beta_3$ hexamer, resulting in the recycling of cellular ATP from ADP and inorganic phosphate (Boyer, 1997; Yoshida *et al.*, 2001).

The bovine enzyme comprises at least 15 different subunits, with $\alpha_3\beta_3\gamma\delta\epsilon$ assigned to the F_1 domain and $c_8abF_6dOSCpegfA_6L$ assigned to the F_o domain (Walker *et al.*, 1991). With 22 X-ray structures of subcomplexes in the PDB and two holoenzyme entries in the EMDB, the bovine enzyme is arguably the best structurally characterized F-ATP synthase. This set of 22 X-ray structures includes the first high-resolution X-ray structure of the F_1 domain (Abrahams *et al.*, 1994), the F_1 domain in complex with a partial peripheral stalk (Rees *et al.*, 2009), the F_1 - c_8 complex (Watt *et al.*, 2010) and an electron cryo-microscopy structure of the detergent-solubilized intact complex in vitrified ice at 18 Å resolution (Baker *et al.*, 2012). However, no crystal structures of an intact F-ATP synthase have yet been reported.

The lack of high-resolution crystal structures of the intact F-ATP synthase complicates the verification of the currently accepted two-half-channel model for the generation of directed rotation in F_o by a proton motive force (Vik & Antonio, 1994; Junge *et al.*, 1997). It also leaves open questions regarding the molecular nature of the interaction in F_o that ensures stability during synthesis of ATP without impeding the relative rotation of the proton-translocating rotor ring to the proton-access half-channels harbouring a subunit (Gerle, 2010, 2011).

Electron crystallography of two-dimensional crystals is a powerful approach for studying the structure of membrane proteins in a near-physiological environment (Fujiyoshi, 2011; Raunser & Walz, 2009). Although most successful in the study of relatively small membrane proteins, recent advances in the study of gap junctions (Oshima *et al.*, 2007) and the proton-pumping P-type H,K-ATPase (Abe *et al.*, 2011) provide a strong indication that electron crystallography of

Figure 1

(a) Electron micrograph of a two-dimensional crystal of bovine F-ATP synthase in negative stain, (b) its Fourier transform and (c) a projection map calculated from a single two-dimensional crystal with unit-cell parameters $a = 185.0$, $b = 170.3$ Å, $\gamma = 92.5^\circ$ (outlined in yellow).

two-dimensional crystals can be applied to large complexes such as the mitochondrial F-ATP synthase.

Previous attempts to conduct electron crystallographic studies of the structures of various membrane-reconstituted F-ATP synthases from sources ranging from spinach to bovine (Böttcher *et al.*, 1995; Lévy *et al.*, 1999; Arechaga & Fotiadis, 2007; Dubachev *et al.*, 1993) did not yield a three-dimensional reconstruction. Only the study of Dubachev and coworkers provided data on the functionality of the membrane-reconstituted F-ATP synthase.

Here, we report the two-dimensional crystallization of membrane-reconstituted intact F-ATP synthase from bovine heart mitochondria in its intact and active oligomycin-sensitive form.

2. Materials and methods

2.1. Purification of intact F-ATP synthase from bovine heart mitochondria

Submitochondrial particles were isolated from bovine heart as described previously (Shinzawa-Itoh *et al.*, 2010). A suspension of submitochondrial particles in 40 mM HEPES pH 7.8, 2 mM MgCl_2 , 0.1 mM EDTA, 0.1 mM DTT was solubilized on ice by the addition of deoxycholate and decylmaltoside to final concentrations of 0.7 and 0.4% (w/v), respectively. Thereafter, the suspension was centrifuged at 176 000g for 50 min. The supernatant was layered onto a sucrose step gradient [40 mM HEPES pH 7.8, 0.1 mM EDTA/DTT, 0.2% (w/v) decylmaltoside and 2.0, 1.1, 1.0 or 0.9 M sucrose] and subjected to equilibration centrifugation at 176 000g for 15.5 h. Fractions exhibiting ATPase activity were loaded onto a Poros-20HQ ion-exchange column and subsequently eluted by a linear concentration gradient from 0 to 240 mM KCl in 40 mM HEPES pH 7.8, 2 mM MgCl_2 , 0.1 mM EDTA, 0.1 mM DTT. Finally, fractions containing F-ATP synthase were concentrated to 10 mg ml⁻¹ using an Amicon concentrator.

2.2. ATPase activity measurement and mass spectroscopy

To determine the enzymatic activity of the isolated F-ATP synthase, an ATP-regenerating enzyme-coupled assay was employed (Pullman *et al.*, 1960). Using this assay system, hydrolysis of ATP by the F-ATP synthase accompanied by NADH oxidation was monitored at 340 nm at 293 K. Further experimental conditions are

given in the Supplementary Material¹. To examine the subunit composition of the present bovine F-ATP synthase preparation, the purified enzyme was examined using a MALDI-TOF mass spectrometer (Voyager DE PRO system).

2.3. Membrane reconstitution and two-dimensional crystallization

Immediately after isolation and concentration, bovine F-ATP synthase was mixed with decylmaltoside-solubilized 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) at a lipid:protein ratio of 0.2 and kept on ice overnight. Reconstitution into lipid membranes and two-dimensional crystallization was achieved by dialysis-mediated detergent removal against 500 ml detergent-free buffer [40 mM Tris-HCl pH 8.2, 100 mM NaCl, 0.02% (w/v) NaN_3 , 0.5 mM ADP, 5 mM MgCl_2 , 0.1 mM DTT, 0.1 mM EDTA] for 10 d with daily buffer exchange using 20 µl dialysis buttons (Hampton Research) covered by a SpectraPore dialysis membrane with a molecular-weight cutoff of 15 000 Da.

2.4. Electron microscopy and image analysis

Samples from dialysis buttons (2.5 µl) were applied onto freshly glow-discharged, carbon-coated 400 mesh copper grids (Veco). After brief blotting (Advantec), the samples were then stained using a 2% uranyl acetate solution and air-dried. Images were taken with a JEM1010 transmission electron microscope (Jeol) equipped with a 2K × 2K slow scan CCD camera (Gatan, Pleasanton, California, USA) at 100 kV and 12 pA cm⁻², an exposure time of 2 s and a magnification of 40 000×, corresponding to a pixel size of 6 Å. Collected CCD images were transformed to MRC format. All images were processed with the MRC two-dimensional crystal processing package (Crowther *et al.*, 1996) to correct lattice distortions (Henderson *et al.*, 1986).

3. Results and discussion

Starting from 1100 g of fresh bovine heart muscle, approximately 200 mg highly purified F-ATP synthase was obtained. The preparation typically has ATPase activity of 0.3 µmol mg⁻¹ min⁻¹ and high

¹ Supplementary material has been deposited in the IUCr electronic archive (Reference: US5043).

oligomycin sensitivity (>90%). Mass spectrometry performed on freshly purified F-ATP synthase showed the presence of 18 different subunits, including all of the subunits considered to be essential components of the intact and functional bovine F-ATP synthase (a mass spectrogram is shown in Supplementary Fig. S1).

The Fourier-transformed images taken from the negatively stained two-dimensional crystals (Fig. 1a) prepared as described above exhibit optical diffraction spots to better than 25 Å resolution (Fig. 1b). A projection map calculated from a single two-dimensional crystal gave a unit cell (outlined in Fig. 1b) with parameters $a = 185.0$, $b = 170.3$ Å, $\gamma = 92.5^\circ$ and shows two strong densities that are likely to originate from the extramembranous F_1 domain. This suggests the presence of two F-ATP synthase complexes in the unit cell. However, the deduction of molecular packing in two-dimensional crystals from the interpretation of projection maps alone tends to be error-prone. This is especially true for determination of the molecular packing of membrane proteins that include large extramembranous domains, such as the F-ATP synthases or V-ATPases (Gerle *et al.*, 2006; Tani *et al.*, 2013). At this point, we therefore refrain from postulating a model of the molecular packing in the crystalline sheet. The inherent instability of F-ATP synthase is a major challenge in any crystallization attempt. Therefore, it is prudent to suspect that crystals grown from preparations of intact F-ATP synthase may not contain intact and functional complexes, but rather functionally impaired subcomplexes. On the other hand, no clear hexagonal density is detectable in the calculated projection map. Hexagonal density is likely to originate from a dominance of the $\alpha_3\beta_3$ hexamer in the overall mass, *i.e.* in a subcomplex missing large parts of F_0 . Furthermore, the minimum distance of 117 Å between the centre of the putative F_1 densities is in excess of the distance between closely packed $\alpha_3\beta_3$ hexamers. This suggests that the two-dimensional crystals contain more than the F_1 domain, or the F_1 - c_8 subcomplex.

To further confirm the intactness and functionality of the F-ATP synthase in the two-dimensional crystals, we measured its specific ATPase activity and oligomycin sensitivity. After the addition of dodecylmaltoside to a concentration of one critical micelle concentration [0.01% (w/w)], the enzyme activity of the two-dimensional crystal suspension increased to a level similar to the activity level observed before crystallization (90–100%). Furthermore, the detergent-treated two-dimensional crystals have an oligomycin sensitivity of 75–85%, which is close to the sensitivity observed before two-dimensional crystallization (>90%). These results strongly suggest that the two-dimensional crystal is composed of intact and non-impaired F-ATP synthase complexes (further details regarding the present experimental results are given in the Supplementary Material). Tests on the suitability of this type of two-dimensional crystal for data collection using unstained specimens in vitrified ice will be conducted. Considering the large unit-cell size of more than 150 Å and the lack of symmetry in the F-ATP synthase complex itself, the suitability of the present two-dimensional crystal for electron diffraction seems unlikely. Consequently, structural analysis by electron crystallography will have to rely on images alone; obtaining a density map at a resolution sufficient to build an atomic model will thus be very challenging, as seen in the case of the nicotinic acetylcholine receptor (Miyazawa *et al.*, 2003). Apart from the two-dimensional crystal quality, another possible challenge to obtaining high-resolution image data from the F-ATP synthase two-dimensional crystals could stem from the susceptibility of large

hydrophilic domains to damage during cryogenic sample preparation, as reported for a two-dimensional crystal of gastric P-type H,K-ATPase (Yang *et al.*, 2013). However, as described in the same report, a remedy for this challenge will be likely to involve application of the recently developed carbon-sandwich technique (Gyobu *et al.*, 2004).

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