Two-dimensional crystallization and preliminary electron crystallographic result of partially purified F_0 from porcine mitochondria

ZHANG Xujia[1], SUN Jingchuan[1], YANG Hui[1], LI Huilin[2], HUANG Youguo[1] & YANG Fuyu[1]

1 Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China;
2 Life Science Division, Berkeley National Laboratory, Berkeley, CA 94720, USA

Correspondence should be addressed to Zhang Xujia (e-mail: xjzhang@sun5.ibp.ac.cn)

Abstract **After removal of cytoplasmic sector F_1 from submitochondrial particles of F_0F_1-ATP synthase complex with guanidine hydrochloride, the transmembrane sector F_0 was specifically extracted from the stripped membranes in the presence of detergent CHAPS and partially purified. Two-dimensional crystals were produced by the reconstitution of the partially purified F_0 into asolectin and microdialysis. The obtained crystals are able to diffract to 2 nm. The projection map of the negatively stained crystal shows that the crystal has $p42_12$ symmetry, lattice constant, $a = b = 14.4$ nm. A unit cell contains four F_0 molecules.**

Keywords: membrane protein, proton channel F_0, two-dimensional crystal, electron microscopy.

Mitochondrial H^+-ATPase is an F-type ATPase (F_0F_1-ATPase), serves to generate ATP by using the proton gradient force across the mitochondrial membrane. F_0F_1-ATPase consists of two sectors: cytoplasmic sector F_1, containing $\alpha_3\beta_3\gamma$ subunits, transmembrane sector F_0, containing abc_{9-12} subunits. F_1 is responsible for the ATP synthesis while F_0 for the proton translocation across the mitochondrial membrane, which energizes F_1. The atomic resolution structure of F_1 was determined by X-ray crystallography[1], and proved the conformational changes mechanism proposed by Boyer[2]. The structural details of F_0, however, are still very limited. It has been suggested that the a and b subunits are on the outside of a multimeric ring of c subunits within the membrane, with the interface between a and c subunits providing the pathway for proton translocation. An electron density map obtained from 3-D crystals of a subcomplex F_1c_{10} of yeast mitochondrial ATP synthase shows a ring of 10 c subunits[3], implying that the possible rotation of the c ring generates the conformational changes in F_1. So far, the arrangement of F_0 subunits is not clear.

We have been working on the structure and function of F_0F_1-ATPase from porcine heart mitochondria[4—6]. To get the whole picture of F_0F_1-ATPase, especially the details of F_0, the electron crystallography is established by 2-D crystallization of membrane proteins. The F_0 sector was isolated and partially purified from the inner membrane of porcine heart mitochondria. Two-dimensional crystals were formed by the microdialysis method. The obtained projection map of negatively stained crystal of the F_0 provides important structural information.

1 Materials and methods

(i) F_0 purification. Porcine heart mitochondria were prepared according to Smith[7]. Submitochondrial particles were prepared from mitochondria by sonication as described by Lutter[8]. The submitochondrial particles were suspended in a PA buffer (0.15 mol/L KH_2PO_4, pH 7.9, 1 mmol/L ATP, 25 mmol/L EDTA, 0.5 mmol/L DTT, 5% ethylene glycol, 0.001% PMSF). F_0 was purified according to McEnery[9] with a modification. A solution of guanidine hydrochloride (3.3 mol/L) in the PA buffer was added to submitochondrial suspension to give a final concentration of 2.6 mol/L and protein concentration 3 mg/mL. After incubating 5 min on ice, the guanidine was diluted 3 times by the PA buffer. The stripped membranes were harvested by centrifugation at 142000×g for 30 min.

The above stripped membranes were resuspended in PA buffer containing 1% detergent of CHAPS to a final protein concentration of 3.5 mg/mL. The membrane suspension was incubated on ice for 15 min, followed by the centrifugation at 105000×g for 1 h. The solution containing F_0 was pooled and precipitated with $(NH_4)_2SO_4$. An SDS-PAGE revealed that the partially purified F_0 does not contain F_1 subunits. All the experiments were performed at 4℃ without otherwise stated.

(ii) Two-dimensional crystallization. Two-dimensional crystallization was performed by the microdialysis method. The F_0 solution (1 mg/mL) in the presence of 1% CHAPS was mixed with asolectin solution at a ratio of 1 : 1 (*w*/*w*). The mixture was dialyzed against buffer (10 mmol/L Mes (pH 6.0), 20 mmol/L NaCl, 1 mmol/L $CaCl_2$, 1 mmol/L NaN_3) at 20℃. The crystals were formed in one week.

(iii) Electron microscopy. 3 μL aliquots of samples were applied to freshly prepared and glow-discharged carbon films. After 30 s, excess solution was blotted off from the side of the grid with one layer of filter paper. Specimens were negatively stained with uranyl acetate (1% (*w*/*v*)) containing 0.1% Triton X-100. Specimens were routinely examined with a Phillips 120 electron microscope operated at 120 keV. The images were recorded at a magnification of 27500 times and ~500 nm defocus.

(iv) Image processing. Suitable micrograph areas were selected by optical diffraction and digitized with a Zeiss SCAI scanner using a 21 μm step-size. Images were processed using the MRC image processing package on Unix workstation[10].

2 Results and discussion

By screening detergents, it was found that detergent CHAPS was able to specifically extract F_0 from the inner membrane of mitochondria. The purified F_0 maintained higher proton translocation activity and contained most of the subunits. CHAPS is also suitable for 2D crystallization due to its higher CMC and easier to be removed by dialysis.

F_0 was reconstituted into asolectin bilayer in the course of detergent CHAPS dialysis. Membrane protein reconstitutions by dialysis is widely used to study the structure and function of membrane proteins. In order to produce 2D crystals, lower lipid/protein ratio was used. Meanwhile, ionic strength and pH are also the key factors for the crystal formation. Overview pictures of negatively stained specimens were taken at 3000 magnification. The crystal appeared to be darker than the background. The lattice array of F_0 molecules could be seen at higher magnification, i.e. above 20000 magnification. The crystal patches did not have a certain shape, but had clear edges. The lattice lines are perpendicular to each other. Fig. 1 is a typical 2D crystal of the F_0. Its maximum diameter is 1 μm, and diffracts to 2 nm.

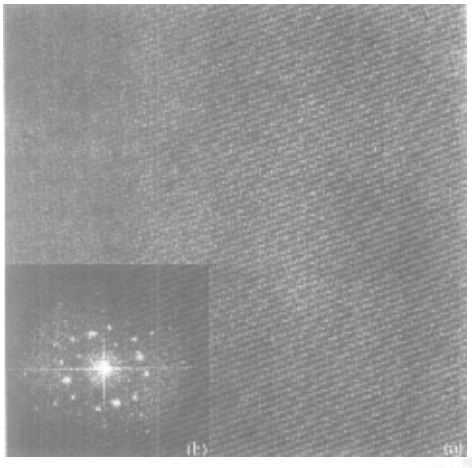

Fig. 1. (a) An electron micrography of negatively stained two-dimensional crystal of partially purified F_0 from porcine heart mitochondria; (b) power spectrum, fourth order diffraction spots are observed, corresponding to 2 nm.

MRC package for data processing of 2D crystals is used worldwide. Recently, we installed this package and used it for data processing in this work. Unlike 3D crystals, 2D crystals of proteins can only have 17 possible plane groups. The symmetry elements found can suggest p4 or $p42_12$ symmetry out of the 17 plane groups listed in table 1. Considering the missing reflections of (2h, 0) and (0, 2k) from the power spectrum (fig. 1(b)), we conclude $p42_12$ symmetry. It is noted that the phase residue of $P12_1$_b is the lowest, but it is triclinic, i.e. $a \neq b$, $\gamma \neq 90°$. However, the projection map (fig. 2) clearly shows that $a = b$, $\gamma = 90°$. Furthermore, each unit cell contains four molecules shown in fig. 2. The power spectrum (fig. 1(b)) also shows tetragonal, not orthorhombic. Together, the crystal obtained in this study is assigned as $p42_12$ symmetry.

Table 1 Phase residual of 17 kinds of plane group

	Plan group	Phase residual
1	P1	24.4
2	P2	23.1*
3b	P12_b	73.1
3a	P12_a	71.2
4b	$P12_1$_b	12.7*
4a	$P12_1$_a	17.8*
5b	c12_b	73.1
5a	c12_a	71.2
6	P222	54.0
7b	$P222_1$b	54.7
7a	$P222_1$a	52.4
8	$P22_12_1$	18.3*
9	c222	54.0
10	P4	16.4*
11	P422	47.0
12	$P42_12$	16.5*
13	P3	53.0
14	$P3_12$	43.1
15	$P32_1$	42.5
16	P6	44.
17	P622	40.2

* Acceptable.

Fig. 2 gives the projection map with $p42_12$ symmetry applied with MRC program. The projection map indicates that the crystal has $p42_12$ symmetry, and each unit cell contains four F_0 molecules. The lattice constant is $a = b =$ 14.4 nm. Meanwhile, F_0 molecule shows an internal pseudo two fold symmetry at 2 nm resolution. F_0 subunits arrange an elliptical structure with a central hole. The short axis (X axis) is along the pseudo two fold symmetry, while a strong and two weak densities are on the one side of long axis (Y axis), and some weaker densities are found on the other.

So far, there are no structural details of F_0 reported. Even though, models of F_0 have been proposed based on the biochemical experiments[11]: c subunits, which consists of two α helixes with a hairpin shape, form an oligermeric ring in the membrane providing the pathway for proton translocation. a subunit, which consists of five α helixes, is the interface of the c ring. The interaction between a subunit and c ring is crucial for the proton translocation. b subunit, which consists of two α helixes, spans into membrane. Its big hydrophilic domain expends into cytoplasm,

Fig. 2. A projection map of F_0 from porcine heart mitochondria The crystal has p42.2 symmetry. Inset: unit cell with lattice constant 14.4×14.4 nm.

and possibly interacts with α/β subunit. It has been suggested that b subunit is a bridge between a subunit and F_1 sector. Other subunits of F_0, e.g. d, g and f, etc. have not been defined yet.

We partially purified F_0 complex from porcine heart mitochondria. It reasonably agreed with the purified F_0 complex from bovine heart mitochondria reported by Walker et al.[12], which revealed by SDS-PAGE. Our partially purified F_0 complex contains most of F_0 subunits, such as c, a, b, etc., also the stalk portion, OSCP. Because of the interference of these subunits, we are not able to see a circle ring, instead we got an elliptical ring (fig. 2). Based on the current model of F_0, it could be postulated that the strongest density could be a subunit, while the weaker densities on both sides of a subunit could be b, d, f and OSCP, etc. The densities opposite a subunit along the *Y* axis could be c subunits. To our knowledge, no project map of F_0 complex containing a, b, c subunits has been reported.

To yield 2D crystals, partially purified F_0 was obtained by a modified method. After screening crystallization conditions, we obtained the 2D crystal of F_0. Although impurities are still in the sample, we believe that the crystal is from F_0 based on the following evidence: i) protein purification was done according to the traditional F_0 purification procedure. SDS-PAGE reveals that there is no F_1 subunits contamination. Meanwhile, we tested the activities of other complex in the respiratory chain of mitochondria with specific inhibitor, and no activities were observed. ii) the projection map (fig. 2) indicates that the molecule has round shape with a central hole. This structure basically agreed with the current model of c subunit forming a ring.

In summary, the transmembrane sector F_0 of F_0F_1-ATPase from porcine heart mitochondria was partially purified, and two-dimensional crystals were yielded. The crystal diffracted up to 2 nm, and the projection map shows $p42_12$ symmetry. This work provides an example to study membrane structure by electron crystallography in China. Meanwhile, the projection map gives some clues for the arrangement of F_0 subunits, and provides very important information of F_0 structure. We have taken the images of crystals at different tilt angles, and 3D reconstruction from 2D crystal is underway.

Acknowledgements We are very grateful to Prof. Xu Wei for critical reading of this manuscript. This work was supported by the Key Project of Chinese Academy of Sciences (Grant No. KJ951-A1-601), the National Natural Science Foundation of China (Grant No. 39730130) and the Fund of the Chinese Academy of Sciences to Zhang Xujia (Grant No. KSCX2-2-05).

References

1. Abrahams, J. P., Leslie, A. G., Lutter, R. et al., Structure at 2.8Å resolution of F_1-ATPase from bovine heart mitochondria, Nature, 1994, 370(6491): 621.
2. Boyer, P. D., The ATP synthase——a splendid molecular machine, Annu. Rev. Biochem., 1997, 66: 717.
3. Stock, D., Leslie, A. G. W., Walker, J. E., Molecular architecture of the rotary motor in ATP synthase, Science, 1999, 286: 1700
4. Yang, F. Y., Huang, Y. G., Tu, Y. P., Divalent cation and lipid-protein interactions of biomembranes, Bioscience Reports, 1993, 13(3): 143.
5. Yang, H., Zhang, L., Zhang, X. et al., Purification, reconstitution and H^+ translocation activity of porcine heart mitochondrial F_0, Prog. Biochem. Biophys., 2001, 28(2): 227.
6. Yang, H., Huang, Y. G., Zhang, X. J. et al., Cardiolipin is essential for higher proton translocation activity of reconstituted F_0, Science in China, Ser. C, 2001, 44: 146.
7. Smith, A. L., Preparation, properties and conditions for assay of mitochondria:slaughterhouse material, small-scale, Methods Enzymol, 1967, 10: 81.
8. Lutter, R., Abrahams, J. P., Van Raaij, M. J., Crystallization of F_1-ATPase from bovine heart mitochondria, J. Mol. Biol., 1993, 229(3): 787.
9. McEnery, M. W., Hullihen, J., Pedersen, P. L., F_0 "proton channel" of rat liver mitochondria. Rapid purification of a functional complex and a study of its interaction with the unique probe diethylstilbestrol, J. Biol. Chem., 1989, 264: 20 12029
10. Henderson, R., Baldwin, J. M., Downing, K. H. et al., Structure of purple membrane from *Halobacterium Halobium*: recording, measurement and evaluation of electron micrographs at 3.5Å resolution, Ultramicroscopy, 1986, 19: 147
11. Fillingame, R. H., H^+ transport and coupling by the F_0 sector of the ATP synthase: insights into the molecular mechanism of function, J. Bioenerg. Biomembr., 1992, 24: 5485
12. Collinson, I. R., Runswick, M. J., Buchanan, S. K. et al., F_0 membrane domain of ATP synthase from bovine heart mitochondria: Purification, subunit composition and reconstitution with F_1-ATPase, Biochemistry, 1994, 33: 7971.

(Received July 17, 2001)