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Crystallization and preliminary X-ray diffraction study of the catalytic subunit of archaeal H^+-transporting ATP synthase from *Pyrococcus horikoshii* OT3

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H^+-transporting ATP synthase (H^+-ATPase) is a multi-subunit complex which acts to produce ATP molecules. The catalytic subunit A of the archaeal-type H^+-ATPase from *Pyrococcus horikoshii* OT3 was cloned, expressed in *Escherichia coli*, purified and crystallized by the hanging-drop vapour-diffusion method with MPD as a precipitant. X-ray intensity data were collected to 2.55 Å resolution at beamline BL41XU of SPring-8. The crystals belong to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 128.0$, $c = 104.7$ Å, and contain one molecule per asymmetric unit.

1. Introduction

H^+-transporting ATP synthase/hydrolase (H^+-ATPase; EC 3.6.3.14) is a multi-subunit enzyme that exists in every organism and acts to produce ATP molecules. F-type ATPase (also called F-ATPase or F_0F_1-ATP synthase) acts as a functional ATP synthase in a rotational mode by the electrochemical potential gradient of H^+ (Yasuda *et al.*, 2001). The V-type enzyme in vacuoles and clathrin-coated vesicles (V-ATPase) pumps H^+ rather than synthesizing ATP under physiological conditions, although it has a similar structure to the F-type enzyme (Futai *et al.*, 1998; Forgac, 1999). Archaeal A-type ATPase, also called A-ATPase or A_0A_1-ATP synthase, is the third class of the H^+-translocating ATPase superfamily (Schafer & Meyering-Vos, 1992). A-ATPase operons and genes encoding structural proteins are highly conserved in archaeal species. Each enzyme of the three classes mentioned above consists of two sectors: a hydrophilic catalytic headpiece sector ($F_1/V_1/A_1$) and a membrane sector ($F_O/V_O/A_O$). The extramembrane $F_1/V_1/A_1$ sectors (containing a $\alpha_3\beta_3$ subcomplex in F_1; A_3B_3 in V_1/A_1) are connected *via* the F_1 γ subunit or the V_1/A_1 D subunit to the $F_O/V_O/A_O$ sectors. The major nucleotide-binding subunits are α and β in F_1-ATPase and A and B in V_1/A_1-ATPase. The subunits β and A are catalytic in each ATPase. A-type enzymes function as ATP synthases, as reported in *Methanosarcina mazei* Go1 (Becher & Muller, 1994), *Halobacterium salinarium* (*halobium*) (Mukohata *et al.*, 1987; Ihara & Mukohata, 1991), *H. saccharovorum* (Hochstein, 1992) and *Pyrodictium abyssi* (Dirmeier *et al.*, 2000). On the basis of its subunit composition and the primary sequences of its subunits, however, the A-type enzyme is more closely related to the V-type than to the F-type (Muller *et al.*, 1999). Therefore, A-ATPase is thought to have chimeric properties of F-ATPase and V-ATPase with regard to structure and function.

The three-dimensional structures of the A-ATPase complex or any component of A-ATPase are still unknown. Structural studies of A-ATPase will provide important information on the evolutionary relationship between F-, V-, and A-ATPases. As part of our interest in the structural biology of H^+-ATPase, especially A-ATPase, we crystallized the catalytic subunit of A-ATPase. The gene (*ph1975*) encoding ATPase subunit A in the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 (Kawarabayasi *et al.*, 1998) is predicted to contain a self-splicing protein element called intein coded by 1128 nucleotides (42 kDa) from its nucleotide sequence (Perler, 2002). The intein-removed mature subunit A consists of 588 amino-acid residues with a molecular weight of 66 kDa.

Here, we report the crystallization conditions and preliminary analysis of the crystallographic data from the catalytic subunit A of A-ATPase from *P. horikoshii*.

2. Materials and methods

2.1. Construction of expression vector

The gene encoding *P. horikoshii* A-type ATPase catalytic subunit A was amplified by PCR according to the method of Cann *et al.* (2001) with four primers: F1 with an *Nde*I restriction site (5′-GAGGTGAGTA**CATATG**-GTGGCGAAGGGGAG), R1 with a *Sal*I restriction site (5′-CTTGCTCA**GTCGAC**T-CACGCCCCATACTTC), F2 (5′-GGCCTTT-CGGCAGCGGTAAGACGGTGACTCAGC-ATCAGC) and R2 (5′-GCTGATGCTGAG-TCACCGTCTTACCGCTGCCGAAAGGCC). The DNA fragments encoding the N-terminal extein (723 nucleotides) and C-terminal extein (1044 nucleotides) were amplified by PCR from the genomic DNA of *P. horikoshii* with

the primers F1-R2 and F2-R1, respectively. The amplified fragments coding the N-terminal and C-terminal exteins were fused together *via* a second PCR using the primers F1 and R1. The fused DNA fragment was digested with *Nde*I and *Sal*I and ligated into *Nde*I/*Sal*I-digested pET22b(+) vector (Novagen).

2.2. Purification

The plasmid was transformed into *Escherichia coli* B834 Codon Plus (DE3)-RIL cells (Stratagene). The cells were grown at 310 K in 2 l LB medium containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. At an OD_{600} of 0.6, the cells were induced by the addition of 1 m*M* IPTG and growth continued at 310 K for 5 h. The cultured cells were resuspended in Tris–HCl buffer (50 m*M* Tris–HCl pH 8.5, 50 m*M* NaCl, 1 m*M* DTT, 1 m*M* EDTA) and disrupted using a French pressure cell (Aminco). The cell lysate was incubated at 343 K for 30 min, kept on ice and then centrifuged at 40 000*g* for 30 min. Ammonium sulfate was added to the supernatant to 60% saturation. The solution was centrifuged at 20 000*g* for 20 min. The protein pellet was resuspended and dialyzed against buffer *A* (50 m*M* Tris–HCl pH 9.0, 1 m*M* DTT, 50 m*M* NaCl). The solution was applied onto a HiTrap Q-XL column (Amersham Bioscience) equilibrated with buffer *A*. The bound protein was eluted with a linear NaCl gradient (0.05–1.0 *M*). The fractions containing ATPase subunit A were applied onto a column of HiLoad 26/60 Superdex200pg (Amersham Bioscience) equilibrated with buffer *B* (50 m*M* Tris–HCl pH 8.0, 1 m*M* DTT, 200 m*M* NaCl). The peak fractions were dialized against buffer *A* and loaded onto a Resource Q column (Amersham Bioscience) equilibrated with buffer *A*. The bound protein was eluted from a linear NaCl gradient (0.05–1.0 *M*). ATPase subunit A eluted at a concentration of 0.3 *M* NaCl. The peak fractions were combined and dialyzed against crystallization buffer (50 m*M* Tris–HCl pH 7.5) and concentrated to a final concentration of 10 mg ml^{-1}.

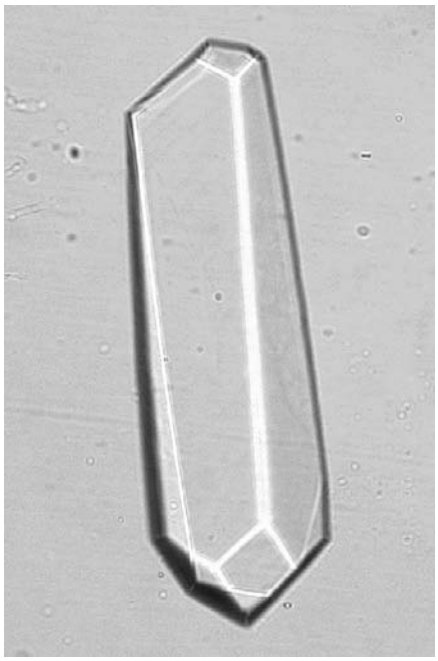

Figure 1
A crystal of subunit A. The dimensions of this crystal are 0.2 × 0.2 × 0.65 mm.

2.3. Crystallization and data collection

Initial crystallization screening was performed by the sitting-drop vapour-diffusion method with Wizard Screens I and II (Emerald BioStructures) and Crystal Screens I and II (Hampton Research). Each drop consisted of 1 µl protein solution and 1 µl reservoir solution and was equilibrated against 100 µl reservoir solution at 293 K. Small crystals of *P. horikoshii* ATPase subunit A were obtained from a reservoir solution containing 35%(*v*/*v*) MPD and 0.1 *M* acetate buffer pH 4.5 (Wizard II No. 21) after 1 d. Further optimization of this condition was performed to improve the size of the crystals. Larger subunit A crystals suitable for X-ray diffraction measurement were obtained by the hanging-drop vapour-diffusion method at 293 K from a solution containing 46–50%(*v*/*v*) MPD and 0.1 *M* acetate pH 4.5–4.7. 2 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 1.0 ml reservoir solution. These crystals were flash-cooled in a stream of nitrogen gas and diffraction data were collected at 100 K. An X-ray intensity data set from the native crystal of subunit A was collected using synchrotron radiation with a MAR CCD detector at beamline BL41XU of SPring-8, Japan. Data processing was performed using *MOSFLM* (Leslie, 1993), *SCALA* and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results

The high expression level of subunit A and the application of heat treatment facilitated the process of purification with no affinity tag, as described above. In a gel-filtration experiment, subunit A eluted at a retention time corresponding to about 66 kDa, which suggests that subunit A exists as a monomer in solution. The typical yield of purified subunit A was 9.5 mg per litre of culture.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell (2.64–2.55 Å).

Beamline	SPring-8 BL41XU
X-ray wavelength (Å)	0.9000
Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	a = 128.0, c = 104.7
Resolution limits (Å)	38.6–2.55
Observed reflections	311493
Unique reflections	28944
Completeness (%)	99.9 (99.9)
Redundancy	10.8 (11.1)
Average $I/\sigma(I)$	3.9 (2.0)
$R_{\rm meas}$† (%)	11.8 (38.3)

† $R_{\rm meas} = \sum_h [m/(m-1)]^{1/2} \sum_j |\langle I\rangle_h - I_{h,j}| / \sum_h \sum_j I_{h,j}$, where $\langle I\rangle_h$ is the mean intensity of the symmetry-equivalent reflections and m is the redundancy.

The size of the subunit A crystals was improved by optimizing the crystallization conditions. By further adjusting the MPD concentration, larger crystals were obtained that diffracted well. Interestingly, a high concentration of precipitant (up to 50% MPD) seems to be preferable for reducing the number of crystals in a drop reproducibly; *i.e.* for the production of high-quality large crystals of ATPase subunit A. Within a week, the subunit A crystals had grown to dimensions of 0.2 × 0.2 × 0.65 mm (Fig. 1). The native crystals belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 128.0$, $c = 104.7$ Å. The asymmetric unit contained one subunit A molecule. The crystal volume per unit molecular weight, $V_{\rm M}$, was calculated to be 3.3 Å^3 Da^{-1}, which corresponds to a solvent content of 62.1% (Matthews, 1968). The native data set was collected and processed to 2.55 Å. The crystallographic parameters and data statistics are given in Table 1.

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