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Purification, crystallization and preliminary crystallographic analysis of the vacuole-type ATPase subunit E from *Pyrococcus horikoshii* OT3

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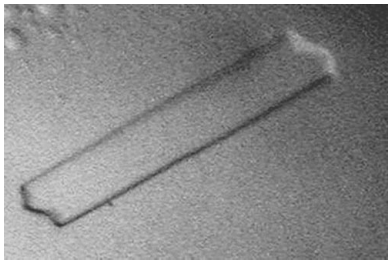

The vacuole-type ATPases in eukaryotic cells translocate protons across various biological membranes including the vacuolar membrane by consuming ATP molecules. The E subunit of the multisubunit complex V-ATPase from *Pyrococcus horikoshii* OT3, which has a molecular weight of 22.88 kDa, has been cloned, overexpressed in *Escherichia coli*, purified and crystallized by the microbatch method using PEG 4000 as a precipitant at 296 K. A data set to 1.85 Å resolution with 98.8% completeness and an $R_{\rm merge}$ of 6.5% was collected from a single flash-cooled crystal using synchrotron radiation. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 52.196, b = 55.317, c = 77.481 Å, and is most likely to contain one molecule per asymmetric unit.

1. Introduction

The vacuolar ATPases (V-ATPases) are found in many organisms and are involved in various physiological processes (Nishi & Forgac, 2002). They play major roles in endomembrane and plasma-membrane proton transport in eukaryotes, are present in the membranes of yeast vacuoles, clathrin-coated vesicles, lysosomes and plant vacuoles, and pump protons into vesicles (Yokoyama *et al.*, 2000). In eukaryotic cells, V-ATPases translocate protons across the membrane by consuming ATP. Eukaryotic V-ATPases account for various cell functions including the acidification of intracellular compartments, renal acidification, bone resorption and tumour metastasis (Yoshida *et al.*, 2001). V-ATPases and the F-type ATP synthases (F-ATPases) are evolutionarily related and share the rotary method of coupling ATP synthesis/hydrolysis and proton translocation across the membrane (Yoshida *et al.*, 2001; Imamura *et al.*, 2003; Yokoyama, Nakano *et al.*, 2003). Like F-ATPases, V-ATPases are oligomeric complexes composed of a water-soluble set of subunits (V_1) and membrane-integral subunits (V_0). However, the subunit composition and structure of V-ATPases are reported to be significantly different from those of F-ATPases (Yokoyama, Nakano *et al.*, 2003).

V-ATPases are also found in some eubacteria (prokaryotic V-ATPases) and archaea (Yokoyama *et al.*, 1998). The V-ATPase from *Pyrococcus horikoshii*, which lacks F-ATPase, is responsible for ATP synthesis in this archaeon (Yokoyama *et al.*, 2000). It is composed of nine different subunits, G-I-L-E-C-F-A-B-D, which are proteins with molecular sizes of 13, 71, 8, 20, 35, 12, 64, 54 and 25 kDa, respectively (Yokoyama, Nagata *et al.*, 2003). The V_1 region consists of four subunits with stoichiometry $A_3B_3D_1F_1$ (Yokoyama *et al.*, 1990), whereas the V_0 region, which is involved in proton-channel activity, is composed of five different subunits, two typical membrane-spanning subunits I and L and three hydrophilic subunits E, G and C. Understanding the structural and functional role of the V-ATPase subunits is essential as they may provide the uniqueness that distinguishes them from the F-ATPases. The precise arrangement of the subunits in the V-ATPase remains an important unclarified issue. Recently, the structure of V-ATPase subunit C from *Thermus thermophilus* was determined using X-ray crystallography (Iwata *et al.*, 2004) and a solution study of the yeast enzyme (Armbrüster *et al.*, 2004) has also been reported. Three-dimensional structures of the other V-ATPase subunits have not yet been reported. Here, we report

the purification and preliminary crystallographic study of V-ATPase subunit E from *P. horikoshii* OT3. V-ATPase subunit E was predicted to be a highly hydrophobic α-helical protein and one of the candidates for the F_1 γ-subunit homologue (Grüber *et al.*, 2000; Chaban *et al.*, 2002). Biochemical studies strongly suggest that the E subunit is a stator subunit rather than a rotor subunit (Arata *et al.*, 2002; Yokoyama, Nagata *et al.*, 2003). The three-dimensional structure of this subunit would help in understanding the molecular mechanism of the V-ATPase. The V-ATPase subunit E from *P. horikoshii* OT3 has a molecular weight of 22.88 kDa and consists of 198 amino-acid residues.

2. Experimental

2.1. Protein expression and purification

The plasmid encoding the V-ATPase subunit E was digested with *Nde*I and *Bgl*II and the fragment was inserted into the expression vector pET-11a (Novagen) linearized with *Nde*I and *Bam*HI. *Escherichia coli* BL21 Codon Plus (DE3)-RIL cells were transformed with the recombinant plasmid and grown at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin for 20 h. The cells were harvested by centrifugation at 4500*g* for 5 min at 277 K and were subsequently suspended in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl and 5 m*M* 2-mercaptoethanol; they were then disrupted by sonication and heated at 363 K for 11.5 min. The cell debris and denaturated protein were removed by centrifugation at 18 000*g* for 30 min. The supernatant solution was used as the crude extract for purification. The crude extract was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) and applied onto a Super Q Toyopearl 650 M (Tosoh) column equilibrated with 20 m*M* Tris–HCl pH 8.0 (buffer *A*). After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing V-ATPase subunit E was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) with buffer *A*. The sample was loaded onto a Resource Q column (Amersham Biosciences) equilibrated with buffer *A*. After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing the V-ATPase subunit E was desalted using a HiPrep 26/10 desalting column with 10 m*M* sodium phosphate pH 7.0. The sample was then applied onto a Bio-Scale CHT-5-I column (Bio-Rad) equilibrated with 10 m*M* sodium phosphate pH 7.0 and eluted with a linear gradient of 10–300 m*M* sodium phosphate pH 7.0. The sample was concentrated by ultrafiltration (Vivaspin, 10 kDa cutoff) and loaded onto a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Biosciences) equilibrated with buffer *A* containing 0.2 *M* NaCl. The homogeneity and identity of the purified sample were assessed by SDS–PAGE (Laemmli, 1970) and N-terminal sequence analysis.

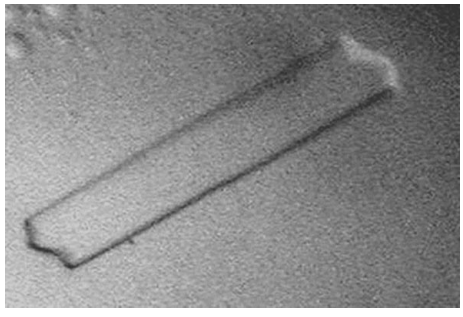

Figure 1
A crystal of V-ATPase subunit E from *P. horikoshii* OT3. The crystal has approximate dimensions of 0.5 × 0.1 × 0.01 mm.

Table 1
X-ray data-collection statistics for the V-ATPase subunit E.

Values in parentheses are for data in the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 52.196, b = 55.317, c = 77.481
Wavelength (Å)	1.000
Resolution range (Å)	30–1.85 (1.92–1.85)
Total No. observed reflections	90376
Total No. unique reflections	19506
Completeness (%)	98.8 (98.6)
Redundancy	4.7(4.8)
Mean $I/\sigma(I)$	11.6 (5.8)
$R_{\rm merge}$† (%)	6.5 (27.7)

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, where $I_j(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of measurement j and the mean intensity for the reflection with indices hkl, respectively.

Finally, the purified V-ATPase subunit E was concentrated by ultrafiltration to 25.43 mg ml^{-1} in buffer *A* containing 0.2 *M* NaCl and the protein stock solution was stored at 203 K.

2.2. Crystallization

Crystals of V-ATPase subunit E were obtained by the microbatch method using Nunc HLA plates (Nalge Nunc International). Initial crystallization conditions were obtained using the TERA (automatic crystallization) system from 144 independent conditions (Sugahara & Miyano, 2002). After optimization, each crystallization drop was prepared by mixing equal volumes (1.0 µl) of precipitant solution [27.5%(*w*/*v*) PEG 4000, 0.1 *M* CHES pH 8.5] and protein stock solution (§2.1). The crystallization drop was then overlaid with a 1:1 mixture of silicon and paraffin oils, allowing slow evaporation of water in the drop, and stored at 296 K.

2.3. X-ray data collection and analysis

Initial tests for the diffraction quality of the crystals were performed using a Rigaku R-AXIS VII image-plate area detector and Cu $K\alpha$ X-rays (Rigaku, Japan). A crystal of V-ATPase subunit E from *P. horikoshii* was flash-cooled in a cryoprotectant solution consisting 27.5%(*w*/*v*) PEG 4000, 0.1 *M* CHES and 30%(*v*/*v*) glycerol pH 8.5 at 100 K. X-ray diffraction data were collected to 1.85 Å from the vitrified crystals at SPring-8 beamline BL26B1 using a Rigaku R-AXIS V image-plate area detector. 140 frames were collected, with 1° oscillation and 30 s exposure time per frame. The wavelength of the synchrotron radiation was 1.000 Å and the distance between the crystal and detector was 250 mm. Data were indexed, integrated and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Well diffracting crystals of the V-ATPase subunit E appeared about 20–22 d after setup and grew to approximate dimensions of 0.5 × 0.1 × 0.01 mm after six weeks (Fig. 1). A complete data set to 1.85 Å resolution was collected at SPring-8 (Japan) beamline BL26B1 and the data-collection statistics are reported in Table 1. A total of 90 376 measured reflections in the resolution range 30–1.85 Å were merged into 19 506 unique reflections with an $R_{\rm merge}$ of 6.5%. The native crystal belongs to the orthorhombic space group $P2_12_12_1$. The asymmetric unit is most likely to contain a single chain of the V-ATPase subunit E; the crystal volume per unit molecular weight, $V_{\rm M}$, was calculated to be 2.5 Å^3 Da^{-1}, corresponding to a solvent content of 50.4% (Matthews, 1968). Crystals of the selenomethionine-labelled protein have been obtained under the same conditions as the native protein and appear to have similar diffraction proper-

ties. The structure will be determined using the MAD method (Hendrickson *et al.*, 1990).

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