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Received 6 June 2012
Accepted 17 July 2012

Crystallization and preliminary X-ray crystallographic analysis of subunit F (F_{1-94}), an essential coupling subunit of the eukaryotic V_1V_O -ATPase from *Saccharomyces cerevisiae*

V-ATPases are very complex multi-subunit enzymes which function as proton-pumping rotary nanomotors. The rotary and coupling subunit F (F_{1-94}) was crystallized by the hanging-drop vapour-diffusion method. The native crystals diffracted to a resolution of 2.64 Å and belonged to space group $C222_1$, with unit-cell parameters $a = 47.21$, $b = 160.26$, $c = 102.49$ Å. The selenomethionyl form of the F_{1-94} I69M mutant diffracted to a resolution of 2.3 Å and belonged to space group $C222_1$, with unit-cell parameters $a = 47.22$, $b = 160.83$, $c = 102.74$ Å. Initial phasing and model building suggested the presence of four molecules in the asymmetric unit.

1. Introduction

Eukaryotic V-ATPases are ubiquitous proton pumps which use the energy of ATP hydrolysis to translocate protons from the cytosol to intracellular compartments or extracellular space. V-ATPase is one of the key proteins that maintain pH homeostasis at both the cellular and the whole organism level (Saroussi & Nelson, 2009; Beyenbach & Wiczorek, 2006). The enzyme has been described to be an important component of the endosomal sensing machinery (Marshansky, 2007). Eukaryotic V_1V_O -ATPases consist of a water-soluble V_1 subcomplex and an integral membrane V_O subcomplex (Fig. 1; Lolkema *et al.*, 2003; Grüber & Marshansky, 2008). Hydrolysis of ATP is carried out in the V_1 headpiece consisting of an A_3B_3 hexamer, and the energy released is transmitted to the membrane-bound V_O domain to drive proton translocation. The coupling of energy occurs *via* the stalk structure, which is an assembly of V_1 subunits C–H and V_O subunits a and d that forms the structural and functional interface. The proposed subunit stoichiometries of the V_1 and V_O domains are $A_3B_3C_1D_1E_1F_1G_2H_x$ (Grüber & Marshansky, 2008) and $a_1d_1c_{4-5}c'_1c''_1$ (Nishi & Forgac, 2002), respectively. To date, the structures of subunits C (Drory *et al.*, 2004), G (Rishikesan *et al.*, 2009, 2010) and H (Sagermann *et al.*, 2001) of the stalk region of the *Saccharomyces cerevisiae* V_1V_O -ATPase have been determined to high resolution. Subunit C is formed by an upper head domain, a large globular foot and an elongated neck domain (Drory *et al.*, 2004). The elongated subunit G is mainly α -helical (Rishikesan *et al.*, 2009, 2010), whereas stalk subunit H is characterized by a large primarily α -helical N-terminal domain that forms a shallow groove and a C-terminal domain connected by a four-residue loop (Sagermann *et al.*, 2001).

Subunit F, together with subunit D, which is a part of the central stalk, is directly involved in the hydrolytic cleavage of ATP in the A_3B_3 headpiece of the V_1 sector, with the proton pumping in the V_O part (Radermacher *et al.*, 2001). It has been proposed that subunit F makes structural alterations during catalysis by way of interacting with subunits A, D and E in a nucleotide-dependent manner (Grüber *et al.*, 2000; Coskun *et al.*, 2004). Furthermore, in the free V_1 -ATPase it has also been shown that subunit F interacts with subunit H in an inhibitory mode by reducing ATP hydrolysis (Parra *et al.*, 2000; Jefferies & Forgac, 2007). Recently, we determined the solution shape of the *S. cerevisiae* V-ATPase subunit F (118 amino acids) using small-angle X-ray scattering, revealing subunit F to be a long egg-shaped protein which is connected *via* a linker to a hook-like segment (Basak *et al.*, 2011). By producing and determining the solution shape of a

C-terminally truncated form of subunit F (F_{1-94}), the hook-like region could be identified as being formed by the very C-terminus of subunit F (Basak *et al.*, 2011). The NMR solution structure of this C-terminal peptide F_{90-116} has been solved, displaying a loop region connected to an α -helical segment (Basak *et al.*, 2011). In order to understand the function of the essential coupling and rotation of subunit F, a high-resolution structure of F_{1-94} is needed. Here, we have produced, purified and crystallized F_{1-94} ; the crystals diffracted to 2.64 Å resolution. Since subunit F_{1-94} has only one methionine as the first amino acid, a selenomethionyl form of the F_{1-94} I69M mutant was produced and crystallized. These crystals diffracted to a resolution of 2.3 Å.

2. Materials and methods

2.1. Cloning and overexpression

The native F_{1-94} protein was produced as recently described (Basak *et al.*, 2011; Swiss-Prot accession No. P39111). The I69M mutant of *S. cerevisiae* F_{1-94} (with a calculated molecular mass of 10 656.03 Da and an isoelectric point of 4.59) was produced by the overlap extension polymerase chain reaction (PCR) method using a subunit F_{1-94} insert in pET9d1 vector (Basak *et al.*, 2011) as a template. In two PCR reactions, flanking primers 5'-GTAAGTAA-ACCATGGCTGAGAAACGTACTCTTATAGCTGTG-3' (forward primer *a*) and 5'-ATCGAGCTCTTAAATTTCTAAAATAGCAGG-GAACGC-3' (reverse primer *d*) incorporating *NcoI* and *SacI* (bold) restriction sites, respectively, were used that hybridized at each end of the F_{1-94} sequence. In addition, the internal primers 5'-GATATTG-CCATCCTTCTAATGAACCAACATATCGC-3' (forward primer *b*) and 5'-GCGATATGTTGGTTCATTAGAAGGATGGCAATATC-3' (reverse primer *c*) were used to hybridize at the site of the mutation

and contain mismatched bases (bold). In separate PCRs, two fragments of the subunit F_{1-94} gene were amplified using primer pairs *a/b* and *c/d*. The overlap allowed one strand from each fragment to act as a primer on the other, and extension of this overlap using the flanking primers (*a* and *d*) resulted in the mutant product. Following digestion with *NcoI* and *SacI*, the PCR product was ligated into the pET9d-His₃ vector (Grüber *et al.*, 2002). The resulting coding sequence included an N-terminal MKHHHHHHP tag. The mutation was verified by DNA sequencing. The verified plasmids were finally transformed into *Escherichia coli* BL21 (DE3) cells (Stratagene), which were grown on 30 µg ml⁻¹ kanamycin-containing Luria-Bertani (LB) agar plates. The selenomethionine protein was produced in *E. coli* strain BL21 (DE3) cells by growing a 5 ml culture overnight at 310 K in LB medium containing kanamycin. The cells were pelleted by centrifugation and were washed twice with M9 minimal medium. The cell suspension was used to inoculate 1 l pre-warmed (310 K) M9 minimal medium and the cells were grown at 310 K. When the OD₆₀₀ reached 0.6, the minimal medium was supplemented with 100 mg l⁻¹ lysine, phenylalanine and threonine, 50 mg l⁻¹ isoleucine, leucine and valine, and 50 mg l⁻¹ selenomethionine. This medium was then shaken for a further 15 min. Thereafter, protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM and growth was continued at 293 K overnight.

2.2. Protein purification

The native F_{1-94} protein of *S. cerevisiae* V-ATPase (VMA7p) was purified as recently described (Basak *et al.*, 2011). The SeMet F_{1-94} I69M mutant was purified using a similar method to the native protein, except that dithiothreitol (DTT) was used in all buffers to avoid the oxidation of selenium. The cells were pelleted by centrifugation at 8500g for 12 min at 279 K. Subsequently, they were lysed on ice by sonication for 3 × 1 min in buffer A [50 mM HEPES pH 7.0, 300 mM NaCl, 0.8 mM DTT, 2 mM PMSF, 2 mM Pefabloc SC [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; Biomol]]. Precipitated material was separated by centrifugation at 10 000g for 35 min. The supernatant was filtered (0.45 µm; Millipore) and passed over a 3 ml Ni²⁺-NTA resin column to isolate subunit F_{1-94} . The His-tagged protein was allowed to bind to the matrix for 1.5 h at 277 K and was eluted with an imidazole gradient (25–400 mM) in buffer A. Fractions containing F_{1-94} were identified by SDS-PAGE (Laemmli, 1970), pooled and concentrated as required using Centricon YM-3 (3 kDa molecular-mass cutoff) spin concentrators (Millipore). Imidazole was removed by gel-filtration chromatography using a Superdex 75 HR 10/30 column (GE Healthcare) and a buffer consisting of 50 mM HEPES pH 7.0, 300 mM NaCl, 1 mM DTT, 5 mM EDTA. The purity and homogeneity of the protein samples were analyzed by SDS-PAGE (Laemmli, 1970) and the gels were stained with Coomassie Brilliant Blue G250. Protein concentrations were determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, Illinois, USA).

2.3. Crystallization conditions

The purified native F_{1-94} was concentrated to 3 mg ml⁻¹ in buffer consisting of 50 mM HEPES pH 7.0, 300 mM NaCl, 5 mM EDTA using a 3 kDa cutoff concentrator. Initial crystallization screening was carried out by the hanging-drop vapour-diffusion method at 291 K using Crystal Screen and Crystal Screen 2 from Hampton Research, USA (Jancarik & Kim, 1991) and Wizard I and II from Emerald BioSystems, USA in 48-well VDX plates (Hampton Research, USA). Small crystals appeared after 3–4 d in Crystal Screen condition No. 6 [30%(w/v) polyethylene glycol (PEG) 4000, 0.2 M MgCl₂, 0.1 M

Figure 1
Schematic diagram of the V₁ (blue) and V_O sections (orange) of the eukaryotic vacuolar ATPases. In eukaryotic V₁V_O-ATPases the V₁ part can be reversibly disassembled from the V_O section as indicated by the arrow.

Tris-HCl pH 8.5]. The crystals were optimized by systematically performing grid screens by varying the concentrations of the precipitant and salt, the buffer pH and the protein concentration. Further optimization was performed by using various additives. The best crystals appeared in 30% PEG 4000, 0.1 M MgCl₂, 0.1 M Tris-HCl pH 8.8, 10% glycerol. The SeMet F₁₋₉₄ I69M mutant protein gave small crystals under the same conditions as used for the native F₁₋₉₄ and was therefore further optimized. A good crystal of diffraction quality appeared within a day in 30% PEG 4000, 0.05 M MgCl₂, 0.1 M Tris-HCl pH 8.8, 10% glycerol with a 5 mg ml⁻¹ protein concentration at 291 K.

2.4. X-ray data collection and analysis

The native crystals were quick-soaked in a cryoprotectant solution containing 25% glycerol in mother liquor and flash-cooled in liquid nitrogen. The SeMet F₁₋₉₄ I69M mutant crystals were transferred to stabilizing solution (32% PEG 4000, 0.05 M MgCl₂, 0.1 M Tris-HCl pH 8.8, 10% glycerol) and the crystals were cryoprotected in the same stabilizing solution containing 22% glycerol and flash-cooled in liquid nitrogen. A single-wavelength data set was collected from a native F₁₋₉₄ crystal in-house at 100 K on a Rigaku R-AXIS IV image-plate detector with a Rigaku RA-Micro 7 HFM rotating copper-anode generator (Rigaku/MSO). 360 frames were collected with an oscillation range of 1° and an exposure time of 5 min per frame. For the SeMet F₁₋₉₄ I69M mutant protein crystal, a three-wavelength multi-wavelength anomalous diffraction (MAD) data set was collected at 100 K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC), Hsinchu, Taiwan using an ADSC Quantum 315 CCD detector. A single crystal was used to collect data at appropriate inflection, peak and high remote wavelengths based on the selenium absorption spectrum. Data were collected as a series of 0.5° oscillation images with 5 s exposure time, covering crystal rotation ranges of 360° for the peak wavelength and 180° for the inflection and high remote wavelengths. The inflection and high remote wavelength data sets were initially collected, followed by the peak wavelength data set. All diffraction data were indexed, integrated and scaled using the *HKL-2000* suite of programs (Otwinowski & Minor, 1997). The results of data processing and the data statistics are summarized in Table 1.

Figure 2 Chromatogram of the gel-filtration analysis at 280 nm absorbance for the SeMet F₁₋₉₄ I69M mutant. Insert, SDS gel (17% total acrylamide and 0.4% cross-linked acrylamide) of the purified SeMet F₁₋₉₄ I69M mutant protein of the V-ATPase from *S. cerevisiae* after purification on Ni²⁺-NTA resin (lane 1) and the indicated fraction from the elution peak after Superdex 75 purification (lane 2).

Table 1

Statistics of data collection and processing for F₁₋₉₄ and the SeMet F₁₋₉₄ I69M mutant protein.

Values in parentheses are for the highest resolution shell.

	F ₁₋₉₄	SeMet F ₁₋₉₄ I69M mutant		
		Peak	Inflection	Remote
Wavelength (Å)	1.54178	0.978683	0.978836	0.963626
Space group	C222 ₁	C222 ₁	C222 ₁	C222 ₁
Unit-cell parameters (Å)				
<i>a</i>	47.206	47.223	47.146	47.153
<i>b</i>	160.264	160.833	160.312	160.334
<i>c</i>	102.494	102.738	102.429	102.439
Molecules in asymmetric unit	4	4	4	4
Solvent content (%)	46.03	46.28	45.86	45.87
Resolution limits (Å)	30.0–2.64	50.0–2.45	50.0–2.33	50.0–2.29
	(2.73–2.64)	(2.54–2.45)	(2.41–2.33)	(2.37–2.29)
No. of images	360	720	360	360
No. of reflections	26881	33722	38421	40195
Unique reflections	11788	14946	17096	17958
Multiplicity	6.4 (6.3)	12.7 (6.4)	6.5 (4.6)	6.4 (3.7)
Completeness (%)	98.9 (99.0)	99.4 (95.9)	99.5 (96.7)	98.8 (89.5)
<i>R</i> _{merge} † (%)	10.2 (28.0)	9.4 (58.6)	8.4 (43.4)	8.4 (45.9)
<i>I</i> /σ(<i>I</i>)	13.7 (7.1)	23.4 (1.8)	18.8 (2.2)	17.1 (1.6)

† $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $\langle I(hkl) \rangle$ is the mean intensity for reflection *hkl*.

(a)

(b)

Figure 3

Crystal photographs of (a) native F₁₋₉₄ (0.4 × 0.07 × 0.03 mm) and (b) SeMet F₁₋₉₄ I69M mutant protein (0.45 × 0.08 × 0.04 mm) from the *S. cerevisiae* V-ATPase.

3. Results and discussion

3.1. Protein characterization

The *S. cerevisiae* V-ATPase subunit F (F_{1-94}) and the SeMet F_{1-94} I69M mutant protein were purified initially by Ni^{2+} -NTA affinity chromatography using an imidazole gradient and subsequently by size-exclusion chromatography to isolate a pure protein of about 11 kDa (Fig. 2). Good diffraction-quality crystals were obtained by modifying the initial condition by varying the precipitant, salt and

protein concentrations and the buffer pH. Typical crystals of F_{1-94} and the SeMet F_{1-94} I69M mutant are shown in Fig. 3.

3.2. Crystallization and preliminary X-ray analysis

The native F_{1-94} crystallized in the orthorhombic space group $C222_1$, with unit-cell parameters $a = 47.21$, $b = 160.26$, $c = 102.49$ Å, and the crystals diffracted to a maximum resolution of 2.64 Å. A representative diffraction pattern is presented in Fig. 4(a). Assuming the presence of four molecules in the asymmetric unit, the solvent content is 46.03% and the Matthews coefficient (V_M) is $2.28 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968). Initial attempts at phasing by molecular replacement using the structures of subunit F of the related A-ATP synthases from *Pyrococcus furiosus* (PDB entry 2qai; Northeast Structural Genomics Consortium, unpublished work) and *Archaeoglobus fulgidus* (PDB entry 2i4r; Northeast Structural Genomics Consortium, unpublished work) were unsuccessful. A fluorescence scan of the selenomethionine-substituted native F_{1-94} crystals yielded a weak signal at the selenium absorption edge with no useful anomalous signal for phase determination. This is most probably owing to the fact that the only available methionine in this protein is at the very N-terminus of the amino-acid sequence. Therefore, the F_{1-94} I69M mutant was generated and the selenomethionyl form was produced. The SeMet F_{1-94} I69M mutant crystals diffracted to a resolution of 2.3 Å (Fig. 4b) and belonged to space group $C222_1$, with unit-cell parameters $a = 47.22$, $b = 160.83$, $c = 102.74$ Å. Based on the solvent content of 46.28% and the V_M of $2.29 \text{ Å}^3 \text{ Da}^{-1}$, four molecules per asymmetric unit were assumed. Phasing is being carried out by a three-wavelength MAD technique.

We thank the staff of beamline 13B1 at the National Synchrotron Radiation Research Centre (NSRRC), a national user facility supported by the National Science Council of Taiwan, ROC, for expert help with data collection. The Synchrotron Radiation Protein Crystallography Facility at NSRRC is supported by the National Research Program for Genomic Medicine. SB is grateful to Nanyang Technological University for the award of a research scholarship. This study was supported by A*STAR BMRC (09/1/22/19/609) to GG.

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Figure 4
Representative diffraction images of (a) native F_{1-94} and (b) the SeMet F_{1-94} I69M mutant from the *S. cerevisiae* V-ATPase. Resolution circles corresponding to 4.5 and 3.0 Å are shown.

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