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Purification and crystallization of the entire recombinant subunit E of the energy producer A_1A_o ATP synthase

Asha Manikkoth Balakrishna,‡ Cornelia Hunke‡ and Gerhard Grüber*

Nanyang Technological University, School of Biological Sciences, 60 Nanyang Drive, Singapore 637551, Singapore

‡ These authors made equal contributions.

Correspondence e-mail: ggrueber@ntu.edu.sg

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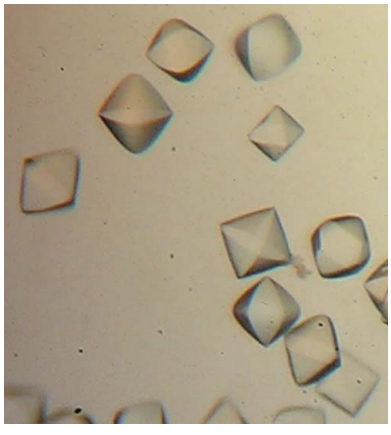
A_1A_o ATP synthases are the major energy producers in archaea. Subunit E of the stator domain of the ATP synthase from *Pyrococcus horikoshii* OT3 was cloned, expressed and purified to homogeneity. The monodispersed protein was crystallized by vapour diffusion. A complete diffraction data set was collected to 3.3 Å resolution with 99.4% completeness using a synchrotron-radiation source. The crystals belonged to space group *I*4, with unit-cell parameters a = 112.51, b = 112.51, c = 96.25 Å, and contained three molecules in the asymmetric unit.

1. Introduction

Archaeal-type ATP synthases (A_1A_o ATP synthases) combine adenosine diphosphate and inorganic phosphate to produce adenosine triphosphate using an electrochemical ion gradient (Deppenmeier & Müller, 2006). The membrane-integrated enzyme is composed of subunits A–F, H, *a* and *c* in the stoichiometry A_3:B_3:C:D:E:F:H_2:*a*:c_x. Like the related bacterial F_1F_o ATP synthase (F-ATP synthase; α_3:β_3:γ:δ:ε:a:b_2:c_x), it possesses a water-soluble A_1 domain containing the catalytic sites and an integral membrane A_o domain that is involved in ion translocation (Müller & Grüber, 2003). ATP is synthesized or hydrolyzed on the A_1 headpiece, which consists of an A_3:B_3 domain, and the energy provided for or released during this process is transmitted to the membrane-bound A_o domain. The energy coupling between the two active domains occurs *via* the so-called stalk or stator sector (Grüber & Marshansky, 2008).

The crystallographic and NMR structures of subunits C (Numoto *et al.*, 2004) and F (Gayen *et al.*, 2007), respectively, provided significant insight into the stalk domain. The low-resolution structure of the peripheral stalk subunit H has also been determined from small-angle X-ray scattering data, revealing a boomerang-like shape which is divided into two arms of 120 and 60 Å in length (Biuković *et al.*, 2007). An NMR solution structure of the 60 Å long N-terminal arm, which is composed of the N-terminal residues 1–47 and called $H_{1–47}$, has been solved, revealing an α-helical feature (Biuković *et al.*, 2009). Previous NMR titration and fluorescence correlation spectroscopy experiments performed in our laboratory suggested that $H_{1–47}$ interacts with the N-terminal segment of subunit E *via* proposed helix–helix formation (Gayen *et al.*, 2008). The association of subunits E and H is an important factor in the stabilization and formation of the stator in A-ATP synthases (Lokanath *et al.*, 2007; Grüber & Marshansky, 2008). To date, the crystallographic structure of the C-terminal part (residues 81–198) of subunit E of the A-ATP synthase from *Pyrococcus horikoshii* OT3 has been reported, showing the C-terminal domain of E to be composed of four antiparallel β-strands and six α-helices (Lokanath *et al.*, 2007). The N-terminal domain was not observed in this structure. Here, we describe the cloning, production and crystallization of the entire E subunit of the A_1A_o ATP synthase from *P. horikoshii* OT3, including residues 1–198.



2. Materials and methods

2.1. Cloning and overexpression

The coding region for subunit E was amplified by PCR using genomic DNA of *P. horikoshii* OT3 (ATCC JCM 9974). The amplified products, incorporating *Nco*I and *Sac*I restriction sites, were digested and ligated to pET 9-d1-His3 vector (Grüber *et al.*, 2002). The forward primer 5′-GCG **CCA TGG** CTA ACG GTG CTG AGC TAA TAA TTC AAG AA-3′ and the reverse primer 5′-GGC **GAG CTC** TCA CCC GAA TAA TAC CTT AGC TAT TGT-3′ were used to amplify and clone subunit E. The plasmid was transformed into *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene). To express the respective proteins, liquid cultures were grown at 310 K in LB medium containing kanamycin (30 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}) until an optical density OD_{600} of 0.6–0.7 was reached. To induce the production of proteins, cultures were supplemented with isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 m*M*, followed by incubation for a further 4 h at 310 K.

2.2. Protein purification

Bacterial cells containing recombinant subunit E were harvested from 2 l cultures by centrifugation at 8000*g* for 10 min at 279 K. The cells were lysed on ice in buffer *A* (50 m*M* Tris pH 7.5, 150 m*M* NaCl, 1 m*M* PMSF, 4 m*M* Pefabloc SC) by sonication with an ultrasonic homogenizer (Bandelin, KE76 tip) for 3 × 1 min. After sonication, the cell lysate was heated for 20 min at 343 K, followed by centrifugation at 10 000*g* for 35 min at 277 K. The resulting supernatant was passed through a filter (0.45 µm pore size) and supplemented with Ni^{2+}–NTA resin. The His-tagged protein was allowed to bind to the matrix for 2 h at 277 K by mixing on a sample rotator (Neolab) and eluted with an imidazole gradient (0–250 m*M*) in buffer *A*. Fractions containing the required protein were identified by SDS–PAGE (Laemmli, 1970), pooled and concentrated using a Millipore spin concentrator with a molecular-mass cutoff of 3 kDa. The sample was applied onto a Superdex HR75 gel-filtration column (10/30, GE Healthcare). Respective fractions were concentrated in Millipore spin concentrators. The purity of the protein sample was analyzed by SDS–PAGE (Laemmli, 1970). The SDS–PAGE was stained with Coomassie Brilliant Blue R250.

2.3. Circular-dichroism (CD) spectroscopy

Steady-state CD spectra were measured in far-UV light (185–260 nm) using a Chirascan spectropolarimeter (Applied Photophysics) according to Biuković *et al.* (2007).

2.4. Crystallization conditions

The purified protein was concentrated to 6 mg ml^{-1} in 50 m*M* Tris pH 7.5, 150 m*M* NaCl buffer using a 3 kDa cutoff concentrator. Preliminary screening for initial crystallization conditions was performed by the sitting-drop vapour-diffusion method using Wizard I and II screens (Emerald BioSystems, USA) at 298 K by mixing 1 µl droplets of concentrated protein solution with an equal volume of reservoir solution in 24-well Cryschem plates (Hampton Research, USA).

2.5. X-ray data collection and analysis

Prior to data collection, the crystals were quick-soaked in a cryoprotectant solution containing 25% ethylene glycol in mother liquor and flash-cooled in a nitrogen stream at 100 K. Data sets for subunit E crystals were collected at 140 K on beamline 13B1 at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) using an ADSC Quantum 315 CCD detector. All diffraction data were indexed, integrated and scaled using the *HKL*-2000 suite of programs (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1.

3. Results and discussion

3.1. Protein characterization

The SDS–PAGE of recombinant subunit E revealed a prominent band of about 23 kDa found entirely within the soluble fraction, which was purified by chromatography (Fig. 1*a*). The secondary structure of this subunit was determined from circular-dichroism

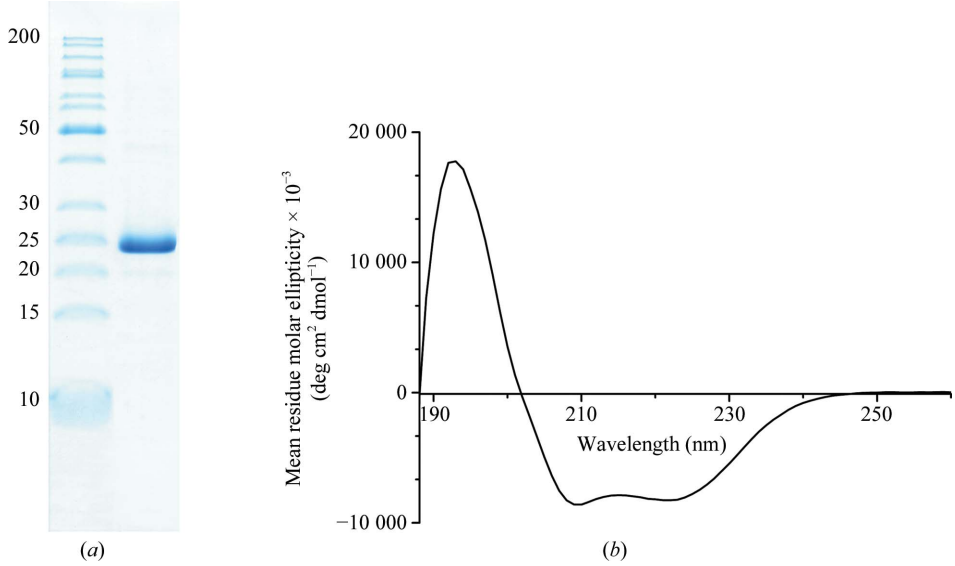


Figure 1
(*a*) SDS gel (17% total acrylamide and 0.4% cross-linked acrylamide) of the purified recombinant subunit E of the A-ATP synthase from *P. horikoshii* OT3 (lane 2). Lane 1, molecular-weight markers (kDa). (*b*) Far-UV CD spectrum of subunit E (2 mg ml^{-1}).

spectra (Fig. 1*b*). The overall spectrum was characteristic of a protein with a mixed α/β-structure. The average secondary-structure content was 67% α-helix, 16% β-sheet and 17% random coil, which was consistent with secondary-structure predictions based on the amino-acid sequence of subunit E.

3.2. Crystallization and preliminary X-ray analysis

Crystals of good diffraction quality appeared in about 5 d using a condition from the Wizard I screen consisting of 10% 2-propanol, 100 m*M* 2-morpholinoethanesulfonic acid pH 6.0 and 200 m*M* calcium acetate and this condition was optimized by systematically adjusting the protein concentration and buffer. Finally, optimized native crystals were grown at 13 mg ml^{-1} protein concentration in 10% 2-propanol, 100 m*M* 2-morpholinoethanesulfonic acid sodium salt pH 6.0 and 200 m*M* calcium acetate. A typical crystal is shown in Fig. 2 with dimensions of 0.1 × 0.1 × 0.05 mm. Analysis of the crystal by SDS–PAGE and MALDI mass spectrometry revealed a mass of 23.8 kDa, indicating that the entire subunit is present in the crystal.

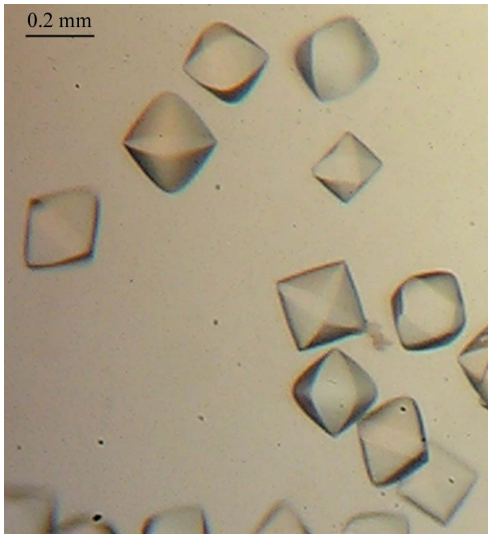


Figure 2
Crystals of subunit E of *P. horikoshii* OT3 A-ATP synthase. The crystals are approximately 0.2 × 0.3 × 0.3 mm in size.

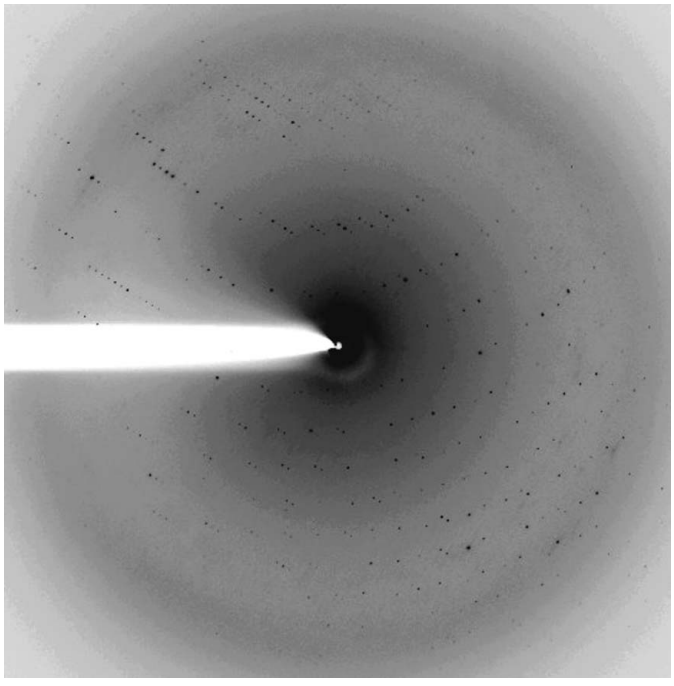


Figure 3
Diffraction image of a subunit E crystal.

Table 1
Statistics of crystallographic data collection for subunit E.

Values in parentheses are for the highest resolution shell (3.37–3.25 Å).

Wavelength (Å)	1
Space group	*I*4
Unit-cell parameters (Å, °)	a = 112.51, b = 112.51, c = 96.25, $\alpha = \beta = \gamma$ = 90
Resolution range (Å)	30–3.25
No. of unique reflections	9112
Total No. of reflections	54539
Completeness (%)	99.4 (99.6)
$R_{\rm merge}$† (%)	5.6 (46.5)
Multiplicity	6.0 (5.8)
Mean $I/\sigma(I)$	29.19 (3.39)
Mosaicity	0.7

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

These crystals diffracted to 3.3 Å resolution (Fig. 3) and belonged to space group *I*4, with unit-cell parameters a = 112.51, b = 112.51, c = 96.25 Å. Assuming the presence of three molecules in the asymmetric unit, the solvent content was 46.06% and $V_{\rm M}$ was 2.28 Å^3 Da^{-1} (Matthews, 1968). An initial solution was obtained by *MOLREP* (Vagin & Teplyakov, 1997) with an *R* factor of 42.7% and a correlation coefficient of 50.0% using the crystal structure of the C-terminal domain of subunit E from *P. horikoshii* OT3 (PDB code 2dm9; Lokanath *et al.*, 2007) as a model.

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