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Crystallization and preliminary X-ray analysis of the complex of the ε-subunit and the central domain of the γ-subunit from the *Escherichia coli* ATP synthase

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A complex of the ε-subunit and the central domain of the γ-subunit from the ATP synthase of *Escherichia coli* has been purified and crystallized and preliminary X-ray analysis has been carried out. The crystals belong to space group $C222_1$, with unit-cell parameters a = 76.7, b = 176.1, c = 67.1 Å (at 100 K). Determination of the structure of this protein complex promises to greatly improve the understanding of energy coupling between the F_0 and F_1 sectors within the enzyme complex.

1. Introduction

ATP synthases (F_0F_1-ATPases) are highly conserved enzyme complexes located in inner bacterial and mitochondrial membranes and chloroplast thylakoid membranes. Their function is to harness energy released by the movement of protons down a transmembrane electrochemical gradient to drive the synthesis of ATP, the universal biological energy currency (Senior, 1990). The enzyme consists of two major portions. In *E. coli*, the water-soluble portion F_1 has subunit stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$, with the catalytic sites located on the three β-subunits (Abrahams *et al.*, 1994), while the membrane-bound portion F_0 consists of one a-subunit, two b-subunits and 10–14 c-subunits. Proton flow at the interface between a ring of c-subunits; the a-subunit in the F_0 sector drives rotation of the ring (Vik & Antonio, 1994; Junge *et al.*, 1997; Dimroth *et al.*, 1999; Rastogi & Girvin, 1999) and a complex of the γ- and ε-subunits to which it is attached (Watts *et al.*, 1996; Schulenberg *et al.*, 1999). This in turn brings about cyclical conformational changes in the three catalytic sites in the F_1 sector that are required for catalysis (Boyer, 1993).

Crystal structures of large portions of F_1 purified from bovine heart (Abrahams *et al.*, 1994), rat liver (Bianchet *et al.*, 1998) and the bacterium *Bacillus* PS3 (Shirakihara *et al.*, 1997) have been determined and show that the three α- and three β-subunits are arranged alternately around a coiled pair of helices formed by the N- and C-termini of the γ-subunit. A partially complete low-resolution (3.9 Å) structure of ATP synthase from yeast mitochondria (Stock *et al.*, 1999) reveals the structural architecture of the c-subunit ring from F_0 and the overall shape of the central stalk joining the two sectors of the enzyme. However, the ε-subunit and the central domain of the γ-subunit, which together comprise the bulk of the mass of the central stalk, are not resolved to atomic resolution in any F_1 structure published thus far. In the intact complex, this portion of the enzyme communicates rotational conformational changes in F_0 to F_1 in order to bring about catalysis (Capaldi *et al.*, 2000). Structures of the isolated ε-subunit have been solved by NMR (Wilkens *et al.*, 1995) and by X-ray crystallography (Uhlin *et al.*, 1997). Crystallization and preliminary X-ray analysis of a complex of the ε-subunit and the entire γ-subunit from *E. coli* have been reported previously (Cox *et al.*, 1993). However, these crystals proved to be insufficiently stable for structure determination (J. M. Guss, personal communication). In the crystal structure of bovine F_1, the N- and C-termini of the γ-subunit are buried in a hydrophobic sleeve within the $\alpha_3\beta_3$ hexamer (Abrahams *et al.*, 1994). We therefore considered the possibility that crystals of the isolated γ/ε complex might be more stable if these segments of the γ-subunit were removed by genetic means. Here, we report the purification of a complex of the ε-subunit and the central domain of the γ-subunit from which the 11 N-terminal amino-acid residues and 29 C-terminal residues have been removed (γ'). This complex has been purified and crystallized and preliminary X-ray analysis has been carried out. Determination of the structure of these proteins will significantly advance the understanding of energy coupling in ATP synthase.

2. Materials and methods

2.1. Construction of bacterial overexpression plasmid

Plasmid pAN698, described by Cox *et al.* (1993), expresses a fusion of *Schistosoma japonicum* glutathione-S-transferase (GST)

and the γ-subunit of the *E. coli* ATP synthase, along with the ε-subunit, under the control of the IPTG-inducible *tac* promoter. This plasmid possesses a *Bam*HI restriction site at the junction between the *Sj26* gene encoding GST and the *uncG* gene encoding the γ-subunit and a second *Bam*HI site 277 bp downstream from the termination codon of the *uncC* gene encoding the ε-subunit. To facilitate removal of this latter *Bam*HI site, the plasmid was digested with *Xba*I and *Sst*I, which have unique restriction sites immediately upstream and downstream of the *Bam*HI site, respectively. The overhanging ends were filled using dNTPs and the Klenow fragment of *E. coli* DNA polymerase I. The blunt ends were religated using T4 DNA ligase, creating the plasmid pAN1310. Using oligonucleotide 5′-GATCGGATCCGTCCAGAACACGCAA-3′ as (+)-stranded primer and oligonucleotide 5′-CCGTCCGCGGTTAAATCAGGCTGCC-3′ as (−)-stranded primer, PCR was carried out using the plasmid pAN698 as a template. The resulting 760 bp fragment encompasses a portion of the *uncG* gene encoding amino acids 11–257 of the full-length γ-subunit. A *Bam*HI site is introduced covering codons for residues 11 and 12, thereby replacing Ala11 with Gly and retaining Ser at position 12. A stop codon replaces the codon for Lys258, immediately followed downstream by a restriction site for *Sal*II. Plasmid pAN1310 was digested with *Bam*HI and *Sal*II and the ∼5.6 kbp vector fragment was purified. The PCR product was also digested with *Bam*HI and *Sal*II and ligated into the vector to create plasmid pAN4005. This plasmid expresses a fusion of GST and the central portion of the γ-subunit (total protein MW of 54.1 kDa), along with the ε-subunit (MW of 15.1 kDa), both under the control of the *tac* promoter.

2.2. Purification of versions of the γ'/ε protein complex

Plasmid pAN4005 was used to transform *E. coli* strain XL-1 Blue (Stratagene, La Jolla, CA, USA). 2 l batches of the resulting strain were grown in 5 l conical flasks with orbital shaking in Luria broth supplemented with 1% glycerol and 100 µg ml^{-1} ampicillin at 310 K. When the density of the cultures reached an OD at 595 nm of 1.0, IPTG was added to a final concentration of 0.1 m*M* to induce expression of the GST-γ' and ε proteins. The cultures were then allowed to grow for a further 3 h before the bacteria were harvested.

The cells were resuspended in purification buffer (50 m*M* Tris–HCl pH 7.9, 20 m*M* $MgCl_2$) and disrupted in a French pressure cell. The cell debris and membranes were removed by ultracentrifugation at $3.6 \times 10^5 g$ for 3 h. The supernatant was incubated for 4 h at 277 K with 5 ml (wet volume) of glutathione cross-linked to agarose beads (Sigma, St Louis, MO, USA). The beads were washed thoroughly and resuspended in 20 ml purification buffer. Human thrombin (Sigma) was then added to a final concentration of 0.1 NIH units ml^{-1} and incubated overnight at 295 K to cleave the γ'/ε complex from the GST. The beads were then removed by centrifugation. The purity of the γ'/ε complex in the supernatant was estimated from SDS–PAGE to be approximately 95% (data not shown).

The crude γ'/ε complex was loaded onto a BioRad (Hercules, CA, USA) UNO-Q1 anion-exchange column equilibrated in purification buffer using the BioRad BioLogic DuoFlow chromatography system. The protein was eluted from a linear NaCl gradient in a single peak at 570 m*M* NaCl and then loaded onto a BioRad SE-1000 gel-filtration column equilibrated in purification buffer. The γ'/ε complex eluted as a single peak from the gel-sieve chromatography. The protein was concentrated to 40 mg ml^{-1} using a Centriprep concentrator (Amicon) with a 10 kDa cutoff and stored in 50 µl aliquots at 193 K.

2.3. Crystallization

Using the hanging-drop vapour-diffusion method, initial screens of crystallization conditions were carried out employing a sparse-matrix kit (Hampton Research, CA, USA). Crystals were obtained using condition 29 of Crystal Screen I (0.1 *M* HEPES, initial pH 7.5, plus 0.8 *M* sodium potassium tartrate). Optimum conditions for crystal growth were subsequently found with buffer containing 0.1 *M* Tris–HCl, 0.9 *M* sodium tartrate, 20 m*M* $MgCl_2$ and 20% glycerol at a final pH of 7.9. The presence of the glycerol in the crystallization buffer allowed the crystals to be cryofrozen without the need for any additional reagents. Crystals appeared after 2 d, but would change morphology after 4 d. Once the crystals changed morphology they did not diffract. We were unable to find any conditions that would stabilize the crystals. However, once cryofrozen, the crystals would continue to diffract for at least 5 d on a rotating-anode X-ray source. The following crystal-annealing procedure was necessary to attain diffraction data to 2.1 Å. Crystals were cryofrozen and then removed from the Cryostream for 3–5 s before being once again cryofrozen. Without this freeze/thaw/freeze step the diffraction limit was never beyond 8 Å.

Table 1
X-ray data-collection statistics.

Resolution shell (Å)	No. of reflections	Completeness (%)	R_{sym}	$I/\sigma(I)$
50.0–4.30	2959	90.7	0.033	31.2
4.30–4.42	3092	98.0	0.041	21.9
3.42–2.99	3112	99.0	0.054	24.9
2.99–2.71	3102	100.0	0.083	13.8
2.71–2.52	3102	100.0	0.117	10.4
2.52–2.37	3070	100.0	0.173	8.3
2.37–2.25	3045	99.5	0.250	5.8
2.25–2.17	2774	89.9	0.270	3.7
2.17–2.10	1925	63.1	0.310	2.7
Overall	26181	87.1	0.061	18.5

2.4. Collection of crystallographic diffraction data

Diffraction data from crystals of the γ'/ε complex were collected at 100 K using a MAR Research image-plate detector (240 mm) and Cu $K\alpha$ radiation from a Rigaku rotating-anode generator operating at 40 kV and 100 mA. Reflections could be measured to a resolution of 2.1 Å (see Table 1). Data were collected for up to 5 d, over which time no significant reduction in the quality of diffraction was observed.

3. Results and discussion

Crystallization of a complex of the ε-subunit and the central domain of the γ-subunit from the *E. coli* ATP synthase has been achieved in conditions markedly different from those reported previously for the complex with the full-length γ-subunit (Cox *et al.*, 1993). Cox and co-workers describe the use of a low-pH crystallization buffer [0.1 *M* sodium citrate, 0.1 *M* ammonium sulfate, 30%(*w*/*v*) PEG 8000 pH 5.6]. In our

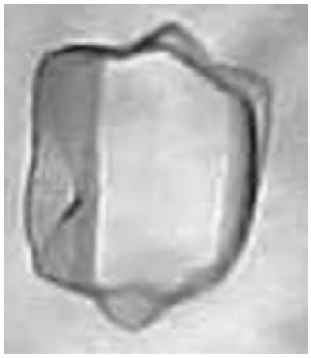

Figure 1
Crystal of the complex of γ'/ε-subunits of *E. coli* ATP synthase.

hands, however, crystallization could be achieved in the pH range 7.3–8.9, with optimum diffraction observed with crystals grown at pH 7.9. The γ'/ε complex shows markedly improved resistance to proteolysis during purification and the crystals are more stable both in the mother liquor and in the Cryostream during exposure to the X-ray beam, in comparison to the complex with the full-length γ-subunit (J. M. Guss, personal communication). The typical sizes of γ'/ε crystals were $0.2 \times 0.2 \times 0.3$ mm (see Fig. 1) and they belong to the *C*-centred orthorhombic space group $C222_1$, with unit-cell parameters $a = 76.7$, $b = 176.1$, $c = 67.1$ Å.

Molecular replacement using only the ε-subunit (PDB code 1aqt) as a search model was not successful. We are currently screening heavy-atom derivatives. The γ- and ε-subunits, which together form a central structural element within the enzyme, are responsible for communicating the conformational changes between the F_0 and F_1 sectors that are necessary for catalysis. Determination of the structure of the complex of these proteins will therefore significantly advance our understanding of energy coupling in ATP synthase.

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