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The expression, purification, crystallization and preliminary X-ray analysis of a subcomplex of the peripheral stalk of ATP synthase from bovine mitochondria

Jocelyn A. Silvester,[a] Veronica Kane Dickson,[a] Michael J. Runswick,[a] Andrew G. W. Leslie[b] and John E. Walker[a]*

[a]The Medical Research Council Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2XY, England, and [b]The Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Correspondence e-mail: walker@mrc-dunn.cam.ac.uk

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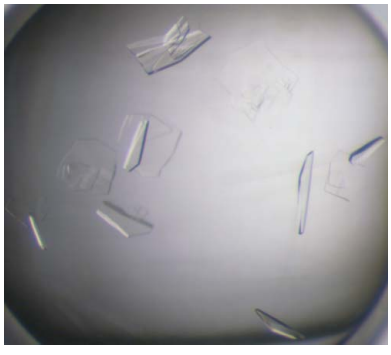
A subcomplex of the peripheral stalk or stator domain of the ATP synthase from bovine mitochondria has been expressed to high levels in a soluble form in *Escherichia coli*. The subcomplex consists of residues 79–184 of subunit b, residues 1–124 of subunit d and the entire F_6 subunit (76 residues). It has been purified and crystallized by vapour diffusion. The morphology and diffraction properties of the crystals of the subcomplex were improved by the presence of thioxane or 4-methylpyridine in the crystallization liquor. With a synchrotron-radiation source, these crystals diffracted to 2.8 Å resolution. They belong to the monoclinic space group $P2_1$.

1. Introduction

The F-ATP synthase in mitochondria, eubacteria and chloroplasts is a multisubunit complex consisting of a globular F_1 catalytic domain attached to an intrinsic membrane F_o domain by a central stalk and a peripheral stalk. The structure of the F_1 catalytic domain in the enzyme from bovine heart mitochondria has been studied in great detail (Abrahams *et al.*, 1994; Gibbons *et al.*, 2000; Menz *et al.*, 2001; Kagawa *et al.*, 2004). It is an assembly of the α-, β-, γ-, δ- and ε-subunits in the ratio 3:3:1:1:1 (Walker *et al.*, 1985). The γ-, δ- and ε-subunits form the central stalk, in which an elongated α-helical coiled coil in the γ-subunit penetrates into the $\alpha_3\beta_3$ subcomplex along its axis of threefold pseudo-symmetry. The lower region of the central stalk is exposed between the $\alpha_3\beta_3$ subcomplex and F_o and its foot interacts extensively with a ring of c-subunits buried in F_o (Stock *et al.*, 1999). Synthesis of ATP in the catalytic β-subunits at interfaces with non-catalytic α-subunits is driven by the rotation of the central stalk and the c-ring as an ensemble (Boyer, 1998; Walker, 1998). This rotation is impelled by the passage of protons from the inter-membrane space into the matrix *via* channels at the interface between the external surface of the c-ring and a hydrophobic membrane subunit known as a (or ATPase-6). The a subunit is linked to the external surface of the F_1 domain by the peripheral stalk, which is thought to counter the tendency of the $\alpha_3\beta_3$ subcomplex to follow the rotation of the central stalk (Collinson *et al.*, 1996; Walker & Kane Dickson, 2006). In bovine mitochondria, the peripheral stalk is an assembly of single copies of subunits OSCP (oligomycin-sensitivity conferral protein), b, d and F_6 (factor 6), consisting of 190, 214, 160 and 76 residues, respectively (Collinson *et al.*, 1996). Its membrane-extrinsic region consisting of subunits OSCP, b′ (residues 79–214 of the subunit), d and F_6 has been reassembled as a water-soluble subcomplex *in vitro* from recombinant proteins (Collinson *et al.*, 1994) and shown to form a 1:1 complex with F_1-ATPase (Collinson *et al.*, 1996). The subcomplex has been produced also in *Escherichia coli* by co-expression of the bovine subunits from a single operon in an expression plasmid (M. J. Runswick & J. E. Walker, unpublished results) and regions of the subcomplex have been deleted systematically to find subcomplexes that were suitable for X-ray crystallographic analysis (J. A. Silvester & J. E. Walker, unpublished work). The overexpression, purification and crystallization of one such subcomplex are described here. The subcomplex consists of residues 79–184 of subunit b, residues 1–124 of subunit d and the entire F_6 subunit (76 residues).



2. Materials and methods

2.1. Cloning and overexpression

The coding regions for subunits b (residues 79–184), d (residues 1–124) and the entire F_6 subunit (76 residues) of bovine ATP synthase were amplified by PCR from existing vectors (Walker, Gay *et al.*, 1987; Walker, Runswick *et al.*, 1987; Collinson *et al.*, 1994). The forward primers consisted of the 5′ sequence TAGGAATTC followed by an *Nde*I site and the first 24 bases of the 5′ end of the coding sequence. The reverse primers have the 3′ overhang CGA, a *Hin*dIII site, an *Nhe*I site and 24 bases (including the stop codon) of the 3′ end of the coding sequence. The amplified sequences were cloned sequentially into the pRUN expression vector, which is designed to facilitate the cloning of coding regions for multisubunit complexes into a single operon (M. J. Runswick, unpublished work). It is derived from pMW172 (Way *et al.*, 1990), which is itself a derivative of pRK172 (Studier *et al.*, 1990). The order from the promoter of the coding regions for the peripheral stalk subunits was F_6, d1–124 and b79–184. Then the expression plasmid was introduced into the host, *Escherichia coli* C41 (DE3) (Miroux & Walker, 1996), which was grown at 298 K in 2×TY medium containing ampicillin (100 µg ml^{-1}). Overexpression of the subcomplex was induced with isopropyl β-D-thiogalactopyranoside (150 µg ml^{-1}). The level of expression of the soluble subcomplex was ∼15–20 mg per litre of culture.

2.2. Purification

All procedures were performed at 277 K. Bacterial cells containing the overexpressed subcomplex were harvested from two 1 l cultures by centrifugation (2000*g*, 10 min) and resuspended in ∼50 ml of purification buffer [10 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 2 m*M* dithiothreitol, 10%(*v*/*v*) glycerol] containing two Complete protease-inhibitor tablets (Roche Diagnostics, Germany). The resuspended cells were broken by passage at 6.9 MPa through a French Pressure cell (SLM Instruments, Urbana, IL, USA). The broken cells were centrifuged (30 000*g*, 20 min; 260 000*g*, 60 min). The supernatant contained the subcomplex. Then the protein solution was dialysed twice in a SpectraPor dialysis membrane with a molecular-weight cutoff of 3500 Da for 4 h against purification buffer (4 l) containing two Complete protease-inhibitor tablets (Roche Biochemicals). The subcomplex was purified by ion-exchange chromatography in the purification buffer, firstly on S-Sepharose (15 × 2.6 cm internal diameter) with a salt gradient from 0 to 0.4 *M* and then, following dialysis of appropriate fractions for 8 h, on a Q-Sepharose column (10 × 2.6 cm internal diameter) with a salt gradient from 0 to 0.4 *M*. The subcomplex eluted from the S- and Q-Sepharose columns at 200 and 150 m*M* NaCl, respectively. Finally, the protein was concentrated to ∼1.0 ml on a Vivaspin membrane and passed in 0.5 ml aliquots through a column of Superdex-75 (30 cm × 10 mm internal diameter) in the same purification buffer described above containing 150 m*M* NaCl. It eluted in a single symmetrical peak at 10.5 ml. The combined peak fractions were concentrated to a final protein concentration of 14.0 mg ml^{-1} with a Vivaspin concentrator (2 ml capacity) and either stored at 277 K or used immediately to set up crystallization trials.

The purified subcomplex was analysed by SDS–PAGE and proteins were stained with Coomassie Brilliant Blue R-250.

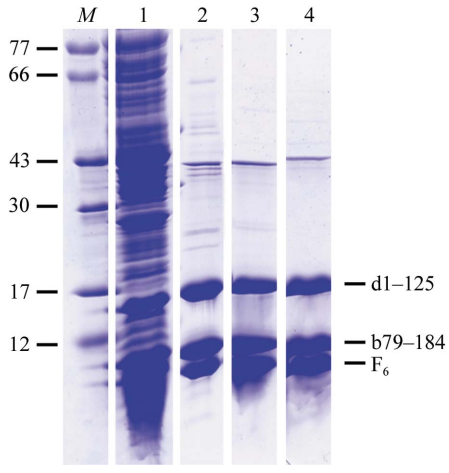


Figure 1
Purification of the subcomplex of the peripheral stalk from the F-ATPase from bovine mitochondria. Samples taken during the purification process were analysed by SDS–PAGE. Lane *M*, molecular-weight markers (kDa); lane 1, supernatant obtained by centrifugation of cells of *E. coli* C41(DE3) after overexpression of the subcomplex from an expression vector; lanes 2, 3 and 4, subcomplexes after successive purification on S-Sepharose, Q-Sepharose and Superdex-75, respectively. The positions of the molecular-weight markers are shown on the left and of the subunits of the subcomplex on the right. The band at 43 kDa in samples of the subcomplex was identified by peptide mass mapping as a multimeric form of subunit d which forms during SDS–PAGE analysis.

2.3. Crystallization

The initial crystals of the subcomplex formed spontaneously during storage at 277 K after concentration of the protein, as described above, in buffer lacking glycerol but containing 0.001% phenylmethylsulfonyl fluoride. In the standard conditions that were developed subsequently, the storage buffer contained 10%(*v*/*v*) glycerol but lacked phenylmethylsulfonyl fluoride. Immediately before setting up crystallization trials, the solution of the subcomplex was centrifuged (62 500*g*, 40 min) to remove any aggregated protein. Crystals were grown by vapour diffusion in sitting drops (CrystalClear Strips, Douglas Instruments). Crystals were harvested with cryoloops (Hampton Research) and passed through cryoprotectant solutions [100 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA, 150 m*M* NaCl, 15–19%(*w*/*v*) PEG 5000 MME] containing 15–25%(*v*/*v*) glycerol increased in 5% steps. Then the crystals were vitrified in liquid nitrogen.

2.4. Characterization of crystals

The diffraction properties of cryoprotected crystals were evaluated with a Rigaku RuH3R rotating-anode X-ray source equipped with a MAR 345 image-plate detector. Crystals that diffracted beyond 4 Å were examined at the European Radiation Synchrotron Facility (ESRF), Grenoble, France.

3. Results and discussion

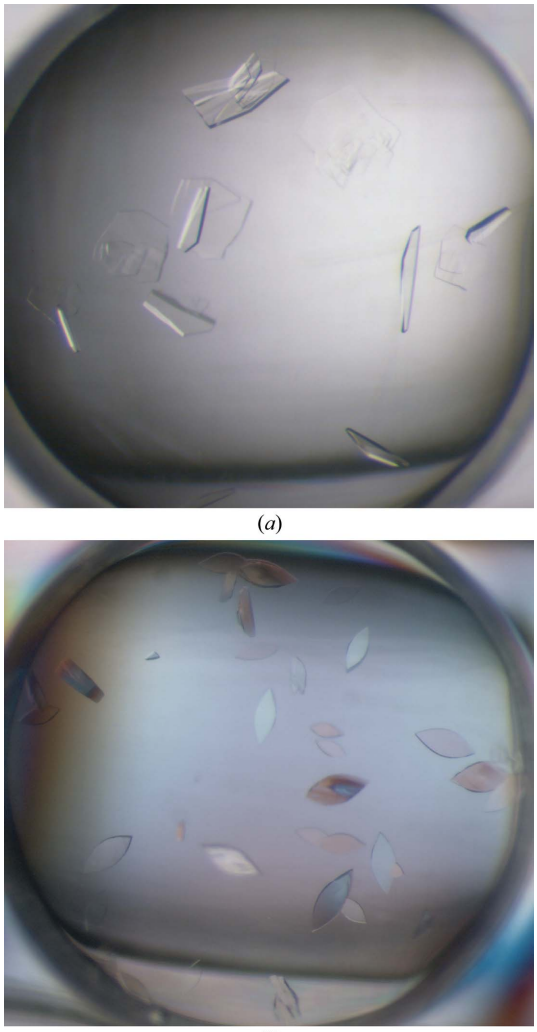
3.1. Protein overexpression and characterization

The subcomplex was overexpressed in *E. coli* C41(DE3) at ∼10–20 mg per litre of bacterial culture. It was found almost entirely in the water-soluble cytoplasmic fraction, from which it was purified by chromatography (Fig. 1), yielding ∼30–40 mg of purified material from two 1 l cultures. Only subunit F_6 retained its initiator methionine residue (denoted residue −1).

3.2. Crystallization

When a sample of the purified subcomplex was stored at 277 K in purification buffer lacking glycerol and containing phenylmethylsulfonyl fluoride (see §2.3) after concentration of the protein to ∼80 mg ml^{-1}, small crystals formed within 10 d. Based on these

conditions, the parameters influencing crystal formation (pH, temperature, buffer and protein concentrations, type and concentration of polyethylene glycols as precipitants, divalent cations) were examined. Crystals of substantially improved quality were obtained at 283 K in 4 µl sitting drops formed from 2 µl protein solution plus 2 µl of reservoir solution buffer consisting of 100 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 1 m*M* EDTA, 5%(*v*/*v*) glycerol and 11–17.5%(*w*/*v*) PEG 5000 monomethyl ether with a 100 µl reservoir. Therefore, the final concentration of glycerol in the drop was 7.5%. These crystals were clusters of thin hexagonal plates with typical dimensions of 150 × 100 × 30 µm. The inclusion of glycerol [final concentration 7.5%(*v*/*v*)] reduced the tendency for crystals to grow in clusters, as did the addition of 1,4-dioxane [1.8%(*v*/*v*)], which also improved crystal morphology. The presence of glycerol and 1,4-dioxane together gave many single crystals with increased thickness (>50 µm). In a further round of optimization of conditions, the influence of a number of cyclic compounds chemically related to 1,4-dioxane was investigated. They were piperazine, piperidine, pyrimidine, 1,4-dithiane, tetrahydro-2*H*-pyran-4-ol, 1,4-thioxane, 4-hydroxypiperidine, morpholine, 1,3-dioxane, 2-(2-bromoethyl)-1,3-dioxane, 4-methylpyridine (or γ-picoline), tetrahydro-2-(1*H*)-pyrimidinone and cyclo-isopropylidenemethylmalonate. The presence of either 1.0%(*v*/*v*) 4-methylpyridine or 2.0%(*v*/*v*) thioxane gave crystals of equivalent or better quality to those grown with 1,4-dioxane and the crystallization experiments were more reproducible. Therefore, the final optimal crystallization conditions were a reservoir solution (100 µl) consisting of 100 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 1 m*M* EDTA, 5.0%(*v*/*v*) glycerol, 11–17.5%(*w*/*v*) PEG 5000 MME and either 1.0%(*v*/*v*) 4-methylpyridine or 2.0%(*v*/*v*) thioxane, with a sitting drop consisting of 2 µl protein solution (14 mg ml^{-1}) and 3 µl reservoir solution. Crystals grew within 12 h and were formed completely in 72 h. The dimensions of the largest crystals were ∼300 × 200 × 80 µm. Both irregular and almond-shaped plates were observed (Fig. 2). The diffraction properties of crystals of both morphologies were very similar.



(*a*)

(*b*)

Figure 2
Crystals of the subcomplex of the peripheral stalk of the F-ATPase from bovine mitochondria. (*a*) and (*b*) show the 'hexagonal' and 'almond-shaped' morphologies, respectively. The scale bar represents 0.5 mm.

Table 1
X-ray diffraction data and processing statistics.

Values for the highest resolution bin (2.95–2.80 Å) are shown in parentheses. Note that data beyond 2.9 Å were measured only in the corners of the detector, hence the low completeness for the highest resolution bin.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 50.50, b = 79.35, c = 115.66, β = 93.1
Mosaic spread (°)	0.7
Resolution range (Å)	65–2.8
Unique reflections	21257 (2534)
Multiplicity	11.0 (9.6)
Completeness (%)	94.1 (77.4)
$R_{\rm merge}$†	0.10 (0.94)
$R_{\rm p.i.m.}$‡	0.03 (0.31)
$\langle I/\sigma(I)\rangle$	19.7 (2.4)
Wilson B factor (Å^2)	72.0

† $\sum_h \sum_i |I(h) - I(h)_i| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the mean weighted intensity after rejection of outliers. ‡ $R_{\rm p.i.m.}$ is the precision-indicating merging R factor (Weiss, 2001): $R_{\rm p.i.m.} = \sum_h [1/(N-1)]^{1/2} \sum_i |I(h)_i - I(h)| / \sum_h \sum_i I(h)_i$, where N is the redundancy.

3.3. Preliminary X-ray analysis

A complete data set was collected to 2.8 Å resolution from a single cryocooled crystal on beamline ID23 at the ESRF using a MAR Mosaic 225 3×3 CCD detector. The images were integrated with *MOSFLM* (Leslie, 1992) and scaled with *SCALA* (Evans, 2006). The X-ray diffraction data and the processing statistics are summarized in Table 1. The crystals are of sufficient quality to permit the structure of the complex to be obtained. They belong to the monoclinic space group $P2_1$ and the Matthews coefficient suggests the presence of two copies of the subcomplex in the asymmetric unit ($V_{\rm M}$ = 3.2 Å^3 Da^{-1}; $V_{\rm s}$ = 61.5% solvent). The diffraction was anisotropic, being strongest in the c direction.

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