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Crystallization and preliminary crystallographic studies of the mitochondrial F_1-ATPase from the yeast *Saccharomyces cerevisiae*

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A genetically modified (His_6-tagged) form of the mitochondrial F_1-ATPase (MW = 370 kDa) has been purified from the yeast *Saccharomyces cerevisiae* and crystallized in the presence of polythelene glycol (PEG) 6000 as a precipitant, 1 m*M* $NiCl_2$, 1 m*M* Mg AMP-PNP and 50 µ*M* Mg ADP. X-ray diffraction data were obtained on three separate occasions using synchrotron radiation, with a progression in the quality of the diffraction data, which improved from 3.3 to 3.0 to 2.8 Å. On the second occasion, the diffraction was improved by a crystal-annealing procedure. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 110.6, b = 294.2, c = 190.4 Å, β = 101.6°. The asymmetric unit contains three molecules of yeast F_1, with a corresponding volume per protein weight (V_M) of 2.8 Å^3 Da^{-1} and a solvent content of 55%.

1. Introduction

Mitochondrial ATP synthase (EC 3.6.3.14) is the enzyme responsible for the synthesis of 90% of cellular ATP under aerobic conditions. The enzyme is composed of a water-soluble portion, F_1-ATPase (EC 3.6.1.34; MW = 370 kDa), and a membrane portion, F_0 (MW = 190 kDa). F_1-ATPase is composed of five different subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ (Walker *et al.*, 1985). The catalytic sites are located in the β-subunits, but have some contributions from the α-subunits (Abrahams *et al.*, 1994). As a result, there are three catalytic sites composed of chemically identical α/β pairs within the enzyme. The subunit stoichiometry of F_0 is not as certain, but $a_1b_1c_{9-12}$ (Collinson *et al.*, 1994; Stock *et al.*, 1999; Walker *et al.*, 1995) appears to form a minimal structure on which a functional F_0 is based; in the yeast enzyme, there are ten copies of subunit *c* (Stock *et al.*, 1999).

The high-resolution crystal structure of F_1-ATPase was first determined for the bovine enzyme at a resolution of 2.8 Å (Abrahams *et al.*, 1994). This study provided critical evidence in support of the binding-change mechanism of ATP synthesis (Boyer *et al.*, 1973), including the clear asymmetry of the enzyme. Although the three catalytic sites are composed of identical subunits, the conformations of the catalytic sites are not identical: one site was filled with AMP-PNP (the β_{TP} subunit) and the second with ADP (the β_{DP} subunit), while the third was empty (the β_E subunit). The structure also provided a clear insight into the role of the γ-subunit, which was within the α/β core of F_1, and provided the first details of the asymmetric associations of the γ-subunit with the catalytic β-subunits. This suggested a mechanism by which rotation of the γ-subunit within the core of F_1 results in sequential conformational changes at the catalytic sites. Indeed, ATP-dependent rotation of the γ-subunit was later demonstrated by single-molecule fluorescent studies (Hirono-Hara *et al.*, 2001; Noji *et al.*, 1997).

Since the initial publication of the structure of bovine F_1-ATPase (Abrahams *et al.*, 1994), ten additional high-resolution structures of the bovine enzyme in the presence of a variety of natural and artificial inhibitors have been determined by the same laboratory (for examples, see Walker, 1998) and a few lower resolution structures of the enzyme or subcomplexes of the enzyme from rat liver (Bianchet *et al.*, 1998), spinach chloroplast (Groth & Pohl, 2001), thermophilic *Bacillus* PS3 (Shirakihara *et al.*, 1997) and *Escherichia coli* (Hausrath *et al.*, 1999) have also been obtained. With the exception of the *E. coli* structure (which is only available at low resolution), none of the structures clearly displayed the asymmetric features of the bovine F_1-ATPase, either because these were averaged by the crystallographic symmetry (in the case of the rat and chloroplast enzymes) or (in the case of the PS3 enzyme) because the complex did not contain the single-copy subunits (γ, δ, ε) or nucleotides and is therefore likely to be symmetric. Because it has not proved possible to reconstitute the bovine enzyme from recombinantly expressed subunits, no model system was available that allowed the analysis of genetically modified forms of F_1-ATPase by X-ray crystallographic

analysis. For this reason, an effort was undertaken to crystallize the yeast F_1-ATPase with the future goal of analysis of mutant-enzyme structures. This report presents the purification and crystallization of the yeast F_1-ATPase and the initial analysis of the diffraction data. The diffraction data were improved from nominal resolution limits of 3.3 to 2.8 Å.

2. Experimental

2.1. Expression and purification

The gene encoding the β-subunit was genetically modified by the addition of six His codons to the region encoding the mature N-terminus, thereby allowing purification of the F_1-ATPase by Ni-chelate affinity chromatography. This gene was integrated into the genome of a yeast strain, DMY301, which contained a null mutation in the gene encoding the β-subunit (ATP2). Details of the genetics and molecular biology will be presented elsewhere.

Yeast was grown at room temperature in YPEG medium (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol, 30 l) in 50 l carboys for 48 h. The washed yeast was suspended in two volumes of breaking buffer (0.65 *M* sorbitol, 0.1 *M* Tris–HCl, 5 m*M* EDTA, 5 m*M* ε-aminocaproic acid, 5 m*M* *p*-aminobenzamidine, 0.2% bovine serum albumin pH 8.0). The yeast was broken using glass beads (0.45 mm) and the Bead Beater as described in Mueller (1988). The mitochondria were isolated by centrifugation and washed with wash buffer (0.65 *M* sorbitol, 0.02 *M* Tris–HCl, 1 m*M* EDTA, 5 m*M* ε-aminocaproic acid, 5 m*M* *p*-aminobenzamidine pH 7.5). The mitochondria were suspended in wash buffer to a protein concentration of approximately 30 mg ml^{-1} and stored at 193 K.

Submitochondrial particles (SMP) were produced essentially as described by Mueller (1988). Mitochondria were suspended in sonication buffer (0.25 *M* sucrose, 0.05 *M* phosphate, 5 m*M* ε-aminocaproic acid, 5 m*M* *p*-aminobenzamidine, 1 m*M* EDTA pH 7.5) to a protein concentration of 10 mg ml^{-1}. After centrifugation at 100 000*g*, the SMP were washed twice with sonication buffer minus the EDTA (SB). The particles were suspended in SB at a protein concentration of 15–20 mg ml^{-1}.

The F_1-ATPase was released from the membrane by extraction with chloroform (Ryrie, 1977). The F_1-ATPase was purified at 277 K on an Ni–NTA Superflow (Qiagen, Valencia, CA, USA) column equilibrated with 97% solution *A* and 3% solution *B* (solution *A*, 10% methanol, 0.3 *M* NaCl, 0.25 *M* sucrose, 50 m*M* phosphate, 5 m*M* ε-aminocaproic acid, 5 m*M* *p*-aminobenzamidine, 1 m*M* PMSF pH 7.5; solution *B*, 10% methanol, 0.3 *M* NaCl, 0.3 *M* imidazole, 0.25 *M* sucrose, 50 m*M* phosphate, 5 m*M* ε-aminocaproic acid, 5 m*M* *p*-aminobenzamidine, 1 m*M* PMSF pH 7.5). The column was washed with 300 ml buffer *A* containing 3% buffer *B*, eluted with buffer *B* and collected in 3 ml fractions. The fractions containing the enzyme were pooled and adjusted to 2 m*M* ADP.

The F_1-ATPase was concentrated using a centrifugal concentrator (100 kDa molecular-weight cutoff, Vivascience, Hanover, Germany) and purified by gel filtration at room temperature on a 1.6 × 100 cm Superdex 200 column equilibrated with SDX buffer (0.25 *M* sucrose, 0.2 *M* NaCl, 0.05 *M* Tris, 1 m*M* EDTA, 1 m*M* ATP, 1 m*M* PMSF pH 8.0) at 1 ml min^{-1}. The fractions containing the non-proteolyzed enzyme were pooled, precipitated with 70% saturated ammonium sulfate and stored at 277 K.

2.2. Crystallization and collection of X-ray diffraction data

The enzyme was collected by centrifugation and dissolved in HEM buffer (50 m*M* HEPES pH 7.3, 2 m*M* EDTA, 4 m*M* $MgCl_2$, 5 m*M* *p*-aminobenzamidine, 5 m*M* ε-aminocaproic acid, 0.1 m*M* thymol, 1 m*M* AMP-PNP and 0.05 m*M* ADP) at a protein concentration of 8 mg ml^{-1}. For the initial data set, the enzyme was crystallized by vapor diffusion using the sitting-drop technique at 296 K in a 50% (1 + 1 µl) mixture of 11% PEG 6000, 20% glycerol, 4% ethanol, 0.1 *M* sodium acetate, 0.5 m*M* $NiSO_4$. The crystals grew to about 0.05 × 0.05 × 0.1 mm in size and were cryoprotected in Paratone-N oil (Hampton Research, Aliso Viejo, CA, USA) and plunged into liquid nitrogen. For the second and third data sets, the enzyme was treated in the same way and crystallized by the sitting-drop method in a 50% mixture (3 + 3 µl) of 11% PEG 6000, 20% glycerol, 8% methanol, 0.1 *M* sodium acetate, 1 m*M* $NiSO_4$. After 24 h at 296 K, the samples were seeded with pieces of crystals grown under similar conditions except that they contained 12% PEG 6000. These crystals grew to dimensions of 0.5 × 0.3 × 0.2 mm. The crystals were frozen as before, except that the crystals were first placed in a mixture of 70% Paratone-N oil with 30% paraffin oil before freezing in 100% Paratone-N. The lighter oil mixture was necessary to prevent the crystals from cracking.

All data sets were collected at 100 K using synchrotron radiation. The initial data set was collected from three crystals (crystals 105, 110 and 111) at the European Synchrotron Radiation Facility (ESRF, Grenoble, France), beamline ID14-4. 30° of data (oscillation angle 0.3° per image) were collected from each crystal using an ADSC CCD detector. The second data set was obtained from a single crystal (crystal 308) at the Advanced Photon Source (APS, Argonne, IL, USA) beamline ID-22 using a MAR 165 CCD detector. Over 180° of data were collected using an oscillation angle of 0.3°. Prior to data collection, the crystal was annealed by blocking the Cryostream for 7 s. This annealing process greatly reduced the mosaic spread (defined as the full width of a reflection) from an initial value estimated visually as 0.6–1.0° to a value of 0.28° as estimated by *MOSFLM* from a single image. The final data set was obtained from a single crystal (crystal 304) at the APS (beamline ID-5) using a MAR 225 mosaic CCD. In this case, annealing was not required. More than 180° of data were collected using an oscillation angle of 0.3°. For all three data sets, the collection strategy was determined using the 'Strategy' option in *MOSFLM* (Leslie, 1992). In the first case, in which three crystals were used, Strategy provided the φ range required to complete the data set (given that data had already been obtained), while in the second and third data sets Strategy provided the starting point in φ for the data collection in order to collect the maximum completeness for a 90° rotation in case radiation damage was high (the crystal was in a random orientation). Furthermore, in the final data set, data collection was suspended owing to beamline problems, the crystal was recovered and the collection was completed one month later. *MOSFLM* was used to determine the range in φ required to complete the data. The crystal suffered no apparent damage from dismounting and remounting and little radiation damage from the initial images taken.

3. Results

The His_6 tag on the β-subunit of yeast F_1-ATPase allowed affinity chromatography on a Ni–NTA column with elution of the enzyme with imidazole. This gave a fully active enzyme, contrary to a prior report in which the enzyme was eluted from the column using EDTA (Bateson *et al.*, 1996). The final purification step was by gel filtration, which eliminated aggregates and

Table 1
Data-collection statistics for yeast F_1-ATPase.

Values in parentheses are for the highest resolution shell. For the third data set, the two values given correspond to the 2.91–2.74 and 2.74–2.60 Å resolution shells, respectively.

X-ray wavelength (Å), beamline	0.93940, ESRF (ID14-4)	1.000, APS (ID-22)	1.000, APS (ID-5A)
Temperature (K)	100	100	100
Resolution range (Å)	30.0–2.98 (3.15–2.98)	30.0–3.02 (3.10–3.02)	30.0–2.60
Space group	$P2_1$	$P2_1$	$P2_1$
No. crystals	3	1	1
Unit-cell parameters (Å, °)	Crystal 105, a = 110.9, b = 293.1, c = 189.4, β = 101.8 Crystal 110, a = 111.1, b = 293.0, c = 189.7, β = 102.0; Crystal 111, a = 111.4, b = 292.3, c = 188.4, β = 102.0	Crystal 308, a = 111.8, b = 294.4, c = 190.8, β = 101.7	Crystal 304, a = 110.6, b = 294.2, c = 190.4, β = 101.6
Observed/unique reflections	503806/207254	811824/231840	1242188/346124
Completeness (%)	86.9	98 (98)	94 (92.3, 67.3)
Mean multiplicity	2.4	3.5	3.6
Mean $I/\sigma(I)$	5.6 (0.9)	11.6 (1.7)	11.7 (2.6, 1.4)
$R_{\rm merge}$† (%)	17.9 (87.1)	6.1 (35.8)	7.4 (40.6, 52.6)

† $R_{\rm merge} = \sum\sum_i |I_{hi} - \langle I_h\rangle| / \sum\sum I_{hi}$, where $\langle I_h\rangle$ is the weighted mean intensity for all observations of reflection h.

proteolytic products. The final product was pure based on SDS–gel electrophoresis with a specific activity of about 80 units per milligram of protein. Typically, 50 mg of purified protein was obtained from the 800 g of yeast derived from a 30 l culture.

The crystals were grown under two similar but different conditions. For the first data set, the crystals were grown in a small-format sitting-drop tray (3 µl drops) (Douglas Instruments, UK). The crystals grew to dimensions of 0.05 × 0.05 × 0.1 mm within one week, but did not grow larger. The crystals were frozen using Paratone-N oil, but good diffraction was only observed if the crystals were at least three weeks old. This requirement for aging may arise from the need to partially dehydrate the crystallization medium, thereby increasing the glycerol concentration.

The first data set was obtained by merging the diffraction data from three different crystals named 105, 110 and 111. The crystals were small and only about 30° of data could be obtained before considerable radiation damage occurred. Crystal 105 diffracted the best of these three crystals, with reflections observed to 3.0 Å. The diffraction images were indexed in a primitive monoclinic Bravais lattice, with unit-cell parameters as shown in Table 1. The data were integrated with *MOSFLM* (Leslie, 1992) and scaled and merged with *SCALA* contained within the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The space group was determined to be $P2_1$. The unit-cell parameters of the three crystals were quite similar, but there was a significant variation (1.3 Å) in the c parameter. The data from the three crystals did not merge as well as expected, giving an $R_{\rm merge}$ of 7.3% at low resolution (36–9.4 Å) compared with values of 4–5% for data from individual crystals. The variation in unit-cell parameters and the high $R_{\rm merge}$ suggests that there are slight differences in the packing of the molecules in the three crystals, making them non-isomorphous. It was clear from these data that a single larger crystal was required in order to obtain a good high-resolution data set.

The second and third data sets were obtained from crystals 308 and 304, respectively. These crystals were grown and frozen under similar conditions as 105, 110 and 111, but with three changes. Firstly, the crystals were grown from a larger drop size (6 µl) in a larger sitting-drop tray (Hampton Research, Aliso Viejo, CA, USA). Secondly, the crystals were grown by microseeding. These changes gave larger crystals, but the crystals cracked when placed directly into Paratone-N oil for cryoprotection. The third change was to place the crystals first in a mixture of Paratone-N and paraffin oils (7:3) for 1–5 min and then into 100% Paratone-N oil. The crystals did not crack under these conditions. These three changes gave single crystals suitable for collection of a high-resolution data set.

Initially, diffraction images of crystal 308 showed a high mosaic spread, but this was improved by annealing, which reduced the mosaic spread to less than 0.3°. Annealing/dehydration has been used successfully in the past to improve the resolution limit and mosaic spread (Harp *et al.*, 1998; Yang *et al.*, 2002). The $R_{\rm merge}$ of 35.8% for the high-resolution shell (Table 1) was much better than the previous data set because the data were obtained from a single crystal and the radiation damage was less severe.

The final data set was obtained from crystal 304. This data set was collected as for crystal 308 except that a larger detector was used, the MAR 225 CCD. This larger detector allowed the crystal-to-detector distance to be increased, thereby increasing the separation of the reflections. This was important as one of the unit-cell parameters was quite large (b = 293 Å). Data were collected and processed to a resolution of 2.6 Å (Table 1). The data were not reliable in the highest resolution shell, with an $R_{\rm merge}$ of 52.6% and an $I/\sigma(I)$ of only 1.4. However, the data were quite good in the 2.91–2.74 Å resolution shell, with an $R_{\rm merge}$ of 40.6% and an $I/\sigma(I)$ of 2.6.

The structure of the yeast F_1-ATPase was solved by molecular replacement with *AMoRe* (Navaza, 2001) using the native structure of the bovine enzyme (PDB code 1e1q; Braig *et al.*, 2000) minus the nucleotides as a starting model. The cross-rotation function using data in the 20–4 Å resolution range gave two clear solutions with Patterson correlation coefficients of 0.213 and 0.172 (the next highest solution was 0.104). The corresponding correlation coefficients on F (CC_F) were 0.307, 0.307 and 0.271. The translation function for the first molecule gave a single solution (with a cutoff set to half the maximum peak value) with a CC_F value of 0.218. The translation function for the second molecule (with the first molecule fixed) also gave a single solution and increased the correlation coefficient to 0.410. Rigid-body refinement of the two molecules gave a final CC_F value of 0.532 and an R factor of 44.5% for data in the 20–4 Å resolution range.

The resulting model gave sensible packing in the monoclinic unit cell and corresponded to an estimated solvent content of 70%, which did not seem unreasonable in view of the weak diffraction. However, initial refinement of the model only reduced the $R_{\rm free}$ to 37% and significant additional density became visible between molecules in the asymmetric unit, suggesting that in fact there were three molecules present rather than two. The translation function was therefore calculated separately for the rotation-function solutions ranked third to sixth (CC_F values 0.271, 0.264, 0.262, 0.257), keeping molecules one and two fixed. Only one of these, corresponding to the fourth-ranked solution of the rotation function, gave an increase in the CC_F value from the translation function (0.436 compared with 0.410 for molecules one and two alone). Rigid-body refinement of the

three molecules resulted in a final CC_*F* value of 0.574 and an *R* factor of 43.0% for data in the 20–4 Å resolution range. The third molecule was a good fit to the additional density seen between molecules 1 and 2. The presence of three molecules of yeast F_1 in the asymmetric unit gives a crystal volume per protein weight (V_M) of 2.8 Å^3 Da^{-1}, which corresponds to a solvent content of 55% (Matthews, 1968).

The model is currently being refined using data to 2.8 Å resolution. However, at this stage it is clear that the structure is generally similar to the bovine enzyme (Abrahams *et al.*, 1994), but has some distinctive differences. This advance now provides a tool to couple mutagenesis with structure determination of the F_1-ATPase.

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