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Crystallization note

Purification, crystallization, and properties of F_1-ATPase complexes from the thermoalkaliphilic *Bacillus* sp. strain TA2.A1

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Abstract

Recently, we reported the cloning of the *atp* operon encoding for the F_1F_0-ATP synthase from the extremely thermoalkaliphilic bacterium *Bacillus* sp. strain TA2.A1. In this study, the genes encoding the F_1 moiety of the enzyme complex were cloned from the *atp* operon into the vector pTrc99A and expressed in *Escherichia coli* in two variant complexes, F_1-wt consisting of subunits $\alpha_3\beta_3\gamma\delta\varepsilon$ and $F_1\Delta\delta$ lacking the entire δ-subunit as a prerequisite for overproduction and crystallization trials. Both F_1-wt and $F_1\Delta\delta$ were successfully overproduced in *E. coli* and purified in high yield and purity. $F_1\Delta\delta$ was crystallized by micro-batch screening yielding three-dimensional crystals that diffracted to a resolution of 3.1 Å using a synchrotron radiation source. After establishing cryo and dehydrating conditions, a complete set of diffraction data was collected from a single crystal. No crystals were obtained with F_1-wt. Data processing of diffraction patterns showed that $F_1\Delta\delta$ crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell parameters of $a = 121.70$, $b = 174.80$, and $c = 223.50$ Å, α, β, $\gamma = 90.000$. The asymmetric unit contained one molecule of bacterial $F_1\Delta\delta$ with a corresponding volume per protein weight (V_M) of 3.25 Å^3 Da^{-1} and a solvent content of 62.1%. Silver staining of single crystals of $F_1\Delta\delta$ analyzed by SDS–PAGE revealed four bands α, β, γ, and ε with identical M_r-values as those found in the native F_1F_0-ATP synthase isolated from strain TA2.A1 membranes. ATPase assays of $F_1\Delta\delta$ crystals exhibited latent ATP hydrolytic activity that was highly stimulated by lauryldimethylamine oxide, a hallmark of the native enzyme.

Keywords: Crystallization; X-ray; F_1-ATPase; Thermoalkaliphile; TA2.A1

1. Introduction

F_1F_0-ATP synthase is the universal rotary motor for the production of ATP, the source of cell energy in living organisms (Senior, 1988). The enzyme is found in the inner membranes of mitochondria, the thylakoid membranes of chloroplasts and the cytoplasmic membranes of bacteria (Boyer, 1993). Irrespective of its origin, the ATP synthase shares a common molecular architecture covering two unique rotary motor elements, F_1 and F_0, that are functionally coupled to each other (Duncan et al., 1995; Noji et al., 1997; Sabbert et al., 1996). The membrane-embedded F_0 motor is an electro-mechanical energy converter that uses the flux of protons or Na^+ ions across the membrane down the transmembrane electrochemical gradient to generate torque (Aksimentiev et al., 2004; Feniouk et al., 2004; Meier et al., 2005; Rastogi and Girvin, 1999; Xing et al., 2004). The mechanical energy thus created is transmitted to the F_1 motor by a rotating central stalk including the γ subunit (Noji et al., 1997). Its rotation within the surrounding subunits of F_1 induces cyclic conformational changes in the three catalytic sites in F_1 which enable them to form ATP from ADP and P_i (Abrahams et al., 1994, 1993; Lucken et al., 1990). The reaction catalyzed by the ATP synthase is fully reversible, so ATP hydrolysis generates torque in F_0 that reverses the flux of ions across the membrane (Boyer, 1997).

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During growth on non-fermentable carbon sources, alkaliphilic bacteria synthesize ATP using a proton-coupled F_1F_0-ATP synthase (Cook et al., 2003; Hicks and Krulwich, 1990; Hoffmann and Dimroth, 1990; Hoffmann and Dimroth, 1991; Keis et al., 2004). At high alkaline pH values where these bacteria thrive, the electrochemical proton gradient driving ATP synthesis appears to be suboptimal and how the ATP synthases function under these conditions remains enigmatic. Further to this unresolved thermodynamic problem, the ATP synthases of alkaliphilic bacteria seem to have acquired unique properties for survival at high environmental pH. A peculiarity of the alkaliphilic ATP synthases is their specific blockage or latency in the ATP hydrolysis direction (Cook et al., 2003; Hicks and Krulwich, 1990; Hoffmann and Dimroth, 1990; Keis et al., 2004). We hypothesize that this latency is a built-in safety device required to enable survival at high pH when respiration slows. The most critical parameter for survival under these conditions is to maintain the cytoplasmic pH near neutral and therefore an unregulated ATP synthase that pumps protons outwards, if the proton motive force drops below the phosphorylation potential, would be detrimental for cell viability (Olsson et al., 2003). Recent work from our group has identified that the latency in ATP hydrolysis activity is intrinsic to F_1 (Cook et al., 2003).

An important step towards the elucidation of how the F_1F_0-ATP synthase from strain TA2.A1 is blocked in the ATP hydrolysis direction and how its ATP synthetic activity is regulated will be the provision of high resolution structural information. To date, no structure of an alkaliphilic ATP synthase has been solved and success in crystallizing ATP synthases from bacterial sources in general has been limited, therefore hindering high resolution structural data to be obtained for bacterial F_1 complexes. To date, the only structures of soluble subcomplexes of bacterial ATP synthases reported are a 4.4 Å resolution (PDB code 1JNV) $F_1\Delta\delta$ structure from *Escherichia coli* (Hausrath et al., 2001) and the 3.2 Å resolution structure of the $\alpha_3\beta_3$ subcomplex of F_1 from the thermophilic *Bacillus* sp. PS3 (PDB code 1SKY) (Shirakihara et al., 1997). Neither X-ray structure provides information on side-chain features of the γ and ε subunits which are needed to understand how bacterial ATP synthases regulate ATP hydrolysis and synthesis.

As a first step towards this goal, we report here on the purification, biochemical characterization and crystallization of recombinant F_1-ATPase from the thermoalkaliphilic *Bacillus* sp. strain TA2.A1 and the initial analysis of its diffraction data.

2. Strains and growth conditions

For general cloning purposes, *E. coli* DH10B (Hanahan et al., 1991) was used. For overexpression of recombinant F_1 proteins, *E. coli* RNE41(DE3) (gift of B. Miroux, Meudon, France) was routinely used. Recombinant RNE41(DE3) cells were routinely grown at 30 °C with shaking at 180 rpm in baffled 5 L Erlenmeyer flasks containing 2 L of Luria Bertani (LB) medium supplemented with 100 μg/ml ampicillin. The medium was inoculated with an overnight culture (1%). At an OD_{600} of 0.5, the cultures were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM). Cells were collected by centrifugation for 30 min at 5500*g*, washed once in 20 mM Tris–HCl (pH 8.0) containing 1 mM $MgCl_2$ and 10% glycerol, and frozen at −76 °C.

For the isolation of the native F_1F_0-ATP synthase, strain TA2.A1 was grown at 65 °C in 200 L of alkaline medium (pH 9.2) as described previously (Olsson et al., 2003). Briefly, cells were grown overnight (18 h) in 20 ml of rich alkaline medium supplemented with 8 g/L L-glutamate and 2.5 g/L yeast extract, at 160 rpm and 65 °C. The inoculum was then used for a second starter culture (1.5 L of rich medium) and grown with shaking overnight. The overnight culture was used to inoculate a 200 L fermenter which was operated at 220 rpm and constantly aerated with pressurized air at 65 °C. The OD_{600} was measured at intervals of 1 h. The fermenter was rapidly cooled to 12 °C before the end of exponential growth (OD_{600} at 0.8) and cells were harvested by centrifugation and frozen without further treatment at −76 °C.

3. Biochemical techniques

Protein concentration was determined by the BCA method (Pierce) using bovine serum albumin as a standard. SDS–PAGE was performed as described (Schagger and von Jagow, 1987). Gels were stained with Coomassie brilliant blue R 250 or with silver (Wray et al., 1981). ATP hydrolysis was measured with an ATP-regenerating assay as described previously (Cook et al., 2003).

3.1. Construction of expression plasmids

In a previous study (Keis et al., 2004), the *Bacillus* sp. TA2.A1 genes encoding for the F_1 subunits were cloned in pUC8 as two overlapping fragments in plasmids pB9 and pA3. To assemble all of the F_1-encoding genes, plasmid pB9 was digested with *Sac*I and *Eco*RI and the remainder of the F_1 genes were inserted as a 1.5-kb *Sac*I–*Eco*RI fragment from pA3 to create plasmid pTA2F1. Subsequently, the F_1-encoding genes were cloned as a *Sal*I fragment from pTA2F1 into the expression vector pTrc99A (Amersham Biosciences) under the control of the strong tryptophan promoter to create plasmid pTrcF1. To construct an expression plasmid (pTrcα–ε) containing all of the F_1 genes except for *atpH*, the start of *atpA* was amplified using a primer containing a *Nco*I site (underlined) (5′-AAATT TCCATGGGCATCAGACCTGAAGAAATC-3′) and a second primer (5′-GCAGAAGCTGTCACCACAAT-3′) homologous to sequence upstream from a unique *Ase*I site, and plasmid pB9 as template. The amplified fragment was digested with *Nco*I and *Ase*I, and simultaneously cloned with a 3.79-kb *Ase*I–*Sal*I fragment, containing the remainder of the F_1 genes, into pTrc99A digested with *Nco*I and

*Sal*I. PCR was carried out with the Phusion high-fidelity PCR kit (Finnzymes) according to the manufacturer's instructions. The region amplified by PCR was confirmed by DNA sequencing. Due to the introduction of the *Nco*I site at the start of *atpA,* the second amino acid in the α subunit was changed from a serine to a glycine.

3.2. Purification of recombinant F_1-ATPases and native F_1F_o–ATP synthase

RNE41(DE3) cells containing either the plasmid pTrcF1 or pTrcα–ε were grown in 6 L batch cultures and harvested as described above. Cells were thawed out and resuspended in 100 ml of 20 mM Tris–HCl (pH 8.0) containing 10% glycerol and 1 mM $MgCl_2$. Diisopropylfluorophosphate (DFP) and DNase I were added to the suspension at a final concentration of 0.1 mM and 2.4 mg/ml, respectively. Cells were disrupted by two passages through a pre-cooled French-pressure cell at 82 MPa and unbroken cells and cell debris were removed by a 30 min centrifugation at 150 000*g*. The cell-free supernatant was heated for 30 min at 60 °C to denature any contaminating *E. coli* proteins, and then centrifuged for 45 min at 150 000*g*. The heat-treated supernatant was brought to 5 mM $MgCl_2$ and 0.5 M NaCl before applying to a 50 ml column of chelating Sepharose fast flow (Amersham Biosciences). Before applying the F_1 sample, the chelating Sepharose column was loaded with 80 ml of 0.3 M $CuSO_4$, washed with five column volumes of ddH_2O and equilibrated with five column volumes of 20 mM Tris–HCl (pH 8.0) containing 0.5 M NaCl, 5 mM $MgCl_2$, and 10% glycerol. After loading the F_1 sample (120 ml) the column was washed with three column volumes of the same buffer. The bound F_1 was eluted with one column volume of the same buffer containing 100 mM imidazole. The eluted F_1 was dialyzed (6000 Da MWCO) against 20 mM Tris–HCl (pH 8.0) containing 1 mM $MgCl_2$ and 10% glycerol to remove excess salt. The sample (approximately 200 mg protein) was applied to a 60 ml POROS HQ 50 column (Applied Biosystems, Foster City, USA), which was equilibrated with the dialysis buffer. Bound proteins were eluted with 12 column volumes of a 0–600 mM linear gradient of NaCl in the same buffer. The F_1-ATPase-containing fractions were pooled and concentrated to 30 mg/ml using Amicon Ultra centrifugal filter devices (100 kDa MWCO). The F_1-ATPase sample (1 ml) was then applied in aliquots of 0.2 ml to a gel permeation chromatography (GPC) Superose six column (Amersham Biosciences) and fractionated with buffer containing 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA and 100 mM NaCl. The peak at 370 kDa was pooled and immediately used for crystallization trials.

Purification of the native F_1F_0-ATP synthase from *Bacillus* sp. strain TA2.A1 was carried out as described previously (Cook et al., 2003) The F_1F_0-ATP synthase-containing fractions were pooled and concentrated to 10 mg/ml protein using Amicon Ultra centrifugal filter devices (100 kDa MWCO) in 20 mM Tris–HCl (pH 8.0) containing 1 mM $MgCl_2$, 100 mM NaCl, 10% glycerol, and 0.05% β-dodecyl–maltoside.

3.3. Crystallization

The purified F_1F_0, F_1-wt, and $F_1\Delta\delta$ enzymes (at approximately 10 mg/ml protein) were used for crystallization trials. Initial conditions for crystal growth were identified for each protein by micro-batch trials performed at the high-throughput crystallization laboratory at the Hauptmann–Woodward Institute (Buffalo, NY) (Luft et al., 2001). Initial conditions obtained were further optimized in the case of $F_1\Delta\delta$ crystals by micro-batch trials using handmade screens. Typically, for each condition, 2 μl of protein solution (6–10 mg/ml), was mixed with 2 μl of the well solution in 96-well plates containing 70 μl of dry paraffin oil. Cryo-protection of the $F_1\Delta\delta$ crystals prior to data collection was achieved by soaking the crystals in mother liquor with increasing concentrations of polyethylene glycol (PEG) 400 through four steps: 5, 10, 15, and 20% (w/v). The equilibration time at each concentration was at least 10 min. These crystals were flash-frozen in liquid nitrogen before shipment to the synchrotron facility.

4. Data collection

X-ray diffraction data from single crystals were collected using an Oxford Cryojet at the SLS PX-beamline at the Paul Scherrer Institute in Villigen, Switzerland. Data were collected with monochromatic X-rays ($\lambda = 1.0000$ Å) at a detector to crystal distance of 300 mm on an MAR225 mosaic CCD detector at an oscillation range of 1° and an exposure time of 1 s. To reduce radiation damage, the beam intensity was reduced to 26.9% of the initial intensity by insertion of filters. Indexing, integration, scaling, post-refinement, and reduction of the data were carried out using the XDS package (Kabsch, 1993).

5. Subunit composition and catalytic activity of $F_1\Delta\delta$ crystals

Twenty crystals of $F_1\Delta\delta$ having an average dimension of 0.15 mm × 0.15 mm × 0.3 mm were transferred one by one with a cryo-loop into 50 μl of fresh mother liquor. Care was taken not to co-transfer traces of protein precipitate with the crystals. After repeating the transfer of crystals four times using fresh mother liquor, the washed crystals were centrifuged at 15 000*g* for 5 min. The supernatant was removed and the crystal pellet was dissolved in 20 mM Tris–HCl (pH 8.0). The crystal pellet was analyzed by SDS–PAGE and ATPase activity was determined as previously described (Cook et al., 2003).

The genes encoding the subunits for the F_1-ATPase of *Bacillus* sp. strain TA2.A1 (viz. *atpHAGDC*) were cloned from the *atp* operon of strain TA2.A1 as described previously (Keis et al., 2004). Overexpression of the two plasmids pTrcF1 and pTrcα–ε in RNE41(DE3) cells yielded highly stable recombinant complexes of F_1-wt and $F_1\Delta\delta$, respectively. Following cell disruption and centrifugation, the *E. coli* supernatant was heated for 30 min at 60 °C reducing the amount of endogenous *E. coli* proteins by

more than 90%. The subsequent three-step chromatographic procedure, encompassing high-affinity purification by Cu^{2+}-chelate chromatography, anionic exchange chromatography, and final purification by GPC, yielded highly pure F_1-ATPase complexes (Fig. 1A). The progress of the purification was followed by comparing the latent and LDAO-stimulated ATP hydrolytic activity. Typically, 28–35 mg of ATPase with a specific activity (LDAO-stimulated) of 28–32 U/mg of protein were obtained from 6 L batches of recombinant RNE41(DE3) cultures.

A notable problem in previous preparations of bacterial F_1-ATPases has been the contamination by cytochrome b_0, that could be overcome by using cytochrome b_0–deficient strains (Gruber et al., 1997). Due to the stability of the F_1-ATPase complexes from *Bacillus* sp. TA2.A1, a heating step overcame this contamination as evidenced by SDS–PAGE. Moreover, based on the staining intensities of the α, β, γ, δ, and ε bands, F_1-ATPase complexes appear to have the correct molar ratio of 3:3:1:1:1 for F_1-wt and 3:3:1:1 for $F_1\Delta\delta$ and an identical subunit pattern compared with the native F_1F_0-ATP synthase from strain TA2.A1 (Fig. 1B).

Characteristics of recombinant F_1-wt and $F_1\Delta\delta$ were determined with the ATP-regenerating assay at 45 °C as described previously (Cook et al., 2003). Michaelis–Menten plots and kinetic analyses using the Lineweaver–Burk equation indicated that the apparent K_m values for ATP of recombinant F_1-wt and $F_1\Delta\delta$ (0.48 and 0.70 mM, respectively) were in good agreement with those previously determined for the native F_1F_0-ATP synthase (Cook et al., 2003). The latent ATP hydrolytic activity of both forms could be stimulated 30- to −45-fold by the addition of 0.4% LDAO (data not shown). These data confirm earlier findings, that the blockage of ATP hydrolysis is an intrinsic property of the F_1 moiety of the ATPase.

Initial attempts to crystallize recombinant F_1-wt and $F_1\Delta\delta$, as well as native F_1F_0-ATP synthase using commercial crystal screens (Hampton Research) in sitting-drop vapor diffusion experiments did not yield any promising crystallization conditions. It was therefore decided to prepare micro-batch crystal-growth screening experiments in 1536-well microassay plates at the high-throughput crystallization laboratory at the Hauptman Woodward Institute (Buffalo, New York). Micro-batch screening with 1536 distinct cocktails and using the three samples F_1-wt, $F_1\Delta\delta$, and F_1F_0 yielded a cluster of promising conditions solely in the case of $F_1\Delta\delta$ (Luft et al., 2001). All conditions found for $F_1\Delta\delta$ were mixtures of salt with PEG at rather basic pH values spanning a range from 7.5 to 9.5. The most promising condition that yielded three-dimensional cube-shaped crystals contained 0.1 M LiCl, 0.1 M Tris–HCl (pH 8.0), and 20% (w/v) PEG 8000. The condition was optimized in our laboratory with 1 M LiCl, 100 mM Tris–HCl (pH 8.8), and 20% (w/v) PEG 6000. Protein (6 mg/ml) in 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and reservoir buffer were mixed 1:1 in 70 µl of dry paraffin oil. Crystals with dimensions 0.15 × 0.15 × 0.3 mm and well-defined edges and faces were obtained at 23 °C within 2 days (Fig. 2).

To address the question if the $F_1\Delta\delta$ crystals represented a functional state of the bacterial F_1 motor and contained all components of the starting material, single crystals were subjected to SDS–PAGE analysis and their ATPase activity determined (Fig. 3). After silver staining, the apparent molecular masses and intensities of the α, β, γ, and ε bands of $F_1\Delta\delta$ crystals were similar to the corresponding subunits of the starting material (see Fig. 1A for comparison),

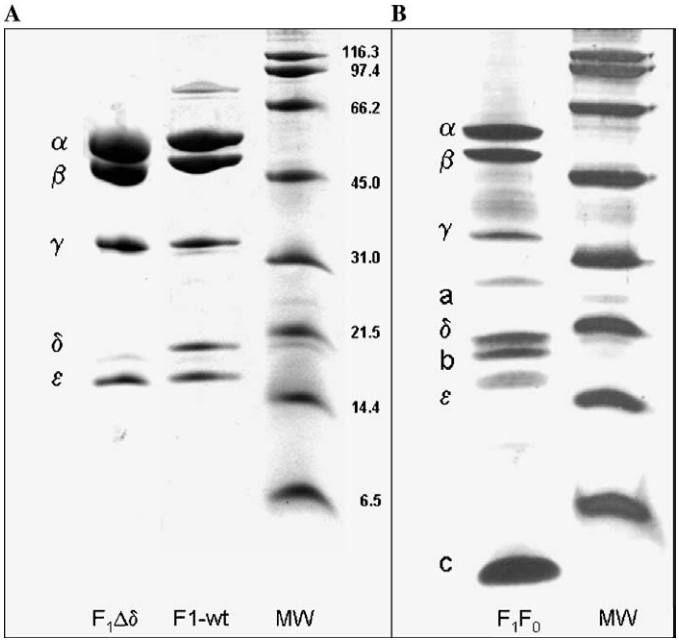

Fig. 1. (A) SDS–PAGE of recombinant ATPase complexes from the thermoalkaliphilic *Bacillus* sp. TA2.A1 stained with Coomassie brilliant blue. $F_1\Delta\delta$ (30 µg) and F_1-wt (30 µg) were isolated from *E. coli* clones as described in Materials and methods. (B) SDS–PAGE of native F_1F_0-ATP synthase (2 µg) isolated from *Bacillus* sp. strain TA2.A1 cells stained with silver. Samples were resolved on 12% SDS–PAGE gels by the method of Schägger et al. (Schagger and von Jagow, 1987). Also shown are migrations of broad-range molecular mass markers (MW, Bio-Rad) with molecular masses indicated in kDa.

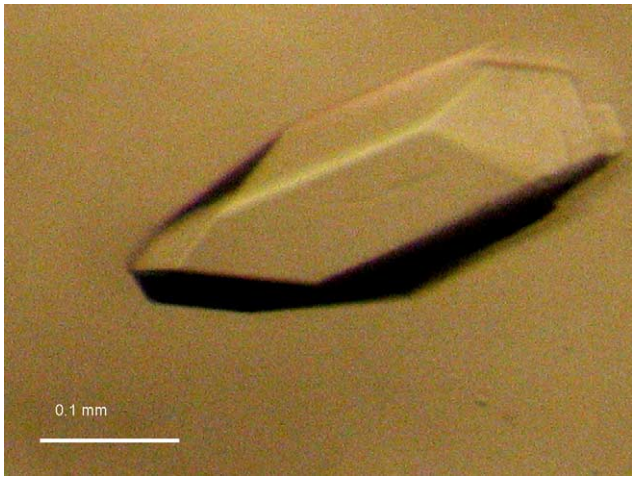

Fig. 2. A crystal of $F_1\Delta\delta$ obtained by micro-batch crystallization.

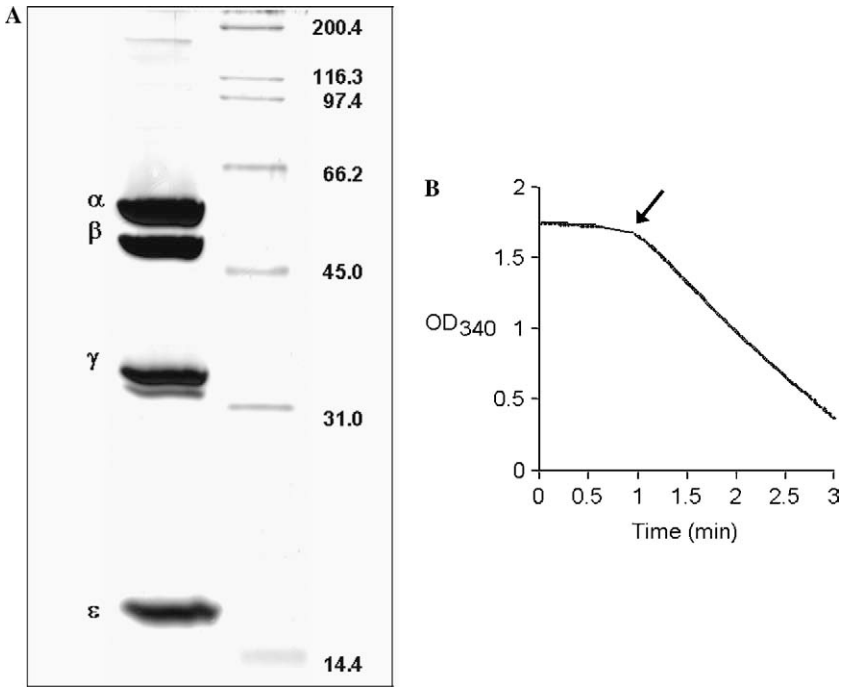

Fig. 3. (A) SDS–PAGE of $F_1\Delta\delta$ crystals (2 μg). Samples were resolved on 12% SDS–PAGE gels by the method of Schägger et al. (Schagger and von Jagow, 1987) and were stained with silver. The position of the subunits is indicated. Also shown is the migration of the broad-range molecular mass marker (Bio-Rad) with molecular masses in kDa. (B) ATPase activity of the dissolved $F_1\Delta\delta$ crystals and activation by LDAO. The velocity increase was due to addition (indicated by arrow) of 0.4% (w/v, final concentration) LDAO. OD_{340}, optical density at 340 nm.

indicating a correct subunit stoichiometry in the crystals. Accordingly, crystals of $F_1\Delta\delta$ were indistinguishable from soluble $F_1\Delta\delta$ in the ATPase assay, both exhibiting latent ATPase activity that was highly stimulated by LDAO (Fig. 3B).

The crystals diffracted between 8 and 6 Å at RT using capillaries and a copper rotating anode X-ray source. Similar results were obtained at 100 K using synchrotron radiation and flash-freezing in cryo-buffers containing glycerol or 2-methyl-2,4-pentanediol (MPD). Improved crystal diffraction between 4.0 and 3.1 Å at 100 K was achieved by dehydrating the $F_1\Delta\delta$ crystals prior to flash-cooling in mother liquor with increasing concentrations of PEG 400 up to 20% (w/v) (Fig. 4). Analysis of the collected data (Table 1) indicated that $F_1\Delta\delta$ crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell parameters of $a=121.70$, $b=174.80$, and $c=223.50$ Å, $\alpha,\beta,\gamma=90.000$ giving a volume per protein weight (V_M) of 3.25 $Å^3$ per Da (62.1% estimated solvent content) for a crystal containing one molecule of $F_1\Delta\delta$ per asymmetric unit. A total of 711 472 observations were obtained at 3.09 Å resolution giving 87 190 unique reflections (98.2% completeness, $R_{merge}=0.19$). Using these data, we are now in the process of determining the high resolution structure by molecular replacement using bovine mitochondrial F_1-ATPase as a search model.

Diffraction quality is essential for high resolution X-ray crystallographic studies of macromolecular protein complexes. In this communication, we show substantial improvement of diffraction resolution by adding PEG 400 to single $F_1\Delta\delta$ crystals. Similar cases of post-crystallization treatments

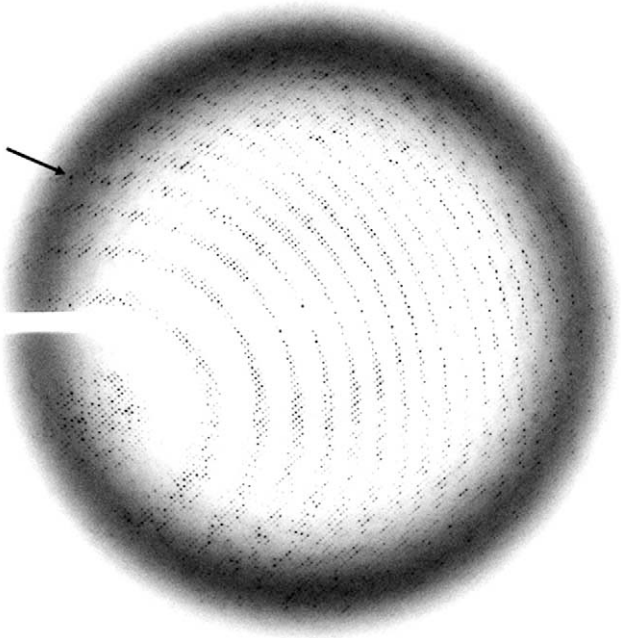

Fig. 4. Oscillation diffraction image of a frozen crystal of F1Δδ taken by the use of synchrotron radiation. The oscillation range was 1° and the crystal-to-detector distance was 300 mm. The diffraction spot indicated by an arrow is at resolution of 3.1 Å.

have been previously reported in the literature where dramatic improvement of poorly diffracting crystals has been achieved by controlled dehydration as reviewed (Heras et al., 2003). Interestingly, our comparative crystallization trials

Table 1
Crystal data and diffraction statistics

Space group	$P2_12_12_1$	
Cell parameters	$a = 121.70$, $b = 174.80$, and $c = 223.50$ Å	
Resolution range	50.0–3.09 Å	3.56–3.09 Å
Total reflections	711 472	238 116
Unique reflections	87 190	29 287
I/SIGMA	7.62	3.16
R_{merge} [a]	0.19	0.45
Completeness (%)	98.2	95.2

[a] $R_{merge} = (\sum_h \sum_j |I_j(h) - \langle I_{(h)} \rangle|)/(\sum_h \sum_j I_j(h))$, where $I_j(h)$ is the jth measurement of the intensity of reflection h and $\langle I_{(h)} \rangle$ is the mean intensity of equivalent reflections.

indicate that $F_1\Delta\delta$ crystallizes more readily, while F_1-wt seems to have a latent tendency to aggregate. These findings are in agreement with a recent review showing that only F-type ATPases lacking the δ subunit have been crystallized (Weber and Senior, 2003).

We propose that the work presented here provides the necessary prerequisites to solve the first structure of an F_1-ATPase from an alkaliphilic bacterium and that such a structure will shed light on the molecular mechanism responsible for the latent ATP hydrolysis activity by this enzyme. Features of this structure might also advance our knowledge on the regulation of ATP synthesis or hydrolysis activities by F_1F_0-ATP synthases.

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