An improved purification of ECF_1 and ECF_1F_0 by using a cytochrome *bo*-deficient strain of *Escherichia coli* facilitates crystallization of these complexes

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Abstract A novel strategy, which employs a cytochrome *bo*-lacking strain (GO104) and a modified isolation procedure provides an effective approach for obtaining much purer preparations of ECF_1F_0 than described previously, as well as for isolating homogeneous and protein-chemically pure ECF_1. ECF_1 obtained in this way could be crystallized by vapor-diffusion using polyethylene glycol (PEG) as a precipitant in a form suitable for X-ray diffraction analysis. The crystals belong to the orthorhombic space group $P2_12_12_1$, with lattice parameters $a=110$, $b=134$, and $c=269$ Å, and diffract to a resolution of at least 6.4 Å.

Key words: F_1-ATPase; F_1F_0-ATP synthase; Crystal

1. Introduction

F_1F_0 type ATPases or ATP synthases catalyze oxidative or photo-phosphorylation by using a transmembrane proton-motive force to drive ATP synthesis. In the reverse direction, these enzymes use ATP to generate a proton gradient that can be used in ion transport processes. They are large, multisubunit complexes. For example, the *Escherichia coli* enzyme ECF_1F_0 is composed of 8 different subunits. Five of these, the α, β, γ, δ and ε subunits are present in the stoichiometry 3:3:1:1:1, respectively, and form the membrane extrinsic F_1 part which contains the (three) catalytic sites. The other three different subunits, a, b and c in the molar ratio 1:2:9–12, form the membrane bilayer intercalated F_0 part which includes the proton pore. The entire complex has a molecular weight of approximately 530 000 [1–4].

A major advance in our understanding of these complex enzymes has come with the crystallization of MF_1 and a structure determination of the $\alpha_3\beta_3$ domain and part of the γ subunit at 2.8 Å resolution [5,6]. So far, there has been limited success in crystallizing enzyme from bacterial sources. The only report of such crystals is of a preparation reconstituted from α and β subunits ($\alpha_3\beta_3$) from TF_1 [7]. High resolution structural data for the isolated δ and ε subunits of ECF_1 have been obtained in our laboratory by NMR [8,9]. However, until recently we (and others) have failed to obtain X-ray diffraction quality crystals of either ECF_1 or ECF_1F_0, despite prolonged efforts.

A serious problem has been that preparations contain low but significant amounts of impurities, and are not homogenous. Therefore, we have looked for improved ways of purifying ECF_1 and ECF_1F_0. We were aware that a major impurity of ECF_1F_0 was cytochrome *bo* under the conditions of relatively high aeration at which we grow *E. coli* for large-scale purification of the enzyme complex. Therefore, we have examined isolation of both ECF_1 and ECF_1F_0 from cytochrome *bo*-deficient strains of *E. coli*. This novel genetic removal of impurities has allowed us to obtain highly pure ECF_1 and ECF_1F_0, which should be useful for a range of biochemical and biophysical studies. As we show for ECF_1, these preparations are pure and homogenous enough that crystals of the enzymes can now be obtained.

2. Materials and methods

2.1. Purification of ECF_1

E. coli cells (100 g) were resuspended in 150 ml of 200 mM Tris, pH 8.0 and 1 mM EDTA added prior to dilution with a further 150 ml of buffer containing 200 mM Tris, pH 8.0, 1 M sucrose and 60 mg lysozyme. The suspension was stirred for 20 min at room temperature, and then 300 ml distilled water, 5 mg DNase I and 50 mM $MgCl_2$ added with subsequent stirring for 20 min at 4°C. After centrifugation (11 000×*g*, 15 min), the pellet was resuspended in 450 ml of 0.1 M TES, pH 7.0, 20 mM $Mg(C_2H_3O_2)_2$, 0.25 M Sucrose, 0.25 mM EGTA and 40 mM EACA, and 5 mM PAB, 1 mM DTE, 1 mM PMSF, 5 mg DNase I added before disruption of cells using a French pressure cell press. The suspension after French pressing was centrifuged at 165 000×*g* for 2 h. The pellet containing the membranes was washed three times in 400 ml of 50 mM TES, pH 7.0, 15% (v/v) glycerol, 40 mM EACA, 6 mM PAB (buffer A) and subsequently in modified buffer A with 5 mM TES with centrifugation at 165 000×*g* for 90 min after each wash.

Detachment and precipitation of the ECF_1 was carried out according to Senior et al. [10], using PEG 8000 as a precipitant. After centrifugation of the precipitated enzyme (13 200×*g*, 1 h) the pellet was dissolved in 74 ml buffer B (50 mM Tris, pH 7.4, 20% (v/v) glycerol, 2 mM EDTA, 1 mM ATP, 1 mM DTT, 40 mM EACA and 1 mM PMSF) and centrifuged at 39 200×*g* for 15 min. The pellet was resuspended in buffer B with the addition of 0.2 M NaCl and stirred for 1 h before loading onto a DEAE Sepharose CL-6B-200 column. The column-bound protein was washed with the same buffer before eluting with 0.8 M NaCl at a flow rate of 0.6 ml/min. The peak of enzyme, as determined by ATPase activity, was pooled and precipitated with 70% $(NH_4)_2SO_4$, then applied to a Sephacryl S-300-HR column, according to Gogol et al. [11]. Enzyme eluting from this column was located by enzyme activity, precipitated for 1 h in 70% $(NH_4)_2SO_4$, centrifuged at 15 000×*g* for 20 min and then dissolved in 50 mM MOPS, pH 7.0, 0.5 mM EDTA and 10% glycerol (v/v).

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Abbreviations: DTE, erythro-1,4-dimercapto-2,3-butanediol; DTT, dithiothreitol; EACA, 6-aminohexanoic acid; ECF_1, soluble portion of the *Escherichia coli* F_1F_0-ATPase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethanedioxybis-(ethylamine) tetra-acetate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAB, *p*-aminobenzamidine; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; TES, *N*-tris[Hydroxymethyl]methyl-2-aminoethane-sulfonic acid

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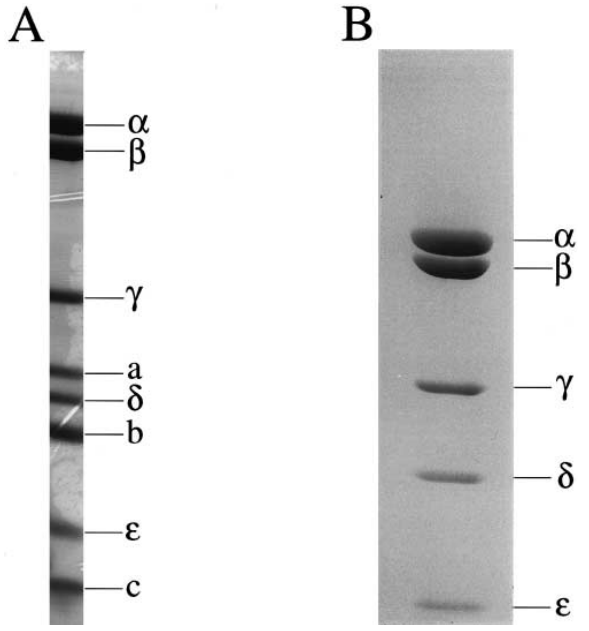

Fig. 1. SDS polyacrylamide gel electrophoresis of ECF_1F_0 and ECF_1. (A) 35 μg [4 mg/ml] of ECF_1F_0 and (B) 60 μg of ECF_1 [20 mg/ml] were separated by a 16–22% and 10–18% linear gradient SDS-PAGE, respectively. The gels were stained with Coomassie Brilliant Blue R.

2.2. Removal of bound nucleotides for crystallization of ECF_1

ECF_1 isolated as described above is heterogeneous with respect to the nucleotide content of both catalytic and non-catalytic sites. Thus, bound nucleotides were removed by passing enzyme samples through three consecutive Sephadex G-50 centrifuge columns in the MOPS buffer described above. Analysis of residual bound nucleotide was made with 30 μg aliquots of ECF_1 (15 mg/ml) denatured with 10 μl of 14% perchloric acid. Samples were centrifuged at 5000 rpm for 2 min, neutralized with 6 μg of 5 M K_2CO_3, and then incubated at 4°C for 15 min before centrifugation at 5000 rpm for 2 min [12]. Nucleotide content of the supernatant was determined by HLPC using a Brownlee C-18 (Spheri-5) reversed-phase column eluted with 50 mM KH_2PO_4, pH 5.0, 2.5 mM tetrabutylammonium phosphate at a flow rate of 1 ml/min at 35°C. The relationship between the integrated peak area and nucleotide amounts was determined from nucleotide standards of known concentration. After this treatment, it was found that ECF_1 preparations retained 0.1–0.3 mol of ATP per mole enzyme.

2.3. Crystallization conditions for ECF_1

Purified and nucleotide-depleted ECF_1 concentrated to 10 mg/ml using Amicon Centron 100 concentrators was used in crystallization experiments. Crystals were grown by the hanging drop vapor diffusion method [13] at 20°C. Each drop consisted of 4 μl of well solution containing 25% PEG 400, 0.1 M HEPES, pH 7.0, 3 mM $MgCl_2$, 75 μM AMP-PNP and 1.5 μM ADP. Crystals were mounted in tapered glass capillaries according to Abrahams and Leslie [14]. X-ray data were collected on a R-AXIS IIc imaging plate area detector using Cu K_α radiation generated by a Rigaku RU 300 rotating anode, operating at 40 kV and 100 mA. The crystal-to-imaging plate distance was 450 mm, and the collimator size, 0.7 mm. Oscillations of 1° per 120 mm were used. R-AXIS data were processed with DENZO [15].

2.4. Other methods

Subunit compositions of ECF_1 and ECF_1F_0 preparations were analyzed using SDS containing polyacrylamide gradient gels, according to Laemmli [16]. Gels were stained with Coomassie Brilliant Blue R as described by Downer et al. [17]. Protein concentrations were determined with the BCA assay system from Pierce Chemical Co.

3. Results and discussion

Preparations of ECF_1F_0 have traditionally been made from strains of *E. coli* such as AN1460 [18], AN888 [19] and RF-7

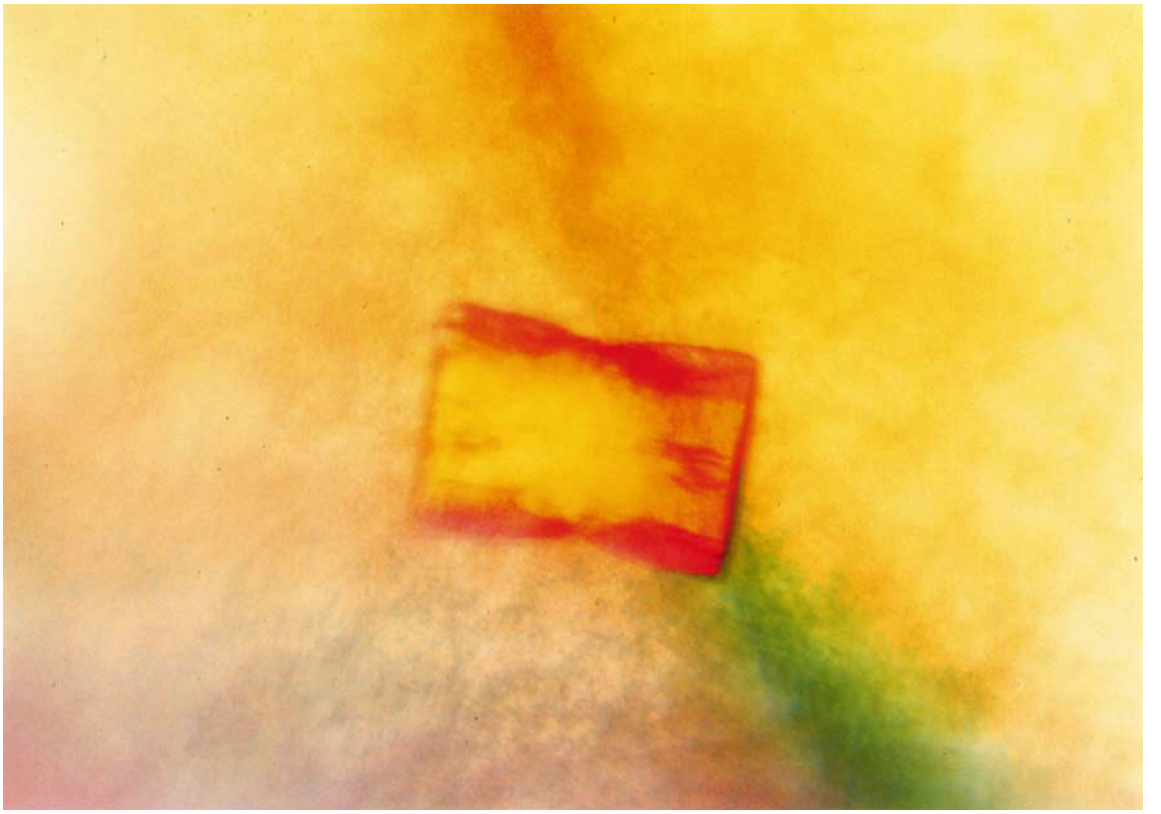

Fig. 2. Crystal of ECF_1. The crystal has approximately dimensions of 0.5×0.4×0.2 mm.

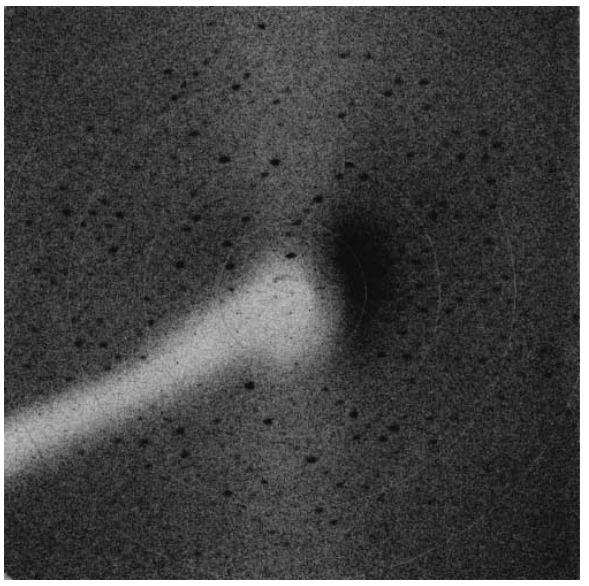

Fig. 3. A 1° oscillation image of crystals of ECF_1. The image was recorded on an R-AXIS IIc imaging-plate area detector, with an exposure time of 120 min. Reflections at the extreme right and extreme left of the image correspond to a resolution of 5.4 Å.

[20] that contain the *unc* operon on an overexpressing plasmid. Our best preparations of enzyme from these strains always contain impurities that can be clearly observed even at low protein loading of SDS polyacrylamide gels. These preparations often appear slightly brown and spectral analyses show the presence of cytochrome *bo*. Therefore, to improve the purification of ECF_1F_0, we decided to isolate the enzyme from a strain of *E. coli* that was unable to make this cytochrome. Thus, the *unc*-containing plasmid, pAN45 [18], was introduced into the *E. coli* strain GO104 which has cytochrome *bo* genetically deleted [21]. Fig. 1A presents a 16–22% gradient (SDS) polyacrylamide gel of a typical preparation of enzyme from this strain, purified by the standard technique of Foster and Fillingame [20], and shown here after the sucrose gradient step and before reconstitution with added lipids, which itself improves purity to a small extent. The preparation is considerably more pure than our best preparations of enzyme using previous strains (see, for example, figs. 4 and 2, respectively, in Aggeler and Capaldi, 1995 [22]; Watts et al., 1996 [23] and Fig. 1A and C and Fig. 2, respectively [24–26], for preparations from other laboratories). Subsequent ion exchange chromatography steps have recently yielded an ECF_1F_0 preparation from which 3D crystals have been obtained. On preliminary analysis these diffract to at least 10 Å, as will be described in detail elsewhere.

Surprisingly, the use of this strain also yielded improved preparations of ECF_1 as evident in Fig. 1B, which shows a Coomassie blue-stained SDS polyacrylamide gel loaded with 60 μg of enzyme. The degradation product of δ subunit often seen in ECF_1 preparations running slightly above the ε subunit, is not seen in these new preparations. Other minor impurities often resolved on gels when equally heavily loaded are also not seen. Moreover, the molar ratio of γ, δ and ε subunits appears to be 1:1:1, based on a quantitation of the staining intensity of the three bands of these subunits, respectively (result not shown). Additional evidence that the ε subunit is in stoichiometric amounts comes from activity measurements. The ε subunit is an inhibitor of the ATPase activity of ECF_1. In most preparations, addition of exogenous ε subunit reduces ATPase activity, indicating that a fraction of enzyme molecules are devoid of ε subunit, or that this subunit is shifted from its inhibitor site. However, ECF_1 from strain GO104 has relatively low ATP hydrolysis rates as isolated (10 μmol ATP hydrolyzed mg^{-1} min^{-1}) and is not further inhibited by addition of excess ε subunit.

An additional and important indication of the improved purity of ECF_1 from the cytochrome *bo*-deficient strain is our recent success in obtaining 3D crystals of the enzyme. A typical crystal is shown in Fig. 2. These contain all five subunits (α, β, γ, δ and ε) in the same stoichiometries as in the starting isolated enzyme, based on SDS polyacrylamide gels of dissolved crystals. They grow to a size of $0.5\times0.4\times0.2$ mm within 6–8 weeks, are stable to X-ray radiation for at least 36 h, and diffract to at least 6.4 Å resolution (Fig. 3). Analysis of the diffraction data collected so far reveals an orthorhombic lattice with $a=110$, $b=134$ and $c=269$ Å, and a space group of $P2_12_12_1$, assuming one molecule (molecular weight 380 000) in the asymmetric unit gives a solvent content of 51.6% and a V_m of 2.56 $Å^3$ per Da, which is well within the normal range of V_m values for protein crystals [27].

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