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Large-scale purification and characterization of the five subunits of F_1-ATPase from pig heart mitochondria

Dominique Gagliardi, François Penin and Danièle C. Gautheron

Laboratoire de Biologie et Technologie des Membranes et des Systèmes Intégrés du CNRS, Université Claude Bernard de Lyon I, Villeurbanne (France)

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A large-scale purification procedure was developed to isolate the five subunits of F_1-ATPase from pig heart mitochondria. The previously described procedure (Williams, N. and Pedersen, P.L. (1986) Methods Enzymol. 126, 484–489) to dissociate the rat liver F_1-ATPase by cold treatment followed by warming at 37°C has been adapted for the pig heart enzyme. Removal of endogenous nucleotides from that enzyme before dissociation led to the efficient separation of the α and γ subunits from β, δ and ϵ subunits. The β subunit was purified in the hundred-milligram range by anion-exchange chromatography in the absence of any denaturing agent. This subunit was free from any bound nucleotide and almost no ATPase and adenylate kinase-like activities were detected. The δ and ϵ subunits were purified by reversed-phase chromatography (RP-HPLC) in the milligram range. As recently reported (Penin, F., Deléage, G., Gagliardi, D., Roux, B. and Gautheron, D.C. (1990) Biochemistry 29, 9358–9364), these purified subunits kept biophysical features of folded proteins and their ability to reconstitute the tight $\delta\epsilon$ complex. The α and γ subunits remained poorly soluble and required dissociation by 8 M guanidinium chloride prior to their purification by RP-HPLC. In addition, characterizations of the five subunits by IEF and SDS-polyacrylamide gel electrophoresis are reported, as well as ultraviolet spectra and solubility properties of the β, δ and ϵ subunits.

Introduction

ATP synthesis in oxidative phosphorylation is catalysed by the ATPase-ATPsynthase (F_0F_1) complex. This ubiquitous enzyme contains two main parts: the F_0 part channelling the transfer of protons across the membrane and the F_1 part containing the nucleotide sites for ATP synthesis and ATP hydrolysis. F_1-ATPase is composed of five different subunits organized according to a $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ stoichiometry (for reviews see Refs. 1–4). To study the structure-function relationships of F_1-ATPase and of its components, the purified five subunits must be available in large amounts, well characterized and as native as possible. Until recently, the F_1-ATPase subunits have most reliably been isolated at a preparative level with a combination of anion- and cation-exchange chromatographies in the presence of 8 M urea, as well for mitochondria [5–7], as for chloroplasts [8] and bacteria [9]. However, poor resolution and low yields are often associated with these time-consuming methods. Moreover, carbamoylation of primary amino group of protein may occur when urea is used as a dissociating agent. Several authors have thus developed RP-HPLC procedures to rapidly purify the F_1-ATPase subunits. In the case of the mitochondrial enzyme, this was achieved by loading purified F_1-ATPase onto RP columns either directly [10,11] or after treatment with guanidinium chloride [12] or F_3Ac [13]. Unfortunately, these procedures are only suitable for micropreparative amounts of subunits, especially for primary structure studies. Moreover, RP-HPLC is known to be potentially denaturing for

Abbreviations: ATPase, adenosine-5′-triphosphatase (EC 3.6.1.34); F_1-ATPase, solubilized mitochondrial ATPase; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; Ap_5A, P_1,P_5-di(adenosine-5′-)pentaphosphate; HPLC, high-performance liquid chromatography; F_3Ac, trifluoroacetic acid; RP-HPLC, reversed-phase chromatography; BSA, bovine serum albumin.

Correspondence: F. Penin, Laboratoire de Biologie et Technologie des Membranes et des Systèmes Intégrés du CNRS, Université Claude Bernard de Lyon I, 43 Bd du 11 Novembre 1918, F-69622 Villeurbanne Cedex, France.

proteins and it is worth checking that biochemical and biophysical features of purified and native proteins are identical.

Purification of the five subunits of F_1-ATPase in the absence of any denaturing agent has only been achieved in the case of bacteria [14–16]. For the mitochondrial enzyme, only the β subunit has been purified under non-denaturing conditions using the ability of F_1-ATPase to dissociate either in the presence of LiCl [17,18] or by cold treatment [19,20].

In the present paper, we report a large-scale purification procedure by HPLC in order to prepare all the five subunits from a single batch of pig heart mitochondrial F_1-ATPase. The protocol described here is simple, rapid and reproducible. This is the first method ever reported for the purification of δ and ϵ subunits in the milligram range. Another particular interest is the high recovery of β subunit (i.e., at least 75% of the β subunit content of the starting F_1-ATPase) allowing the preparation of highly-purified β subunit in the hundred-milligram range without any denaturing process.

Materials and Methods

Materials. AP-1 glass column packed with Accell QMA anion-exchanger medium (500 Å, 37–55 μm, 1×16 cm), gel-filtration HPLC column Protein PAK Glass 200SW were from Waters and DEAE MemSep 1010 chromatography cartridge was from Millipore. RP-HPLC column Delta PAK C4 (300 Å, 15 μm, 3.9×300 mm) was from Waters, Ultrabase C4 cartridge (300 Å, 10 μm, 4.6×30 mm) was from the Société Française de Chromatographie and Aquapore C4 cartridge (300 Å, 7 μm, 4.6×30 mm) was from Brownlee. HPLC was performed on a Waters apparatus consisting of two M510 pumps, an U6K injector and a 990 Photodiode Array Detector. Anion-exchange chromatographies were achieved using a 650 System from Waters. Ultraviolet spectra were recorded with a Beckman-DU7 spectrophotometer. Centriprep concentrators equipped with a YM10 ultrafiltration membrane were from Amicon. Minitan S Ultrafiltration System was from Millipore. Molecular mass marker proteins were from Serva.

Biological preparations. The previously described procedure was used to prepare purified nucleotide-depleted F_1-ATPase from pig heart mitochondria [21]. The cold dissociation procedure described by Williams and Pedersen [20] for rat liver F_1-ATPase was adapted to the pig heart enzyme as follows: the glycerol concentration of F_1-ATPase solution was lowered from 50% to 2% by washing with phosphate buffer (250 mM potassium phosphate, 5 mM EDTA (pH 7.5)) using a Minitan S Tangential Ultrafiltration system equipped with a polysulfone membrane (cut off 30000). The F_1-ATPase was then concentrated to about 15 mg/ml and precipitated by addition of solid ammonium sulfate up to 60% saturation at room temperature. After centrifugation for 10 min at $20000 \times g$, the pellet was dissolved in the above phosphate buffer at 2 mg protein per ml and dialysed overnight at 4°C against the same buffer. After incubation at 37°C for 45 min, the precipitated proteins were pelleted by centrifugation (15 min, $100000 \times g$). The pellet was named 'precipitated fraction' (numbered 2 in Fig. 1) and the supernatant 'soluble fraction' (numbered 3 in Fig. 1).

Analytical methods. The protein content was estimated either by the method of Lowry et al. [22] or by the previously described electrophoretic method [23]. SDS-polyacrylamide gel electrophoresis (15% acrylamide) was performed according to Ref. 24, as detailed in Ref. 25. Densitometric integration of Coomassie blue stained gel was performed using a Vernon PHI 6 gel densitometer. Isoelectric focusing was achieved either in the presence or in the absence of 9 M urea on a 4% polyacrylamide gel (0.5 mm thick) with a mixture of ampholines 3.5–9.5. The conditions described in the LKB Laboratory Manual (1986, pp. 56–58) were followed using a Multiphor II electrofocusing apparatus (LKB). To determine the pH gradient profile after migration, a 1 cm band of gel was cut and sectioned into 4 mm segments. Each segment was incubated overnight in 400 μl of 10 mM KCl prior to pH reading at room temperature [26]. Measurements of ATPase activity were performed by incubating the β-subunit preparation (7 μM) in 50 mM Tris-acetate, 2.5 mM ATP, 5 mM $MgSO_4$ (pH 7.5) at 30°C. At intervals, aliquot samples and controls (without β subunit) were withdrawn and their content in AMP, ADP and ATP were determined by HPLC as previously described [27]. The rate of ATPase activity was constant over a period of at least 20 h. To estimate adenylate kinase activity in the β-subunit preparation, the same method was used except that the incubation medium (50 mM Tris-acetate, 5 mM $MgSO_4$, pH 7.5) contained either 2.5 mM ADP or 2 mM ATP and 0.5 mM AMP, in the presence or in the absence of 0.2 mM Ap_5A [28].

Results

The overall purification procedure of the five subunits of F_1-ATPase is summarized in Fig. 1A and is further detailed below. Each intermediate protein fraction and purified subunits are numbered from 1 to 9 and the SDS electrophoresis patterns of these fractions are presented in Fig. 1B, using the same numbering for the lanes.

Dissociation of F_1-ATPase

F_1-ATPase was depleted from endogenous nucleotides by gel filtration on Ultrogel AcA 34 in the

presence of 50% glycerol as previously described [21]. The resulting nucleotide-depleted F_1-ATPase was submitted to cold dissociation and then incubated at 37°C as described under Material and Methods. After centrifugation, electrophoretic analysis shows that the 'soluble fraction' (Fig. 1B, lane 3) was essentially composed of β, δ and ϵ subunits, while the 'precipitated fraction' mainly contained α and γ subunits (Fig. 1B, lane 2). The relative amounts of α and β subunits in each fraction were estimated by densitometric integration of Coomassie blue stained electrophoresis gel. Considering the amount of the β subunit as the 100% reference in the 'soluble fraction', the slight α subunit contamination represented less than 1%. Conversely, the low β subunit content in the 'precipitated fraction' did not exceed 5% as compared to the α subunit

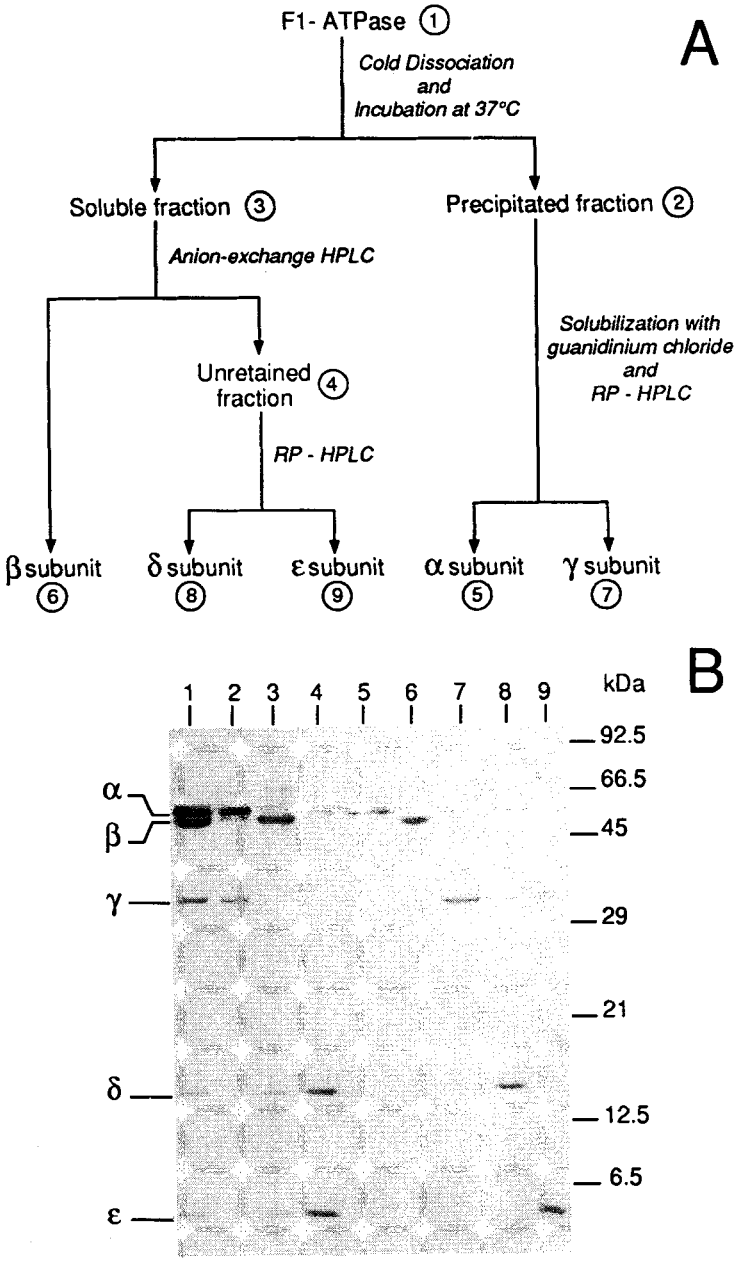

Fig. 1. Schematic purification procedure of the five subunits of F_1-ATPase and electrophoretic analysis. (A) The purification scheme describes the successive steps to purify each subunit from F_1-ATPase. Each step is detailed in the text. For clarity, the protein fractions are numbered from 1 to 9. (B) SDS polyacrylamide gel electrophoresis of purified subunits of F_1-ATPase and intermediate purification fractions. Lane numbering corresponds to that of protein fractions in (A). Lane 1, F_1-ATPase (14 μg); lane 2, precipitated fraction (6 μg); lane 3, soluble fraction (6 μg); lane 4, unretained fraction (5 μg); lane 5, purified α subunit (3 μg); lane 6, purified β subunit (3 μg); lane 7, purified γ subunit (2 μg); lane 8, purified δ subunit (3 μg); lane 9, purified ϵ subunit (2 μg). Migrations of known molecular mass proteins are indicated on the right of the figure. SDS polyacrylamide gel electrophoresis was performed according to Ref. 24 as detailed in Ref. 25 on 15% acrylamide gel (Coomassie blue staining, lowest detection limit about 0.1 μg).

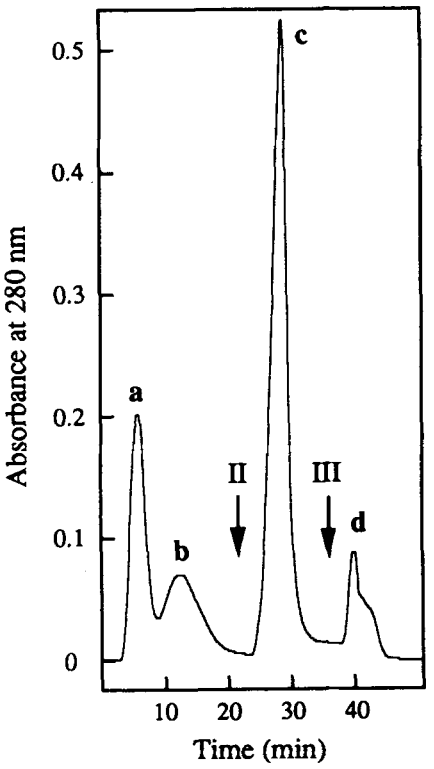

Fig. 2. Purification of β subunit by anion-exchange chromatography on Accell QMA phase: The phosphate concentration of the 'soluble fraction' (numbered 3 in Fig. 1) was lowered to 150 mM (pH 7.5) by addition of water and proteins were concentrated by ultrafiltration on YM 10 membrane (Centriprep device). A sample of 5 ml containing 20 mg of protein was loaded on the Accell QMA column (1×16 cm) equilibrated in 150 mM potassium phosphate (pH 7.5) (buffer I). The flow rate was held at 2 ml/min. The chromatography was developed with buffer II (250 mM potassium phosphate, 50 mM potassium chloride (pH 7.5)) and buffer III (250 mM potassium phosphate, 1 M potassium chloride (pH 7.5)) as indicated by arrows. Peak a: 'unretained fraction' (numbered 4 in Fig. 1) containing δ and ϵ subunits; peak b: EDTA and nucleotides; peak c: purified β subunit; peak d: aggregated protein and impurities. See the text for details.

content. When the enzyme was not depleted from its endogenous nucleotides by gel filtration in the presence of 50% glycerol (i.e., when it contained about 2.1 mol of nucleotides per mol of F_1-ATPase, [21]), the dissociation of F_1-ATPase was much less efficient. Indeed, as much as 30% β subunit remained associated with α and γ subunits in the 'precipitated fraction' (data not shown).

Purification of β subunit

The 'soluble fraction' (numbered 3 in Fig. 1) was diluted to lower the phosphate concentration to 150 mM and protein concentrated to about 4 mg per ml by ultrafiltration using a Centriprep device equipped with a YM10 membrane. Samples containing about 20 mg protein were applied to an Accell QMA anion-exchange column. Fig. 2 shows a typical elution profile. The two first peaks a and b were eluted by washing the column with 150 mM potassium phosphate (pH 7.5) (buffer I). Peak a, named 'unretained fraction' and numbered 4 in Fig.1, contained δ and ϵ subunits and trace amounts of α subunit, as shown by electrophoresis (lane 4, Fig. 1B). Peak b contained EDTA and some nucleotides. The β subunit was eluted in a single peak (peak c) by 250 mM potassium phosphate, 50 mM KCl (pH 7.5) (buffer II). By washing the column with 1 M KCl in the same buffer (buffer III) some aggregated proteins and some impurities coming from the phosphate buffer were eluted (peak d). The β-subunit fractions obtained from several chromatographies were pooled, concentrated by ultrafiltration on a YM10 membrane (Centriprep device), precipitated by addition of solid ammonium sulfate up to 60% saturation and stored at 4°C. Alternatively, the concentrated solution of β subunit (at about 5 mg protein /ml) was stored in liquid nitrogen. The electrophoretic pattern presented in Fig. 1B, lane 6 shows the high purity of the β subunit. Considering the amount of β subunit present in the loaded fraction as 100% reference, more than 90% of this subunit were recovered after chromatography. Small-scale purification of β subunit could also be performed on Mono Q anion exchanger or MEM-SEP DEAE 1010 under similar conditions (data not shown).

Purification of δ and ϵ subunits

Several 'unretained fractions' (numbered 4 in Fig. 1) obtained above by anion exchange chromatography were pooled and concentrated by ultrafiltration on a YM10 membrane (Centriprep device) to about 0.5 mg/ml and were stored in liquid nitrogen. From this fraction, the $\delta\epsilon$ complex could be purified by gel filtration as described previously [23]. The purification of δ and ϵ subunits was performed by RP-HPLC using a linear gradient of acetonitrile in 0.1% F_3Ac. Fig. 3 shows a typical profile when 0.8 mg of protein were applied on a small cartridge (4.6 × 30 mm) packed with Ultrabase C4 phase.

The ϵ subunit was eluted as a single peak at 32% acetonitrile. After lyophilisation, the ϵ subunit was dissolved in a minimal volume of 100 mM potassium phosphate, 2 mM dithiothreitol (pH 6.0), and stored in liquid nitrogen. The electrophoretic pattern presented in Fig. 1B (lane 9) shows the high purity of the ϵ-subunit preparation. More than 80% of the ϵ subunit present in the loaded fraction were recovered by this procedure (the 100% reference is given by the amount of this subunit in the loaded fraction).

The elution profile in Fig. 3 shows that the thin peak b eluted at 42% acetonitrile was followed by a long tail. Peak b exclusively contained pure δ subunit as shown by electrophoresis (lane 8, Fig. 1B). Electrophoretic analyses not reported here revealed that the tail contained some δ and ϵ subunits and some

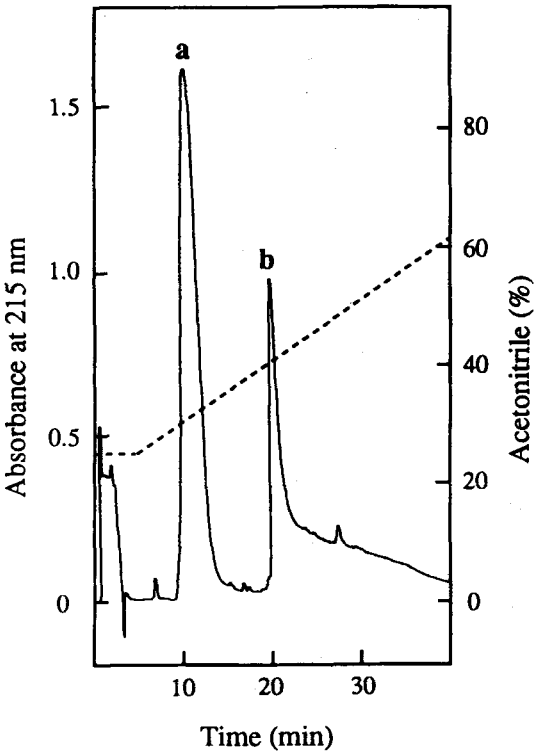

Fig. 3. Purification of δ and ϵ subunits by reversed-phase chromatography: The 'unretained fraction' (peak a in Fig. 2, number 4 in Fig. 1) was concentrated up to about 0.5 mg protein per ml by ultrafiltration on YM 10 membrane (Centriprep device). A sample containing 0.8 mg of protein was loaded on the column (cartridge of 4.6×30 mm packed with Ultrabase C_4 phase) previously equilibrated in 25% acetonitrile, 0.1% F_3Ac. A linear gradient of acetonitrile in 0.1% F_3Ac was performed as indicated. Peak a: purified ϵ subunit; peak b: purified δ subunit. See the text for details.

contaminating proteins. The δ subunit present in the thin peak b was lyophilised, dissolved in a minimal volume of 100 mM potassium phosphate (pH 7.5), and stored in liquid nitrogen. Considering the δ subunit amount in the loaded fraction as 100% reference, about 40% of this subunit were recovered after chromatography. This rather low yield could be explained by the poor solubility of the δ subunit in acidic media. The amplitude of peak a (ϵ subunit) appears much larger than that of peak b (δ subunit), whereas protein amounts in each peak are roughly the same. This great difference is mainly due to the higher ultraviolet absorption coefficient of ϵ subunit as compared to that of δ subunit (see below).

The purification of δ and ϵ subunits by RP-HPLC has also been performed either on cartridges (4.6 × 30 mm) packed with Aquapore C4 or C8 phases, or on a Delta PAK C4 column (3.9 × 300 mm) under similar chromatographic conditions (data not shown). The elution profiles and the yields of purified subunits were very close to the above ones.

Purification of α and γ subunits

The α and γ subunits present in the 'precipitated fraction' obtained after cold dissociation of F_1-ATPase (numbered 2 in Fig. 1) were poorly soluble in the absence of denaturing agents. Hence, the 'precipitated fraction' was treated for 16 h at room temperature with 8 M guanidinium chloride, 50 mM dithiothreitol, 20 mM Tris-H2SO_4 (pH 8.0), at 5 mg protein per ml. Samples of about 1 mg protein were applied on a reversed-phase column (cartridge of 4.6 × 30 mm packed with Ultrabase C4 phase) equilibrated with 40% acetonitrile in 0.1% F_3Ac, and the chromatography was developed as indicated in Fig. 4. The elution peaks a (44% acetonitrile) and b (47% acetonitrile) contained purified γ and α subunits respectively, as shown by the electrophoretic patterns presented in Fig. 1B (γ subunit, lane 7; α subunit, lane 5). Other electrophoretic analyses, not shown here, revealed that: (i) the two successive shoulders following peak a also contained γ subunit, (ii) the proteins eluted between peak b and c were a mixture of α and γ subunits, (iii) peak c mainly contained β subunit and small amounts of α and γ subunits. Recoveries of α and γ subunits

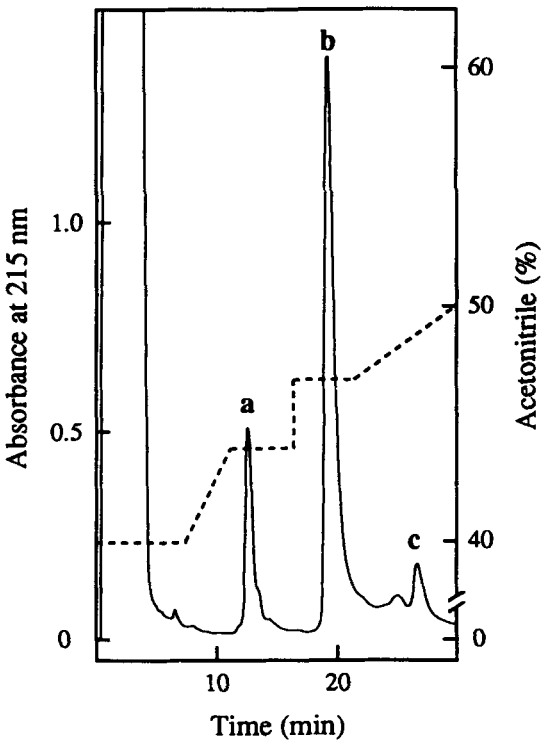

Fig. 4. Purification of α and γ subunits by reversed-phase chromatography: The 'precipitated fraction' (numbered 2 in Fig. 1) was incubated for 16 h at room temperature with 8 M guanidinium chloride, 50 mM dithiothreitol, 20 mM Tris-H_2SO_4 (pH 8.0) at 5 mg protein per ml. Samples of 0.75 mg protein were loaded on the column (cartridge of 4.6×30 mm packed with Ultrabase C_4 phase) previously equilibrated in 40% acetonitrile, 0.1% F_3Ac. The separation was carried out using the indicated gradient of acetonitrile in 0.1% F_3Ac. Peak a: purified γ subunit; peak b: purified α subunit; peak c: mixture of α, β and γ subunits. See the text for details.

were around 60% and 50%, respectively. Results of the same kind were obtained under similar chromatographic conditions using Delta PAK C4 phase (data not shown). Purified α and γ subunits were very poorly soluble in classical protein buffers in the absence of denaturing agents such as urea or guanidinium chloride.

Properties of the purified subunits

Purity of every isolated subunit was checked by electrophoresis, RP-HPLC and IEF. The electrophoretic patterns presented in Fig. 1B show that a single band was obtained for each purified subunit (lanes 5 to 9) without any detected contamination under the used conditions where the lowest detectable protein amount was about 0.1 μg. Moreover, when larger amounts of purified α, β or γ subunits (up to 30 μg) were analysed by electrophoresis, contaminating proteins represented less than 1% (not shown). These contaminations seemed essentially due to subunit degradation. In addition, no isolated subunit was ever found significantly contaminated by the others. In the case of δ and ϵ subunits, neither contamination nor degradation have ever been detected. When large amounts (100–300 μg) of purified subunits were submitted to RP-HPLC, contaminations represented less than 1% of the detected peak and were not necessarily proteins (data not shown). Each purified subunit was also analysed by IEF. Table I shows that several molecular species were observed for α, β and γ subunits while only one was detected for δ subunit. The ϵ subunit migrated near the cathode in the pH range used and it could thus not be determined whether it was homogeneous. However, both native and reconstituted $\delta\epsilon$ complexes exhibited only one focused band when analysed by IEF (p*I* 5.9 [23]). This indicates that the ϵ subunit probably exists as a single molecular species. Identical patterns were observed for the five subunits when the whole F_1-ATPase was subjected to IEF under denaturing conditions. These results indicate that the purification procedures described above did not modify the electrophoretic properties of F_1-ATPase subunits. Table I also shows that α and γ subunits are rather neutral proteins, β and δ subunits are acidic proteins while ϵ subunit is a very basic one.

TABLE I

Isoelectric focusing analysis of the purified subunits of F_1-ATPase

Isoelectric focusing in the presence or in the absence of 9 M urea and p*I* estimation were performed as described in Materials and Methods. When several isoelectric focused bands were present for one subunit, the p*I* and the relative amounts of each molecular species are indicated. These relative amounts were determined by densitometric integration of IEF gel, taking the sum of all molecular species for a given subunit as 100%

Purified subunit	Isoelectric point	Relative amounts of molecular species (%)
α [a]	6.5	5
	6.6	10
	6.9	85
β [b]	4.7	50
	4.8	50
γ [a]	8.0	30
	8.1	70
δ [b]	4.4	100
ϵ [b]	$\geqslant 9.5$	(100) [c]

[a] IEF in the presence of 9 M urea.
[b] The same data were obtained when IEF was performed either in the presence or absence of 9 M urea.
[c] Deduced from IEF analysis of the $\delta\epsilon$ complex, see text.

β subunit. The ultraviolet absorption spectrum of pure β subunit in 100 mM potassium phosphate (pH 7.5) (solid line, Fig. 5A) exhibited a maximum at 276.5 nm. An absorption coefficient of 3.7 ± 0.1 could be calculated ($A_{1\%,1\ cm}$, 276.5 nm) using the Lowry procedure to determine protein concentration and taking BSA as the standard. This absorption peak was due only to tyrosine residues, since it was previously checked by intrinsic fluorescence that no tryptophan residue was present in β subunit [23]. Under alkaline conditions (0.1 M NaOH), the absorption maximum was shifted to 293 nm (Fig. 5A, dotted line) and the amplitude of the absorption peak was increased. On the basis of a molecular mass for β subunit of 51000 and a molar extinction coefficient for tyrosine residue of 2390 at 294.4 nm [29], a value of 11.2 tyrosine residues per β subunit was calculated.

The β subunit was highly soluble at neutral pH in phosphate or Tris buffers (at least up to 20 mg protein/ml), even when the subunit was solubilized from an ammonium sulfate precipitate. When analysed by gel filtration on a Protein PAK 200SW column equilibrated in 100 mM potassium phosphate (pH 7.5), the β subunit was eluted as a single peak with an apparent molecular mass of 50000 Da (data not shown). The β subunit did not contain any bound nucleotide as checked by the HPLC method used to determine the content of nucleotide in F_1-ATPase [27]. The ATPase activity of the β subunit preparation was very low: less than 1 nmol/min per mg protein. In addition, some adenylate kinase-like activity was detected (about 0.8 nmol/min per mg protein) which was only partially inhibited by Ap_5A (70% inhibition in the presence of 200 μM Ap_5A).

δ subunit. The ultraviolet spectrum of δ subunit in 100 mM potassium phosphate (pH 7.0), is presented in Fig. 5B (solid line). It exhibits several maxima at 252, 258.5, 264.5, 268 and 276 nm. This multiplicity of maxima was certainly due to a high content in phenylalanine residues as compared to tyrosine ones. Using

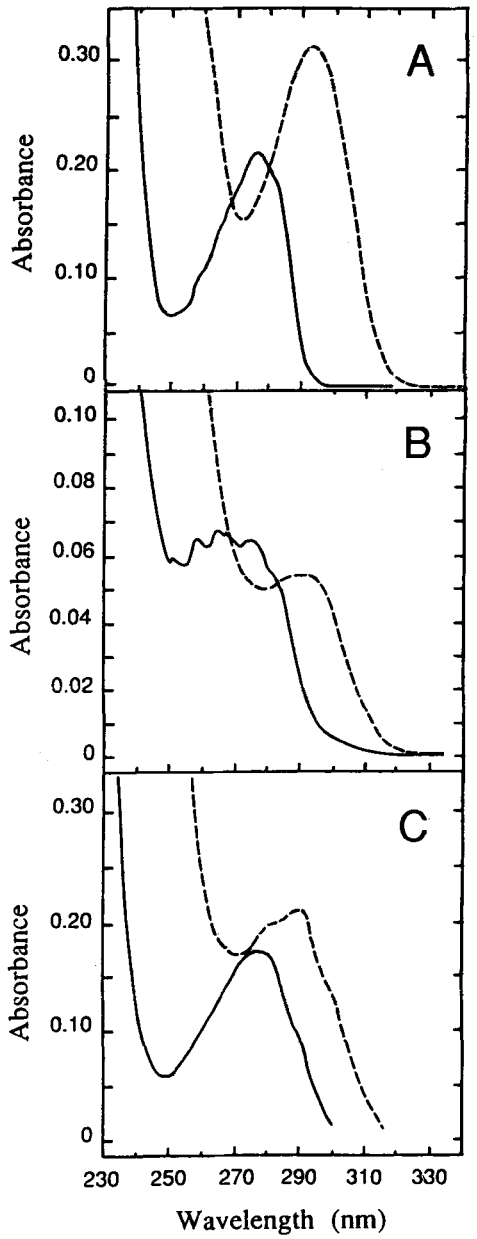

Fig. 5. Ultraviolet absorption spectra of β, δ and ϵ subunits of F_1-ATPase: The ultraviolet absorption spectra of β, δ and ϵ subunits were recorded either in 100 mM potassium phosphate (solid line) or in 0.1 M NaOH (dotted line). The protein concentration were determined by the Lowry procedure [22] for β subunit and by the electrophoretic procedure [23] for δ and ϵ subunits. (A) β subunit (0.58 mg/ml); the phosphate buffer was at pH 7.5. (B) δ subunit (0.35 mg/ml); the phosphate buffer was at pH 7.0. (C) ϵ subunit (0.1 mg/ml); the phosphate buffer was at pH 6.0 and contained 2 mM dithiothreitol.

the electrophoretic procedure previously described to estimate protein content [23], an absorption coefficient of 1.8 ± 0.1 ($A_{1\%,1\ cm}$, 276 nm) could be calculated for the δ subunit. In 0.1 M NaOH, the amplitude of the absorption peak was decreased and the wavelength of maximal absorption was shifted to 289 nm (Fig. 5B, dotted line). The δ subunit did not contain any tryptophan, as checked previously by intrinsic fluorescence studies [23]. Thus, on the basis of a molecular mass for δ subunit of 15000 Da and a molar extinction coefficient for tyrosine residues of 2390 at 294.4 nm [29], a value of 1.2 tyrosine residue per δ subunit was calculated.

Lyophilized δ subunit was easily solubilized in phosphate buffer at pH above 6.5 while it was poorly soluble in acidic media. This is consistent with the acidic nature of δ subunit as shown in Table I. When analysed by gel filtration on a Protein Pak 200SW column equilibrated in 100 mM potassium phosphate (pH 7.0), the δ subunit was eluted as a single peak with an apparent molecular mass of 23000 Da (data not shown).

ϵ subunit. Fig. 5C shows that the ultraviolet spectrum of ϵ subunit in 100 mM potassium phosphate, 2 mM dithiothreitol (pH 6.0) (solid line) exhibited a maximum at 276 nm. An absorption coefficient ($A_{1\%,1\ cm}$, 276 nm) of 17.4 ± 0.2 was calculated, using the electrophoretic procedure to estimate the protein concentration [23]. Under alkaline conditions (0.1 M NaOH), the wavelength of maximal absorption was 291 nm and the amplitude of the peak was slightly increased (Fig. 5C, dotted line). From absorbance at 294.4 nm, a total content of tyrosine and tryptophan of 4.6 was estimated since at this wavelength the molar extinction coefficients of tyrosine and tryptophan equalled 2390 [29] and since the molecular mass of ϵ subunit was 5600. In addition, a molar ratio of tyrosine to tryptophan of 2.8 was calculated using the formula described in Ref. 29. The combination of all data allowed to estimate that ϵ subunit contained 1.2 tryptophan residues and 3.4 tyrosine residues.

Lyophilised ϵ subunit was soluble in buffers at pH $\leqq 6.0$ but poorly at pH above 6.0. This was consistent with its highly basic nature (Table I). When analysed by gel filtration on a Protein Pak 200SW column equilibrated in 100 mM potassium phosphate, 2 mM dithiothreitol (pH 6.0), the ϵ subunit was eluted as a single peak. However, the retention time was out of range of the calibration curve established with proteins of known molecular masses. Moreover, under more acidic condition (i.e., 50 mM sodium acetate, 100 mM sodium sulfate (pH 5.0)), the retention time decreased. These results indicated that interactions between ϵ subunit and stationary phase occurred.

Discussion

The general procedure described here to purify all the five subunits of F_1-ATPase from pig heart mitochondria brings several great improvements as compared to other published methods. Indeed, large amounts of the five subunits are highly purified from a single F_1-ATPase preparation with high yields. Consid-

ering the content of each subunit in F_1-ATPase as 100% reference before cold dissociation, one can purify 55% of α subunit, 75% of β subunit, 40% of γ subunit, 30% of δ subunit and 70% of ϵ subunit. Typically, starting from 300 mg of purified nucleotide-depleted F_1-ATPase, as much as 90 mg β subunit, 5 mg δ subunit and 3 mg ϵ subunit can be easily purified within two days. As checked by SDS electrophoresis and RP-HPLC, each purified subunit appears more than 99% pure and homogeneous on the basis of its molecular mass. IEF analysis revealed several molecular species for the purified α, β and γ subunits. However, since the same heterogeneities are observed for these subunits when the whole starting F_1-ATPase is analysed under the same conditions, they are not produced during the purification procedure. Such heterogeneities might be due to ragged N-terminal extremities of the subunits, as previously reported for the beef heart F_1-ATPase [7]. In addition, the p*I* determined for the five subunits of pig heart F_1-ATPase is in agreement with the results reported for beef heart F_1-ATPase [6,7].

One essential step for the efficiency of subunit purification is the cold dissociation of F_1-ATPase, previously described for the rat liver enzyme [20]. In order to make this treatment highly efficient, F_1-ATPase must be first depleted as much as possible from its endogenous nucleotides. Indeed, when endogenous nucleotides are still present, some undissociated F_1-ATPase remains in the 'soluble fraction' and is eluted just before the β subunit during anion-exchange chromatography. In contrast to data reported by Williams et al. [19] for rat liver F_1-ATPase, the 'soluble fraction' from pig heart enzyme does not contain any γ subunit and subsequently no $\beta\gamma$ complex can be purified.

As yet, substantial quantities of α and γ subunits from mitochondria can only be solubilized in the presence of denaturing agents. This requirement limits structural and functional studies of these subunits. Nevertheless, the availability of large quantities of α and γ subunits purified as described here, allows to find the best conditions for their study.

Our preparation of β subunit appears to be monodispersed when analysed by gel filtration and does not contain any nucleotide. The latter feature is consistent with results reported for purified β subunits from *Escherichia coli* [30,31] and from beef heart mitochondria [32]. However, preparations of β subunit containing about 0.1 mole nucleotide/mole β subunit have been described for *Rhodospirillum rubrum* [33,34] and chloroplast [35]. Our β subunit preparation was able to bind nucleotides (ATP and ADP) and fluorescent analogues. However, we have observed that the β subunit tends to form polymers (and/or aggregates) when it is incubated with ATP or ADP at concentration as low as 50 μM, either in the presence or in the absence of magnesium ions (data not reported here). The use of rather concentrated phosphate buffer to purify β subunit might prevent any nucleotide binding and consequently any possible aggregation. This lack of aggregation probably explains our high recovery of purified β subunit.

Various isolated β subunits have been found to have either no measurable or very low ATPase activity [9,15,36–40] and it remains unclear whether these very low activities are an intrinsic β-subunit property or are due to contamination. The ATPase activity of our β-subunit preparation was lower than 1 nmol/min per mg protein, which is among the weakest activities reported for β subunit preparation from various species [36–39,41]. Although no contamination of β subunit by the other F_1-ATPase subunits are detected by electrophoretic analysis, this ATPase activity could be due to trace amount of F_1-ATPase, especially since the behavior of F_1-ATPase in anion-exchange chromatography is very close to that of β subunit. However, taking into account the specific activity of purified F_1-ATPase (120 μmol/min per mg [21]), it can be calculated that no more than $8 \cdot 10^{-6}$ mg of F_1-ATPase could be present per mg of β subunit. Alternatively, traces of α subunit (not detectable by electrophoresis analysis) might be responsible of this ATPase activity since it has been reported that complexes of α and β subunits exhibit such activity at least in bacteria [38,39,41] and in chloroplasts [40]. Besides, the very low, only partially sensitive Ap_5A adenylate kinase-like activity detected in our β subunit preparation might also be due to some enzymatic contamination.

We have recently reported that δ and ϵ subunits are associated in a tight $\delta\epsilon$ complex [23] which can be purified as a molecular entity by gel filtration from the 'unretained fraction' (numbered 4 in Fig. 1). All attempts to efficiently break the δ–ϵ interaction by ion-exchange chromatography have failed. Up to now, the only reliable way to dissociate $\delta\epsilon$ complex and to purify δ and ϵ subunits remains RP-HPLC in acidic medium (0.1% F_3Ac). Although these conditions are rather drastic, the purified δ and ϵ subunits exhibit circular dichroism spectra typical of folded proteins and retain their potentiality to reconstitute the $\delta\epsilon$ complex [23]. Moreover, the reconstituted $\delta\epsilon$ complex exhibits the same chromatographic behavior, circular dichroism spectra and intrinsic fluorescence features as the isolated $\delta\epsilon$ complex shows. Thus, the δ and ϵ subunits purified as described here appear suitable for the study of their functional role inside the F_0F_1 complex.

All estimations of δ and ϵ protein concentrations with conventional procedures, as proposed by Lowry et al. [22] or Bradford [42] or with bicinchoninic acid [43], have been found unsatisfactory since the ϵ subunit amount is largely overestimated whereas the δ subunit

is underestimated. This is probably due to the particular aminoacid composition of these small proteins. An accurate estimation of the δ and ϵ protein concentrations is obtained by using the previously described electrophoretic procedure [23] and taking the amount of these subunits in F_1-ATPase as the reference. This allows the determination of specific protein absorption coefficients (see Results) which greatly simplify the protein content estimation for purified δ and ϵ subunits. In addition, the tyrosine contents determined for purified β, δ and ϵ subunits from pig heart are in good agreement with those reported for these subunits from beef heart F_1-ATPase [7].

From a technical point of view, the Accell QMA anion-exchange phase used to purify the β subunit in large quantities appears to be as effective as a MonoQ column. A preparative column can be packed at very low price. The reversed-phase chromatographic profiles presented here (Figs. 3 and 4) demonstrate that an efficient protein separation can be performed on small cartridges (4.6 × 30 mm) even when as much as 1 mg of protein is loaded. Their much lower cost as compared to classical columns allows to use one cartridge for each type of application, thus avoiding any contamination by residual protein from previous runs. Nevertheless, regeneration of reversed-phase media could efficiently be performed by repeated injections of 8 M guanidinium chloride in 50 mM Tris-H_2SO_4, 20 mM dithiothreitol (pH 8.0) while washing with 60% acetonitrile in 0.1% F_3Ac.

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