The δ - and ϵ -subunits of bovine F_1 -ATPase interact to form a heterodimeric subcomplex

George L. ORRISS, Michael J. RUNSWICK, Ian R. COLLINSON, Bruno MIROUX, Ian M. FEARNLEY, J. Mark SKEHEL and John E. WALKER*

The Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

The δ -subunit of bovine F_1 -ATPase was expressed from a bacterial vector at fairly high level in *Escherichia coli*, but the yield of bovine ϵ -subunit was rather low under similar conditions. However, co-expression of the proteins from a dicistronic operon δ - ϵ in the same expression vector, produced both of them in good yield in a soluble form in the bacterial cytoplasm, and by chromatography it was found that the δ - and ϵ -subunits were associated in a stable complex. The amino groups in the complex were labelled exhaustively by chemical reaction under denaturing conditions with ethyl-[1- 14 C]acetimidate. The α -amino groups of the proteins were unmodified, but complete reaction of all ϵ -amino groups in both proteins was demonstrated by deter-

mination of the molecular masses of the modified proteins by electrospray MS. The modified subunits were separated by denaturing gel electrophoresis, and from measurements of the ratio of incorporated radioactivities and the lysine contents of the proteins, it was calculated that the subcomplex contains equimolar amounts of the two proteins. As the apparent molecular mass of the complex determined by gel filtration was 29 kDa, it appears that the complex contains one copy of each protein. It is likely that the δ - and ϵ -subunits are associated in a similar manner in the bovine F_1 -ATPase complex, and that, like a bacterial homologue of the δ -subunit, they interact with the γ - and β -subunits.

INTRODUCTION

The catalytic domain of ATP synthase in eubacteria, chloroplasts and mitochondria is a peripheral membrane assembly known as F_1 -ATPase (for reviews see [1,2]). It contains five different polypeptides in the ratio $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$. In the 2.8 Å-resolution electron density map obtained by X-ray analysis of crystals of F_1 -ATPase from bovine heart mitochondria, the positions of 2983 amino acids have been determined [3]. With the exception of short disordered regions at their N-terminals, the sequences of the three α - and the three β -subunits have been traced, together with three segments of sequence in the single γ -subunit, but the positions of the remainder of the γ -subunit (about 150 amino acids) and the entire sequences of the δ - and ϵ -subunits (150 and 50 amino acids respectively) were not determined, as they are disordered in the crystal lattice.

In order to facilitate study of the locations and structures of the bovine δ - and ϵ -subunits, as described below, both proteins have been produced by heterologous overexpression in *Escherichia coli*. The δ -subunit was quite well expressed alone, and accumulated in a soluble form in the bacterial cytoplasm. In contrast, similar attempts to overexpress the bovine ϵ -subunit alone were relatively unsuccessful, but when it was co-expressed with the bovine δ -subunit from a recombinant dicistronic operon, both proteins were well expressed and formed a stable water-soluble complex in the cytoplasm. The purified complex contained equimolar quantities of each subunit. It is likely that the two subunits are associated in a similar way in bovine F_1 -ATPase.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). *Pyrococcus furiosus* DNA polymerase

was obtained from Stratagene (La Jolla, CA, U.S.A.). Ethyl-[1- 14 C]acetimidate (specific radioactivity 27 mCi/mmol) was supplied by Amersham Radiochemicals (Amersham, Bucks., U.K.). Streptomycin sulphate was obtained from Sigma Chemical Company (Poole, Dorset, U.K.).

Analytical methods

Protein concentrations were estimated by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL, U.S.A.). Polyacrylamide gels containing a 12–22% acrylamide gradient (acrylamide/bisacrylamide: 30:0.8, w/w) were cast in minigel format (10 cm $\times$ 10 cm $\times$ 0.6 cm) and run in the buffer system described by Laemmli [4]. Proteins were detected by staining with PAGE Blue 83 dye. After transferring the proteins by electrophoresis to a poly(vinylidene difluoride) membrane, N-terminal sequences of proteins were determined by automated Edman degradation in an Applied Biosystems Procise model 494 protein sequencer. For electrospray mass spectrometric analysis in a Perkin-Elmer Sciex API III⁺ triple-quadrupole mass spectrometer, the δ - and ϵ -subunits were separated from pure $\delta\epsilon$ -complex by reverse-phase HPLC. Chromatography was conducted on a Vydac C8 column [150 mm $\times$ 1.0 mm internal diameter (i.d.)] in an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Auburn, CA, U.S.A.). The solvent (flow rate of 50 μ l/min) was either 0.1% (v/v) aqueous trifluoroacetic acid, followed by a linear gradient of acetonitrile containing 0.09% (v/v) trifluoroacetic acid (chromatography of ϵ sample a; see Table 1), or 0.25% formic acid followed by a linear gradient of acetonitrile containing 0.2% formic acid (all other samples). The eluate was split with a Valco T-fitting, and a portion (approx. 10% or 5 μ l/min) was transferred to the electrospray ionization interface of the mass spectrometer via coated fused silica capillary tubing (75 μ m i.d.). The mass spectrometer employs pneumatically assisted electro-

Abbreviation used: i.d., internal diameter.

* To whom correspondence should be addressed.

Table 1 Molecular masses of δ - and ϵ -subunits of bovine F_1 -ATPase

Molecular masses measured by electrospray (esi) MS were compared with values calculated from the protein sequences. The δ - and ϵ -subunits contain 4 and 8 lysine residues, respectively, and each has a free α -amino group.

Protein sample	Molecular mass (Da)		
	By esi-MS	Calculated	Amino acids
Purified δ	15 065.2	15 064.9	1–147
Purified ϵ (sample a)*	5781.6	5782.8	1–51
	5651.2	5651.6	2–51
	5551.5	5552.5	3–51
Purified ϵ (sample b)†	5781.3	5782.8	1–51
	5651.1	5651.6	2–51
	5551.1	5552.5	3–51
Acetimidated δ	15 232.7	15 230.2‡	1–147
Acetimidated ϵ †	6118.5	6112.33‡§	1–51
	5987.7	5981.1‡§	2–51

* The three values arise from the complete chain with initiator methionine-1 present, by partial removal of the initiator methionine-1, and by initiation at codon-2 (GTG) and removal of the corresponding initiator methionine.

† Produced from the modified dicistronic vector in which codon-2 (GTG) was replaced by GTT. The two values arise from translational initiation at codon-1 and partial removal of the initiator methionine.

‡ The calculations assume that the α -N-terminals were unmodified by acetimidation, as N-terminal sequencing of modified subunits indicated.

§ The difference between the measured and calculated values arises at least in part because the mass measurements were made with protein that had been modified with ^{14}C -labelled acetimidate. According to information received from the manufacturers, the ratio of ^{14}C : ^{12}C in the reagent is approximately 1:8.

spray ionization. It was operated in positive-ion mode, with both quadrupoles (Q1 and Q3) tuned with a mixture of poly(propylene glycols), and calibrated to give unit mass resolution at 2000 mass to charge ratios. The protein ions produced by electrospray ionization were analysed in quadrupole Q1 by scanning from 600 to 1600 Da in 5.3 s, with a step size of 0.2 Da and a dwell time of 1 ms.

Construction of bacterial expression plasmids

(a) Separate expression of the δ - and ϵ -subunits

The DNA sequences encoding the δ - and ϵ -subunits of bovine F_1 -ATPase were amplified from total heart cDNA (10 ng) by 30 cycles of PCR with appropriate oligonucleotide primers based on the cDNA sequences [5,6]. The forward primers for the δ - and ϵ -subunits were synthesized with additional 5' linker sequences TAGGAATTCATATG (containing *Eco*RI and *Nde*I sites), and TAGGGATCCCATATG (containing *Bam*HI and *Nde*I sites). The 5' linker (TAGAAGCTTTTA) of the reverse primer for the δ - and ϵ -subunits contained a *Hind*III site. The amplified products were fractionated on 1.4% (w/v) agarose gels, purified by the 'GeneClean' procedure (BIO101, La Jolla, CA, U.S.A.), and cloned into appropriately predigested M13 mp8 vectors. DNA sequences were verified by the modified dideoxy procedure [7,8]. Double-stranded fragments coding for the bovine δ - and ϵ -subunits were released from the replicative form of M13, and recloned, as described before [9], into the high-copy-number expression vector pMW7 [10], which is a close relative of pRK172 derived from the low-copy-number pET vectors [11]. Plasmids were amplified in *E. coli* TG1 grown at 37 °C for 5 h. Colonies containing the requisite coding sequences were identified by amplifying the insert by PCR using a forward primer specific for the 5' flanking region of the plasmid and reverse primers

Figure 1 Arrangement of the coding sequences for the δ - and ϵ -subunits of bovine F_1 -ATPase in a bacterial expression vector

The coding sequences of the δ - and ϵ -subunits are separated by an intergenic sequence (IS) AATAATTTTGTAACTTAAGAAGGAGATATA. This sequence duplicates the sequence in the vector and immediately preceding the coding sequence of the δ -subunit. Both sequences contain a Shine and Dalgarno sequence (SD), GAAGGAGA. The arrows show the direction of transcription.

complementary to the 3' end of the coding sequences for the δ - and ϵ -subunits. The sizes of the products were then estimated by gel electrophoresis. Plasmids prepared from these colonies were used to transform *E. coli* C41(DE3), a mutant of BL21(DE3) that permits higher expression levels of heterologous proteins (B. Miroux and J. E. Walker, unpublished work).

(b) Co-expression of the δ - and ϵ -subunits

Vectors were constructed containing the coding sequences of both the bovine δ - and ϵ -subunits, in the order δ - ϵ and vice versa, with a ribosome-binding site in the intergenic sequence (see Figure 1). The $\delta\epsilon$ -vector was generated as follows. First, the DNA sequences for the bovine δ - and ϵ -subunits were amplified from the δ - and ϵ -expression plasmids by 30 cycles of PCR using reverse and forward primers respectively, containing the complement and the sequence of the intergenic region (see Figure 1). In addition, the reverse primer for the δ -subunit was extended in a 3' direction by the complement of 21 bases at the 3' end of the coding sequence of the δ -subunit, and at the 3' end of the forward primer for the ϵ -subunit were the first 21 bases of its coding sequence. The forward primer for the δ -subunit consisted of the linker sequence TAGGGTACCCATATG (containing *Kpn*I and *Nde*I sites) and bases 1–21 of the δ -coding sequence, and the reverse ϵ -primer consisted of the linker TAGAAGCTTTTA (containing a *Hind*III site) and the complement of 21 bases of the 3' end of the ϵ -coding sequence. In a second PCR of 30 cycles, the forward primer for the δ -subunit and the reverse primer for the ϵ -subunit were used with the mixture of products of the first reactions as template to amplify the desired fragment containing the coding sequences for the δ - and ϵ -subunits separated by the intergenic sequence. The amplified products were cloned into M13 mp19 vector which had been digested with *Kpn*I and *Hind*III. When the δ - and ϵ -subunits were expressed from a clone containing this sequence, it was found that translation of the ϵ -subunit had initiated at both the

Figure 2 Chromatographic purification of the bovine $\delta\epsilon$ -subcomplex

Upper panel: ion-exchange chromatography on DEAE-Sephacrose CL-6B in TEPD buffer. The elution position of the $\delta\epsilon$ -subcomplex is shown. Solid line, absorbance at 280 nm; broken line, salt gradient. Middle panel: ion-exchange chromatography on CM-Sephacrose CL-6B in CEPD buffer. The position of the $\delta\epsilon$ -subcomplex is marked. Lower panel: gel-filtration chromatography on Sephacryl S-100 (HR), in TEPDN buffer. The fraction volumes in the upper, middle and lower panels were 4.1 ml, 2.5 ml and 2.0 ml respectively.

expected site and from the GTG codon for valine-2. By changing this codon to GTT, translational initiation of the ϵ -subunit was restricted to the expected ATG (see below). The $\epsilon\delta$ -vector was generated in a similar manner to the $\delta\epsilon$ -vector.

Overexpression of the δ - and ϵ -subunits of bovine F_1 -ATPase in bacteria

A colony of recently transformed cells of *E. coli* C41(DE3) was inoculated into growth medium (50 ml) containing ampicillin (100 μ g/ml). The cells were grown at 37 °C overnight. A sample (2 ml) of this preculture was added to 1 litre of medium containing ampicillin (100 μ g/ml) and the bacteria were grown at 37 °C until their absorbance at 600 nm had reached 0.6. Expression of the bovine proteins was induced by addition of solid isopropylthiogalactopyranoside (150 mg). Further additions of the same amount of inducer followed 1, 2 and 3 h later. Growth of the cells was continued for a further 15 h. The cells were centrifuged (7000 *g*, 10 min), resuspended in a buffer containing 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF), 20 μ M amastatin and 0.002 % (w/v) PMSF, and broken by two passages of the suspension through a French pressure cell at 4 °C. The broken cells were centrifuged (180 000 *g*, 1 h, 4 °C). Streptomycin sulphate solution (10 %, w/v) was added dropwise to the supernatant to a final concentration of 1 % (w/v). The suspension was stirred for 30 min at 4 °C, and then centrifuged (45 000 *g*, 20 min). The supernatant was dialysed twice at 4 °C in 3500 Da cut-off dialysis membrane, against buffer (TEPD; 4 l) consisting of 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 2 mM dithiothreitol and 0.002 % (w/v) PMSF. A precipitate was removed by centrifugation (45 000 *g*, 20 min).

Purification of the bacterially expressed $\delta\epsilon$ -subcomplex of bovine F_1 -ATPase

All of the purification steps were carried out at 4 °C, and 3500 Da cut-off dialysis membrane was used throughout. The solution containing the $\delta\epsilon$ -complex was applied to a DEAE-Sephacrose CL-6B column (20 cm $\times$ 5 cm i.d.) equilibrated in TEPD buffer. The $\delta\epsilon$ -subcomplex eluted at about 150 mM NaCl on a linear salt gradient 0–0.3 M NaCl in the same buffer [see Figures 2 (upper panel) and 3 (upper panel)]. Pooled fractions containing the complex were dialysed overnight against 4 l of CEPD buffer, consisting of 10 mM sodium citrate, pH 6.0, 1 mM EDTA, 2 mM dithiothreitol and 0.002 % (w/v) PMSF. The dialysate was applied to a CM-Sephacrose CL-6B column (20 cm $\times$ 2 cm i.d.) equilibrated in the same buffer. The $\delta\epsilon$ -subcomplex was found in the break-through fractions following free δ -subunit [see Figures 2 (middle panel) and 3 (middle panel)]. Appropriate fractions were pooled, dialysed overnight against 4 l of TEPDN buffer, consisting of TEPD containing 150 mM NaCl. The dialysate was concentrated to 3 ml by ultrafiltration with an Amicon YM3 membrane. The concentrated solution was applied to a Sephacryl S-100 (HR) column (60 cm $\times$ 2.6 cm i.d.) equilibrated in TEPDN buffer [Figures 2 (lower panel) and 3 (lower panel)]. The final recovery of purified $\delta\epsilon$ -subcomplex was about 1.5 mg per litre of bacterial culture.

Estimation of the molecular mass of the $\delta\epsilon$ -subcomplex

The following proteins, with their molecular masses in parentheses, were employed to calibrate a column of Sephacryl S-100 HR (60 cm $\times$ 2.6 cm i.d.) packed in TEPDN buffer: BSA (67 kDa), hen ovalbumin (43 kDa), bovine chymotrypsin

Figure 3 Analysis of column fractions during the purification of the bovine $\delta\epsilon$ -subcomplex

The fractions were analysed by SDS/PAGE and bands were stained with PAGE Blue 83 dye. In the upper, middle and lower panels fractions from the DEAE-Sepharose, CM-Sepharose and Sephacryl S-100 columns are shown respectively (see Figure 2). The positions of the subunits of bovine F_1 -ATPase are indicated on the left of each panel. SM indicates the starting material applied to the columns. The numbers along the top correspond to the fractions that were analysed from the columns in Figure 2.

(25 kDa), and bovine RNase I (13.7 kDa). The elution volume of a solution of the $\delta\epsilon$ -subcomplex was measured in the same buffer, and its molecular mass was estimated from the calibration line.

Reaction of the $\delta\epsilon$ -subcomplex with ethyl-[1- 14 C]acetimidate

A sample of the purified $\delta\epsilon$ -subcomplex was dialysed overnight in 3500 Da cut-off membrane into a buffer consisting of 0.2 M sodium borate, pH 10.0, 6 M guanidine hydrochloride, 2 mM EDTA, 0.02 % sodium azide and 0.002 % (w/v) PMSF. To the dialysate (1 ml; 48 nmol of complex containing 670 nmol of amino groups) was added a solution {36 μ l containing 6.7 μ mol of ethyl-[1- 14 C]acetimidate (specific radioactivity 27 mCi/mmol); a 10-fold molar excess over amino groups} in 0.2 M sodium borate, pH 10.0. The solution was kept at room temperature for 4 h, dialysed overnight at 4 °C against 1 % acetic acid, and freeze-dried. The radiolabelled proteins were separated by gel electrophoresis under denaturing conditions, and then rapidly fixed and stained. The gel was vacuum-dried on Whatman 3MM paper, exposed to an image plate for 18 h, and then the intensities

of the bands were measured with a Molecular Dynamics phosphor imager (Molecular Dynamics Ltd., Kemsing, U.K.).

Search for interactions of the $\delta\epsilon$ -subcomplex with other components of bovine ATP synthase

The bovine stalk subcomplex, containing one copy of each of subunits OSCP, F_6 and d and one copy of the b' fragment of subunit b, was reconstituted *in vitro* from recombinant proteins [9]. Equimolar quantities of the 'stalk' (2mg) and the $\delta\epsilon$ -subcomplex (0.6mg) were mixed together, and left to stand at 20 °C for 1 h. Then the mixture was applied to a Sephacryl S-100 (HR) gel-filtration column (100 cm $\times$ 1.6 cm i.d.) equilibrated at 4 °C in a buffer consisting of 20 mM Tris/HCl (pH 7.5), 50 mM sucrose, 100 mM NaCl, 5 mM dithiothreitol, 1 mM EDTA, 0.001 % PMSF and 10 % (w/v) glycerol. The absorbance of the effluent was monitored at 280 nm, and protein-containing fractions were analysed by SDS/PAGE.

RESULTS AND DISCUSSION

Bacterial expression of the δ - and ϵ -subunits of bovine F_1 -ATPase

When its expression was induced, the δ -subunit of bovine F_1 -ATPase accumulated at fairly high levels in a soluble form in the cytoplasm of *E. coli* [see Figure 4, lane (b)], whereas lower levels of the bovine ϵ -subunit formed under similar conditions [Figure 4, lane (c)]. The induction of expression of neither protein impaired the growth of the cells significantly, and the most likely reason for the poor bacterial expression of the bovine ϵ -subunit, which is a basic protein of only 50 amino acids, is rapid degradation after synthesis. Therefore, in order to try and overcome this problem, expression plasmids were constructed with dicistronic operons containing both δ - and ϵ -subunits in both possible orders, in the hope that the ϵ -subunit might be

Figure 4 Expression of the recombinant δ - and ϵ -subunits of bovine F_1 -ATPase in *E. coli*

Bacterial cells were analysed by SDS/PAGE and stained with PAGE Blue 83 dye. Lane (a), bovine F_1 -ATPase; lane (b), bovine δ -subunit; lane (c), bovine ϵ -subunit; lanes (d) and (e), bovine δ - and ϵ -subunits co-expressed from vectors containing GTG and GTT at ϵ codon-2 respectively; lane (f), expression of bovine δ - and ϵ -subunits from the dicistronic operon ϵ - δ .

stabilized by forming a complex with the δ -subunit. Induction of expression from the $\delta\epsilon$ operon produced amounts of both proteins that were easily detectable by gel electrophoresis of cell contents [see Figure 4, lane (d)]. Their presence was also confirmed by N-terminal sequencing. However, for unknown reasons, neither protein was present at a significant level in induced cells containing the cistrons in the order $\epsilon\delta$ [see Figure 4, lane (f)].

It was evident from N-terminal sequencing and from mass spectrometric measurements that the bacterially expressed ϵ -subunit was heterogeneous in its N-terminal sequence (see Table 1). This was due to a combination of the partial removal of the initiator methionine corresponding to the ATG codon-1, resulting in chains starting at methionine-1 and valine-2, and by translational initiation at the GTG codon-2, producing truncated chains with methionine at their N-terminals in the place of valine-2 found in the other chains. Therefore, the GTG codon in the $\delta\epsilon$ operon was changed to GTT, preventing translational initiation at this site, without adversely affecting the expression of either protein [see Figure 4, lane (e)].

Subcomplex formation by the bovine δ - and ϵ -subunits

Both of the co-expressed proteins were soluble in the cytoplasmic fraction of the bacterial cells, as was the individually expressed δ -subunit. In contrast, the individually expressed ϵ -subunit formed inclusion bodies. The co-expressed δ - and ϵ -subunits purified together on cation and anion ion-exchangers (see Figures 2 and 3), suggesting that they were stably associated in the pH range 6.0–8.0 at least. Additional evidence of association of the two proteins was obtained by gel-filtration chromatography, as the two proteins co-eluted at a position corresponding to a higher molecular mass (estimated from the elution positions of standard proteins to be 29 kDa) than was observed for either the bovine δ - or ϵ -subunit separately.

Molar ratio of the proteins in the subcomplex

In order to estimate the molar ratio of the bovine δ - and ϵ -subunits in the subcomplex, the proteins in the purified subcomplex were denatured in guanidine hydrochloride and their amino groups were reacted with a 10-fold molar excess of ethyl-[1- 14 C]acetimidate. Methyl acetimidate has been used successfully in similar experiments in the past [12,13], but it is less stable than ethyl acetimidate and the radiolabelled form is not available from a commercial source.

An essential prerequisite of this method of estimation of molar ratios of proteins is that the protein amino groups must be completely amidinated. In order to demonstrate that this condition had been fulfilled in the reaction of ethyl-[1- 14 C]acetimidate with the bovine δ - and ϵ -subunits, the amidinated subunits were separated by reverse-phase HPLC. They eluted from the column at 52.2% and 37.8% acetonitrile respectively, whereas unmodified proteins in a control sample eluted at 48.6% and 37% acetonitrile respectively. No unmodified subunits were detected in the samples that had been exposed to the reagent. The yields of N-terminals in sequencing experiments conducted with comparable samples of unmodified and modified ϵ - and δ -subunits were the same. Therefore, there was little or no reaction of the reagent with the α -amino groups and only ϵ -amino groups were modified. In other experiments it has been found that α -amino groups react subsequent to ϵ -amino groups under prolonged or more stringent reaction conditions (I. M. Fearnley and J. E. Walker, unpublished work). The modified δ - and ϵ -subunits gave unique masses by electrospray MS. The determined masses (see

Figure 5 Incorporation of radioactivity into the bovine δ - and ϵ -subunits in the $\delta\epsilon$ -subcomplex by reaction with ethyl-[1- 14 C]acetimidate

The subunits were separated by SDS/PAGE and the incorporated radioactivity in each track was recorded with a phosphor imager. Twice as much sample was loaded in lane 4 as in lanes 1–3. For quantification, see Table 2.

Table 2 Molar ratios of bovine δ - and ϵ -subunits in the $\delta\epsilon$ -subcomplex

Incorporated radioactivities, given in arbitrary units, were measured from the intensities of subunits, radiolabelled by acetimidation, recorded from four tracks of a polyacrylamide gel with a phosphor imager (see Figure 5). The δ -subunit has four ϵ -amino groups/chain and ϵ -subunits have eight.

Track	Radioactivity		Molar ratio ($\delta:\epsilon$)
	δ -subunit	ϵ -subunit	
1	19099	32164	1.0:0.84
2	16295	28726	1.0:0.88
3	17076	30981	1.0:0.91
4	34225	55768	1.0:0.81

Table 1) were also in accord with complete amidination of all ϵ -amino groups, but not α -amino groups in both subunits. There was no evidence in the mass spectra of heterogeneity arising, for example, from under-reacted species.

After separation of the chemically modified subunits by PAGE (Figure 5), the ratio of radioactivity incorporated into each protein was measured. Since the number of ϵ -amino groups in each chain is known from their sequences, the molar ratio of the two proteins can be calculated. In four acetimidation experiments conducted on independently isolated samples of the $\delta\epsilon$ complex, both chains were present in equimolar quantities, suggesting that the interaction between the proteins is specific. The results of one such experiment are shown in Table 2.

No attempt has been made to investigate the secondary structures of the complexed proteins by spectral measurements, except that preliminary NMR studies indicate the presence of α -helices in the recombinant bovine $\delta\epsilon$ protein complex (D. Neuhaus, G. L. Orriss and J. E. Walker, unpublished work). This suggestion agrees with secondary structure predictions (results not shown), which suggest that both proteins are made predominantly of a single α -helix. This would be likely to give

Figure 6 Investigation of possible interactions between the bovine $\delta\epsilon$ -subcomplex and the bovine stalk complex

Upper panel: gel filtration of a mixture of $\delta\epsilon$ - and stalk subcomplexes through a column of Sephacryl S-100 (see the Materials and methods section for further details). Lower panel: SDS/PAGE analysis of column fractions.

rise to an elongated structure, and so estimation of the molecular mass of the $\delta\epsilon$ -subcomplex by gel filtration would tend to give high values. As the molecular mass of the complex estimated by gel-filtration chromatography was 29 kDa, and the combined mass of the two proteins is 20848 Da, given that the ratio $\delta:\epsilon$ is 1:1, it is likely that the subcomplex contains one copy of each protein.

Implications for the structure of ATP synthase

The δ - and ϵ -subunits and about 50% of the γ -subunit were not detected in the electron-density map obtained by X-ray crystallography of bovine F_1 -ATPase. However, uninterpretable density is present in the map adjacent to a region referred to as 'the stem', that has been shown to be composed of other regions of the γ -subunit and is thought to be part of the stalk connecting the F_1 and F_0 domains in the intact ATP synthase complex [3]. This uninterpretable density, which is readily accommodated in the crystal lattice, probably arises from disordered polypeptide in the γ -, δ - and ϵ -subunits, although this remains to be proved. The work described in this paper provides evidence that the δ - and ϵ -subunits associate with each other to form a 1:1 complex,

but as yet there is no biochemical evidence to show how this subcomplex interacts with the other subunits in F_1 -ATPase.

Clues about these interactions are provided by *E. coli* F_1 -ATPase. Bovine and eubacterial γ -subunits are closely related [14]. The eubacterial ϵ -subunit is the homologue of the bovine δ -subunit, and there is no equivalent of the bovine ϵ -subunit in bacteria [15]. Some experiments on *E. coli* F_1 -ATPase have demonstrated interactions between bacterial γ - and ϵ -subunits, and others between the ϵ - and β -subunits. First, the bacterial γ - and ϵ -subunits form a stoichiometric subcomplex containing one copy of each protein [16]. Secondly, the bacterial ϵ -subunit can be chemically cross-linked with the C-terminal region of the β -subunit by the zero-length cross-linking reagent, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide [17]. Thirdly, substitution of glutamate-381 by cysteine in the β -subunit of the *E. coli* enzyme allows a disulphide to form under oxidizing conditions with cysteine-108 in the ϵ -subunit [18]. Therefore, it is likely that in bovine F_1 -ATPase similar interactions form between the δ -subunit and the γ - and β -subunits. The present work suggests that the bovine ϵ -subunit will be present in the same region of the enzyme as the δ -subunit. These notions can be explored once refolded recombinant bovine β - and γ -subunits and fragments of them become available.

In one reconstitution experiment carried out as part of the present work, the $\delta\epsilon$ -subcomplex and the bovine 'stalk' complex [9] were mixed together in equimolar amounts. No stable interaction was detected by gel filtration between the $\delta\epsilon$ -subcomplex and the bovine 'stalk' subcomplex. The two complexes eluted separately from the column (see Figure 6).

G.L.O. is supported by an M.R.C. Research Studentship, I.R.C. by a Beit Memorial Fellowship, and B.M. by a Fellowship from the Human Capital and Mobility program of the E.C. We thank K. Nagai and M. Way for advice concerning construction of the operon.

REFERENCES

- Senior, A. E. (1988) *Physiol. Rev.* **68**, 177–231
- Fillingame, R. H. (1990) *The Bacteria* **12**, 345–391
- Abrahams, J. P., Leslie, A. G. W., Lutter, R. and Walker, J. E. (1994) *Nature (London)* **370**, 621–628
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Runswick, M. J., Medd, S. M. and Walker, J. E. (1990) *Biochem. J.* **266**, 421–426
- Viñas, O., Powell, S. J., Runswick, M. J., Iacobazzi, V. and Walker, J. E. (1990) *Biochem. J.* **265**, 321–326
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Biggin, M. D., Gibson, T. J. and Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963–3965
- Collinson, I. R., van Raaij, M. J., Runswick, M. J., Fearnley, I. M., Skehel, J. M., Orriss, G. L., Miroux, B. and Walker, J. E. (1994) *J. Mol. Biol.* **242**, 408–421
- Way, M., Pope, B., Hawkins, M. and Weeds, A. G. (1990) *EMBO J.* **9**, 4103–4109
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W. (1990) *Methods Enzymol.* **185**, 60–89
- Bates, D. L., Harrison, R. A. and Perham, R. N. (1975) *FEBS Lett.* **60**, 427–430
- Gray, J. C., Hooper, E. A. and Perham, R. N. (1980) *FEBS Lett.* **114**, 237–239
- Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M. and Tybulewicz, V. L. J. (1985) *J. Mol. Biol.* **184**, 677–701
- Walker, J. E., Runswick, M. J. and Saraste, M. (1982) *FEBS Lett.* **146**, 393–396
- Cox, G. B., Cromer, B. A., Guss, J. M., Harvey, I., Jeffrey, P. D., Solomon, R. G. and Webb, D. C. (1993) *J. Mol. Biol.* **229**, 1159–1162
- Dallman, H. G., Flynn, T. G. and Dunn, S. D. (1992) *J. Biol. Chem.* **267**, 18953–18960
- Aggeler, R., Haughton, M. A. and Capaldi, R. A. (1995) *J. Biol. Chem.* **270**, 9185–9191