ATP synthase from bovine heart mitochondria: identification by proteolysis of sites in F_0 exposed by removal of F_1 and the oligomycin-sensitivity conferral protein

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The exposure to trypsinolysis of subunits of F_1F_0 -ATPase and of its F_0 domain have been compared in everted inner membrane vesicles (submitochondrial particles) made from bovine mitochondria. Treatment of submitochondrial particles with guanidine hydrochloride removed the subunits of F_1 -ATPase and the oligomycin-sensitivity conferral protein (OSCP), and exposed sites that were occluded in the intact F_1F_0 -ATPase complex. These sites were identified by purifying the subunits from the isolated F_0 and F_1F_0 -ATPase complexes before and after proteolysis of the vesicles, and by characterizing them by N-terminal sequencing and electrospray-ionization mass spectrometry. In the stripped vesicles, subunit F_6 was completely digested away by either trypsin or chymotrypsin. Trypsin also cleaved subunit b, first at the bond arginine-166–glutamine-167, and then at the consecutive linkages, lysine-120–arginine-121 and arginine-121–histidine-122. Chymotrypsin-sensitive sites were observed after the adjacent methionines 164 and 165. Trypsin also removed

amino acids 1–3 of subunit d, and minor cleavage sites were observed in subunit d between amino acids 24 and 25, in subunit g between amino acids 5 and 6, and after amino acid 40 in subunit e. The other subunits remained protected from proteolysis. In membrane-bound F_1F_0 -ATPase, the N-terminus of subunit d was also accessible to trypsin, and subunit e was more susceptible to proteolysis than in F_0 . Otherwise the F_0 subunits and the OSCP were protected. Subunits α and β were cleaved by trypsin at the same sites in their N-terminal regions as in purified F_1 -ATPase. The trypsinized F_0 was incapable of binding F_1 -ATPase in the presence of the OSCP. These experiments and *in vitro* re-assembly experiments described elsewhere, that were guided by the results of the proteolysis experiments, have helped to establish a central role for subunit b in the formation of the stalk connecting the F_1 and F_0 domains of the F_1F_0 -ATPase complex.

INTRODUCTION

The slender stalk linking the intrinsic membrane domain of F_1F_0 -ATPases to the extrinsic domain, F_1 -ATPase, plays a central role in transmitting energy generated by oxidative or photosynthetic electron transport to the catalytic sites in F_1 . The energy is produced in the form of a chemical potential gradient for protons across the energy-transducing membrane. Proton transport through the membrane by F_0 probably induces long-range conformational changes that pass via the stalk to the catalytic sites to drive ATP synthesis (Boyer, 1993). Alternatively, the stalk could be a conduit conducting the protons to the catalytic sites in F_1 , where they could participate directly in ATP formation (Williams, 1961; Mitchell, 1966). Therefore, knowledge of the structure of the stalk and of its interactions with both F_1 and with the membrane domain is crucial for understanding the process of energy transduction. If the stalk is disrupted by physical or chemical means, intact F_1 -ATPase is released. In the bovine enzyme, this release can be accompanied by liberation of a protein known as the oligomycin-sensitivity conferral protein (OSCP) (Kagawa and Racker, 1966), which is required for the reassembly of F_1 with the membrane domain and is therefore probably a component of the stalk. Other parts of the stalk are in the stem region of the F_1 particle, which protrudes about 40 Å from the approximately 100 Å diam. spherical F_1 (Abrahams et al., 1993, 1994). The stem is likely to be composed of the δ and ϵ subunits of F_1 and part of the γ subunit.

The remaining components of the stalk stay with the stripped membranes. If these membranes are treated with silicotungstate, a protein called coupling factor 6 (F_6) is removed (Knowles et al., 1971; Fessenden-Raden, 1972), and this protein must be added back, together with OSCP, for correct reassociation of F_1 -ATPase with these membranes. Subunit b is another membrane-associated subunit involved in binding F_1 . It is bound to the membrane via its N-terminal region, and the remainder of its chain forms an extensive extrinsic domain (Walker et al., 1987). The C-terminal region of subunit b is exposed to proteolysis by trypsin in membranes from which F_1 has been removed, but the sites of proteolysis have not been identified unambiguously (Papa et al., 1989; Hekman et al., 1991). In keeping with the important role of b in the stalk, a fully functional F_1F_0 -ATPase cannot be reconstituted from the proteolysed membranes with F_1 and OSCP (Papa et al., 1989). Recently, a simple procedure has been devised for efficient stripping of F_1 and OSCP from bovine mitochondrial membranes and for purifying the F_0 from the stripped membranes (Collinson et al., 1994a). The purified F_0 has been reconstituted with F_1 -ATPase in the presence of the OSCP to re-form a functional F_1F_0 -ATPase complex (Collinson et al., 1994a). Bovine F_0 is a complex of nine different subunits, amongst them F_6 and b. As described in the present paper, the stripped membranes from bovine heart mitochondria have been exposed to proteases. The resulting proteolysed F_0 has been purified and its component subunits have been isolated by reverse-phase h.p.l.c. Each of them has been characterized by

Abbreviations used: OSCP, oligomycin-sensitivity conferral protein; F_6 , coupling factor 6; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene difluoride).

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protein sequencing and electrospray-ionization mass spectrometry, and the sites of proteolysis have been identified. For comparison, similar studies have been carried out on membranes containing the intact F_1F_0 -ATPase. These studies have shown that removal of F_1 and OSCP exposes F_0 and the C-terminal region of subunit b. Subunit F_0 was extensively proteolysed, and no fragment of it remained associated with the membrane. In subunit b, the primary cleavage site is between amino acids 166 and 167, 48 amino acids from the C-terminus. A secondary cleavage site is between amino acids 120–121. The N-terminal region of subunit d was also affected, but otherwise the other subunits in F_0 were resistant to cleavage. A similar pattern of exposure of the subunits was suggested by chymotryptic treatment of F_0 in stripped membranes.

MATERIALS AND METHODS

Analytical methods

Protein concentrations were estimated by the bicinchoninic acid assay (Pierce Chemicals, Rockford, IL, U.S.A.). PAGE (Laemmli, 1970) was performed using 12–22% acrylamide gradients with 0.1% (w/v) SDS in minigels (10 cm × 10 cm). Proteins were detected in gels by staining with PAGE Blue 83 dye or by Western-blot analysis (see below).

Chromatographic supports

Q-Sepharose, and Sepharose S-300 were purchased from Pharmacia Biotech Ltd., Milton Keynes, U.K. Immobilized Reactive Yellow 3 dye was purchased from Sigma Chemical Company, Poole, Dorset, U.K.

Purification of mitochondrial proteins

Bovine F_0 , F_1 -ATPase and F_1F_0 -ATPase were purified from heart mitochondria as described before (Collinson et al., 1994a; Lutter et al., 1992; S. K. Buchanan and J. E. Walker, unpublished work). Bovine ATPase subunits OSCP, F_0 and d and fragment b' (amino acids 79–214 of subunit b) were produced by over-expression of the proteins in *Escherichia coli*. Except for F_0 , which was soluble in the bacterial cytoplasm, the proteins formed inclusion bodies. The OSCP was refolded and purified from inclusion bodies, and F_0 was purified from the cytoplasmic fraction (Collinson et al., 1994b). Inclusion bodies containing subunit d and fragment b' were dissolved in 10% SDS and run on denaturing polyacrylamide gels. The proteins were recovered by electroelution and ethanol precipitation.

Limited proteolysis of F_0 and F_1F_0 -ATPase from bovine heart mitochondria

Submitochondrial particles (everted inner membrane vesicles), which had been stripped with guanidine hydrochloride (Collinson et al., 1994a), were suspended at a concentration of 2.5 mg of protein/ml in a buffer consisting of 20 mM Tris/HCl (pH 7.5), 50 mM sucrose and 10% (w/v) glycerol. Trypsin (1:20, w/w) was added and the suspension was kept at 28 °C for 40 min. Proteolysis was terminated by addition of soybean trypsin inhibitor (5-fold excess by wt). The suspension was cooled to 4 °C, and the membranes were harvested by centrifugation (142000 g, 30 min). Modified F_0 was extracted from the trypsinized membranes and purified as described for the preparation of intact bovine F_0 (Collinson et al., 1994a), except that a smaller Q-Sepharose HP column (15 cm × 2.6 cm i.d.) was used, and the S-Sepharose chromatography step was omitted. The purified

proteolysed F_0 was concentrated by ultrafiltration through an Amicon YM100 ultrafiltration membrane to a protein concentration of 1–2 mg/ml.

A similar experiment was performed at 28 °C for 30 min by adding chymotrypsin (7.5 mg) to stripped membranes (150 mg of protein in 60 ml of buffer). Proteolysis was stopped by addition of protease inhibitors [final concentrations 0.33 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 0.001% phenylmethanesulphonyl fluoride (PMSF), 2 µg/ml aprotinin, 1 mM EDTA and 1 mM benzamidin]. The sample was cooled to 4 °C, the membranes were harvested by centrifugation as above, and were washed twice with a buffer consisting of 20 mM Tris/HCl (pH 7.5), 10% glycerol, and 50 mM sucrose, and containing the protease inhibitors. The modified F_0 was purified as described above for trypsinized F_0 .

Untreated submitochondrial particles (370 mg of protein, resuspended at 5 mg/ml) were exposed to trypsin (1:2, w/w) at 37 °C for 40 min. The proteolysed F_1F_0 -ATPase was purified by a procedure similar in the detergent-extraction and Q-Sepharose-chromatography steps to the one described for proteolysed F_0 . This partially purified material was then passed unbound through a Reactive Yellow 3 dye column. It was concentrated to approx. 5 ml by ultrafiltration on an Amicon YM100 membrane, and was then applied to a Sephacryl S-300 HR column (60 cm × 2.6 cm i.d.) equilibrated in a buffer containing 20 mM Tris/HCl (pH 8.0), 50 mM sucrose, 100 mM NaCl, 2 mM $MgSO_4$, 1 mM EDTA, 0.001% PMSF, 0.02% sodium azide and 10% glycerol. The peak corresponding to proteolysed F_1F_0 -ATPase was concentrated to approx. 2 mg/ml by ultrafiltration.

Purification of subunits of bovine F_0 and F_1F_0 -ATPase after proteolysis

Samples of F_0 and F_1F_0 -ATPase, purified after treatment with trypsin or chymotrypsin, were dissolved in 6 M guanidine hydrochloride. The subunits were separated by reverse-phase chromatography on a C_8 Aquapore RP-300 column (7 µm particles, 300 Å pore size, 100 mm × 2.1 mm i.d.; from Applied Biosystems, Warrington, Cheshire, U.K.), equilibrated in 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient. Subunits a and c were not recovered from any complex by reverse-phase chromatography.

Mass spectrometric and N-terminal sequence analysis of proteins

Subunits of bovine F_0 and F_1F_0 -ATPase, of both complexes treated with trypsin, and of F_0 treated with chymotrypsin were recovered from h.p.l.c. and dissolved in a mixture of 0.1% trifluoroacetic acid and acetonitrile. Samples of these solutions were introduced into a VG BIO-Q triple-quadrupole mass spectrometer with electrospray ionization (VG Biotech, Altrincham, Cheshire, U.K.). Electrospray mass spectra were measured as described previously (Walker et al., 1992), except that the delivery solvent was 50% aq. acetonitrile. The subunits of the same proteolysed complexes were also separated by PAGE under denaturing conditions, transferred to a poly(vinylidene difluoride) (PVDF) membrane (Pilkington et al., 1991) and, after staining with PAGE Blue 83 dye, their N-terminal sequences were determined (Fearnley et al., 1989). The α-N-formyl groups on subunits a and A6L were removed on the membranes with methanol/HCl before sequencing (Collinson et al., 1994a).

Preparation and use of antisera

Purified samples (about 400 µg) of subunits F_0 and d, and of fragment b' were redissolved in PBS (135 mM NaCl, 2.5 mM

KCl and 12 mM sodium phosphate, adjusted to pH 7.2) diluted 1:2 (v/v) with Freund's complete adjuvant, and injected subcutaneously into New Zealand White rabbits. After 6–7 weeks, a second injection was made of the same amount of protein diluted in PBS in incomplete Freund's adjuvant. About 10 days later, the sera were assayed on PVDF membranes to which had been transferred about 1 μ g of the protein antigen. The membrane was placed for 30 min in 'blocking buffer' consisting of 5% (w/v) BSA and 1% (w/v) Marvel skimmed-milk powder (Premier Brands, Birmingham, U.K.) in PBS. The sera were diluted 1:1000 in blocking buffer and added to the membrane. The antigen-antibody complexes were visualized by following the test serum with biotinylated anti-(rabbit IgG), preformed avidin-biotinylated horseradish peroxidase complex, and diaminobenzidine and a nickel salt (Hsu et al., 1981a,b). All of the antibodies gave a strong signal. Subunits of trypsinized and chymotrypsinized F_0 were separated by PAGE in the presence of SDS, transferred to PVDF membranes and reacted with subunit-specific antisera and detection reagents as described above.

Reconstitution of trypsinized F_0 with F_1 -ATPase and OSCP

Bovine F_1 -ATPase and OSCP were added to trypsinized F_0 (2.0 mg; molar ratios 3:3:2 respectively), dissolved in a buffer (1 ml) consisting of 20 mM Tris/HCl (pH 7.5), 50 mM sucrose, 10% glycerol, 1 mM EDTA, 0.001% PMSF and 8 mM dithiothreitol. The mixture was kept at 28 °C for 45 min and was then loaded on a column of Sephacryl S-300 HR equilibrated at 4 °C in a buffer containing 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 50 mM sucrose, 10% glycerol, 1 mM EDTA and 0.001% PMSF. The flow rate of the elution buffer was 1 ml/min. The fractions were analysed by PAGE. A similar experiment was conducted with proteolysed F_0 (0.7 mg) and a 1.5-fold molar excess of each of OSCP, F_1 -ATPase and F_0 .

RESULTS AND DISCUSSION

Trypsinolysis of bovine F_0

Samples of bovine F_0 , isolated from stripped inner membranes of bovine heart mitochondria that had been treated briefly with trypsin, were compared by SDS/PAGE with a control sample of untreated F_0 [Figure 1, lanes (b) and (c)]. No intact subunit b could be seen in the proteolysed sample, and two new bands with apparent molecular masses of 18 and 9 kDa had been generated. Both of these bands reacted with an antibody raised against amino acids 79–214 of bovine subunit b [Figure 1, lane (d)]. No intact F_0 was visible in the PAGE Blue 83-stained gel, and only a trace amount was detected by immunological staining using the anti-(bovine F_0) antibody.

The subunits in the proteolysed F_0 and in the non-proteolysed control sample were separated by reverse-phase h.p.l.c. (see Figure 2). Each peak from the resulting chromatograph was analysed by N-terminal sequence analysis and by electrospray-ionization m.s. This combination of N-terminal sequence and exact molecular mass characterizes each fragment precisely. These analyses (summarized in Tables 1 and 2) confirmed that the 18 kDa and 9 kDa bands in the trypsinized sample were both derived from subunit b. The former is amino acids 1–166 of subunit b, and the latter is a mixture of amino acids 1–120 and 1–121 of the same protein. Papa et al. (1989) have observed that trypsin cleavage of mitochondrial membranes stripped with urea generated an 18 kDa product from subunit b. They concluded that the cleavage had occurred after lysine-202 (or possibly after lysine-205 or lysine-206, only 8–12 amino acids from the C-

terminus of the subunit). In view of our findings, it is unlikely that this suggestion is correct.

In another trypsinolysis experiment conducted with stripped membranes (results not shown), the proportion of the 9 kDa fragment to the 18 kDa fragment was greater. It was apparent that the 18 kDa band was generated first and that the 9 kDa band appeared subsequently, as observed previously by Hekman et al. (1991).

No peak corresponding to intact F_0 was present in the proteolysed sample, and no fragment derived from this subunit was detected. Therefore, membrane-bound F_0 was extensively digested by trypsin, and the tryptic fragments did not remain

Figure 1 Effect of trypsin on subunits of F_0 in inner membrane vesicles from bovine heart mitochondria

After proteolysis, F_0 was isolated by ion-exchange chromatography. The subunits were separated by SDS/PAGE (Laemmli, 1970) and detected with either PAGE Blue 83 dye [lanes (a)–(c)], or by Western-blot analysis using an antibody raised against amino acids 79–214 of bovine subunit b. Lane (a), purified bovine F_1F_0 -ATPase; lane (b), purified F_0 ; lane (c), F_0 isolated after treatment of stripped membranes with trypsin; lane (d), Western blot of same material as in lane (c).

Figure 2 Separation by reverse-phase h.p.l.c. of subunits of F_0 from bovine heart mitochondria before and after trypsinolysis

The upper (dotted) and lower (solid) traces correspond to the separations of subunits in a control sample of F_0 and a sample of F_0 that was exposed to trypsin respectively. F_0 , e, d, f, g, b and A6L indicate the positions of the intact subunits; e' is the form of subunit e present in both the untreated and treated samples that lacks the N-terminal valine, and (b)₂ is a dimer of b observed by electrospray-ionization m.s. The unmodified subunits and the products of proteolysis e-1, d-1, d-2, g-1 and b-1 are described in Tables 1 and 2. The hydrophobic F_0 subunits, a and c, were not recovered from the h.p.l.c. column.

Table 1 Characterization of subunits of bovine F_0

For purification of the subunits, see Figure 2. The peaks were analysed by electrospray-ionization m.s. (esi-m.s.) and N-terminal sequence analysis. Abbreviation: N.D., not determined.

Subunit	Mass		N-terminal sequence	Amino acids
	Esi-m.s.	Calculated		
b	24671.9	24670	PVPPLP	1–214
d	18604.5	18604	None	1–160
a	N.D.	—	MNENL†	1–226
F_6	8959.2	8959	NKELD	1–76
e	8189.4*	8190	VPPVQ	1–70
f	10209.2	10209	None	1–87
g	11328.0	11329	None	1–102
A6L	7964.1	7965	MPQLD†	1–66
c	N.D.	—	DIDTAA	1–75

* Subunit e has a ragged N-terminus and contains minor amounts of chains either lacking residue 1 or with an additional methionine before residue 1.

† In this Table and in subsequent ones, the N-terminal sequences of subunits a and A6L were determined after removal of the N- α -formyl group.

Table 2 Characterization of subunits of bovine F_0 after mild trypsinolysis

For separation of the subunits by reverse-phase h.p.l.c., see Figure 2, and for details of the analyses see the Materials and methods section. Abbreviations: N.D., not determined; esi-m.s., electrospray-ionization m.s.

Protein	Mass		N-terminal sequence	Identity
	Esi-m.s.	Calculated		
b-1	19152.8	19152	PVPPLP	Residues 1–166
b-2¶	13490.8	13491	PVPPLP	Residues 1–121
b-3¶	13333.7	13335	PVPPLP	Residues 1–120
d-1	18150.8	18150	KLALKT*	Residues 4–160
d-2‡	15858.8	15855	N.D.	Residues 25–160
a	N.D.	N.D.	MNENL	Intact subunit
F_6 †	—	—	—	—
e	8191.8	8190	VPPVQ	Intact subunit
e-1	4583.2	4583	N.D.	Residues 1–40
f	10209.3	10209	None	Intact subunit
g	11327.0	11329	None	Intact subunit
g-1‡	10686.7	10685	N.D.	Residues 6–102
A6L	7964.6§	7965	MPQL	Intact subunit
c	N.D.	N.D.	DIDTAA	N-terminal intact

* A species lacking the N-terminal lysine was also observed.

† Neither F_6 nor any fragment of it was recovered from the h.p.l.c. column.

‡ Insufficient material to permit N-terminal sequence analysis.

§ A minor series of peaks corresponding to a mass of 5154.8 was also present in this peak. This value could be derived from amino acids 1–43 of A6L (calculated mass 5156).

|| See footnotes to Table 1.

¶ Observed after prolonged trypsinolysis.

associated with the membrane complex. Among the remaining subunits of bovine F_0 , only subunits d, g, e and A6L were affected by trypsin. Residues 1–3 were removed from subunit d, and a minor cleavage was observed between residues 24 and 25. Other minor trypsin cleavage sites were observed at the bond arginine-5–asparagine-6 of subunit g, between arginines 40 and 41 in subunit e, and at the bond lysine-43–methionine-44 in subunit A6L. Subunit A6L contains a hydrophobic region from amino acids 8–28 that could form a transmembrane α -helix, and it has been suggested previously that its N-terminal region is exposed to the outer surface of the inner mitochondrial membrane

Figure 3 Effect of trypsin on subunits of bovine F_1F_0 -ATPase in submitochondrial particles from bovine heart mitochondria

The enzyme was purified by chromatography, and its subunits were separated by SDS/PAGE and stained with PAGE Blue 83 dye. Lanes (a) and (e), F_1F_0 -ATPase treated with trypsin; lane (b), trypsinized F_0 ; lane (c), chymotrypsinized F_0 ; lane (d), untreated F_1F_0 -ATPase. In lanes (a) and (e), note the absence of the 18 kDa band found in (b) and (c), and a doublet in the position of subunit d.

Figure 4 Separation by reverse-phase h.p.l.c. of subunits of bovine F_1F_0 -ATPase after exposure to trypsin

The upper (dotted) line corresponds to the separation of subunits of a control sample of F_1F_0 -ATPase, and the lower (solid) line to the separation of subunits in a similar sample after trypsinolysis. The elution positions of intact subunits F_6 , e, e-1, e-2, α , γ , δ , g, β , b and A6L are shown. The recoveries of α , β and γ were less than 10% of the starting material. The peak labelled with a query contained aggregates of α , β and other subunits. The peaks labelled f contained substantial quantities of the detergent associated with the F_1F_0 -ATPase, which prevented m.s. analysis. The peaks in the lower trace labelled e-1, e-2 and g-1 contained proteolytic fragments (see Table 3).

(Oda et al., 1989; Hekman et al., 1991). Trypsin had no evident effect on the N-terminal regions of the hydrophobic subunits c and a. Their electrospray-ionization mass spectra have not been recorded, but neither protein has a potential tryptic cleavage site near to its C-terminus, and it is therefore unlikely that any cleavage occurred in this region of either subunit.

Table 3 Characterization of the subunits of bovine F_1F_0 -ATPase after mild trypsinolysis

For separation of the subunits by reverse-phase h.p.l.c., see Figure 4. Details of the analyses are given in the Materials and methods section.

Protein	Mass		N-terminal sequence	Conclusion
	Esi-m.s.	Calculated		
α	N.D.	—	ILGAD	1–15 removed
β	N.D.	—	AGATT	1–7 removed
γ	30144.8	30142	ATLKDIT	No effect
δ^*	N.D.	—	AEEAA	N-terminal intact
ϵ	5652.5	5651	VAYWR	No effect
a	N.D.	—	MNENL	N-terminal intact
b	24673.9	24670	PVPP	No effect
c	N.D.	—	DIDTAA	N-terminal intact
d	18604.5	18604	None	No effect
d-1	18286	18278	KLAL	Residues 1–4 removed
e-1*	4582.9	4583	VPPV	Residues 41–70 removed
e-3	3968.8	3969	N.D.	Residues 1–35 remain
f†	N.D.	—	None	N-terminal intact
g	11327.9	11329	None	No effect
g-1	10688	10685	XLAXKAPA	Residues 6–102 remain
OSCP	20934.2	20931	N.D.	No effect
F_6	8960.2	8959	NKEL	No effect
A6L	7966.1	7965	MPQL	No effect

* See footnotes to Table 1.

† In the h.p.l.c. separation, the peak containing subunit f was obscured by detergent (see Figure 4).

Figure 5 Effect of chymotrypsin on subunits of F_0 in inner membrane vesicles from bovine heart mitochondria

For further details, see the Materials and methods section and the legend to Figure 1. Lane (a), F_0 ; lane (b), chymotrypsinized F_0 ; lane (c), F_1F_0 -ATPase. Note the absence of F_6 in lane (b).

Trypsinolysis of bovine F_1F_0 -ATPase

In the intact bovine F_1F_0 -ATPase in inner mitochondrial membrane vesicles, subunits b, OSCP and F_6 were all protected from the effects of trypsin (see Figures 3 and 4, and Table 3). As in F_0 , the N-terminal regions of subunits d and g were accessible to the protease. Somewhat surprisingly, the C-terminal region of subunit e (and possibly the N-terminal region of g) in F_1F_0 -ATPase was more susceptible to trypsinolysis than in F_0 . The N-terminal regions of the F_1 subunits γ , δ and ϵ were also unaffected by

Figure 6 Separation by reverse-phase h.p.l.c. of subunits of F_0 from bovine heart mitochondria after exposure to chymotrypsin

The upper (dotted) and lower (solid) traces correspond to the separations of subunits in a control sample of F_0 and a sample of F_0 that was exposed to chymotrypsin respectively. In the lower trace, the positions of two cleavage products of subunit b are designated b-C1 and b-C2. For other details, see the legends to Figures 2 and 4. The characterization of the subunits and the cleavage products are described in Table 4.

trypsin, whereas the N-terminal regions of subunits α and β were exposed, and were cleaved at the same sites as in F_1 -ATPase (Walker et al., 1985).

Chymotrypsinolysis of bovine F_0

A sample of stripped membrane vesicles was treated with chymotrypsin under similar conditions to those described for trypsin. The modified F_0 was isolated and analysed by SDS/PAGE [see Figures 5(b) and 6]. Again it appeared that subunit b had been affected, and a new band appeared at about 18 kDa. By analysis of the isolated subunits (see Table 4) two adjacent

Table 4 Characterization of the subunits of bovine F_0 after mild chymotrypsinolysis

For separation of the subunits by reverse-phase h.p.l.c., see Figure 6. Details of the analyses are given in the Materials and methods section. Abbreviations: esi-m.s., electrospray-ionization m.s.; N.D., not determined.

Protein	Mass		N-terminal sequence	Identity
	Esi-m.s.	Calculated		
b-C1	18 999.7	18 996	PVPLP	1–165
b-C2	18 867.9	18 865	PVPLP	1–164
d	18 605.5	18 604	No sequence	Intact protein
a	N.D.	—	MNENL	Intact protein
e*	8 190.2	8 190	VPPVQ	Intact protein
f	10 209.1	10 209	No sequence	Intact protein
g	11 328.8	11 329	No sequence	Intact protein
A6L	7 965.6	7 965	MPQLD	Intact protein
c	N.D.	—	DIDTAA	N-terminal intact

* See footnotes to Table 1.

chymotryptic cleavage sites were identified in subunit b after amino acids 164 and 165 (both of them methionine residues). A fragment with an apparent molecular mass of 9 kDa was detected with the anti-b' antibody, but it was not detected in the mass spectrometric analysis. As in the trypsinolysis experiments, almost all of F_0 was degraded, and no fragments of F_0 were found in association with the isolated F_0 . None of the other F_0 subunits was affected by chymotrypsin digestion. Since there are numerous potential tryptic and chymotryptic sites in subunit b that were not cleaved in these proteolysis experiments, they must be relatively shielded and inaccessible in the F_0 complex.

Attempted reconstitution of F_1F_0 -ATPase from trypsinized F_0

Bovine F_1 -ATPase did not bind to trypsinized F_0 in the presence of OSCP, or in the presence of both OSCP and F_0 , whereas in a control experiment, F_1F_0 -ATPase was successfully reconstituted from untreated purified bovine F_0 , OSCP and F_1 -ATPase, as demonstrated before (Collinson et al., 1994a). Therefore, the complete removal of amino acids 167–214 of subunit b and the partial removal of amino acids 120–166 prevents important intersubunit interactions in the stalk.

Topography of subunit b from bovine heart mitochondrial F_0

The hydrophobic profile of bovine subunit b has two hydrophobic regions from residues 30–53 and 58–78 that could form transmembrane α -helices (Walker et al., 1987). Therefore, this region near to the N-terminus of the protein may form a hairpin structure consisting of two antiparallel α -helices linked by a short loop (see Figure 7). The remainder of the subunit from amino acids 79–214 has 19 acidic amino acids, 22 basic amino acids (arginine plus lysine) and four histidines in its sequence. Therefore, it was suggested that this latter region is outside the lipid bilayer, where it could interact with other globular subunits of F_1F_0 -ATPase and form part of the stalk (Walker et al., 1987).

The experiments described above have shown that when the F_1 -ATPase and the OSCP are stripped away, two sites in bovine subunit b following amino acids 166 and 120 become accessible to trypsin (see Figure 7) and adjacent sites to chymotrypsin. The cleavage sites are found in a region with the highest charge

Figure 7 Model of bovine subunit b summarizing the sites of proteolysis

The subunit is proposed to be anchored in the membrane by two hydrophobic α -helices made of amino acids 30–53 and 58–78. The remainder of the protein (amino acids 79–214) lies outside the lipid bilayer. This extramembranous domain is referred to as b'. The primary site of trypsin cleavage in subunit b in stripped inner membrane vesicles from bovine heart mitochondria is the bond arginine-166–glutamine-167, and secondary cleavages take place subsequently at lysine-120–arginine-121 and arginine-121–histidine-122. Chymotrypsin cleaves the subunit after the adjacent methionine residues 164 and 165. In the intact F_1F_0 -ATPase complex, it is thought that the C-terminal region of b interacts with the OSCP, and that subunits d and F_6 interact with amino acids 120–165 and 120–214 respectively. Each bovine F_1F_0 -ATPase complex contains one copy of subunit b (Collinson et al., 1994b).

density in the subunit. These findings have guided experiments described elsewhere (Collinson et al., 1994b) in which the entire extramembranous region of subunit b from amino acids 79–214 (fragment b' in Figure 7), and the domains consisting of amino acids 79–165, 121–214 and 165–214 (see Figure 7) were all produced by heterologous overexpression in *E. coli*. By *in vitro* assembly experiments conducted with these fragments, together with the OSCP, F_6 , subunit d and F_1 -ATPase, their major intersubunit interactions have been studied. These experiments have shown that the C-terminal region of subunit b interacts predominantly with OSCP. There was no evidence of direct strong interaction of subunit b with F_1 -ATPase. Subunits d and F_6 appear to interact mainly with amino acids 120–165 and 120–214 of subunit b respectively, and to some extent with each other. Trypsinolysis of membranes stripped so that OSCP remains associated with F_0 have suggested that F_6 is shielded by OSCP (Joshi et al., 1986), and in chemical cross-linking experiments F_6 and OSCP were linked to subunit b *inter alia* (Joshi and Burrows, 1990). Subunits d, OSCP, F_6 and fragment b' together form a stable stoichiometric complex containing one copy of each subunit that is representative of the stalk in the intact F_1F_0 -ATPase. This stalk complex is capable of binding with an equimolar quantity of F_1 -ATPase to form a complex referred to as F_1 -stalk (Collinson et al., 1994b). It is hoped that this complex

will be helpful in extending the high-resolution structure of F_1 -ATPase into the stalk region.

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