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Identification of the Subunits of F_1F_O-ATPase from Bovine Heart Mitochondria‡

John E. Walker,* Renê Lutter,§,∥ Alain Dupuis,⊥ and Michael J. Runswick
Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, U.K.
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ABSTRACT: An oligomycin-sensitive F_1F_O-ATPase isolated from bovine heart mitochondria has been reconstituted into phospholipid vesicles and pumps protons. This preparation of F_1F_O-ATPase contains 14 different polypeptides that are resolved by polyacrylamide gel electrophoresis under denaturing conditions, and so it is more complex than bacterial and chloroplast enzymes, which have eight or nine different subunits. The 14 bovine subunits have been characterized by protein sequence analysis. They have been fractionated on polyacrylamide gels and transferred to poly(vinylidene difluoride) membranes, and N-terminal sequences have been determined in nine of them. By comparison with known sequences, eight of these have been identified as subunits β, γ, δ, and ϵ, which together with the α subunit form the F_1 domain, as the b and c (or DCCD-reactive) subunits, both components of the membrane sector of the enzyme, and as the oligomycin sensitivity conferral protein (OSCP) and factor 6 (F_6), both of which are required for attachment of F_1 to the membrane sector. The sequence of the ninth, named subunit e, has been determined and is not related to any reported protein sequence. The N-terminal sequence of a tenth subunit, the membrane component A6L, could be determined after a mild acid treatment to remove an α-*N*-formyl group. Similar experiments with another membrane component, the a or ATPase-6 subunit, caused the protein to degrade, but the protein has been isolated from the enzyme complex and its position on gels has been unambiguously assigned. No N-terminal sequence could be derived from three other proteins. The largest of these is the α subunit, which previously has been shown to have pyrrolidonecarboxylic acid at the N terminus of the majority of its chains. The other two have been isolated from the enzyme complex; one of them is the membrane-associated protein, subunit d, which has an α-*N*-acetyl group, and the second, surprisingly, is the ATPase inhibitor protein. When it is isolated directly from mitochondrial membranes, the inhibitor protein has a frayed N terminus, with chains starting at residues 1, 2, and 3, but when it is isolated from the purified enzyme complex, its chains are not frayed and the N terminus is modified. Previously, the sequences at the N terminals of the α, β, and δ subunits isolated from F_1-ATPase had been shown to be frayed also, but in the F_1F_O complex they each have unique N-terminal sequences. It is now apparent that the fraying of the α, β, and δ subunits arises when the F_1 particle is released from mitochondrial membranes and that the extent of fraying depends upon the method of release; proteolysis is less extensive when sonication rather than shaking with chloroform is employed. The sequences of the subunits of F_1F_O-ATPase show that eight of them are related to the subunits of the bacterial and chloroplast complexes, and presumably they have functions similar to those of their homologues. The ATPase inhibitor, F_6, and subunits ϵ, d, e and A6L have no obvious counterparts in bacteria and chloroplasts. The inhibitor may have a regulatory function, and F_6 is essential for binding F_1 to the membrane sector, but the roles of the other subunits are obscure.

The ATP synthases of eubacteria, chloroplasts, and mitochondria have related structures and mechanisms. They are membrane-bound enzymes that catalyze ATP production from ADP and inorganic phosphate by using the transmembrane potential gradient for protons, $\Delta\tilde{\mu}_{H^+}$, to drive the reaction (Mitchell, 1961; Nicholls, 1982). In bacterial enzymes and in reconstituted mitochondrial enzymes the process is reversible, and the enzymes can hydrolyze ATP and use the energy released to pump protons. The enzymes from these various sources differ in the complexity of their subunits. To date, the simplest ATP synthase to be described is that from *Escherichia coli* (Fillingame, 1981). It has eight different subunits; five of them, α, β, γ, δ, and ϵ, for a globular domain, F_1-ATPase, which lies outside the membrane, and the three others, a , b, and c, comprise the membrane sector of the enzyme to which F_1 is bound. The enzymes from photosynthetic chloroplasts and bacteria are slightly more complex and appear to have nine subunits (Pick & Racker, 1979; Westhoff

‡The genetic sequence reported for the e subunit of F_1F_O-ATPase has been submitted to GenBank under Accession Number J05330.
*To whom correspondence should be addressed.
§Present address: Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands.
∥R.L. was supported by a long-term EMBO Fellowship and also by a European Science Exchange Fellowship from the Netherlands Organization for Pure Research (N.W.O).
⊥Present address: Laboratoire de Biochimie, LBIO, CENG, BP85X, 38041 Grenoble Cedex, France.

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et al., 1985; Cozens & Walker, 1987; Falk & Walker, 1988). They are all homologues of the subunits of the *E. coli* complex, and the extra component arises because two different subunits are both related to the b subunit of the *E. coli* complex.

Mitochondrial ATP synthases are even more complicated. Preparations of the enzyme isolated from bovine heart mitochondria have been described that have between 12 and 18 protein components (Serrano et al., 1976; Stiggal et al., 1978; Ludwig et al., 1980), and a preparation of the enzyme from rat liver has a minimum of 9–10 subunits (McEnery et al., 1984). In both the bovine and rat enzymes the five subunits of the F_1-ATPase could be recognized with some certainty from their mobilities on polyacrylamide gels, but the identities of other bands were uncertain since sequences were not determined. Three other subunits, the oligomycin sensitivity conferral protein (OSCP),[1] factor 6 (F_6) and subunit c, have been purified from bovine mitochondrial membranes (MacLennan & Tzagaloff, 1968; Fessenden-Raden, 1972; Cattel et al., 1970, 1971) but not directly from the isolated enzyme complex. Reconstitution studies have shown that the OSCP and F_6 are bona fide components of the enzyme (MacLennan & Tzagoloff, 1968; Fessenden-Raden, 1972; Ernster et al., 1986), and their identities with bands present in preparations of the enzyme have been inferred from their mobilities on polyacrylamide gels. Nonetheless, until the studies described below, unambiguous assignment of the bands corresponding to these proteins has been difficult. In part this has been not only due to differences in complexity of subunits in preparations of the enzyme but also, in the case of the OSCP in particular, because of differences in its relative migration with respect to other components with similar apparent molecular weights. The evidence for the presence of the c subunit in bovine ATP synthase has been provided previously by experiments that show that it is labeled by dicyclohexylcarbodiimide (Cattell et al., 1970, 1971; Graf & Sebald, 1978), and also by sequence homology with the *E. coli* protein, which mutational evidence shows it to be an essential part of ATP synthase (Sebald & Hoppe, 1981). However, the unmodified monomeric c subunit has not been identified with certainty on polyacrylamide gels. The inability to recognize bands corresponding to this and other subunits has impeded some studies of the mitochondrial ATP synthase. Notably, the stoichiometries of many of the subunits in the mitochondrial complex have not been determined, and only the stoichiometries of the five subunits in the F_1-ATPase complex are known (Walker et al., 1985). The definitive identification of the subunits in the present work opens the way to these measurements.

Other questions arise about the functions and the identities of the extra subunits that have been seen in the various preparations, and in some cases it is unclear whether they really are components of the ATP synthase complex, and the minimal mitochondrial complex has not been defined. An ideal, but difficult, way to answer these questions is to isolate the individual components, to reconstitute the purified subunits into the enzyme complex, and to demonstrate that the activity of the enzyme depends upon their presence. Another approach, that we have followed, is to eliminate subunits from the enzyme by purifying simpler forms of the complex that retain the activities of the enzyme and then to characterize each individual component present in the complex by protein sequence analysis.

[1] Abbreviations: OSCP, oligomycin sensitivity conferral protein; SDS, sodium dodecyl sulfate; PVDF, poly(vinylidene difluoride); PMSF, phenylmethanesulfonyl fluoride.

As described elsewhere (Lutter et al., 1991) we have isolated an oligomycin-sensitive F_1F_O-ATPase complex from bovine heart mitochondria that has been reconstituted and shown to pump protons. By polyacrylamide gel electrophoresis under denaturing conditions this complex contains 14 different subunits. All but one of these bands have been identified by protein chemical methods and correspond to proteins of known sequence. Together with previous studies (Fearnley & Walker, 1986; Walker et al., 1985, 1987a) these experiments identify seven of them as homologues, and therefore presumably the functional equivalents, of the subunits of the bacterial complex. An eighth component is similar in hydrophobic profile to the bacterial b subunit but is not significantly related in primary structure to it and has been proposed to be the equivalent protein in the mitochondrial complex (Walker et al., 1987a). The six other components appear to have no equivalents in bacterial complexes and so they are classified as being supernumerary.

In addition, these experiments have revealed surprising new details about some of the subunits of the enzyme. Earlier studies of the components of F_1-ATPase and of the inhibitor isolated from mitochondria rather than from the F_1F_O-ATPase had shown that the α, β, δ, and inhibitor proteins all have ragged N termini, which in principle could have arisen in the proteolytic removal of mitochondrial import presequences, although other explanations were also considered (Walker et al., 1985; Runswick et al., 1986). It is now evident that the same subunits in the membrane-bound F_1F_O-ATPase are not ragged at their N-terminals and that the fraying appears to arise during or immediately after release of F_1 with chloroform or of the inhibitor from the mitochondrial inner membrane. These findings may be at least of practical importance in helping to finds means of reducing the microheterogeneity of preparations of F_1-ATPase being used in crystallization studies.

Materials and Methods

Protease Inhibitors. Leupeptin was obtained from Boehringer Corporation Ltd., Lewes, U.K.; pepstatin, antipain, chymostatin, and elastatinal were from The Peptide Institute Inc., Osaka, Japan; and bestatin and amastatin were from Sigma Biochemicals, Poole, U.K.

Protein Estimation. This was performed either by the procedure of Bradford (1976) as modified by Macart and Gerbaut (1982) or by the BCA method (Pierce and Warriner Ltd., Chester, U.K.)

Isolation of F_1F_O-ATPase. The isolation of the enzyme is described briefly here and in greater detail elsewhere (Lutter et al., 1991). Mitochondrial membranes were prepared from bovine heart mitochondria by homogenization under hypoosmotic conditions. The membranes were resuspended in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 10% glycerol. The protein concentration (determined by the BCA method) was adjusted to 10 mg/mL, and the F_1F_O-ATPase was solubilized in a buffer consisting of 20 mM Tris-HCl, pH 7.5, 10 mM succinate, 35 mM sodium chloride, 2 mM ATP, 1 mM EDTA, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, and 1.2% dodecyl β-D-maltoside (final concentration). The extract was centrifuged (49000*g*, 15 min, 4 °C), and the supernatant was layered at room temperature onto a DE-52 column (15 cm × 2.7 cm i.d.), which had been preequilibrated in a buffer identical with the solubilization buffer except that the detergent concentration was 0.1%. Turbid material emerged from the column first in the break-through volume and was followed immediately by the F_1F_O-ATPase. Cholate was added to these fractions from a 10% stock solution to a final concentration of 2%, and then

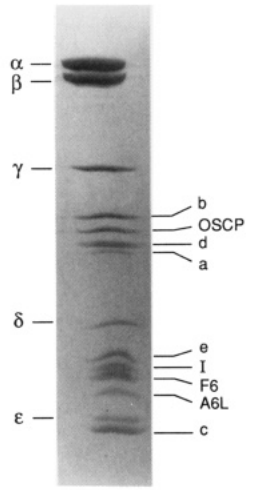

FIGURE 1: The identities of subunits present in a preparation of F_1F_O-ATPase isolated from bovine mitochondria. The proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate on a polyacrylamide gel (10–25% gradient of acrylamide) and stained with Coomassie Blue dye. For details of the identification of subunits see the text.

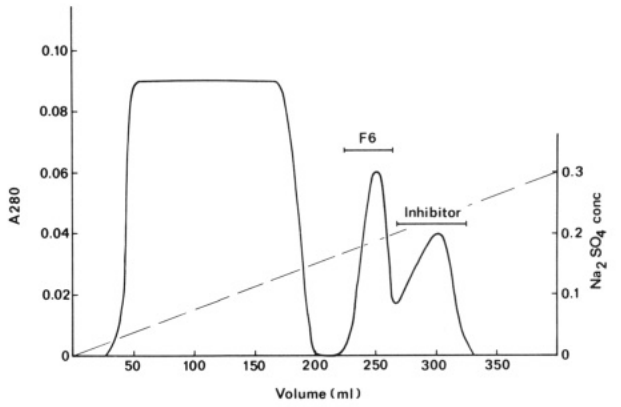

FIGURE 2: Purification of the F_6 and ATPase inhibitor proteins from isolated bovine mitochondrial F_1F_O-ATPase (75 mg): —, absorbance at 280 nm; – –, sodium sulfate gradient. For the isolation of the OSCP from F_1F_O-ATPase see Walker et al. (1987a). For other experimental details see Materials and Methods.

the F_1F_O-ATPase was precipitated with solid ammonium sulfate added with stirring at 0 °C to a final concentration of 45% saturation. The precipitate was collected by centrifugation at 49000*g* for 15 min and then resolubilized in 300–500 μL of a solution containing 20 mM Tris-HCl, pH 8.0, 50 mM sucrose, 1 mM magnesium sulfate, 0.5% (w/v) PMSF, and 7.1 mM 2-mercaptoethanol. Any particulate material was removed by centrifugation for 5 min at 13 000 rpm in a microfuge, and the supernatant was layered onto a TSK G4000 SW preparative HPLC column (60 × 2.1 cm i.d.), which had been preequilibrated in a buffer consisting of 20 mM Tris-HCl, pH 8.0, 50 mM sucrose, 1 mM magnesium sulfate, 0.05% (w/v) dodecyl β-D-maltoside, 0.001% (w/v) PMSF, 10% (v/v) glycerol, 0.1 M sodium chloride, and 2 mM 2-mercaptoethanol. The flow rate was 4 mL/min. F_1F_O-ATPase eluted in the first peak (detected by its absorbance at 280 nm), and this material was layered onto a Mono Q column (Pharmacia Ltd., Milton Keynes, U.K.; 10 cm × 1.0 cm i.d.) equilibrated with the same buffer as was employed with the TSK column. A linear sodium choride gradient was applied and F_1F_O-ATPase eluted at a concentration of about 0.3 M. Fourteen different polypeptides could be detected in this preparation by polyacrylamide gel electrophoresis (see Figure 1). This was performed as described below, and proteins were detected either with Coomassie Blue dye or by silver staining (Morrissey, 1981). About 60% of the activity of the enzyme is sensitive to the antibiotic oligomycin (Lutter et al., 1991). The enzyme has also been reconstituted into phospholipid vesicles and has uncoupler-sensitive ATP hydrolytic activity, indicative of proton-pumping activity (Lutter et al., 1991). In earlier experiments, Triton X-100 rather than dodecyl β-D-maltoside was used as detergent in the isolation of F_1F_O-ATPase (Lutter et al., 1991). Enzyme prepared by this method appeared to a have diminished levels of subunit e. It had an oligomycin-sensitive ATP hydrolase activity but after reconstitution did not pump protons. This could have been due to the presence of residual Triton X-100 in the preparation, although other explanations are possible.

Purification of F_1-ATPase from Bovine Heart Mitochondria. This was done as described by Walker et al. (1985) by use of material released from submitochondrial particles by chloroform (Beechey et al., 1975). The enzyme had a specific ATP hydrolase activity of 50–80 units/mg in the absence of bicarbonate and approximately twice that value in its presence. In order to try to prevent degradation of the N-terminal regions of the α and β subunits of F_1-ATPase, various protease inhibitors were added singly at the final concentrations given in parentheses to the buffer employed in the chloroform release step. These include leupeptin (0.5 μg/mL), pepstatin (0.7 μg/mL), antipain (200 μg/mL), chymostatin (100 μg/mL, dissolved in dimethyl sulfoxide), bestatin (40 μg/mL, in methanol) amastatin (50 μg/mL, in methanol), elastatinal (20 μg/mL) and *o*-phenanthroline (2 mM, in ethanol). Two other samples of F_1-ATPase prepared by the method of Knowles and Penefsky (1972) were generous gifts from Drs. H. S. Penefsky and J. A. Berden.

Purification of Subunits from F_1F_O-ATPase. The purification of subunits b, d, OSCP, a, and A6L from the isolated F_1F_O-ATPase have been described previously (Walker et al., 1987a; Fearnley & Walker, 1986). The ATPase inhibitor protein, F_6, and the OSCP were obtained as byproducts of the purification of subunit d. The starting point of this purification was F_1F_O-ATPase (75 mg) that had been S-alkylated with [2-^{14}C]iodoacetic acid under denaturing conditions in the presence of 6 M guanidine hydrochloride. As described previously, this mixture of subunits was fractionated by gel filtration through Sepharose 6B (145 cm × 4.0 cm i.d.) in 6 M urea at pH 4.0; the ATPase inhibitor protein and F_6 eluted together in the low molecular weight fraction (Walker et al., 1987a). These fractions were dialyzed against 10 mM sodium acetate, pH 5.2. Then the solution was centrifuged briefly to remove traces of insoluble material, and the supernatant was applied to a column of CM-Sephadex C50 (3 cm × 1 cm i.d.). The column was eluted with a linear gradient of sodium sulfate (0–1.0 M). F_6 and the ATPase inhibitor protein emerged at 0.06–0.12 M and 0.12–0.26 M salt, respectively (see Figure 2). The recovery of each protein in this experiment was 5 nmol.

Characterization of Peptides from the ATPase Inhibitor Protein. The protein (1 nmol) was digested at 37 °C for 4 h with chymotrypsin (0.2 μg). The resultant digest was fractionated by HPLC on a C_{18} reversed-phase column (30 mm × 2 mm; made by Brownlee, supplied by Anachem, Luton, U.K.) in 0.1% trifluoroacetic acid and with a linear gradient of acetonitrile. The flow rate was 180 μL/min. Peptides Ch1 and Ch2, which were sequenced (see Figure 3), eluted at 17 and 5% acetonitrile, respectively.

Transfer of Proteins to Poly(vinylidene difluoride) Membranes. Samples of F_1F_O-ATPase (50 μg) were dried in vacuo and then were dissolved in 10% sodium dodecyl sulfate (5 μL).

To this solution were added water (20 μL) and a dye mix solution (2 μL) containing 50% sucrose, 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, 0.3% xylene cyanol FF, and 0.3% bromophenol blue. Eight of these samples were fractionated in separate lanes of a polyacrylamide gradient gel (10–25%; 0.6 mm thick) in the presence of 0.1% sodium dodecyl sulfate (Laemmli, 1970). Then, the proteins were transferred by electrophoresis to a poly(vinylidene difluoride) membrane (Immobilon, Millipore) as described by Matsudaira (1987) except that the transfer was conducted at pH 6.8 in 0.01 M sodium phosphate buffer containing 0.05% sodium dodecyl sulfate. At pH 11.0, the recommended condition for electrophoretic transfer, it was found that most of the γ subunit remained in the gel and was not transferred to the membrane. Proteins were stained on the membrane with 0.1% Coomassie Blue dye in 50% methanol for a maximum period of 5 min, and after a brief destaining with 50% methanol the bands were excised with a scalpel and stored at −20 °C before sequence analysis.

Subunit e was separated from other subunits of F_1F_O-ATPase by electrophoresis in a polyacrylamide gel under denaturing conditions, and cleaved with cyanogen bromide [see Runswick et al. (1990)]. The products were fractionated under similar conditions and transferred to a polyvinylidene membrane as described above. Staining with Coomassie Blue dye revealed a doublet. The upper band was the uncleaved e subunit, and by sequence analysis it was shown that the lower band was the C-terminal cyanogen bromide fragment.

Protein Sequence Analysis. Isolated subunits of F_1F_O-ATPase chymotryptic peptides from the ATPase inhibitor protein, and proteins that had been transferred to poly(vinylidene difluoride) membranes were sequenced in an Applied Biosystems 890B gas-phase protein sequencer with on-line detection of phenylthiohydantoin amino acids. An optical sensor has been incorporated into the sample loop of the HPLC chromatograph. This permits 90% of each sample produced by the sequencer to be analyzed and increases the effective sensitivity of the sequencer by a factor of almost 2. Isolated proteins and peptides were applied to the glass fiber discs in the presence of polybrene (3 mg) and sodium chloride (0.2 mg). Pieces of membrane to which proteins had been transferred by electrophoresis were placed in the reaction chamber of the sequencer in the presence of a Teflon seal only, and neither a glass fiber disc nor polybrene was employed in these experiments. In cases where no sequence was obtained from samples absorbed on poly(vinylidene difluoride) membranes, duplicate samples were treated in an Eppendorf tube with methanolic hydrochloride (1.5 M) for 2 h at room temperature. Subsequently, the supernatant was dried down, the residue was redissolved in 70% formic acid (10 μL), and the solution was spotted onto a piece of the membrane. Then the samples were subjected to Edman degradation as described above.

Amino Acid Analysis. Samples of proteins or peptides were hydrolyzed for 24 h at 110 °C in vacuo in the presence of 6 N hydrochloride acid containing 0.1% phenol. Hydrolysates were analyzed in a Durrum D500 amino acid analyzer.

Cloning and Sequence Analysis of cDNAs Encoding Subunit e. The synthesis and purification of oligonucleotides has been described before (Pilkington et al., 1991). Oligonucleotide primers for use in polymerase chain reactions were made with a linker sequence containing various restriction enzyme sites (*Eco*RI, *Hin*dIII, or *Bam*HI) on their 5′ ends; this facilitated the cloning of products into M13 vectors. Polymerase chain reactions performed with single-stranded bovine heart cDNA as template have been described before and so have the fractionation of products of these reactions, their detection with synthetic oligonucleotides, and their recovery and cloning into M13 vectors (Runswick et al., 1990; Pilkington et al., 1991; Dupuis et al., 1991). When degenerate primers were employed, the first 30 cycles were carried out at a lower primer concentration (50 nM) and were followed by an additional 20 cycles with a primer concentration of 2 μM. The methods used in the screening of a bovine cDNA library (Gay & Walker, 1985) have been given in earlier publications (Walker et al., 1989).

The sequences of cloned products were determined by the modified dideoxy method (Sanger et al., 1977; Biggin et al., 1983), with use of either the LMB2 universal primer (Duckworth et al., 1981) or unique synthetic primers based upon known sequences. All sequences were determined completely in both senses of the DNA, and data were compiled and analyzed with DBUTIL and ANALYSEQ (Staden, 1982, 1985).

Table I: Equivalent Subunits in ATP Synthases in Bacteria, Chloroplasts, and Bovine Mitochondria

type	bacteria	chloroplasts	mitochondria
F_1	α	α	α
	β	β	β
	γ	γ	γ
	δ	δ	OSCP
	ε	ε	δ
	–	–	ε
F_O	a	a (or x)	a (or ATPase-6)
	b[a]	b and b′ (or I and II)	b
	c	c (or III)	c
Supernumerary	–	–	F_6
	–	–	inhibitor
	–	–	A6L
	–	–	d
	–	–	e

[a] ATP synthases in *E. coli* and bacterium PS 3 (both eight-subunit enzymes) have two identical copies of subunit b per complex. Purple non-sulfur bacteria and cyanobacteria appear to have nine different subunits, the extra subunits (known as b′) being a homologue of b (Cozens & Walker, 1987; Falk & Walker, 1988). Similarly, chloroplast enzymes are made of nine nonidentical subunits, and the chloroplast subunits known as I and II are the homologues of b and b′.

Results

Characterization of Subunits Isolated from the F_1F_O-ATPase Complex. The a and A6L subunits were isolated from chloroform/methanol extracts of the F_1F_O-ATPase complex (Fearnley & Walker, 1986), and subunits b and d were obtained by disrupting the complex with chaotropic agents and detergents followed by chromatographic steps (Walker et al., 1987a). Both a and A6L required treatment with methanolic hydrochloride, presumably to remove an α-*N*-formyl group, before the N-terminal sequence could be obtained, and they proved to be the products of overlapping genes in mitochondrial DNA (Fearnely & Walker, 1986). Their sequences deduced from DNA sequence showed that subunit a is a homologue of the bacterial homonym, and that A6L has no obvious bacterial counterpart (see Table I). Subunit d also has a modified N terminus, in this case an *N*-acetylalanine residue, whereas the α-amino group of subunit b is unmodified and extensive sequence could be determined. The sequences of neither protein are evidently related to any of those of the subunits of bacterial ATP synthase, but the similarity of the hydrophobic profiles of the b protein and those of homonyms in bacterial enzymes supports the view that they are structural analogues (Walker et al., 1987a).

In the course of the isolation of subunits b and d, the opportunity arose to isolate the OSCP, F_6, and the inhibitor

Table II: Amino Acid Compositions of the OSCP, F_6 and ATPase Inhibitor Protein from Bovine Mitochondria[a]

	OSCP		F_6		inhibitor	
amino acid	a	b	a	b	a	b
aspartic acid[b]	10.5	10	9.7	10	6.8	7
threonine	12.5	13	3.8	4	0	0
serine	17.1	16	2.4	2	6.8	7
glutamic acid[c]	15.7	19	13.4	13	16.9	18
proline	8.9	8	5.3	6	0	0
glycine	9.8	9	4.4	4	6.7	6
alanine	16.8	16	2.3	2	10.3	10
cysteine	nd[d]	1	nd	0	nd	0
valine	16.3	16	4.5	5	1.6	2
methionine	1.0	8	2.1	2	0	0
isoleucine	9.4	11	1.4	1	4.2	4
leucine	20.3	23	6.1	6	4.2	4
tyrosine	5.0	5	2.6	3	0.8	1
phenylalanine	4.4	4	6.4	6	1.8	2
histidine	1.3	1	0	0	5.2	5
lysine	19.4	21	8.9	9	10.3	10
arginine	10.1	9	4.1	4	7.5	8
tryptophan	nd	0	nd	0	nd	0
no. of residues		190		77		84

[a] Amino acid compositions are given from amino acid analysis (a) and from the sequence (b). [b] Sum of aspartic acid and asparagine. [c] Sum of glutamic acid and glutamine. [d] Not determined.

Table III: Amino-Terminal Sequences Determined on Subunits of F_1F_O-ATPase from Bovine Heart Mitochondria[a]

subunit	sequence[b]
α	no sequence
β	AAQASPSPK...
γ	ATLKD...
b	PVPPLPEHGGY...
OSCP	FAKLV...
d	no sequence
a	no sequence
δ	AEAAAAQAP...
e	VPPVQVSPLIKLGRYSALFLGMAYGAKRY-NYLKPR
ATPase inhibitor	no sequence
F_6	NKELD...
A6L[c]	MPQLD...
ϵ	VAYWRQ...
c	DIDTAAKFIG...

[a] Subunits were separated from non-S-alkylated enzyme by polyacrylamide gel electrophoresis, transferred to poly(vinylidene difluoride) membranes, and sequenced as described in Materials and Methods. [b] The same result has been obtained with subunits, γ, δ, e, inhibitor, and F_6 from the F_1F_O-ATPase complex. [c] After deformylation.

protein from the F_1F_O-ATPase complex, and although all three proteins had been isolated from mitochondrial extracts and had been demonstrated by reconstitution experiments to be components of F_1F_O-ATPase, their isolation from the purified enzyme had not been reported and considerable confusion and uncertainty surrounded their identifications in polyacrylamide gel analyses. The OSCP and F_6 proteins isolated in this way proved to be identical in both amino acid compositions (Table II) and N-terminal sequences (Table III) of the proteins characterized previously. However, Edman degradation of the protein thought to be the ATPase inhibitor generated no N-terminal sequence, although its amino acid composition was indistinguishable from that calculated from its sequence. The N terminus of the inhibitor characterized previously is frayed, and chains are present that commence at residues 1, 2, and 3 (Runswick et al., 1986). Isolation and sequence analysis of two peptides isolated from a chymotryptic digest of the protein obtained from the F_1F_O-ATPase showed that their sequences are identical with parts of the chain of the authentic inhibitor

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        10        20        30        40        50
GSESGDNVRSSAGAVRDAGGAFGKREQAEEERYFRARAKEQLAALKKHHE
                  ----------
                  Ch1

        60        70        80
NEISHHAKEIERLQKEIERHKQSIKKLKQSEDDD
                            -------
                            Ch2
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FIGURE 3: Sequences of peptides obtained from a chymotryptic digest of the ATPase inhibitor protein associated with F_1F_O-ATPase. They are aligned with the sequence of the mature protein isolated from mitochondrial membranes (Runswick et al., 1986).

Table IV: Heterogeneity of N-Terminal Sequences in Subunits of Two Preparations of F_1-ATPase and Lack of Heterogeneity in the Same Subunits in F_1F_O-ATPase from Bovine Mitochondria[a]

complex	subunit	sequence	%
F_1(C)	α	<QKTGTAE...	65
		KTGTAE...	35
F_1(P)	α	<QKTGTAE...	90
		KTGTAE...	10
F_1F_O	α	<QKTGTAE...	100
F_1(C)	β	QASPSPK...	27
		ASPSPK...	25
		SPSPK...	45
F_1(P)	β	AAQASPSPK...	38
		AQASPSPK...	10
		QASPSPK...	19
		ASPSPK...	25
		SPSPK...	8
F_1F_O	β	AAQASPSPK...	100
F_1(C)	δ	AEAAAAQ...	50
		AAAAQ...	50
F_1(P)	δ	AEAAAAQ...	100
F_1F_O	δ	AEAAAAQ...	100

[a] F_1(C) is F_1-ATPase released from mitochondrial membranes with chloroform and further purified according to Walker et al. (1985); F_1-(P) is F_1-ATPase prepared according to Knowles and Penefsky (1972). The experiments concerning the sample provided by Dr. Penefsky are reported here, but substantially the same results were obtained with a second sample provided by Dr. Berden. The data about F_1(C) are taken from Walker et al. (1985). The reduced level of sequences in the α subunit of F_1(P) and the lack of sequence from the same subunit in F_1F_O are assumed to be caused by the cyclized N-terminal glutamine, as found in F_1(C) α subunit (Walker et al., 1985). The figure for the β subunit in F_1(C) is the sum of chains with free N-terminal glutamine and its degradation products pyrrolidone carboxylic acid and glutamic acid. In the experiment to estimate the amounts of the various N terminals present in the α, β, and δ subunits of F_1(P), the total of α and β chains present is assumed to be three times the amount of the δ chains (which have a unique N terminus). The amount of the δ chains is estimated from the yields of PTH amino acids in step 1 of the Edman degradation (the initial yield in the sequencer was measured independently and found to be 80%).

(see Figure 3). So it appears that the inhibitor protein isolated from the enzyme complex is different from the inhibitor characterized previously in that it has a modified N terminus and that the chains probably start at a unique residue. Further investigation is required to clarify this point.

N-Terminal Sequences of Subunits of F_1F_O-ATPase Transferred to PVDF Membranes

Subunits α, β, and δ. As with the inhibitor protein, earlier experiments on these proteins, which had been isolated from the F_1-ATPase, had demonstrated that they all have frayed N terminals (Walker et al., 1985; see also Table IV). In contrast, sequence analyses conducted on the β and δ subunits obtained from the F_1F_O-ATPase complex after transfer of the proteins to PVDF membranes, showed that they have unique N terminals (see Tables III and IV) and, moreover, the sequence of the β subunit is two amino acids longer than the longest chain detected in preparations of this protein obtained

from F_1-ATPase (Walker et al., 1985). The chains of the α subunit also appear to be unique, although in this case no N-terminal sequence was detected and the α-amino group appears to be modified. A pyrrolidone carboxylic acid residue was found in about 65% of the α chains isolated from F_1-ATPase (Walker et al., 1985), and it is likely that this is the blocked N-terminal residue in the α chains of the F_1F_O-ATPase. Thus, the release of the F_1-ATPase from submitochondrial particles with chloroform is accompanied by a rapid proteolytic degradation at the N terminals of the α, β, and δ chains and, as indicated by the experiments described in the previous section, probably of the ATPase inhibitor protein also. It is known that the N-terminal regions of the α and β chains in the F_1 complex are subject to rapid proteolysis by trypsin and chymotrypsin (Leimgruber & Senior, 1976; Hundal & Ernster, 1981; Walker et al., 1985).

These findings suggested the possibility that F_1-ATPase released from mitochondrial membranes by sonication at pH 9.2 as described by Knowles and Penefsky (1972) might not suffer from proteolytic degradation. Therefore, as summarized in Table III, the N terminals in subunits of the enzyme prepared by this alternative procedure were investigated. It was observed in two independently isolated samples that the α and β subunits are somewhat heterogeneous, but to a lesser extent than in the chloroform-released enzyme. Notably, 35% of the β chains from the Knowles and Penefsky enzyme contain the two extra alanine residues at their N terminals, and a further 10% have one extra N-terminal alanine residue. Also, the δ chains appear to be undegraded in this preparation.

We have made several attempts to try to inhibit proteolytic damage during and after release of F_1 particles by chloroform. In addition to phenylmethanesulfonyl fluoride and EDTA, used at higher concentrations than the normal levels in the buffer employed in the release of F_1-ATPase, we have examined the effects of *o*-phenanthroline, leupeptin, pepstatin, antipain, chymostatin, bestatin, amastatin, and elastatinal, but they have little or no inhibitory effect on the proteolytic activity.

Subunits γ and ϵ. There is no evidence of proteolytic damage to the γ and ϵ subunits brought about by release of F_1 particles from submitochondrial membranes by either procedure (see Tables III and IV). Their N-terminal sequences are the same in the membrane-bound complex and in both preparations of F_1-ATPase. This could be interpreted as indicating that their N termini are less exposed in the F_1 complex than are those of the other three subunits.

Subunits b, d, OSCP, and a. These subunits have apparent molecular masses of 19 to 24 kDa, and on polyacrylamide gels they are grouped together. The order of the bands of OSCP, a, and d appears to be dependent upon the experimental conditions, and in some gel systems they are not resolved from each other. In addition, subunit a stains poorly with Coomassie Blue dye. For these reasons there has been considerable confusion about their identities. Resolution of these bands is obtained by the use of 10–25% polyacrylamide gradient gels or, alternatively, under conditions described by Montecucco et al. (1983). The sequences determined on samples of these proteins that had been transferred to PVDF membranes confirmed the position on the gel of the b polypeptide proposed previously (Walker et al., 1987a) and showed that the uppermost protein of the triplet immediately below this band was the OSCP. No N-terminal sequence was obtained from the two other bands nor was any clear sequence revealed by treating the samples with methanolic hydrochloride before sequence analysis. However, it is evident from other experiments on subunits d and a that d is the middle component of the triplet [see previous section and Walker et al. (1987a)] and that the lowest and faintly staining band of this group is subunit a (Fearnley & Walker, 1986). The failure in the present experiment to observe clear sequence originating from the N terminus of subunit a after treatment of the protein with methanolic hydrochloride probably is due to partial hydrolysis of the protein by the reagent.

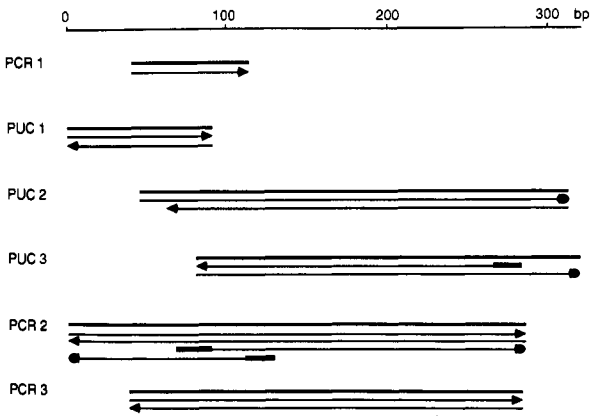

FIGURE 4: Sequence analysis of cDNAs encoding the e subunit of F_1F_O-ATPase. PCR1–PCR3 represent cDNAs made by the polymerase chain reaction, and PUC1–PUC3 are cDNAs isolated from a bovine library. The lengths of the clones are indicated by the heavy lines, and the extents and directions of the determined DNA sequences are marked by arrows. The bars on the ends of arrows indicate the positions of sequencing primers. The scale is in base pairs (bp).

Subunit e, the ATPase Inhibitor Protein, F_6, and A6L. These four proteins gave rise to a quartet on polyacrylamide gels between the δ and ϵ subunits (see Figure 1). The uppermost member of this group, referred to as subunit e, was present in apparent stoichiometric amounts in the F_1F_O-ATPase prepared in the presence of dodecyl β-D-maltoside but was absent from or present in reduced amounts in F_1F_O-ATPase isolated in the presence of Triton X-100. Its N-terminal sequence was determined up to residue 35 (see Table III), and confirmation of the sequence after methionine-22 was obtained by sequencing the C-terminal cyanogen bromide fragment. The N-terminal sequence of the protein was made use of to isolate cDNA clones by a strategy summarized in Figures 4 and 5. First, a partial cDNA encoding amino acids 1–26 was produced by means of the polymerase chain reaction, by priming on single stranded bovine cDNA with mixtures of synthetic oligonucleotides based on amino acids 1–6 and 21–26 (see PCR 1 in Figure 4). The forward and reverse primers were both 17 bases long (plus appropriate linkers; see Materials and Methods) and contained 512 and 128 sequences, respectively. The hybridization probe for recognition of the cDNA, also 17 bases long, was based on amino acids 10–15 and contained 1536 sequences. Since mixed oligonucleotide primers were used in this reaction, the sequence of the partial cDNA was accurate only in the region between them. Second, this partial cDNA was used as a hybridization probe to screen a bovine heart cDNA library for cDNAs encoding the e subunit. Three classes of partial clone were characterized (PUC1–3 in Figure 4). PUC1 covers the 5′ end of the complete cDNA, and PUC2 and PUC3 overlap PUC1 and extend up to the 3′ poly(A) tail. PUC2 and PUC3 differ in the length of polyA sequence at their 3′ ends, but more importantly, they differ in that PUC2 contains an additional 54 bases of sequence in the coding region between the codons for amino acids 11 and 12 of the mature e subunit. In a third set of experiments verification of the cDNA sequence was obtained by sequencing cDNAs produced by polymerase chain reactions with unique primers (see PCR2 and PCR3 in Figure 4). None of the

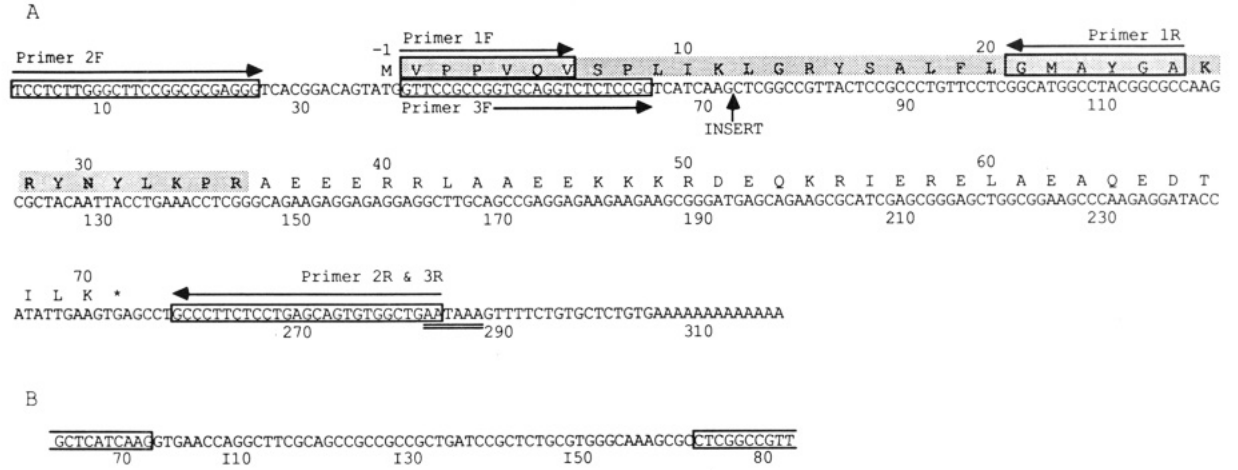

FIGURE 5: The cDNA and amino acid sequences of the e subunit of F_1F_O-ATPase. In part A, the N-terminal sequence of the mature protein determined by direct sequence analysis is shaded. The mature protein sequence is numbered from 1 to 70. Boxes around segments of DNA sequence show the positions of forward (F) and reverse (R) primers used in polymerase chain reactions. A potential signal for polyadenylation is underlined twice. A vertical arrow indicates the position of an insert found in some clones, and its sequence (numbered I1–I60) is shown in part B with flanking sequences, which are boxed.

primers used in these experiments was within the extra 54 base sequence, although both reactions spanned the site of the insert; four clones were sequenced from each reaction, and none of them contained the extra sequence. However, when primer 2R (see Figure 5) was used with a forward primer taken from the insert, a product was obtained that extended from the forward primer through the remainder of the insert and up to the reverse primer, demonstrating the presence in the independent cDNA preparation used in the polymerase chain reaction of species of cDNAs for the e subunit with the same insert as identified in the cDNA library. The most probable explanations is that this extra sequence is an intron in a cDNA arising from a partially spliced transcript, although the two bases at the 3′ extremity of the insert are GC and not AG, the more usual 3′ extremity of an intron. It is clear from the N-terminal protein sequence experiment that the insert does not code for part of the mature e subunit.

The complete cDNA sequence compiled from the shorter cDNAs is 317 nucleotides long (excluding the insert). It is terminated at its 3′ end by a run of A residues separated by 17 intervening nucleotides from the sequence AATAAA, a typical signal for polyadenylation (Proudfoot & Brownlee, 1976). Nucleotides 40 onward encode the mature e subunit and are immediately preceded by an ATG codon. No "in-phase" termination codons are present in the sequence 5′ to it, but in the absence of other information it is assumed to be the translational initiation codon and so the e subunit appears to have no processed mitochondrial import sequence.

The sequence of the mature e subunit is not related to any reported protein sequence, including that of Factor B, a protein that has been suggested to be part of the ATP synthase complex (Kantham et al., 1990; see below). It is a highly charged protein and contains 16 basic and 12 acidic residues in its 70 amino acid sequence. In this respect it is similar to the inhibitor protein (18 basic and 18 acidic amino acids in 84), but there is no obvious extensive sequence relationship between the proteins, and the function of the e subunit is unknown.

No N-terminal sequence was obtained on the second-highest band of the quartet either before or after attempted deformylation with methanolic hydrochloride. However, the experiments described above, and gel electrophoresis experiments in which authentic inhibitor is compared with the F_1F_O-ATPase support the view that this band is the ATPase inhibitor protein. The third band in descending order of the quartet gave an N-terminal sequence identical with that described for F_6 (Grinkevich et al., 1984; Fang et al., 1984), and after deformylation the lowest band had an N-terminal sequence the same as that described for A6L, thereby confirming earlier experiments (Fearnley & Walker, 1986).

Subunit c. Of all of the subunits of bovine mitochondrial F_1F_O-ATPase perhaps the greatest uncertainty has surrounded the location on polyacrylamide gels of this particular subunit. This is because of the pronounced propensity of this protein and of homologues in other species to form oligomers and so to give rise to multiple bands on gels. The picture has been clouded further by the fact that the protein stains poorly with Coomassie Blue dye and by the finding that after reaction with [^{14}C]dicyclohexylcarbodiimide, radioactivity is found to be associated predominantly with proteins with a range of apparent molecular masses (see Discussion). The present protein sequencing experiments (see Table III) demonstrate that, under the experimental conditions used, the c subunit runs faster on the gel than the ϵ subunit, which is a protein of 50 amino acids with a molecular mass of 5652 (Walker et al., 1985). From its migration on polyacrylamide gels, the c subunit has an apparent molecular mass of less than 5 kDa, but its true molecular mass is 7679 (Sebald & Hoppe, 1981). No higher molecular weight aggregates of the protein were detected elsewhere on the gel.

Discussion

Oligomycin-sensitive F_1F_O-ATPase preparations have been obtained previously from bovine heart mitochondria by a number of different procedures and have been reported to contain 12–18 different polypeptides (Serrano et al., 1976; Stiggal et al., 1978; Ludwig et al., 1980). These enzyme preparations hydrolyze ATP and, in contrast to F_1-ATPase preparations, are cold stable and are inhibited by DCCD and oligomycin, albeit to different extents. The current work describes the protein chemical characterization of two different F_1F_O preparations [see Materials and Methods and Lutter et al. (1991)] which both show an oligomycin-sensitive ATP hydrolase activity. However, only the preparation made in the presence of dodecyl β-D-maltoside in which the use of Triton X-100 is avoided, could be shown to pump protons. The latter preparation contains 14 subunits and it appears to be the simplest mammalian F_1F_O-ATPase complex that has been fully characterized. Two possible reasons can be advanced

in explanation of the absence of ATP synthase activity in the former preparation. First, it is possible that the complex is incomplete and, for example, that a subunit is absent or present in substoichiometric amounts. Coupling factor B seems to be in this category. The former protein has been isolated from bovine heart mitochondria and stimulates ATP-driven NAD^+ reduction, ATP-dependent $NAD(P)^+$ transhydrogenase, ATP/Pi exchange, and ADP phosphorylation coupled to succinate or NADH oxidation (Sanadi, 1982). It has an apparent molecular mass of 14.6 kDa, and its N-terminal sequence has been determined (Kantham et al., 1990), and it stains poorly with Coomassie Blue dye. Its presence has been demonstrated in partially purified preparations of ATP synthase but not in homogeneous samples of the enzyme. We have been unable to detect by sequence analysis its presence in our preparation of F_1F_O-ATPase. A second possibility is that appropriate phospholipids are not present in the reconstituted enzyme or are not able to exercise their effect because of the presence of detergent.

Two kinds of experiments have been performed in order to identify the 14 subunits present in the bovine F_1F_O-ATPase complex. These are, first, the isolation and characterization by protein chemical methods of subunits from the purified enzyme complex and, second, N-terminal analysis of subunits that have been separated by polyacrylamide electrophoresis and transferred to PVDF membranes. By these means, all 14 of the subunits of the complex have been identified. Seven of them, α, β, γ, δ, OSCP, a (or ATPase-6), and c (the DCCD reactive proteolipid) are the homologues of the subunits of the simpler *E. coli* complex (see Table I). However, as demonstrated previously, the equivalent subunits in the bovine enzyme of bacterial δ and ϵ subunits are the OSCP and the δ subunit, respectively (Walker et al., 1982; 1985). The bovine ϵ subunit has no homologue in either bacterial or chloroplast enzymes. So it is classified as being supernumerary, and its role in the enzyme complex is unknown. A sequence homologue of the eighth subunit of the bacterial enzyme, the membrane-associated subunit b, is not present in the bovine enzyme, but a membrane-associated protein with a similar hydrophobic profile has been characterized and has been proposed to be the bovine equivalent of bacterial b (Walker et al., 1987a). This is also known as the 24- or 27-kDa protein (Torok & Joshi, 1985), or F_O-1, a component of the membrane sector of the enzyme (Montecucco et al., 1983). More recent topological and functional studies have provided further support for this suggestion (Houstek et al., 1988), and a protein named ATPase-4 that is homologous to both the bovine and *E. coli* b subunits has been characterized as a component of the yeast mitochondrial F_1F_O-ATPase (Velours et al., 1987, 1988).

The identification of a band with an apparent molecular mass of <5 kDa on polyacrylamide gels as the DCCD-reactive proteolipid (or subunit c) deserves comment. Earlier studies of the bovine protein have found that radioactivity from [^{14}C]DCCD was associated with components of 18 and 6.5 kDa (Glaser et al., 1981), of 13 kDa (Kiehel & Hatefi, 1980), and of 10 or 8.5 kDa (depending on the gel system employed in this last case; Ludwig et al., 1980). Also, Graf and Sebald (1978) have commented upon the tendency of the bovine protein to form aggregates. These experiments did not permit the position of the unmodified protein to be identified unambiguously on polyacrylamide gels. This shortcoming has led to uncertainty in the interpretation of other experiments. For example, in their investigation of the topography of the membrane sector of the bovine F_1F_O-ATPase, Montecucco et al. (1983) suggest that a band radiolabeled with photoactivatable phospholipid with an apparent molecular mass of about 8 kDa referred to as F_O-8 is the c subunit, whereas it now appears to be at least as probable that a band with an apparent mass of <5 kDa (F_O-10) in the same experiment may be the c subunit. In our electrophoresis and sequencing experiments with the F_1F_O-ATPase, subunit c has been found only in the position indicated in Figure 1 and never as higher molecular weight aggregates. However, in other experiments conducted on purified subunit c, such aggregates have been observed, and their occurrence depends upon the experimental conditions to which the protein has been subjected (J.E.W. and I. M. Fearnley, unpublished results).

In addition to the bovine ϵ subunit, five other supernumerary subunits have been identified in the bovine complex. These are subunit d, F_6, the ATPase inhibitor protein, A6L, and subunit e. Assuming unit stoichiometries for each of these proteins, they contribute about 60 kDa to a total molecular mass for the entire complex of about 600 kDa (calculated from known stoichiometries for F_1 subunits and stoichiometries of a_1:b_2:c_{12} by analogy with the bacterial complex and molecular weights of subunits). The positions of F_6 and A6L in the enzyme complex fractionated on polyacrylamide gels have been determined by direct sequence analysis, in the case of A6L after a mild treatment to remove an α-*N*-formyl group. The A6L protein, which is a product of the mitochondrial genome (Anderson et al., 1982; Fearnley & Walker, 1986) has been isolated previously and identified as a component of bovine F_1F_O-ATPase (Fearnley & Walker, 1986), and the rat A6L polypeptide has been shown to be identical with chargerin II, which appears to have an important role in energy transduction in oxidative phosphorylation (Higuti et al., 1988). It has been proposed that the A6L protein is the homologue of a smaller hydrophobic protein, aap1 (or ATPase-8), associated with ATPase from yeast mitochondria (Attardi et al., 1984; Michael et al., 1984). This protein is required for the assembly of the yeast complex (Macreadie et al., 1983), and has a phosphate binding activity (Guerin & Napias, 1978; Velours et al., 1982), as the A6L protein has also (Blondin, 1979). Both proteins have a net basic charge, but sequence relationship between them extends over residues 1–4 only (including the initiator methionine) and is statistically insignificant. Also, the proteins have different lengths and in consequence appear to have rather different secondary structures, although both contain a hydrophobic region that could be folded into a membrane-spanning α helix. So while the proposal is tempting, and may well turn out to be true, the evidence in its support is weak. An unfortunate consequence of this proposal is that the bovine and related A6L proteins in other species have become to be referred to as ATPase-8 (Chomyn et al., 1983; Clary & Wolstenholm, 1985; Michael et al., 1984;), this despite the fact that the A6L protein is the twelvth largest protein (by gel mobility) in the bovine complex, and not the eighth largest as the name implies (see Figure 1). Therefore, the use of ATPase-8 as a synonym for the A6L protein is inappropriate.

No sequence was obtained by direct N-terminal analysis from either the subunit d or the ATPase inhibitor protein. The d subunit is known to have an α-*N*-acetyl group (Walker et al., 1987a), and so this result was expected and helps to confirm the assignment of the d band in polyacrylamide gels. It is probably identical with a 20-kDa component of bovine F_1F_O-ATPase that inter alia was labeled by photoactivatable phospholipids and was referred to as F_O-2 by Montecucco et al. (1983). This protein, as the name F_O-2 implies, is thought to be part of the membrane sector of the enzyme, but its sequence does not contain extensive hydrophobic sequences

that could be construed as membrane-spanning segments (Walker et al., 1987a).

The lack of a free N-terminus in the inhibitor protein was surprising since earlier studies on material isolated directly from mitochondria had shown the presence of chains with free α-amino groups starting at residues 1, 2, and 3 (Runswick et al., 1986). The amino acid compositions of the inhibitor protein obtained directly from mitochondria or isolated from the F_1F_O complex are essentially identical and suggest that the protein chains in both preparations are probably the same length. Phenylalanine is found in the mitochondrial import precursor of the F_1F_O-ATPase protein one residue before the start of the mature protein (Walker et al., 1987b), and the amino acid compositions do not indicate an additional phenylalanine in the blocked protein (see Table II). Earlier it had been reported that the inhibitor protein prepared directly from mitochondria had an α-*N*-formyl glycine residue (Dianoux et al., 1984), but this claim was not supported by other studies (Frangione et al., 1981; Runswick et al., 1986). Moreover, the protein isolated from the enzyme complex in the present studies was subjected to a mild acid treatment with methanolic hydrochloride, and subsequently no free N terminus could be detected. So its α-amino group appears not to be modified by a formyl group. The exact nature of this moiety remains to be established as does its effect, if any, on the inhibitory properties of the protein.

The question of whether subunit e is a bona fide component of the enzyme complex remains unanswered at present and requires further investigation. Its presence in the F_1F_O-ATPase complex indicates that it may be, and it appears to purify with the complex in approximately stoichiometric amounts.

We have searched by gel electrophoresis coupled with sequencing experiments for a number of subunits that have been described in earlier preparations of bovine heart mitochondrial ATPase. In addition to factor B, these include the ADP/ATP translocase, detected in their preparations by Montecucco et al. (1983), Glaser et al. (1980), and Joshi and Torok (1984), the uncoupler-binding protein (Hanstein, 1976), a protein with an apparent molecular mass of 30 kDa that is in the preparation of Galante et al. (1979), but which did not immunoprecipitate with the enzyme complex (Ludwig et al., 1980), and the phosphate carrier (Torok & Joshi, 1985). None of these proteins was detected, nor did we detect the presence of subunits of complex I or the core proteins of complex III that Glaser et al. (1980) suggested were present in their preparation. Two protein factors that stabilize and facilitate the binding of the ATPase inhibitor have been characterized from yeast mitochondria (Hashimoto et al., 1983; Okada et al., 1986), but homologues of these proteins were not detected either in our bovine F_1F_O-ATPase.

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Registry No. ATPase, 9000-83-3; ATP synthase, 37205-63-3.

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