The F_1F_o-ATPase Complex from Bovine Heart Mitochondria: The Molar Ratio of the Subunits in the Stalk Region Linking the F_1 and F_o Domains

Ian R. Collinson,‡ J. Mark Skehel, Ian M. Fearnley, Michael J. Runswick, and John E. Walker*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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ABSTRACT: The F_1 globular catalytic domain and the F_o intrinsic membrane domain of the F_1F_o-ATPases in bacteria, chloroplasts, and mitochondria are connected by a slender stalk. In the F_1F_o complex from bovine heart mitochondria, the stalk is thought to contain subunits OSCP, d, and F_6, and the globular part of the membrane bound subunit b, referred to as b′. It has been shown previously that the OSCP, b′, d, and F_6 proteins can be assembled *in vitro* into a water soluble complex named the "stalk". The stalk and F_1-ATPase together form another complex named F_1•stalk. In this paper, the molar ratios of the OSCP, b (or b′), d, and F_6 in the stalk, F_1•stalk, and F_1F_o-ATPase complexes have been investigated by three independent methods. By quantitation of radioactivity incorporated by S-carboxymethylation with iodo-2-[^{14}C]acetic acid into a stalk complex containing a form of F_6 with the mutation Glu_3-Cys, it was shown that the stalk consists of equimolar quantities of its four constituent proteins. In the stalk complex containing the natural F_6 sequence, this conclusion was confirmed both by quantitation of radioactivity incorporated by N^{ϵ}-acetimidation with ethyl [1-^{14}C]acetimidate, and by quantitative N-terminal sequence analysis of subunits. By similar N^{ϵ}-acetimidation experiments, it has been demonstrated that the F_1•stalk complex contains one copy per assembly of the OSCP, b′, d, and F_6 proteins and that the F_1F_o-ATPase contains one copy per enzyme complex of subunits OSCP, b, and d. The presence of one copy per complex of the OSCP, b′ (or b), d, and F_6 proteins in the F_1•stalk and F_1F_o-ATPase complexes, respectively, was confirmed by quantitative sequencing.

The F_1 globular domain and the F_o membrane domain of the F_1F_o-ATPase complexes in bacteria, chloroplasts, and mitochondria are linked by a slender stalk 40−50 Å in length (Fernández-Moran, 1962; Kagawa & Racker, 1962; for reviews see Senior, 1988; Fillingame, 1990, 1992; Penefsky & Cross, 1991; Walker & Collinson, 1994). The F_1 domain contains the three catalytic sites of the enzyme, where ADP is phosphorylated using energy provided by the transmembrane proton motive force. It is thought that one function of the stalk is to couple the biochemical functions of F_o and F_1 by transmitting conformational changes produced by proton transport through F_o, to the catalytic sites in F_1 (Fillingame, 1990) and that an α-helical structure in the γ-subunit provides a key element in the transmission of these conformational changes to the catalytic sites (Abrahams et al., 1994). This α-helical structure is placed centrally in the F_1 domain, and lies on a 6-fold axis of pseudosymmetry, around which the three β-subunits, each containing a catalytic site, are arranged alternately with the three α-subunits. The α-helical structure also protrudes beyond the spherical body of F_1 forming a short stem, and in the intact F_1F_o-ATPase, it is probably part of the stalk. The stalk in the bacterial and chloroplast F_1F_o-ATPases probably also contains the stem region of the γ-subunit, the F_1 subunit ϵ (Wilkens et al., 1995), and possibly the δ-subunit, together with the extrinsic membrane domains of two subunits that are bound to the energy transducing membrane by hydrophobic N-terminal regions, and so are usually considered to be part of F_o. These F_o subunits are two identical b subunits in the *Escherichia coli* enzyme (Walker et al., 1982a; Fillingame, 1990; Dunn, 1992) and are the nonidentical subunits II and IV in the chloroplast assembly (Fromme et al., 1987; Herrmann et al., 1993). In the bovine mitochondrial enzyme, which has more subunits than the bacterial and chloroplast enzymes, the stalk appears to be even more complicated. The γ, OSCP, F_6, b, and d subunits, and possibly also subunits δ and ϵ, all may contribute to its structure (Walker & Collinson, 1994). However, the participation of the δ- and ϵ-subunits in the bovine stalk is uncertain, as a subcomplex consisting of one copy of each protein does not interact *in vitro* with another stable subcomplex known as "stalk" (Orriss et al., 1996), assembled *in vitro* from bacterially expressed bovine proteins OSCP, F_6, d, and b′, a truncated form of subunit b lacking its hydrophobic N-terminal domain. The stalk subcomplex binds to bovine F_1-ATPase *via* OSCP to form a stable stoichiometric F_1•stalk complex (Collinson et al., 1994a). This description of the stalk domains is complicated further by a confusing subunit nomenclature: the bovine proteins OSCP and δ are equivalent to bacterial and chloroplast subunits δ and ϵ, respectively, and there is no bacterial or chloroplast equivalent of the mitochondrial ϵ-subunit (Walker et al., 1982b).

The number of copies of the various subunits forming the stalk domain in intact bovine F_1F_o-ATPase is controversial. By measurement of the radioactivity incorporated into cysteine residues of purified ATPase by S-carboxymethylation, the molar ratio $\alpha_3\gamma_1b_1OSCP_1d_1$ was determined, and the stoichiometry $\alpha_3\gamma_1b_1OSCP_1d_1$ was found in the F_1•stalk complex (Collinson et al., 1994a). The ratios of b:d in

* Author to whom correspondence should be sent. Telephone, 010-44-1223-402239; FAX, 010-44-1223-412178; e-mail, walker@mrc-lmb.cam.ac.uk.

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purified F_o, and of b′:d:OSCP in the stalk, determined by the same procedure were 1:1, and 1:1:1, respectively (Collinson et al., 1994a). Natural bovine F_6 has no cysteine residues, and therefore its stoichiometry could not be determined in these experiments. Based on estimations by an immunological method, Hekman et al. (1991) proposed the molar ratio of 2:1:1:2 for b:OSCP:d:F_6. Lippe et al. (1988) have also proposed that there are 2 mol of subunit b/mol of F_1, and Penin et al. (1985) have suggested that there are 2 mol of OSCP/mol of F_1 in the porcine enzyme. Therefore, in order to try and resolve these controversies, we have determined the molar ratios of these subunits in pure bovine F_1F_o-ATPase, and in the *in vitro* assembled stalk and F_1•stalk complexes. These results strongly support the view that there is one copy of each of subunits OSCP, b, d, and F_6 in all three of these bovine complexes.

MATERIALS AND METHODS

Materials. Iodo-2-[^{14}C]acetic acid (specific activity 56 mCi/mmol; 297 μCi/mg) and ethyl [1^{14}C]acetimidate (specific activity 27 mCi/mmol) were obtained from Amersham Radiochemicals (Amersham, U.K.). Q-Sepharose HP, S-Sepharose HP, Sephacryl S-100 HR, Sephacryl S-300 HR, Superdex 200, Mono-Q HR, and Mono-S HR columns were purchased from Pharmacia Biotech Ltd. (Milton Keynes, U.K.). Spectra-Por dialysis membranes with molecular weight cutoffs of 2 and 3.5 kDa were acquired from Spectrum Medical Industries (Houston, TX). Molecular weight markers for SDS−PAGE[1] were purchased from BDH Laboratory Supplies (Poole, U.K.). F_1F_o-ATPase and F_1-ATPase were purified by established procedures (Buchanan & Walker, 1996; Lutter et al., 1993). The enzymic properties of this preparation of the F_1F_o-ATPase complex have been extensively characterized after reconstitution into unilamellar phospholipid vesicles. The ATP hydrolase activity of the reconstituted enzyme (70 U/mg) is the highest yet reported. It couples ATP hydrolysis to proton pumping and, when co-reconstituted with bacteriorhodopsin, is capable of light driven ATP synthesis. These activities can be almost entirely abolished by oligomycin. Therefore, the enzyme preparation is not only highly pure, but it is functionally intact (Groth & Walker, 1996).

Analytical Methods. Protein concentrations were determined either with bicinchoninic acid (Pierce Chemicals, Rockford, IL; Smith et al., 1985) or by the Bio-Rad assay (Bio-Rad Laboratories GmbH, Münich, Germany; Bradford, 1976). Amino-terminal sequences of proteins were determined with an Applied Biosystems Procise Model 494 protein sequencer. Molecular masses of proteins were measured by ESI-MS with a Perkin Elmer-Sciex API III$^+$ instrument. Denaturing polyacrylamide gels, containing a 12−22% (w/v) acrylamide gradient separating gel and 4% (w/v) stacking gel [acrylamide:bis(acrylamide), 30:0.8 w/w] prepared in minigel format (10 cm × 10 cm × 0.6 mm), were run in the buffer system of either Laemmli (1970) or Schägger and von Jagow (1987). Proteins were detected by staining with 0.2% (w/v) PAGE blue 83 dye dissolved in 50% aqueous methanol containing 10% acetic acid, and by destaining in 10% aqueous methanol containing 10% acetic acid. The positions on the gels of S-carboxymethylated or N^{ϵ}-acetimidated subunits were confirmed by transfer of the proteins from the gel onto pvdf membranes and N-terminal sequence analysis. Subunit d which has an N^{α}-acetyl group was identified by Western blotting with a subunit specific rabbit antiserum.

Bacterial Overexpression and Purification of the Components of the Stalk. Subunits OSCP, F_6, d, and fragment b′ (amino acids 79−214) of the bovine F_1F_o-ATPase were produced by heterologous overexpression in *E. coli* and purified as described before (Collinson et al., 1994a). A mutant of F_6 containing the amino acid substitution Glu_3-Cys was also overexpressed in *E. coli*. It was soluble in the bacterial cytoplasm. The yields of pure OSCP and F_6 and of inclusion bodies containing subunits b′, and d were 80, 25, 260, and 70 mg/L of bacterial cells, respectively. Subunit F_6 and the mutant form were chromatographed on DEAE-Sepharose (Collinson et al., 1994a), concentrated by ammonium sulfate precipitation, and then passed through a column of Sephacryl S-100 (60 cm × 2.6 cm i.d) in a buffer consisting of 50 mM potassium phosphate, pH 7.5, 0.5 M NaCl, 1 mM EDTA, and 0.001% PMSF.

The b′ and d subunits were purified as the b′•d complex as follows. Inclusion bodies containing subunits d (82 mg) and b′ (77 mg) were dissolved together in a solution (20 mL) of 6 M guanidine hydrochloride and 100 mM Tris-HCl (pH 8.0) and diluted with 25 mL of stalk buffer, consisting of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM sucrose, 1 mM EDTA, 0.001% (w/v) PMSF, 8 mM dithiothreitol, 0.02% (w/v) sodium azide, and 10% (v/v) glycerol. When it became apparent that sucrose gave rise to spurious covalent modifications of the OSCP, b′, and d (see Results section), it was omitted from this buffer. The solution was dialyzed three times against stalk buffer (0.5 L) in a 3.5 kDa molecular weight cutoff membrane. A precipitate was removed by centrifugation. The soluble protein was precipitated with ammonium sulfate, redissolved in the same buffer, and applied at 4 °C to a column of Sephacryl S-100 HR (60 cm × 2.6 cm i.d.) equilibrated in stalk buffer. Fractions that contained the b′•d complex were pooled and dialyzed twice against stalk buffer lacking NaCl (500 mL). Then the complex was applied to a column of Q-Sepharose HP (10 cm × 2.6 cm i.d.) equilibrated in stalk buffer lacking NaCl. It eluted at 100 mM NaCl on a linear gradient from 0 to 400 mM NaCl (total volume 600 mL) with a recovery of 15 mg. The N-terminal sequences of pure OSCP and F_6 (and the mutant), and of b′ and d, were verified by automated Edman degradation.

Purification of the Stalk Complex. The stalk was assembled in the stalk buffer (2 mL) from purified b′•d OSCP, and F_6 (1.3, 2.4, and 1.0 mg/mL, respectively, in the molar ratios 1:3:3). The solution was left on ice for 15 min, and then the stalk complex was separated from excess OSCP and F_6 on a column of Sephacryl S-100 HR (60 cm × 2.6 cm i.d.) equilibrated in stalk buffer. A stalk complex containing the mutated form of F_6 (Glu_3-Cys) was prepared by the same procedure. It was stable and formed a stable complex with F_1-ATPase (see below).

Purification of the F_1•Stalk Complex. The stalk complex (1 mg), or the form containing mutated F_6, was added to F_1-ATPase (2.8 mg) in "F_1•stalk" buffer (1 mL) consisting of 20 mM Tris-HCl (pH 7.5), 50 mM sucrose (later omitted),

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; ESI-MS, electrospray ionization mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; AMP-PNP, adenylyl imidodiphosphate; pvdf, poly(vinylidene difluoride).

100 mM NaCl, 2 mM magnesium sulfate, 1 mM EDTA, 0.001% (w/v) PMSF, 8 mM dithiothreitol, 0.02% (w/v) sodium azide, and 10% (v/v) glycerol, to which had been added 1 mM AMP-PNP and 20 μM ADP. The mixture was kept at 25 °C for 15 min and was then applied to a column of Sephacryl S-300 HR (60 cm × 1.6 cm i.d.), equilibrated at room temperature in F_1•stalk buffer. The complex was also assembled on a larger scale (10−50 mg) by mixing the stalk and F_1-ATPase in 5−10 mL of buffer and purified on a column of Sephacryl S-300 HR (60 cm × 2.6 cm i.d.).

S-Carboxymethylation of Cysteines. The bovine stalk complex (OSCP•b′•F_6•d), assembled with F_6 containing the Glu_3-Cys mutation, was reacted with iodo-2-[^{14}C]acetic acid under denaturing conditions. Guanidine hydrochloride (6 M in 0.1 M Tris-HCl, pH 8.0) and dithiothreitol (2-fold molar excess over thiols) were added to the samples (0.5 or 1 mg), to give a final volume of about 2 mL. The reaction vessel was flushed with argon and kept for 90 min at room temperature. Then iodo-2-[^{14}C]acetic acid was added in 10-fold molar excess over total thiols. The reaction vessel was flushed with argon again, and the reaction mixture was kept in the dark at room temperature for a further 90 min. Then the solution was dialyzed three times against 1% (v/v) acetic acid (4 L) in a 2 kDa molecular weight cutoff membrane. The samples were lyophilized, redissolved in 7% (w/v) SDS, and fractionated by SDS−PAGE. The gel was dried, and the positions of radioactively labeled proteins were detected by exposure to a storage phosphor screen for 18 h. The intensities of bands were measured with a phosphor imager (Molecular Dynamics Ltd., Kemsing, U.K.). A sample of pure F_1F_o-ATPase was S-carboxymethlated in a similar way. In order to verify that the S-carboxymethylation of cysteine containing subunits was complete, the subunits were fractionated by reverse phase HPLC, and the molecular masses of the peaks were examined by ESI-MS. In the cases of subunits d, ϵ, and f masses corresponding to fully reacted species were observed, and none corresponding to underreacted subunits.

Acetimidation of Amino Groups. Either 0.5 or 1 mg of the F_1F_o-ATPase, F_1•stalk, and stalk (all containing wild-type F_6) were dissolved in 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 and reacted exhaustively with an 8-fold molar excess over amino groups of ethyl-[1-^{14}C]acetimidate (Orriss et al., 1996). The subunits were separated by SDS−PAGE, and the radioactivity incorporated into the proteins was measured as described above. The subunits in samples of the acetimidated stalk complex and of acetimidated F_1F_o-ATPase were separated by reverse phase HPLC. Their molecular masses were measured by ESI-MS, and their N-terminal sequences were determined by automated Edman degradation.

Quantitative N-Terminal Sequence Analysis of Proteins. Samples were fractionated by SDS−PAGE, and transferred to pvdf membranes with a semidry blotting apparatus (Milliblot Graphite Electroblotter II from Millipore U.K. Ltd., Watford WD1 8YW, U.K.). Transfer of proteins onto a membrane in contact with the gel was carried out by electrophoresis for 40 min with a current of 2.5 mA cm^{-2} of gel. During protein transfer, a second membrane was placed between the electrode and the first membrane that was in direct contact with the gel. The membranes were stained with 0.2% PAGE blue 83 dye dissolved in 50% aqueous methanol containing 1% acetic acid, destained in 50% methanol, rinsed with water, and dried in air. All of the transferred protein was on the first membrane, and no protein was ever detected on the second one. After completion of protein transfer, the polyacrylamide gel was also stained with PAGE blue 83 dye, but no residual proteins were detected. Stained bands were excised from the first membrane, and amino acids 1−20 of each of the proteins were sequenced. From the yields of phenylthiohydantoin amino acids released at each cycle, the initial yield, which is a measure of the total protein in the band, was determined by extrapolation to cycle zero.

RESULTS AND DISCUSSION

Subunit Stoichiometries Estimated by S-Carboxymethylation and by N-Acetimidation. In order to determine the molar ratios of subunits by acetimidate labeling of amino groups or by carboxymethylation of cysteines, it is essential to react all available cysteine or lysine residues as completely as possible. The completeness of carboxymethylation of cysteines and its specificity for cysteine residues under the experimental conditions used have been established in the past with a wide variety of proteins [for example, see: Walker et al. (1980)]. In the present work, complete S-carboxymethylation was demonstrated in subunits d, ϵ, and f in the F_1F_o-ATPase complex (see Materials and Methods). It is reasonable to assume that other cysteine containing subunits in the same sample were also completely S-carboxymethylated. The completeness of N-acetimidation of the δ- and ϵ-subunits of bovine F_1-ATPase has also been investigated by ESI-MS, and under the experimental conditions employed, which are the same as those used in the present work, reaction with N^{ϵ}-amino groups was shown to be complete, and the N^{α}-amino groups were found to have not reacted (Orriss et al., 1996). The extent of N-acetimidation was investigated by ESI-MS in the acetimidated stalk complex and in the F_1F_o-ATPase complex. In both complexes the mass of acetimidated subunit F_6, which has nine lysine residues per chain, corresponded to the complete incorporation of nine acetimidate moieties. By protein sequencing it was shown that the α-amino group of F_6 had not reacted with the reagent, nor had the α-amino groups in subunits OSCP, b′, and d. Additionally, in the F_1F_o complex complete acetimidation of lysine residues was demonstrated in subunits F_6, e, d A6L, and g. It is a reasonable assumption that the lysines in other subunits in the same sample had also reacted completely with ethyl acetimidate.

In the stalk complex the masses of the predominant species in the ESI-mass spectra of acetimidated subunits OSCP, b′, and d corresponded to complete N^{ϵ}-acetimidation of each chain, plus masses of 160 and 320 in each chain. The individual recombinant OSCP, b′, and d subunits had masses corresponding exactly to their sequences, as reported before (Collinson et al., 1994a), and the additions of 160 and 320 mass units to OSCP, b′, and d occurred during *in vitro* assembly of the stalk. These mass differences correspond to the addition of 1 and 2 hexoses, and they were eliminated by carrying out the assembly of the stalk in the absence of sucrose. As species with these additional masses were not associated with F_6, which lacks cysteines, it is likely that the hexose additions arose *via* the single cysteine residues found in OSCP, b′, and d. It should be noted, first, that no such modifications were found in the F_1 or F_1F_o-ATPase

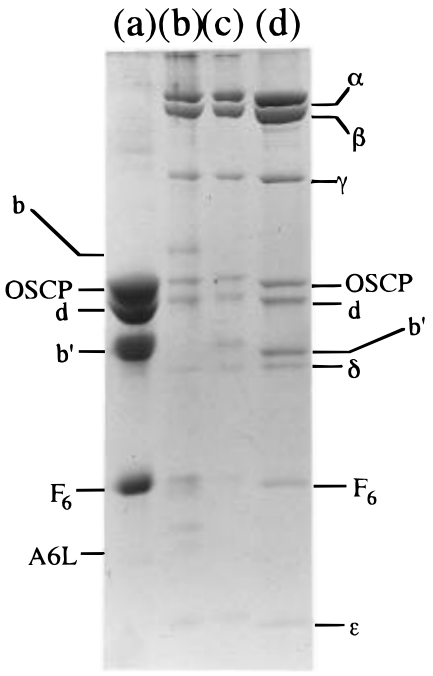

FIGURE 1: Analysis of the covalently modified bovine F_1F_o-ATPase, F_1•stalk and stalk complexes. The complexes were analyzed by SDS−PAGE (Schägger & von Jagow 1987) and stained with PAGE blue 83 dye. Lanes (a), (b), and (c), the [^{14}C]acetimidated stalk, F_1F_o-ATPase, and F_1•stalk complexes, respectively; lane (d), the S-[^{14}C]carboxymethylated F_1•stalk complex containing F_6 with the Glu_3-Cys mutation.

complexes; second, that the presence of such modifications in the acetimidated subunits of F_1•stalk and stalk complexes does not affect the stoichiometry determinations based on the incorporation of radioactivity from ethyl-[1-^{14}C]acetimidate into lysines; and third, that the slight under-reaction of lysines in OSCP, b′, and d does not affect the calculated molar ratios significantly. For the determination of molar ratios of subunits OSCP, b′, and d in the stalk complex by S-carboxymethylation (see below), the complex was assembled and purified in the absence of sucrose.

Samples of the acetimidated stalk, F_1•stalk, and F_1F_o-ATPase complexes, and a sample of the S-carboxymethylated F_1•stalk complex containing F_6 with the Glu_3-Cys mutation were analyzed by SDS−PAGE in the buffer system of Schägger and von Jagow (1987). All of the expected subunits were present in each complex, and in the stalk and F_1•stalk complexes they were resolved from each other completely [Figure 1, parts (a), (c), and (d)]. In the F_1F_o-ATPase complex, several subunits in the vicinity of F_6 were indistinct and poorly resolved [Figure 1(b)].

The introduction of a carboxymethyl or an acetimidate moiety into a protein increases its mass by 58 or 41, respectively, and as there are more lysines than cysteines in each of the proteins being investigated, N-acetimidation of a subunit caused it to migrate more slowly than S-carboxymethylation [compare subunits γ, OSCP, d, b′, F_6, and ϵ in Figure 1 (b), (c), and (d)]. The F_1•stalk complexes containing the wild-type and mutated F_6 subunits containing one additional cysteine residue per F_6 were indistinguishable by SDS−PAGE.

The four subunits of both the S-carboxymethylated and the acetimidated bovine stalk complex were separated in the Schägger and von Jagow gel system [see Figure 2, lanes (b) and (d)], but in the Laemmli system the OSCP and d subunits

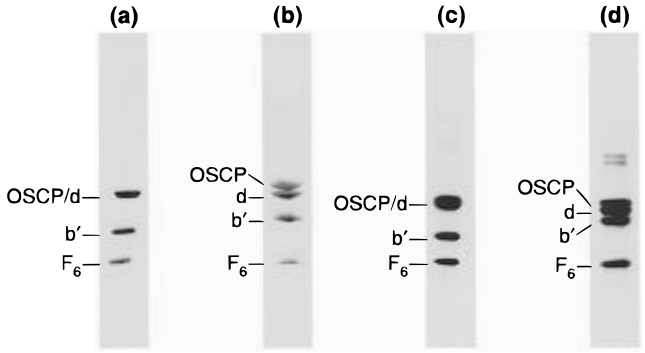

FIGURE 2: Incorporation of radioactivity into the bovine stalk complex by covalent modification with iodo[2-^{14}C]acetic acid and ethyl[1-^{14}C]acetimidate. The subunits were separated by SDS−PAGE in the buffer systems of Laemmli (1970) in lanes (a) and (c), and of Schägger and von Jagow (1987) in lanes (b) and (d). The incorporated radioactivity was recorded with a phosphor imager. For quantitation of incorporated radioactivity, see Table 1. Lanes (a) and (b), the stalk complex containing subunit F_6 with the Glu_3-Cys mutation modified with iodo[2-^{14}C]acetic acid. Lanes (c) and (d), stalk complex modified with ethyl[1-^{14}C]acetimidate.

Table 1: Determination of the Molar Ratios of Subunits of Bovine Stalk Complex by Carboxymethylation and Acetimidation[a]

	carboxymethylation				acetimidation			
	lane (a)		lane (b)		lane (c)		lane (d)	
subunit	radio-activity	ratio	radio-activity	ratio	radio-activity	ratio	radio-activity	ratio
OSCP	4983[b]	1.0	1195	1.1	59 493[b]	0.9	17 575	0.9
d	4983[b]	1.0	1194	1.1	59 493[b]	0.9	20 253	1.1
b′	5213	1.0	1071	1.0	51 392	1.1	13 083	1.0
F_6	3274	0.7	489.9	0.5	30 230	1.0	8 413	1.0

[a] Radioactivity incorporated into the bands in lanes (a)−(d) in Figure 2 was measured in arbitrary units with a phosphor imager (see Materials and Methods). The stalk samples were prepared in the absence of sucrose. [b] the values are 50% of the radioactivity in the unresolved OSCP/d band.

were not resolved [see Figure 2, lanes (a) and (c)]. As the OSCP and d each contains a single cysteine residue, and their lysine contents are each 20 residues per molecule, respectively, half of the the radioactive contents of the bands containing the unresolved subunits in the Laemmli gels was attributed to each subunit. Then these values were used in the calculations of molar ratios [see Table 1, lanes (a) and (c)]. The molar ratios calculated from these experiments and from those experiments where the four constituent subunits were completely resolved [Table 1, lanes (b) and (d)] indicate the presence of equal numbers of subunits OSCP, d, b′, and F_6 in each stalk complex.

All of the subunits of the acetimidated F_1•stalk complex were resolved in the the Schägger and von Jagow gel system, but not in the Laemmli system where, as in the stalk complex, subunits OSCP and d were not resolved (Figure 3). The molar ratios of the stalk subunits in this complex again indicated the presence of equal numbers of subunits OSCP, b′, d, and F_6 in the complex, and the values of incorporated radioactivity in the F_1 subunits with the known stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ strongly suggest that there is one copy of each of subunits OSCP, b′, d, and F_6 in the F_1•stalk complex (Table 2).

In the acetimidated bovine F_1F_o-ATPase complex, subunits OSCP, d, and b were completely resolved from all other subunits in both gel systems (see Figure 4), but subunit F_6

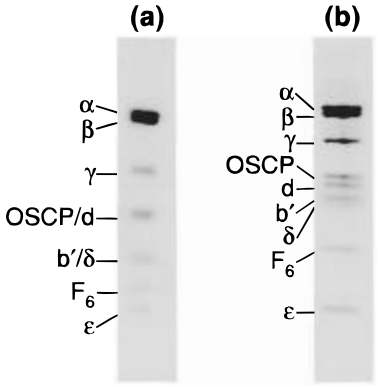

FIGURE 3: Incorporation of radioactivity into the bovine F_1·stalk complex by reaction with ethyl[1-^{14}C]acetimidate. The sample contained the natural F_6 sequence. The subunits were fractionated by SDS−PAGE in the buffer system of either Laemmli (1970), in lane (a), or Schägger and von Jagow (1987), in lane (b). The incorporated radioactivity was recorded with a phosphor imager. The positions of the radioactively acetimidated component subunits are shown at the sides. For quantitation of the incorporated radioactivity, see Table 2.

Table 2: Determination of the Molar Ratios of Subunits of the Bovine F_1·Stalk Complex by Acetimidation[a]

	lane (a)		lane (b)	
subunit	radioactivity	ratio	radioactivity	ratio
α	3490	3.5	6014	3.9
β	2683	3.7	3779	3.5
γ	802.1	1.0	1241	1.0
δ	104[b]	0.8	146.9	0.7
ϵ	260.8	1.0	373.0	0.9
OSCP	507[b]	0.8	992	1.0
b′	364[b]	0.8	611.1	0.9
d	507[b]	0.8	937.8	0.9
F_6	254.1	0.9	408.6	0.9

[a] Radioactivity incorporated into the bands in lanes (a) and (b) in Figure 3 was measured in arbitrary units with a phosphor imager (see Materials and Methods). [b] In the unresolved *δ*/b′ and OSCP/d bands the total radioactivity was distributed between the two components in each band in proportion to their lysine contents [see Figure 3, lane (a)].

could not be separated from other small subunits with similar apparent molecular weights (subunits e, f, g, A6L, and the ATPase inhibitor protein). Therefore, no attempt was made in this experiment to determine the molar ratio of F_6 relative to other stalk subunits OSCP, b, and d, all of which appear to be present in the complex in equal numbers (Table 3). The ratios determined for the F_1 subunits in F_1F_o-ATPase suggest that there is one copy of each of subunits OSCP, b, and d in the complex (Table 3).

Quantitation of Subunits of the Bovine Stalk, F_1·Stalk, and F_1F_o-ATPase Complexes by Protein Sequence Analysis. The measurement of the quantities of each subunit in samples of each complex by protein sequencing offers another independent method of measuring the molar ratios of the components of the complexes. The method depends on separating the subunits by SDS−PAGE and on transferring them quantitatively to a pvdf membrane. Care was taken to ensure that each protein in the three complexes was transferred completely from the gel to the membrane and that the proteins did not pass through this membrane. So that the reproducibility of the values could be assessed, the proteins were sequenced at least three times, and in some cases four times (see Table 4).

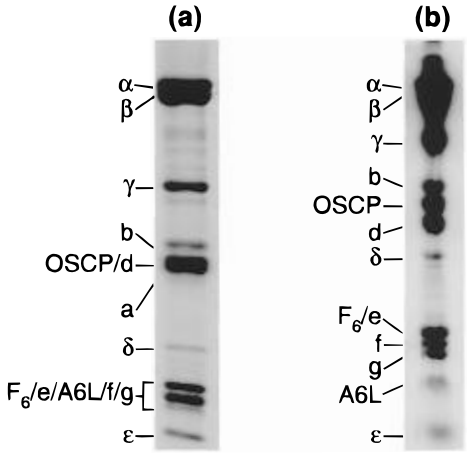

FIGURE 4: Incorporation of radioactivity into the bovine F_1F_o-ATPase complex by covalent modification with reaction with ethyl-[1-^{14}C]acetimidate. The subunits were fractionated by SDS−PAGE in the buffer system of either Laemmli (1970), in lane (a), or Schägger and von Jagow (1987), in lane (b). The incorporated radioactivity was recorded with a phosphor imager. The positions of some of the radioactively labelled component subunits are shown at the sides. For quantitation of incorporated radioactivity, see Table 3.

Table 3: Determination of the Molar Ratios of Some Subunits of Bovine F_1F_o-ATPase by Acetimidation[a]

	lane (a)		lane (b)	
subunit	radioactivity	ratio	radioactivity	ratio
α	6247	3.0	2903	3.0
β	4924	3.3	2415	3.5
γ	1567	0.9	700.8	0.9
δ	180.1	0.7	74.4	0.6
ϵ	529.6	1.0	168.7	0.7
b	794.6	0.6	362.5	0.6
OSCP	1730	1.3	702.6	1.1
d	1586	1.2	689.7	1.1
F_6	nd		nd	

[a] Radioactivity incorporated into the bands in lanes (a) and (b) in Figure 4 was measured in arbitrary units with a phosphor imager (see Materials and Methods). nd, not determined because subunit F_6 was not resolved from other subunits with similar apparent molecular weights.

These sequencing experiments suggest the presence of equal numbers of subunits OSCP, b, d, and F_6 in the stalk complex, and those conducted with the F_1·stalk complex indicate that there is one copy of each of these subunits in this complex (Table 4). Similar conclusions can be drawn from the quantitative sequencing experiments conducted with the F_1F_o-ATPase complex, although the value of 0.6 determined for the molar ratio for subunit F_6 is rather low. That determined for subunit *ϵ* was similarly lower than the expected value of unity. It may be that either these low molecular weight subunits were incompletely captured during transfer onto the pvdf membrane, despite the precautions that were taken to avoid such losses, or that in the isolated F_1F_o-ATPase complex their α-amino groups are partially modified.

The Stoichiometry of Subunits in the Stalk Region of the Bovine F_1F_o-ATPase Complex. The S-carboxymethylation experiments conducted with the stalk complex reconstituted with a form of F_6 containing the Glu_3-Cys mutation are an extension of earlier S-carboxymethylation studies on the stalk and F_o complexes containing the natural cysteine-less F_6 (Collinson et al., 1994a). The earlier experiments demonstrated that the stalk and F_o complexes contain equal numbers

Table 4: Determination of Molar Ratios of Some Subunits in the Stalk, F_1•Stalk, and F_1F_o-ATPase by Protein Sequencing[a]

	stalk[b]	F_1•stalk[b]	F_1F_o-ATPase[c]
subunit	ratio (SD)	ratio (SD)	ratio (SD)
γ	np	1.0(−)	1.0(−)
OSCP	1.0(−)	0.8 (0.1)	1.0 (0.1)
d	1.3 (0.3)	1.2 (0.05)	nd
b	np	np	0.9 (0.1)
b′	1.3 (0.5)	0.8 (0.2)	np
δ	np	1.2 (0.4)	1.0 (0.1)
F_6	1.3 (0.5)	0.9 (0.1)	0.6 (0.1)
ϵ	np	0.6 (0.1)	0.6 (0.1)

[a] The initial yields of various subunits in the stalk, F_1•stalk, and F_1F_o-ATPase complexes were determined by automated Edman degradation (see Materials and Methods). The molar ratios in each complex were calculated from these values by assuming the presence of one OSCP in the stalk complex and one γ-subunit in the F_1•stalk and F_1F_o-ATPase complexes. [b] Mean of three experiments. [c] Mean of four experiments: SD, standard deviation; np, subunit not present in the complex; nd, not determined because natural subunit d has an N^{α}-acetyl group, whereas the recombinant subunit d in the stalk and F_1•stalk complexes does not.

of subunits OSCP, b′, and d, and of b and d, respectively. The present studies show that there are equal numbers of each of subunits OSCP, b′, d, and F_6 in the stalk complex (Table 1). Similar studies of the F_1•stalk and F_1F_o complexes supported the view that both complexes contained one copy of each of the OSCP, b′ (or b), and d subunits (Collinson et al., 1994a). The experiment with F_1F_o-ATPase has been repeated in the present work using a sample in which completeness of S-carboxymethlation of cysteine residues had been established (see above). The stoichiometries of stalk subunits in this sample of the complex were the same as reported before (data not shown; Collinson et al., 1994a). The N^{ϵ}-acetimidation experiments conducted with the stalk, F_1•stalk, and F_1F_o-ATPase (Tables 1−3), and quantitative N-terminal sequencing experiments with the same complexes (Table 4) provided independent corroboration of these conclusions. The conclusions agree with earlier proposals which suggested that the mitochondrial F_1F_o-ATPase contains one copy of subunit d (Hekman et al., 1991) and one copy of the OSCP (Liang & Fisher, 1983; Dupuis et al., 1985a; Dupuis & Vignais, 1987; Hekman et al., 1991), and they disagree with other proposals of two or more copies per complex of OSCP (Hundal & Ernster, 1979; Penin et al., 1985), of b (Hekman et al., 1991; Lippe et al., 1988; Belogrudov et al., 1995), and of F_6 (Hekman et al., 1991).

Implications for the Structure of F_1F_o-ATPases. The *E. coli* enzyme is a complex of eight different polypeptides and is an example of the simplest known F_1F_o-ATPases. The enzymes from photosynthetic bacteria and chloroplasts have 9 subunits, and the bovine heart mitochondrial enzyme is a complex of 16 different proteins (Walker et al., 1990; Collinson et al., 1994b). The sequences of the subunits of F_1F_o-ATPases from various species contain many extensive similarities, and for example, homologues of the α, β, γ, δ, and ϵ subunits that form the F_1 catalytic domain of the *E. coli* enzyme are also in the chloroplast and mitochondrial enzymes. Subunits a, b, and c, the three remaining components of *E. coli* F_1F_o-ATPases, make the F_o membrane sector. Subunits a and c are both hydrophobic proteins that are essential for transmembrane proton transport (Schneider & Altendorf, 1985), and homologues of both of them are also present in the chloroplast and mitochondrial F_1F_o-ATPases (Walker et al., 1990). Subunit b provides an important connection between the F_1 and F_o domains, and there are two identical and closely associated b subunits per *E. coli* complex (Foster & Fillingame, 1982; Dunn, 1992). Each has a hydrophobic N-terminal region, probably folded into a single transmembrane α-helix, followed by a highly charged region of about 120 amino acids outside the lipid bilayer (Walker et al., 1982a).

The b subunit is among the least conserved parts of the F_1F_o-ATPases, and none of the enzymes from photosynthetic bacteria, chloroplasts, and mitochondria contain subunits that are significantly related in sequence to the *E. coli* subunit b. Instead, the enzymes in photosynthetic bacteria and chloroplasts each have two different subunits (called b and b′, and II and IV, respectively), with an overall distribution of hydrophobic and charged residues that is similar to that of *E. coli* b (Cozens & Walker, 1987; van Walraven et al., 1993; Fromme et al., 1987; Herrmann et al., 1993). For this reason, they are thought to be the structural and functional equivalents of the two b subunits in the stalk of the *E. coli* enzyme. It is assumed that one of each of these subunits is found in the photosynthetic bacterial or chloroplast complex. In the bovine mitochondrial F_1F_o-ATPase, the topology of its b subunit resembles that of the bacterial b subunit more closely than those of any of the other subunits in the complex. Like the bacterial b subunit, the bovine b subunit is also anchored in the membrane by a hydrophobic region at its N-terminus, but the latter probably makes two transmembrane spans instead of the single span in bacterial b (Walker et al., 1987). A second similarity with the bacterial b subunit is that the remainder of the sequence of bovine b is highly charged and lies outside the lipid bilayer, where it interacts with F_1 *via* the OSCP (Walker & Collinson, 1994). For these reasons, the bovine b probably fulfils a similar role to that of *E. coli* b. However, the present work has demonstrated that there is only one b subunit per bovine complex, and previous work has shown that the hydrophilic subunits d and F_6 also bind to OSCP and b and contribute to the stalk complex (Collinson et al., 1994a). Therefore, the precise relationships between the bacterial and chloropast stalk subunits on the one hand, and the bovine subunits on the other, are rather unclear.

The sequences of the stalk subunits suggest that α-helical coiled-coils are likely to be an important feature of the various stalk domains. The computer programs COILS (Lupas et al., 1991) and PAIRCOIL (Berger et al., 1995) both predict that amino acids 38−116 in the *E. coli* subunit b have a high probability of forming an α-helical coiled-coil, and the sequence from 38−73 has a 97% probability of doing so. This feature is likely to be the basis of the interaction between the two b subunits, and because both are held in the membrane by their N-terminal regions, the α-helices in this coiled-coil must be parallel. The equivalent regions of the b and b′ subunits in the photosynthetic bacteria and subunits II and IV in chloroplasts have similar tendencies to form coiled-coils. The program COILS, but not PAIRCOIL, predicts that bovine subunits b, d, and OSCP all contain regions that have a high probability of making coiled-coils. Therefore, the interactions between some of the subunits in the stalk region of the bovine enzyme also probably depend on making coiled-coils. By reconstitution experiments it has been demonstrated that the F_1•OSCP interacts and makes a stable complex with b′, but not with d or F_6, and that the regions of b that interact with subunits

d and F_6 are contained within residues 120−166 and 120−214, respectively (Collinson et al., 1994a). At present, it is not known whether the coiled-coils that are proposed to form in the bovine stalk subunits would be parallel or antiparallel. Therefore, the way in which the subunits in the bovine stalk together provide a structure with similar properties to the stalk regions in the bacterial and chloroplast F_1F_o-ATPases remains as an unresolved question that will probably be resolved eventually by structural analysis.

There are indications that the subunits of the stalk region of the F_1F_o-ATPases may not all interact in a single structural unit involved directly in the transmission of energy from F_o to F_1. The γ and bacterial ϵ/bovine δ subunits do seem to be involved in this mechanism, as discussed above, but the bovine stalk complex does not form a stable complex *in vitro* with the bovine $\delta\epsilon$ subcomplex (Orriss et al., 1996), and the bovine b, OSCP, d, and F_6 subunits have been chemically cross-linked to the α- and/or β-subunits, but not to the γ- or δ-subunits (Dupuis et al., 1985b; Belogrudov et al., 1995). The OSCP appears to form the main interaction between the bovine stalk complex and the F_1 sector, and proteolysis experiments suggest that the interaction involves the N-terminal domain of the α-subunits (Hundal et al., 1983), and similar conclusions were reached earlier with the α- and δ-subunits in the *E. coli* enzyme (Dunn et al., 1980). Therefore, the region of interaction between the stalk and F_1 may extend from the region between F_o and F_1, around the external surface of the F_1 particle to the top, where the N-terminal regions of the α-subunits are thought to be (Abrahams et al., 1994). Reconstitution experiments with subunits of F_1-ATPase from bacterium PS3 also show that the bacterial δ-subunits form a stable complex with the α- and β-subunits (Yoshida et al., 1977). Therefore, the function of the "stalk" complex in the bovine enzyme, and of the equivalent parts of the bacterial and chloroplast enzymes, remains a matter of speculation, but one possibility is that it serves as a "stator" to counter the tendency of the $\alpha_3\beta_3$ complex to rotate in response to a rotation of the γ-subunit within $\alpha_3\beta_3$ during catalysis (Abrahams et al., 1994; Aggeler et al., 1995; Duncan et al., 1995; Sabbert et al., 1996).

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