Organization of Subunits in the Membrane Domain of the Bovine F-ATPase Revealed by Covalent Cross-linking*

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The F-ATPase in bovine mitochondria is a membrane-bound complex of about 30 subunits of 18 different kinds. Currently, ~85% of its structure is known. The enzyme has a membrane extrinsic catalytic domain, and a membrane intrinsic domain where the turning of the enzyme’s rotor is generated from the transmembrane proton-motive force. The domains are linked by central and peripheral stalks. The central stalk and a hydrophobic ring of c-subunits in the membrane domain constitute the enzyme’s rotor. The external surface of the catalytic domain and membrane subunit a are linked by the peripheral stalk, holding them static relative to the rotor. The membrane domain contains six additional subunits named ATP8, e, f, g, DAPIT (diabetes-associated protein in insulin-sensitive tissues), and 6.8PL (6.8-kDa proteolipid), each with a single predicted transmembrane helix, but their orientation and topography are unknown. Mutations in ATP8 uncouple the enzyme and interfere with its assembly, but its role and the roles of the other five subunits are largely unknown. We have reacted accessible amino groups in the enzyme with cross-linking agents and identified the linked residues. Cross-links involving the supernumerary subunits, where the structures are not known, show that the C terminus of ATP8 extends about 70 Å from the membrane into the peripheral stalk and that the N termini of the other supernumerary subunits are on the same side of the membrane, probably in the mitochondrial matrix. These experiments contribute significantly toward building up a complete structural picture of the F-ATPase.

The F-ATPase from bovine mitochondria is a membrane-bound protein assembly of ~30 polypeptides of 18 different kinds with a combined molecular mass of ~650 kDa (1, 2). The enzyme has a membrane extrinsic globular F1-, catalytic domain that is attached to the membrane domain by central and peripheral stalks. The F1 domain is an assembly of three α-subunits and three β-subunits arranged in alternation in a spherical complex around an elongated α-helical structure in the γ-subunit (3), and the three catalytic sites of the enzyme lie at interfaces between the α- and β-subunits. The γ-subunit extends from the αβγ-spherical structure to the membrane domain, where it is augmented by the δ- and ε-subunits in the contact region or “foot” with the membrane domain. Together, the γ-, δ-, and ε-subunits constitute the central stalk, and the foot makes extensive contacts with a hydrophobic ring of eight c-subunits in the membrane domain of the enzyme (4). The cγ-ring and the central stalk constitute the rotor of the enzyme. Their rotation as an ensemble at ~100 Hz carries energy into the catalytic sites of the enzyme, and the turning of the rotor brings about conformational changes in the three catalytic sites in the F1 domain that lead to the binding of substrates and the formation of ATP and its release into the matrix. The region of contact between the rotating c-ring and subunit a, another membrane protein of unknown structure, provides the transmembrane pathway for protons to pass from the intermembrane space into the mitochondrial matrix (5), allowing energy from the transmembrane proton-motive force generated by respiration to drive the turning of the rotor. Subunit a forms part of the stator and is in contact with the membrane domain of subunit b, which extends through the peripheral stalk, an elongated subcomplex of single copies of subunits b, d, Fα, and oscp (6–9) to the “top” of the αβγ3 domain (10). Thus, the stator consists of the αβγ3 domain, plus subunits oscp, b, d, Fα, and a, and it remains static relative to the turning of the rotor. Atomic resolution structures have been established for the F1 c-ring (4) and peripheral stalk domains (7, 8), and a mosaic structure of the enzyme has been built within the envelope of a low resolution structure of the entire complex determined by electron cryo-microscopy (11).

Also associated with the membrane domain of the bovine enzyme are six small proteins, ATP8, e, f, g, DAPIT3 (diabetes-associated protein in insulin-sensitive tissues; 6.8PL, 6.8 kDa proteolipid; DSS, disuccinimidyl suberate; 3 The abbreviations used are: DAPIT, diabetes-associated protein in insulin-sensitive tissues; 6.8PL, 6.8 kDa proteolipid; DSS, disuccinimidyl suberate;
associated protein in insulin sensitive tissues), and 6.8 kDa proteolipid (6.8PL), with molecular masses in the range 6.3–11.4 kDa (1, 2, 12–14). Each has a single predicted transmembrane α-helix, and based largely on their staining intensities in gel analyses of the subunit composition of the enzyme, it is assumed that there is one copy of each protein per F-ATPase complex, but there are no definitive quantitative data to support this assumption. These proteins are usually referred to as the “supernumerary” subunits as there are no orthologs in bacterial F-ATPases, and they appear not to be involved directly in the synthesis of ATP. In the yeast enzyme the orthologs of subunits e and g are associated with the formation of dimers of the F-ATPase (15, 16), and they probably play the same role in the mammalian enzyme.

In this study the identification of covalent cross-links introduced into the subunits of the bovine F-ATPase by reaction of exposed amino groups with isotopically labeled bifunctional reagents has been employed as a means of gaining information about the orientation and location of the supernumerary subunits within the bovine enzyme complex.

**Experimental Procedures**

**Analytical Methods**—Protein concentrations were estimated by the bicinchoninic acid assay (17). Protein compositions were analyzed by SDS-PAGE in 12–22% or 4–20% polyacrylamide gradient gels or by blue native PAGE (18, 19). Proteins were analyzed by SDS-PAGE in 12–22% or 4–20% polyacrylamide (21) except that the detergent in the buffers, ose-neopentyl glycol. The ATP hydrolytic activity of the purified bovine F-ATPase, measured by an enzyme-coupled assay (20), was assayed against buffer consisting of 20 mM HEPES, pH 7.3, 0.05% n-dodecyl-β-D-maltose-neopentyl glycol (w/v), 10% glycerol (v/v), 50 mM sodium chloride, 1 mM tris(2-carboxyethyl)phosphine, 2 mM magnesium sulfate, 2 mM ATP, and DSSG(d/d), and DSSG(d/d), (Creative Molecules), the numbers of deuterium atoms, δ, in the two acyl arms of each reagent are indicated by the subscripted numerals and were supplied and used at molar ratios of the two isotopic forms of 1:1 (22, 23). Freshly purified bovine F-ATPase was diluted to a concentration of 1.22 mg/ml in buffer consisting of 20 mM HEPES, pH 7.3, 0.05% n-dodecyl-β-D-maltose-neopentyl glycol (w/v), 10% glycerol (v/v), 50 mM sodium chloride, 1 mM tris(2-carboxyethyl)phosphine, 2 mM magnesium sulfate, 2 mM ATP, 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (100 μg/ml), 1-palmitoyl-2-oleoyl-sn-glycero-phosphoethanolamine (33 μg/ml), and 1-palmitoyl-2-oleoyl-sn-glycero-phospho-(1′-rac-glycerol (33 μg/ml). The reaction conditions were optimized with DSS(d/d) dissolved in dimethyl sulfoxide added at concentrations of 0.1–5 mM. The reactions were carried out at pH values of 6.8, 7.3, and 8.0 for 2, 4, or 20 h and at temperatures of 20 or 37 °C. The optimal cross-linking conditions were 2 μM F-ATPase (1.16 mg/ml) at pH 8.0 reacted with 2 mM DSS(d/d) for 2 h at 20 °C. BS3(d/d), DSG(d/d), and DSSG(d/d) were reacted with the enzyme at the same concentration and under the same conditions. The reactions were terminated by the addition of ammonium bicarbonate to a final concentration of 200 mM. The solutions were kept at 20 °C for 30 min. Insoluble material was removed by centrifugation (10,000 × g, 5 min, 20 °C), and the supernatants were analyzed by SDS-PAGE and blue native-PAGE. The cross-linked F-ATPases were precipitated with ethanol for 18 h at −20 °C, and the precipitates were recovered by centrifugation and redissolved in 6 M guanidinium hydrochloride in 20 mM Tris-HCl buffer. The proteins were reduced with tris(2-carboxyethyl)phosphine, alkylated with iodoacetamide (10 mM), and re-precipitated. These samples of S-alkylated cross-linked F-ATPases were analyzed in three different ways. First, the precipitates were dissolved in 100 mM ammonium bicarbonate and digested at 37 °C for 16 h with trypsin (trypsin:protein, 1:100, w/w). Portions of the digests were diluted with 0.1% formic acid for mass spectrometric analysis. Second, other samples of S-alkylated and cross-linked F-ATPase were fractioned by SDS-PAGE, and gel bands or gel regions were excised and digested with trypsin (24). Third, an S-alkylated sample of F-ATPase cross-linked with DSS(d/d) was dialyzed for 18 h against 100 mM ammonium bicarbonate and digested at 37 °C with trypsin (F-ATPase: trypsin, 100:1, w/w). The digest was dried and fractionated on a column of PolySULFOethyl A (50 mm long × 1.0 mm inner diameter; 300 Å pore size; PolyLC, Columbia, MD) equilibrated at a flow rate of 50 μl/min in 10 mM potassium phosphate buffer, pH 2.7, containing 10% acetonitrile. Peptides were eluted with a gradient of 0–1 M potassium chloride in 20 mM potassium phosphate, pH 3.0, containing 10% acetonitrile (see Fig. 1). Fractions were dried and dissolved in 0.3% trifluoroacetic acid, and salts were removed from the samples by passage through a reverse-phase C18 ZipTip (Millipore).

**Membrane Subunits of Bovine F-ATPase**

**Chemical Cross-linking**—Four deuterium-labeled cross-linking agents were employed. Disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG) are membrane-permeable, whereas di(sulfosuccinimidyld) glutarate (DSSG) and bi(sulfosuccinimidyl) suberate (BS3) are membrane impermeable. In the isotope-labeled versions, DSS(d/d), BS3(d/d), DSG(d/d), and DSSG(d/d), (Creative Molecules), the numbers of deuterium atoms, δ, in the two acyl arms of each reagent are indicated by the subscripted numerals and were supplied and used at molar ratios of the two isotopic forms of 1:1 (22, 23). Freshly purified bovine F-ATPase was diluted to a concentration of 1.22 mg/ml in buffer consisting of 20 mM HEPES, pH 7.3, 0.05% n-dodecyl-β-D-maltose-neopentyl glycol (w/v), 10% glycerol (v/v), 50 mM sodium chloride, 1 mM tris(2-carboxyethyl)phosphine, 2 mM magnesium sulfate, 2 mM ATP, 1-palmitoyl-2-oleoyl-sn-glycero-phosphocholine (100 μg/ml), 1-palmitoyl-2-oleoyl-sn-glycero-phosphoethanolamine (33 μg/ml), and 1-palmitoyl-2-oleoyl-sn-glycero-phospho-(1′-rac-glycerol (33 μg/ml). The reaction conditions were optimized with DSS(d/d) dissolved in dimethyl sulfoxide added at concentrations of 0.1–5 mM. The reactions were carried out at pH values of 6.8, 7.3, and 8.0 for 2, 4, or 20 h and at temperatures of 20 or 37 °C. The optimal cross-linking conditions were 2 μM F-ATPase (1.16 mg/ml) at pH 8.0 reacted with 2 mM DSS(d/d) for 2 h at 20 °C. BS3(d/d), DSG(d/d), and DSSG(d/d) were reacted with the enzyme at the same concentration and under the same conditions. The reactions were terminated by the addition of ammonium bicarbonate to a final concentration of 200 mM. The solutions were kept at 20 °C for 30 min. Insoluble material was removed by centrifugation (10,000 × g, 5 min, 20 °C), and the supernatants were analyzed by SDS-PAGE and blue native-PAGE. The cross-linked F-ATPases were precipitated with ethanol for 18 h at −20 °C, and the precipitates were recovered by centrifugation and redissolved in 6 M guanidinium hydrochloride in 20 mM Tris-HCl buffer. The proteins were reduced with tris(2-carboxyethyl)phosphine, alkylated with iodoacetamide (10 mM), and re-precipitated. These samples of S-alkylated cross-linked F-ATPases were analyzed in three different ways. First, the precipitates were dissolved in 100 mM ammonium bicarbonate and digested at 37 °C for 16 h with trypsin (trypsin:protein, 1:100, w/w). Portions of the digests were diluted with 0.1% formic acid for mass spectrometric analysis. Second, other samples of S-alkylated and cross-linked F-ATPase were fractioned by SDS-PAGE, and gel bands or gel regions were excised and digested with trypsin (24). Third, an S-alkylated sample of F-ATPase cross-linked with DSS(d/d) was dialyzed for 18 h against 100 mM ammonium bicarbonate and digested at 37 °C with trypsin (F-ATPase: trypsin, 100:1, w/w). The digest was dried and fractionated on a column of PolySULFOethyl A (50 mm long × 1.0 mm inner diameter; 300 Å pore size; PolyLC, Columbia, MD) equilibrated at a flow rate of 50 μl/min in 10 mM potassium phosphate buffer, pH 2.7, containing 10% acetonitrile. Peptides were eluted with a gradient of 0–1 M potassium chloride in 20 mM potassium phosphate, pH 3.0, containing 10% acetonitrile (see Fig. 1). Fractions were dried and dissolved in 0.3% trifluoroacetic acid, and salts were removed from the samples by passage through a reverse-phase C18 ZipTip (Millipore).
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Analysis of Cross-linked Peptides by Mass Spectrometry—

Tryptic digests of protein bands from SDS-PAGE gels were analyzed in a MALDI-TOF-TOF mass spectrometer (Model 4800, ABSciex, Warrington, WA1 1RX, UK) with α-cyano-4-hydroxycinnamic acid as matrix. Peptides were fragmented by collision-induced dissociation with air at a collision energy of 1 kV. Trypsin digests of cross-linked F-ATPases (~400 fmol), and fractions of peptides from similar digests that had been obtained by cation exchange chromatography were injected into a nano-scale C18 reverse phase column. The eluate was introduced directly into the nanospray interface of a LTQ Orbitrap XL ETD mass spectrometer (Thermo-Fisher Scientific, Hemel Hempstead, UK), operated in data-dependent acquisition mode. Up to 10 of the most abundant precursor ions of known charge states, but not singly charged, were selected and fragmented by collision-induced dissociation. The m/z values of precursor and fragment ions were measured simultaneously in the Orbitrap and ion-trap analyzers, respectively.

Data Analysis—Data files were processed with Proteome Discoverer (Thermo-Fisher Scientific). The coverage of the sequences of the subunits of the F-ATPase by the tryptic peptides was determined by comparison of the sequences of peptides with the mammalian subsection of the non-redundant database of the National Center for Biotechnology Information with MASCOT (25). "Mono-linked" peptides, where only one arm of the bifunctional cross-linking agent reacted with a protein amino group, were identified by comparison of the data with a FASTA database containing the sequences of the subunits of the enzyme. In these comparisons the light and heavy isotopes of the hydrolysis and aminolysis derivatives of mono-linked α-amino and ε-amino groups, oxidized methionines, and S-propionamido- and S-carbamidomethyl derivatives of cysteine were considered as possible modifications with the following parameters: peptide mass tolerance, ± 5 ppm; fragment mass tolerance, ± 0.5 Da; maximum missed cleavages, 2; peptide confidence, at least medium.

**Identification of Cross-linked and Loop-linked Peptides**—

Doublet peaks corresponding to the light and heavy isotopically labeled cross-linked peptides (including loop-linked peptides) were identified from their mass differences with Hekate (26). For the doublets for DSS(d9/d12) and BS3(d9/d12) the mass difference was 12.0741 ± 0.0375, and for DSG(d9/d6) and DSSG(d9/d6) it was 6.0370 ± 0.0375 Da. No limits were imposed on the intensity ratios of the doublet peaks. To be assigned as an isotopic doublet, peptide ions containing light and heavy isotopes were required to elute within 30 s of each other. The mass threshold for matching the masses of the light and heavy isotope peak of cross-linked peptides with their calculated theoretical values was 2 ppm. The accuracy threshold used in the annotation of fragment ions was ± 0.5 Da. The identified cross-links were ranked according to a modified version of Andromeda (26, 27) with fragment ion data from both the light- and heavy-labeled cross-linked peptides. Mass spectra from cross-linked and loop-linked peptides with a score of >400 were inspected manually. A cross-link was accepted as being significant if both cross-linked peptides had more than five unique fragment ions assigned to them or if the cross-linked peptide had three-five unique fragments ions assigned to one component and more than five to the other. Accepted loop links had three or more unique assigned fragments. Twenty mono-links and six loop links were detected (data not shown). Peptides were discarded when the majority of high intensity fragment ions were not annotated with a theoretical fragment ion from the associated peptide or when fewer than three fragment ions of a peptide component of a cross-link were detected.

**Localization of Cross-links in the Structure of the Bovine F-ATPase**—A structure of the bovine F1-c8-peripheral stalk complex was generated from the structures of the bovine F1-c8- ring (PDB code 2XND (4)), the F1-peripheral stalk (PDB code 2WSS (7)), and the peripheral stalk (PDB code 2CLY (8)), and the position of the peripheral stalk was adjusted according a low resolution structure of the intact complex (11). The distances between Ca atoms of lysine residues in the model of the bovine F1-c8-peripheral stalk complex were estimated with PyMOL and Coot (28, 29). For DSS(d9/d12) and BS3(d9/d12), the maximum permitted inter-Ca distance between connected lysines is 27.4 Å (the 11.4 Å spacer arm plus the length of two lysine side chains, each 6.5 Å, plus twice the coordinate error of 1.5 Å for mobile surface residues). For DSG(d9/d4) and DSSG(d9/d6) with spacer arms of 7.7 Å, the maximum inter-Ca distance is 23.7 Å.

**Analysis of Sequences of F-ATPases**—Sequences of subunits of F-ATPases were aligned with ClustalW (30). Transmembrane α-helices were predicted with HMMTOP (31), and the secondary structures of membrane extrinsic regions were predicted with PSIPRED (32).

**Results**

**Optimization of Reaction Conditions for Cross-linking the F-ATPase**—The concentrations of cross-linkers, and the pH, duration, and temperature of reaction were optimized by varying the conditions and by monitoring the effect of cross-linking on the F-ATPase by SDS-PAGE and blue native-PAGE. The
The number of cross-links identified in each experiment depended upon how the tryptic peptides were generated and fractionated. Fewer cross-linked pairs were identified in samples processed by SDS-PAGE and in-gel digestion than from other samples that were digested with trypsin and analyzed without any further fractionation, probably because of losses of cross-linked peptides in the former procedure during their extraction from gels and subsequent processing. However, there was considerable overlap between the sets of cross-linked peptides that were identified by these means. The most effective procedure was that applied to the tryptic digest of the F-ATPase modified with DSS(d10/d12), where cross-linked peptides were separated from unmodified, mono-linked, and loop-linked peptides by cation exchange chromatography at pH 2.7. At this pH value, cross-linked peptide pairs carry four positive charges (or more if histidine residues are present) and so they bind more strongly to a strong cation exchange resin than unmodified, mono-linked, and loop-linked peptides, which carry a minimum of two positive charges. Thus, the cross-linked peptides were recovered in a separate peak eluting after the less positively charged peptides (Fig. 1). Of the 53 cross-linked peptides identified in this peak, (Tables 1 and 2), 30 contained intrasubunit cross-links, and 23 had intersubunit cross-links. In other experiments where the cation exchange step was omitted, 28 peptides cross-linked with DSS were recovered, and 24, 14, and 17 peptides cross-linked with BS3, DSG, and DSSG were recovered, respectively. There was no significant difference between the number of cross-linked peptides recovered from the membrane subunits of the F-ATPase reacted with the membrane-permeable reagents, DSS and DSG, and with the membrane-impermeable reagents, BS3 and DSSG.

**Cross-links in the Membrane Extrinsic Region of the F-ATPase**—Forty-four cross-linked peptide pairs were identified as originating from membrane-extrinsic subunits of the enzyme. They are summarized in Table 1 together with the Ca distances between the cross-linked lysine residues measured in the structure of the bovine F-ATPase, and the corresponding mass spectrometry data are summarized in Table 2. The lengths of cross-linkers are given in the experimental section. In 38 of them both lysines are resolved in the structure, and the formation of the cross-links is compatible with the inter-Cα distances. For a 39th cross-link between αLys-455 and Lys-24 in subunit d, the inter-Cα distance of 32.3 Å is only slightly above the maximum value of 27.4 Å, and a minor conformational change in the determined structure of the enzyme would allow this cross-link to form. In two other cross-links between the F1 domain and the peripheral stalk, one of the reacted lysine residues is in a region that is locally unresolved in the structure, although adjacent structural elements are well defined. One such cross-link bridges between unresolved residue βLys-9 in the N-terminal region of the subunit and residue βLys-78, which is resolved in the six-stranded β-barrel that forms part of the “crown” of the F1 domain. The Ca distances between the resolved residue βThr-13, which is close to βLys-9 in the sequence of the subunit, and βLys-78 are 10 Å, 10 Å, and 10.2 Å in the βCα, βCβ, and βCγ subunits, respectively. Therefore, the formation of a cross-link bridging between βLys-9 and βLys-78 is compatible with the structure. The second cross-link involves the unresolved residue Lys-2 in subunit F6 and the resolved residue Lys-191 in subunit b. It is clear from the structure of the enzyme that residue Lys-2 in subunit F6 and residue Lys-191 in the C-terminal region of subunit b are in close proximity (the estimated distance is ~25 Å), and the formation of this cross-link is consistent with this conclusion.

**Characterization of Cross-linked Peptides**—The number of cross-links identified in each experiment depended upon how the tryptic peptides were generated and fractionated. Fewer cross-linked pairs were identified in samples processed by SDS-PAGE and in-gel digestion than from other samples that were digested with trypsin and analyzed without any further fractionation, probably because of losses of cross-linked peptides in the presence of BS3 and DSSG.
## Membrane Subunits of Bovine F-ATPase

### TABLE 1

| Subunits | Cross-linked sequences | Lys-Lys$^a$ | Ca-Ca$^a$ |
|----------|-----------------------|-------------|-----------|
| α-α      | VVDALGNATDOSOQIQGSK-STDYQKLVRG | 118–218$^b$ | 14.9      |
| α-α      | GPQIQGSK-DRGKaqi4iydydlsk | 124–262$^b$ | 17.6      |
| α-α      | VASQGGKIP-RKDSVQPAIYVTDQSDFKS | 172–218$^b$ | 25.1      |
| α-α      | FNDDTFDQ-DSAGRQDHYLSKL | 196–262$^b$ | 7.4       |
| α-α      | FNDDTFDQ-TKDSKINGESDAK | 196–488 | 19.3      |
| α-α      | ANPQAGNQ-MDQGQKEDSEDAK | 384–488$^b$ | 8.8       |
| α-α      | GLYKLEPK-LSKQITVSLNLAGFRE | 455–498$^b$ | 15.1      |
| α-α      | FENPLSHIVQHGQALLSKR-TGDKI3ESEDAK | 482–488$^b$ | 8.4       |
| α-β      | VVDALGNATDOSOQIQGSK-ITTTK | 118–304$^b$ | 16.8      |
| α-β      | RSTVVRK-VRTTTR | 218–304$^b$ | 15.0      |
| α-γ      | VGAAQTSRQKQVAGTMK-ALQDQITR | 384–4 | 39.1      |
| α-oscpr  | LIEKGDIVVR-YVIMAKYLV | 84–176 | 21.0      |
| α-b      | VVDALGNATDOSOQIQGSK-EVNR | 118–152 | 21.6      |
| α-d      | GLYKLEPK-NSQAVANSLK | 455–24 | 32.3      |
| β-β      | AAGAAPPSK-FRAGATTG-GRQVVLSDGAP | 9–78$^c$ | $^{b}$ |
| β-β      | VVDALGNATDOSOQIQGSK-ITTTK | 152–439 | 18.2      |
| β-β      | FLRQPQPQAVETPFGHQKLVPLKDZQ-ADLQHAEHHS | 430–476$^b$ | 18.5      |
| β-δ      | KIQOK-ANLQKQRESSLGAADATE | 413–114 | 57.3      |
| γ-γ      | XCLDQITR-SFNQITEQ | 4–14 | 15.3      |
| γ-γ      | SIFTQIR-ITSMK | 14–21$^e$ | 10.8      |
| γ-γ      | ADITKPEED-SEAANLAAGQKV | 58–101 | 14.3      |
| γ-ε      | MVAAKVR-ANAMKTGSGSKT | 30–36$^a$ | 14.4      |
| γ-ε      | ELCQGAR-ANAMKTGSGSKT | 39–36 | 16.3      |
| γ-ε      | THSDQFLTVFKEVOR-1V5KV | 129–46$^b$ | 10.5      |
| δ-ε      | ANLEKQLQGSKDELGADATR-YEQTCAMAR | 114–20$^c$ | 12.7      |
| δ-ε      | ANLEKQLQGSKDELGADATR-TEPKNAMK | 114–31$^c$ | 21.0      |
| δ-ε      | ANLEKQLQGSKDELGADATR-DALKTEPKNAMK | 144–27$^c$ | 18.3      |
| e-ε      | YSQICFARAV-TEPKNAMK | 20–31$^d$ | 13.0      |
| e-ε      | YSQICFARAV-ANAMKTGSGSKT | 20–36$^d$ | 13.8      |
| oscp-oscpr | VQQILKGPQ-SRPQK | 47–65 | 15.5      |
| oscp-oscpr | EPGQlsaLnnPyvk-UKsLSMTAK | 50–67 | 16.5      |
| oscp-oscpr | EPGQlsaLnnPyvk-SLSMTAK | 50–75$^c$ | 16.3      |
| oscp-oscpr | MAASLNNPYPK-VKSLSDMKTAK | 61–67$^c$ | 11.0      |
| b-b      | VQQISQAQASTIK-TCIADLKLSS | 191–202$^b$ | 16.3      |
| b-d      | QCDQDQSSQQALVQK-SACPFLQGQCTR | 112–188$^b$ | 11.8      |
| b-Fα-α  | VQQISQAQASTIK-NEKLDPQVR | 191–2 | $^{a}$ |
| b-Fα-α  | VQQISQAQASTIK-LFVDDKIR | 191–14 | 14.7      |
| d-d      | KLARK-LATLPEPQPAIYDNYVK | 4–47$^c$ | 12.9      |
| d-d      | KLARK-PNLKVPIDPEKD | 4–77 | 18.3      |
| d-d      | LTVGPSEKNPQARKYVNLKVPIDPEKD | 47–77 | 13.0      |
| d-d      | ANVQAGLVDVFDEK-FNLK | 62–72$^b$ | 16.0      |
| d-d      | AGLVDVFDEK-PNLKVPIDPEKD | 71–77 | 12.0      |
| d-d      | SCAPEFLQGQCTR-IQYVEKELK | 108–116 | 12.2      |
| d-Fα-α  | ANVQAGLVDVFDEK-LQMKYOK | 62–47 | 17.8      |

$^a$ Sequence numbers of cross-linked lysines. The hypheon separate the two peptides, and cross-linked residues are bold.

$^b$ Measured in Å in the structure of the bovine F$_{1}$--F$_{0}$ peripheral stalk complex.

$^c$ Cross-link also found in bovine F-ATPase modified with DSG(d$_{4}$d$_{4}$).

$^d$ Cross-link also found in bovine F-ATPase modified with BS$_{3}$(d$_{4}$d$_{4}$).

$^e$ Cross-link also found in bovine F-ATPase modified with DSG(d$_{4}$d$_{4}$).

$^f$ The N-terminal region of β-subunits is disordered.

$^g$ Residue 2 of subunit F$_{6}$ is not resolved.

Three other cross-links are incompatible with the structure of the monomeric bovine F-ATPase, and each was observed only once (see Table 1). One of them bridges between δLys-413 and δLys-114, whereas the Ca distance between the two lysine residues in the structure is 57.3 Å. This cross-link is likely to have formed between two different F-ATPase complexes, and the presence of a small amount of the oligomeric complex in the native gel of the modified complex (Fig. 2, lane j) is consistent with this suggestion. Another incompatible cross-link bridges between αLys-132 and αLys-196, two surface residues in the nucleotide binding domain of the subunit. Although the inter-Ca value given in Table 1 is apparently within the range of acceptable distances, this distance has been measured along the direct path between the two Ca-atoms, which is impeded by a loop involving residues 309–321 of the α-subunit, and the unimpeded Ca distance is considerably longer. The third structurally incompatible cross-link is between αLys-384 and γLys-4. The former residue is exposed at the entrances to catalytic interfaces between the α-subunits and the adjacent β-subunit, whereas the latter residue is in the N-terminal α-helix of the γ-subunit. This α-helix is one of the two α-helical elements in the central shaft of the rotor found in the aqueous cavity in the core of the F$_{1}$ domain. In one of the three catalytic α-β interfaces, between α$_{3p}$ and β$_{p}$, there is a direct unimpeded line of sight between the two residues, but the gap narrows to ~6 Å, and the inter-Ca distance is 38.4 Å. These last two structurally incompatible cross-links could only have formed by reaction with disrupted F-ATPase complexes. Despite these three aberrant cross-links, the overwhelming majority, 93%, of the observed linkages were compatible with the atomic structure of the F-ATPase. Their main value is that they provide reassurance that any cross-links observed in regions of the F-ATPase that are not represented in the current structural model are likely to have validity and that they represent contributions toward defining the organization of subunits in the unresolved region.
Cross-links in the Membrane Domain of the F-ATPase—Eight intersubunit and four intrasubunit cross-links were identified among the tryptic peptides from the F-ATPase that had been cross-linked with the four reagents. The majority of them were identified in the digest of the DSS cross-linked enzyme that was fractionated by ion exchange chromatography. They are summarized in Table 3, and the corresponding mass spectrometry data are summarized in Table 4.

Three of the intersubunit cross-links were between subunit ATP8 and components of the peripheral stalk domain. One of them links residue Lys-54 of subunit ATP8 to residue Lys-73 of the F6 subunit. The two others involve links from residue Lys-46 of ATP8, one to residue Lys-120 of subunit b and the other to residue Lys-24 of subunit d (Fig. 3).

Five of the intersubunit cross-links involved lysine residues in the five supernumerary subunits e, f, g, DAPIT, and 6.8PL (see Fig. 4). All fall in regions of the proteins that are predicted to be membrane extrinsic. Three involve links between residue Lys-49 in the C-terminal region of the 6.8PL subunit with lysine residues in the C-terminal regions of the DAPIT, e, and f subunits. Other cross-links bridge between the C-terminal regions of subunits e and f and the N-terminal regions of subunits f and g.
Discussion

Involvement of Subunit ATP8 in the Bovine Peripheral Stalk—Since its discovery as a subunit of the bovine and yeast F-ATPases (12, 33, 34), subunit ATP8 (also known as A6L in mammals and Aap1 in *Saccharomyces cerevisiae*) has remained a rather mysterious component of the enzyme complex. It is not found in eubacterial and chloroplast enzymes, and therefore, it was classified as a supernumerary subunit, apparently not required for the core ATP synthetic and hydrolytic functions of F-ATPases. The subunit is encoded in the mitochondrial DNA of many, but not all, eukaryotic species (35), and in mammals, the genes for ATP8 and ATPase-6 (or subunit a) overlap (12, 36). The bovine protein is probably folded into a single transmembrane α-helix from residues 8–29 followed by a hydrophilic extension up to its C terminus at residue 66 (Fig. 5A). This region from residue 30 to 66 is predicted to have an
FIGURE 5. Conservation of sequences of ATP8 subunits. Residues are colored as follows: pink, hydrophobic; orange, aromatic; blue, basic; red, acidic; green, hydrophilic; magenta, proline and glycine. Yellow denotes cysteine residues. A, comparison of the sequences of the bovine and yeast ATP8 subunits. The predicted secondary structure of the bovine protein is shown above the sequences. The arrow represents a short predicted β-strand. The regions before, between, and after the α-helices and β-sheet are predicted to be extended.

B, comparison of ATP8 subunits with known secondary structure. Several ATP8 subunits are shown containing transmembrane α-helices. In B and C, the orders represented by the species are shown on the right. B: HOMSA, Homo sapiens; BOSTA, B. taurus; EQUCA, Equus caballus (horse); CANFA, Canis lupus familiaris (dog); MUSMU, Mus musculus (mouse); ORYCA, Oryctolagus cuniculus (rabbit); PHAME, Phakopsora meibomiae (eastern black currant blight); YARLI, Yarrowia lipolytica (yeast); LECMU, Lecanicillium muscariae (fungus). The short horizontal arrow represents a short predicted β-strand.

C, conservation of sequences of subunit ATP8 in selected eukaryotic organisms. Sequences of selected eukaryotic organisms are aligned, and conservation is shown with shading. The sequences are divided into the five major groups of eukaryotic organisms: Ascomycota, Basidiomycota, Fungi, Chytridiomycota, and Zygomycota.

Extended conformation, except for residues 57–61, which may form a short β-sheet. The region consisting of residues 51–63 is well conserved in mammals (Fig. 5B). In contrast, subunit ATP8 in S. cerevisiae has a C-terminal extension that is predicted to be mostly α-helical. It is significantly shorter and poorly related to the same region of the mammalian proteins, although the sequences of ATP8 proteins are well conserved among the fungi (Fig. 5C). On the basis of cysteine scanning mutagenesis and reaction with fluorescein-5-maleimide, residues 1–14 of yeast ATP8 have been proposed to be exposed to the intermembrane space.
brane space followed by a transmembrane α-helix from residues 15–35 (37). A short β-strand is predicted around residue Arg-42, and this residue is conserved throughout fungi (Fig. 5C). Despite these differences, proteolytic digestion studies conducted on bovine mitochondrial membranes (38, 39) and cross-linking studies on the yeast enzyme (40) have shown that the bovine and yeast proteins have a common topography with their N-terminal regions in the intermembrane space and their C-terminal regions on the matrix side of the inner mitochondrial membrane.

In the current work new information about the location of bovine subunit ATP8 in the F-ATPase has come from an extensive study of covalent cross-linking conducted on the intact purified enzyme with bi-functional agents. Among the many
identified cross-links, residues Lys-46 and Lys-54 of ATP8 were found to be linked to three lysine residues, one in each of subunits b, d, and Fp in the peripheral stalk of the enzyme. These cross-links confirm that the C-terminal region of bovine ATP8 is exposed in the matrix of the organelle, and they demonstrate that this region of ATP8 is in the vicinity of the peripheral stalk. The shorter and poorly related C-terminal region of yeastsubunit ATP8 has been proposed to interact with subunits b and d in the yeast enzyme (37). Two of the cross-links in the bovine enzyme involve the structurally defined residues Lys-120 and Lys-24 of subunits b and d, respectively, both of which are found ~60 Å from the membrane domain of the enzyme. Therefore, residues Lys-46 and Lys-54 of ATP8 are nearby (see Fig. 3), and the C-terminal region of bovine subunit ATP8 extends ~60–70 Å upwards from the membrane domain, probably along the axis of the peripheral stalk defined by the long α-helix in subunit b. The third cross-link is between residues Lys-54 in ATP8 and residue Lys-73 in subunit Fp (Fig. 3). Bovine subunit Fp is 76 amino acids long, and in the peripheral stalk, it is folded into two α-helices from residues 4–25 and 33–51 linked by an extended loop region from residues 26–32. Residue 51 is followed by an extended region, which is resolved up to residue Glu-69. The cross-linked residue Lys-73 of Fp is nearby and is estimated to be ~70 Å from the surface of the membrane domain of the enzyme, in accordance with the position deduced from the structurally defined lysine residues in subunits b and d. The only known human pathogenic mutation specific to subunit ATP8 is associated with infantile cardiomyopathy, and it leads to the truncation of the subunit at residue 54 and impairment of ATP synthesis (41). Therefore, in mammalian mitochondrial enzymes subunit ATP8 appears to have a fundamental role in either the synthesis of ATP by mitochondrial F-ATPases or the assembly of the complex or both. If the C-terminal region of subunit ATP8 is, as proposed, an intrinsic drial F-ATPases or the assembly of the complex or both. If the subunit is required for the formation of dimers of the enzyme in the mitochondrial cristae (15, 45, 46), and they are oriented with their N-terminal regions in the mitochondrial matrix and their C-terminal regions in the intermembrane space of the organelle (37, 47). A GXXGG sequence motif in residues 14–18 of the yeast e-subunit forms a homodimeric α-helical coiled-coil involved in holding two F-ATPase complexes together (45). However, the motif is not conserved in the bovine protein. Deletion of either subunit e or subunit f leads to severe disruption of the structure of the mitochondrial cristae, but neither subunit is required for the formation of an enzymatically active complex (15, 48). Like subunits e and g, subunit f is oriented with its N- and C-terminal regions in the mitochondrial matrix and the intermembrane space, respectively (37, 47, 49), but in contrast to subunits e and g, deletion of subunit f disrupted both the assembly and the activity of the complex (44). In addition to subunits ATP8, e, f, and g, the membrane domain of the bovine enzyme also contains two additional subunits, DAPIT and 6.8PL (14, 50), that have been identified also in the human enzyme (51). They are less tightly associated with the bovine complex than the other supernumerary subunits, and they require the presence of phospholipids to remain bound to the complex (14). Subunit DAPIT is encoded only in the genomes of metazoans (Fig. 6), and subunit 6.8PL is restricted to vertebrates (Fig. 7), and so neither subunit is a component of the yeast enzyme. Both have a single predicted transmembrane α-helix (Fig. 4). Conversely, the membrane domains of the yeast F-ATPase additionally contain subunits j (also called i) and k (15, 52, 53), and a recently discovered sub-

**Membrane Subunits of Bovine F-ATPase**

Organized by Supernumerary Subunits—The presence of the supernumerary subunits e, f, and g in the domains of F-ATPases was demonstrated first in the bovine enzyme (2, 13) and subsequently in the enzyme from *S. cerevisiae* (15, 44). All three subunits are predicted to contain a single transmembrane α-helix (see Fig. 4), and the sequences of the bovine and yeast e, f, and g subunits are conserved or conservatively substituted in 26%, 34%, and 46% of their residues, respectively. The predicted secondary structures of the bovine and yeast orthologs are similar but not entirely congruent. Since their discovery, the roles of the yeast subunits have been studied much more extensively than their bovine orthologs. Yeast subunits e and g are involved in the formation of dimers of the enzyme in the mitochondrial cristae (15, 45, 46), and they are oriented with their N-terminal regions in the mitochondrial matrix and their C-terminal regions in the intermembrane space of the organelle (37, 47). A GXXG sequence motif in residues 14–18 of the yeast e-subunit forms a homodimeric α-helical coiled-coil involved in holding two F-ATPase complexes together (45). However, the motif is not conserved in the bovine protein. Deletion of either subunit e or subunit f leads to severe disruption of the structure of the mitochondrial cristae, but neither subunit is required for the formation of an enzymatically active complex (15, 48). Like subunits e and g, subunit f is oriented with its N- and C-terminal regions in the mitochondrial matrix and the intermembrane space, respectively (37, 47, 49), but in contrast to subunits e and g, deletion of subunit f disrupted both the assembly and the activity of the complex (44). In addition to subunits ATP8, e, f, and g, the membrane domain of the bovine enzyme also contains two additional subunits, DAPIT and 6.8PL (14, 50), that have been identified also in the human enzyme (51). They are less tightly associated with the bovine complex than the other supernumerary subunits, and they require the presence of phospholipids to remain bound to the complex (14). Subunit DAPIT is encoded only in the genomes of metazoans (Fig. 6), and subunit 6.8PL is restricted to vertebrates (Fig. 7), and so neither subunit is a component of the yeast enzyme. Both have a single predicted transmembrane α-helix (Fig. 4). Conversely, the membrane domains of the yeast F-ATPase additionally contain subunits j (also called i) and k (15, 52, 53), and a recently discovered sub-
unit l, a homologue of subunit k (54), that are unrelated in sequence to any of the bovine subunits and are not encoded in metazoan genomes. Again, each of subunits j, k, and l has a single predicted transmembrane helical span.

The present studies have revealed a network of cross-links between exposed lysine residues in the N- and C-terminal regions of bovine subunits e, f, g, DAPIT, and 6.8PL (see Fig. 4). Residue Lys-49 in the C-terminal region of 6.8PL is linked to the C-terminal regions of subunits DAPIT, e, and f via cross-links to residues Lys-55, Lys-54, and Lys-79, respectively, and the C-terminal regions of subunits e and f are linked via a cross-link from Lys-47 in subunit e to Lys-85 in subunit f. Finally, the N-terminal regions of subunit f and g are cross-linked from Lys-15 in subunit f to Lys-65 in subunit g. This network of cross-links shows that the N-terminal regions of all five proteins are on the same side of the lipid bilayer in the intact F-ATPase and that, conversely, all of their C-terminal regions are located on the opposite side of the membrane. Although there is as yet no direct information about the orientations of the subunits in the bovine inner mitochondrial membrane, it is reasonable to assume that subunits e, f, and g have the same orientations as their yeast orthologs. In view of the cross-links between

### FIGURE 7. Conservation of the sequences of the 6.8PL subunit in vertebrate F-ATPases.

For details of the color scheme, see the legend to Fig. 5.

| Species | Nomenclature |
|---------|--------------|
| H. sapiens | HOMSA |
| B. taurus | BOSTA |
| P. troglodytes | PANTR |
| R. norvegicus | RATNO |
| M. musculus | MUSMU |
| P. alecto | PTEAL |
| S. scrofa | ORYCU |
| M. domestica | MONDE |
| O. anatinus | ORNA |
| C. livia | TURR |
| S. araneus | GALGA |
| R. norvegicus | ANAPL |
| P. troglodytes | NPIPT |
| S. scrofa | SOUR |
| M. domestica | SOUR |
| O. anatinus | ORNH |
| S. scrofa | MELU |
| R. norvegicus | OPIHO |
| S. scrofa | LEBI |
| M. domestica | STRAU |
| P. alecto | SNBSN |
| S. scrofa | PELS1 |
| M. domestica | ANOSA |
| M. domestica | ALLMT |
| A. platyrhynchos | XENRT |
| A. lucius | XIPMA |
| P. procyon | KURAK |
| P. troglodytes | FUNNE |
| S. aurata | ESOLU |
| M. trachurus | ASTME |
| R. norvegicus | MATZE |
| M. domestica | DAZER |
| M. domestica | STEPA |
| M. niloticus | ORENI |
| M. domestica | ORENI |
| M. domestica | LATCH |

Lys-47 in subunit e to Lys-85 in subunit f. Finally, the N-terminal regions of subunit f and g are cross-linked from Lys-15 in subunit f to Lys-65 in subunit g. This network of cross-links shows that the N-terminal regions of all five proteins are on the same side of the lipid bilayer in the intact F-ATPase and that, conversely, all of their C-terminal regions are located on the opposite side of the membrane. Although there is as yet no direct information about the orientations of the subunits in the bovine inner mitochondrial membrane, it is reasonable to assume that subunits e, f, and g have the same orientations as their yeast orthologs. In view of the cross-links between the
C-terminal regions of the 6.8PL, DAPIT, and e subunits, it seems likely that 6.8PL and DAPIT also will be oriented in a similar fashion, as depicted in Fig. 4.

These studies on the supernumerary subunits of the bovine F-ATPase have little or no apparent relevance to the interpretation of the structure of the dimeric F-ATPase from the mitochondridia of the alga *Polytomella* sp. at 6.5 Å resolution (55). It is known that the F-ATPase from this species and from the related alga *Chlamydomonas reinhardtii* contains the catalytic core subunits α, β, γ, δ, ε, ospc, a, and c plus nine "atypical" subunits (56). Atypical subunits Asa2, Asa4, and Asa7 are thought to be components of the peripheral stalk (57), which is much stouter and more elaborate than the peripheral stalk in the bovine and fungal F-ATPases. The stoichiometry of none of the atypical algal subunits is known, and there is no evident sequence relationship to any of the much more extensively studied peripheral stalk subunits in the bovine enzyme especially (6–10), and there is no relationship either of atypical subunits with any of the supernumerary subunits described here, including ATP8. Indeed, it is not clear whether or not the algal enzymes contain an ATP8 subunit (58). The information in this paper is much more likely to be helpful in establishing relatively high resolution structures of the bovine and fungal enzymes.

References

1. Walker, J. E. (2013) The ATP synthase: the understood, the uncertain, and the unknown. *Biochem. Soc. Trans.* 41, 1–16
2. Walker, J. E., Lutter, R., Dupuis, A., and Runswick, M. J. (1991) Identification of the subunits of F$_{0}$F$_{1}$-ATPase from bovine heart mitochondridia. *Biochemistry* 30, 5369–5378
3. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Structure at 2.8 Å resolution of F$_{1}$-ATPase from bovine heart mitochondridia. *Nature* 370, 621–628
4. Watt, I. N., Montgomery, M. G., Runswick, M. J., Leslie, A. G. W., and Walker, J. E. (2010) Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16823–16827
5. Cherepanov, D. A., Dulkidjianian, A. Y., and Junge, W. (1999) Transient accumulation of elastic energy in proton translocating ATP synthase. *FEBS Lett.* 449, 1–6
6. Collinson, I. R., van Raaij, M. J., Runswick, M. J., Fearnley, I. M., Skehel, J. M., Orriss, G. L., Miroux, B., and Walker, J. E. (1994) ATP synthase from bovine heart mitochondria: in vitro assembly of a stalk complex in the presence of F$_{0}$-ATPase and in its absence. *J. Mol. Biol.* 242, 408–421
7. Rees, D. M., Leslie, A. G. W., and Walker, J. E. (2009) The structure of the membrane extrinsic region of bovine ATP synthase. *Proc. Natl. Acad. Sci. U.S.A.* 106, 21597–21601
8. Dickson, V. K., Silvestre, J. A., Fearnley, I. M., Leslie, A. G. W., and Walker, J. E. (2006) On the structure of the stator of the mitochondrial ATP synthase. *EMBO J.* 25, 2911–2918
9. Walker, J. E., and Dickson, V. K. (2006) The peripheral stalk of the mitochondrial ATP synthase. *Biochim. Biophys. Acta* 1757, 286–296
10. Carbia, M. R., Kellas, F. A., Yang, J.-C., Runswick, M. J., Montgomery, M. G., Walker, J. E., and Neubauer, D. (2007) How the N-terminal domain of the OSCP subunit of bovine F$_{0}$-ATP synthase interacts with the N-terminal region of an α subunit. *J. Mol. Biol.* 368, 310–318
11. Baker, L. A., Watt, I. N., Runswick, M. J., Walker, J. E., and Rubinstein, J. L. (2012) Arrangement of subunits in intact mammalian mitochondrial ATP synthase determined by cryo-EM. *Proc. Natl. Acad. Sci. U.S.A.* 109, 11675–11680
12. Fearnley, I. M., and Walker, J. E. (1986) Two overlapping genes in bovine mitochondrial DNA encode membrane components of ATP synthase. *EMBO J.* 5, 2003–2008
13. Collinson, I. R., Runswick, M. J., Buchanan, S. K., Fearnley, I. M., Skehel, J. M., van Raaij, M. J., Griffiths, D. E., and Walker, J. E. (1994) Membrane domain of ATP synthase from bovine heart mitochondria: purification, subunit composition, and reconstitution with F$_{1}$-ATPase. *Biochemistry* 33, 7971–7978
14. Chen, R., Runswick, M. J., Carroll, J., Fearnley, I. M., and Walker, J. E. (2007) Association of two proteolipids of unknown function with ATP synthase from bovine heart mitochondridia. *FEBS Lett.* 581, 3145–3148
15. Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schägger, H. (1998) Yeast mitochondrial F$_{0}$F$_{1}$-ATP synthase exists as a dimer: identification of three dimer-specific subunits. *EMBO J.* 17, 2121–2130
16. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goede, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bichinchonic acid. *Anal. Biochem.* 150, 76–85
17. Macreadie, I. G., Novitski, C. E., Maxwell, R. J., John, U., Ooi, B. G., McKechnie, I. M., and Williams, A. (2004) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 331, 150–153
18. DeLano, W. L. (2007) "The PyMOL Molecular Graphics System, Version 1.5, Schrodinger, LLC, New York"
19. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 66, 486–501
20. Schmidt, A., Mueller, M., and Aebersold, R. (2008) Identification of cross-linked peptides from large sequence databases. *Proteomics* 8, 4089–4102
21. Buchan, D. W., Minneci, F., Nugent, T. C., Bryson, K., and Jones, D. T. (2013) "In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 8, 3435–3451
22. Holding, A. N., Lammers, M. H., Stephens, E., and Skehel, J. M. (2013) Hekate: software suite for the mass spectrometric analysis and three-dimensional visualization of cross-linked protein samples. *J. Proteome Res.* 12, 5923–5933
23. Cox, J., Neuhauers, N., Michalski, A., Scheltema, R. A., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 1, 2856–2860
24. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probabilistic-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20, 3551–3567
25. DeLano, W. L. (2010) "The PyMOL Molecular Graphics System, Version 1.5, Schrodinger, LLC, New York"
26. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. *Acta Crystallogr. D Biol. Crystallogr.* 66, 486–501
27. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) ClustalW and ClustalX version 2.0. *Bioinformatics* 23, 2947–2948
28. Tushima, N., and Simon, I. (2003) "HMMTOP transmembrane topology prediction server. *Bioinformatics* 19, 849–850
29. Buchan, D. W., Minneci, F., Nugent, T. C., Bryson, K., and Jones, D. T. (2013) Scalable web services for the PSIPRED Protein Analysis Workbench. *Nucleic Acids Res.* 41, W349– W357
30. Macreadie, I. G., Novitski, C. E., Maxwell, R. J., John, U., Ooi, B. G., McKechnie, I. M., and Williams, A. (2004) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 331, 150–153
31. Tusnády, G. E., and Simon, I. (2001) The HMMTOP transmembrane topology prediction server. *Bioinformatics* 17, 849–850
32. Buchanan, D. W., Hynke, N., Griesinger, C., and Grötzner, J. (1983) In-vivo characterization of the mitochondrial F$_{0}$F$_{1}$ ATP synthase subunit 8 in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 11, 4435–4461
Membrane Subunits of Bovine F-ATPase

34. Velours, J., Esparza, M., Hoppe, J., Sebald, W., and Guerin, B. (1984) Amino acid sequence of a new mitochondrialy synthesized proteolipid of the ATP synthase of Saccharomyces cerevisiae. EMBO J. 3, 207–212
35. Gissi, C., Iannielli, F., and Pesole, G. (2008) Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congenic species. Heredity 101, 301–320
36. Anderson, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. I., Staden, R., and Young, I. G. (1981) Sequence and organization of the human mitochondrial genome. Nature 290, 457–465
37. Stephens, A. N., Khan, M. A., Roucou, X., Nagley, P., and Devenish, R. J. (2003) The molecular neighborhood of subunit 8 of yeast mitochondrial F\textsubscript{1}F\textsubscript{0}-ATP synthase probed by cysteine scanning mutagenesis and chemical modification. J. Biol. Chem. 278, 17867–17875
38. Collinson, I. R., Fearnley, I. M., Skehel, J. M., Runswick, M. J., and Walker, J. E. (1994) ATP synthase from bovine heart mitochondria: identification by proteolysis of sites in F\textsubscript{0}, exposed by removal of F\textsubscript{1}, and the oligomycin-sensitivity confering protein. Biochem. J. 303, 639–645
39. Belogrudov, G. I., Tomich, J. M., and Hatefi, Y. (1996) Membrane topology and near-neighbor relationships of the mitochondrial ATP synthase subunits e, f, and g. J. Biol. Chem. 271, 20340–20345
40. Stephens, A. N., Roucou, X., Artika, I. M., Devenish, R. J., and Nagley, P. (2000) Topology and proximity relationships of yeast mitochondrial ATP synthase subunit 8 determined by unique introduced cysteine residues. Eur. J. Biochem. 267, 6443–6451
41. Jonckheere, A. I., Hogeveen, M., Nijtmans, L. G., van den Brand, M. A., Janssen, A. J., Diepstra, J. H., van den Brandt, F. C., van den Heuvel, L. P., Hol, F. A., Hofste, T. G., Kapusta, L., Dillmann, U., Shapmdeen, M. G., Smetink, J. A., and Rodenburg, R. J. (2008) A novel mitochondrial ATP8 gene mutation in a patient with apical hypertrophic cardiomyopathy and neuropathy. J. Med. Genet. 45, 129–133
42. Nagley, P., Farrell, L. B., Gearing, D. P., Nero, D., Meltzer, S., and Devenish, R. J. (1988) Assembly of functional proton-translocating ATPase complex in yeast mitochondria with cytoplasmically synthesized subunit 8, a polypeptide normally encoded within the organelle. Proc. Natl. Acad. Sci. U.S.A. 85, 2091–2095
43. Grasso, D. G., Nero, D., Law, R. H., Devenish, R. J., and Nagley, P. (1991) The C-terminal positively charged region of subunit 8 of yeast mitochondrial ATP synthase is required for efficient assembly of this subunit into the membrane F\textsubscript{0} sector. Eur. J. Biochem. 199, 203–209
44. Spannagel, C., Vaillier, J., Arselin, G., Graves, P. V., and Velours, J. (1997) The subunit f of mitochondrial yeast ATP synthase: characterization of the protein and disruption of the structural gene ATP17. Eur. J. Biochem. 247, 1111–1117
45. Arselin, G., Giraud, M. F., Dautant, A., Vaillier, J., Bréthes, D., Coulary-Salin, B., Schaeffer, J., and Velours, J. (2003) The GXXGX motif of the transmembrane domain of subunit e is involved in the dimerization/oligomerization of the yeast ATP synthase complex in the mitochondrial membrane. Eur. J. Biochem. 270, 1875–1884
46. Bustos, D. M., and Velours, J. (2005) The modification of the conserved GXXGX motif of the membrane-spanning segment of subunit g destabilizes the supramolecular species of yeast ATP synthase. J. Biol. Chem. 280, 29004–29010
47. Velours, J., Spannagel, C., Chaignepain, S., Vaillier, J., Arselin, G., Graves, P. V., Velours, G., and Camougrand, N. (1998) Topography of the yeast ATP synthase F\textsubscript{0} sector. Biochimie 80, 793–801
48. Arselin, G., Vaillier, J., Salin, B., Schaeffer, J., Giraud, M.-F., Dautant, A., Bréthes, D., and Velours, J. (2004) The modularization in subunits e and g amounts of yeast ATP synthase modifies mitochondrial cristae morphology. J. Biol. Chem. 279, 40392–40399
49. Roudeau, S., Spannagel, C., Vaillier, J., Arselin, G., Graves, P. V., and Velours, J. (1999) Subunit f of the yeast mitochondrial ATP synthase: topological and functional studies. J. Bioenerg. Biomembr. 31, 85–94
50. Meyer, B., Wittig, L., Trifilieff, E., Karas, M., and Schägger, H. (2007) Identification of two proteins associated with mammalian ATP synthase. Mol. Cell Proteomics 6, 1690–1699
51. Ohshakya, S., Fujikawa, M., Hisabori, T., and Yoshida, M. (2011) Knockdown of DAPIT (diabetes-associated protein in insulin-sensitive tissue) results in loss of ATP synthase in mitochondria. J. Biol. Chem. 286, 20292–20296
52. Vaillier, J., Arselin, G., Graves, P. V., Camougrand, N., and Velours, J. (1999) Isolation of supernumerary yeast ATP synthase subunits e and i. Characterization of subunit i and disruption of its structural gene ATP18. J. Biol. Chem. 274, 543–548
53. Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schägger, H. (1999) ATP synthase of yeast mitochondria: isolation of subunit j and disruption of the ATP18 gene. J. Biol. Chem. 274, 36–40
54. Liu, S., Charlesworth, T. J., Baso, J. V., Montgomery, M. G., Harbour, M. E., Fearnley, I. M., and Walker, J. E. (2015) The purification and characterization of ATP synthase complexes from the mitochondria of four fungal species. Biochem. J. 10.1042/BJ20150197
55. Allegretti, M., Klusch, N., Mills, D. J., Vonck J., Kühbrandt, W., and Davyes, K. M. (2015) Horizontal membrane-intrinsic α-helices in the stator α-subunit of an F type ATP synthase. Nature 10.1038/nature14185
56. Vázquez-Acevedo, M., Cardol, P., Cano-Estrada, A., Lapaille, M., Remacle, C., González-Halphen, D., et al. (2006) The mitochondrial ATP synthase of chlorophycean algae contains eight subunits of unknown origin involved in the formation of an atypical stator-stalk and in the dimerization of the complex. J. Bioenerg. Biomembr. 38, 271–282
57. Miranda-Astudillo, H., Cano-Estrada, A., Vázquez-Acevedo, M., Colina-Tenorio, L., Downie-Velasco, A., Cardol, P., Remacle, C., Dominguez-Ramírez, L., González-Halphen, D. (2014) Interactions of subunits Asa2, Asa4, and Asa7 in the peripheral stalk of the mitochondrial ATP synthase of the chlorophycean alga Polytomella sp. Biochim. Biophys. Acta 1837, 1–13
58. van Lis, R., Mendoza-Hernández, G., Groth, G., and Attea, A. (2007) New insights into the unique structure of the F\textsubscript{0}F\textsubscript{1}-ATP synthase from the chlamydomonad algae Polytomella sp. and Chlamydomonas reinhardtii. Plant Physiol. 144, 1190–1199