Conservation of Complete Trimethylation of Lysine-43 in the Rotor Ring of c-Subunits of Metazoan Adenosine Triphosphate (ATP) Synthases

Thomas B. Walpole‡, David N. Palmer‡§, Huibing Jiang‡§, Shujing Ding‡, Ian M. Fearnley‡, and John E. Walker‡¶

The rotors of ATP synthases turn about 100 times every second. One essential component of the rotor is a ring of hydrophobic c-subunits in the membrane domain of the enzyme. The rotation of these c-rings is driven by a transmembrane proton-motive force, and they turn against a surface provided by another membrane protein, known as subunit a. Together, the rotating c-ring and the static subunit a provide a pathway for protons through the membrane in which the c-ring and subunit a are embedded. Vertebrate and invertebrate c-subunits are well conserved. In the structure of the bovine F1-ATPase-c-ring subcomplex, the 75 amino acid c-subunit is folded into two transmembrane α-helices linked by a short loop. Each bovine rotor-ring consists of eight c-subunits with the N- and C-terminal α-helices forming concentric inner and outer rings, with the loop regions exposed to the phospholipid head-group region on the matrix side of the inner membrane. Lysine-43 is in the loop region and its ε-amino group is completely trimethylated. The role of this modification is unknown. If the trimethylated lysine-43 plays some important role in the functioning, assembly or degradation of the c-ring, it would be expected to persist throughout vertebrates and possibly invertebrates also. Therefore, we have carried out a proteomic analysis of c-subunits across representative species from different classes of vertebrates and from invertebrate phyla. In the twenty-nine metazoan species that have been examined, the complete methylation of lysine-43 is conserved, and it is likely to be conserved throughout the more than two million extant metazoan species. In unicellular eukaryotes and prokaryotes, when the lysine is conserved it is unmethylated, and the stoichiometries of c-subunits vary from 9–15. One possible role for the trimethylated residue is to provide a site for the specific binding of cardiolipin, an essential component of ATP synthases in mitochondria. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.047456, 828–840, 2015.

The ATP synthase (or F-ATPase)1 embedded in the inner membranes of mitochondria is a multi-protein complex of about thirty polypeptides that couples the transmembrane proton-motive force across the membrane to the synthesis of ATP from ADP and inorganic phosphate in the matrix of the organelle (1). The coupling mechanism involves a mechanical rotation of the enzyme’s rotor at about 100 Hz (2) driven by the proton motive force (3). The rotor itself consists of a hydrophobic ring of c-subunits in the membrane domain of the enzyme plus a central stalk. The central stalk penetrates into the catalytic F1 domain of the enzyme, which protrudes into the matrix space, and the turning of the rotor brings about conformational changes in the three catalytic sites in each F1 domain that lead to the binding of substrates, and the formation of ATP and its release into the matrix (4). The c-subunits that constitute the rotor-ring are among the most hydrophobic proteins in nature, and, because their properties are similar to those of lipids, they have been classified as proteolipids (5, 6). In vertebrates, c-subunits are highly conserved and they are well conserved in invertebrates also (4). In the structure of the bovine F1-c-ring subcomplex, the 75 amino acid c-subunit is folded into two transmembrane α-helices linked by a short loop (4, 7). Each rotor-ring consists of eight c-subunits with the N-and C-terminal α-helices forming concentric inner and outer rings, linked by eight loop regions exposed to the phospholipid head-group region on the matrix side of the inner membrane. Some of the loops are in contact with subunits γ, δ, and ε in the “foot” of the central stalk (4).

1 The abbreviations used are: F-ATPase, ATP synthase; ESI, electrospray ionization; ETD, electron transfer dissociation.
One striking feature of bovine c-subunits is that glutamate-58 in the C-terminal α-helix is exposed to the lipid bilayer around the mid-point of the membrane, and the protonation and deprotonation of this residue via an arginine residue in an adjacent a-subunit in the membrane domain of the enzyme is an essential feature in the generation of rotation (8). Each complete rotation of the rotor produces three ATP molecules, one from each of the three catalytic sites in the F1-domain (9), and requires the translocation through the membrane of one proton per c-subunit (10). Thus, the number of translocated protons required to make each ATP is the number of c-subunits comprising the ring divided by three, and this parameter is referred to as the “energy cost” for making each ATP molecule (4). The identity, or near identity, of the sequences of vertebrate c-subunits makes it highly likely that c₈-rings observed in the bovine enzyme will persist throughout vertebrate F-ATPases, and hence the energy cost in their F-ATPases will be 2.7 translocated protons per ATP, the lowest value so far observed (4). The high conservation of the sequences of c-subunits in invertebrates suggests that their F-ATPases will also have c₈-rings, with an associated energy cost of 2.7 protons per ATP (4). The c-rings in fungi, eubacteria, and plant chloroplasts are larger and are made variously from 10–15 subunits depending on the species, implying that the energy cost in these enzymes is 3.3–5.0 protons per ATP (7, 11–16).

Another striking feature of the bovine c-subunit, and the topic of this paper, is that the ε-amino group of lysine-43 is completely trimethylated (17). In the structure of the bovine c-ring, these residues are in loop regions of each c-subunit near to the boundary between the lipid bilayer and the aqueous phase of the matrix (4). Their role is not known, but if trimethylated lysine-43 plays some important role in the functioning, assembly or degradation of the c-ring, it would be expected to persist throughout vertebrates and possibly invertebrates also. Therefore, as described here, we have isolated F-ATPases and c-subunits from a wide range of metazoans and have characterized the methylation status of lysine-43 in their c-subunits.

**EXPERIMENTAL PROCEDURES**

**Analytical Methods**—Protein concentrations were estimated by the bicinchoninic acid assay (18) (Pierce, ThermoFisher, Rockford, IL) with bovine serum albumin as standard. Proteins were dissolved in 2% SDS at room temperature, and analyzed by SDS-PAGE in 12–22% gradient acrylamide gels, and detected by staining with Coomassie Brilliant blue dye, as described previously (19).

**Animal Samples**—Bovine, porcine, and ovine hearts were purchased from a slaughterhouse. Rabbit hearts and livers from rats and mice were removed in the local animal house. Mitochondria were prepared from vertebrate tissues, from the hepatopancreas of *H. gamarus* and *C. pagurus*, from maggots of *C. vomitoria*, and from *L. terrestris* (22). Mitochondria were isolated by established methods from tissues of *C. gigas*, *M. edulis* and *E. chloroticus* (23), from cultured cells of *T. ni* and *D. melanogaster* (24), and from whole specimens of *A. mollis* (23), *C. incrustans* (25), *A. salina* (25), *C. elegans* (26), and *S. tuberosum* (27).

Mitochondria were washed by resuspension for 30 min at 4 °C in buffer consisting of 50 mM Na₂HPO₄, pH 9.2, 100 mM sucrose, and 0.5 mM EDTA, and centrifugation (47,000 × g, 30 min, 4 °C). This washing process was repeated twice more. The pellet was resuspended at a protein concentration of 10 mg/ml in a solution of 20 mM Tris-HCl, pH 8.0, containing 10% glycerol (v/v). The suspension was stored at −20 °C.

**Affinity Purification of F-ATPases**—Phosphate washed mitochondrial membranes (10 mg/ml) were extracted with a solution of 1% (w/v) n-dodecyl-β-d-maltoside, and the ATPase activity of the F-ATPase in the extract was inhibited by a recombinant protein consisting of residues 1–60 of the bovine F-ATPase inhibitor protein, IF₃, with a glutathione-S-transferase domain and six histidine residues attached to its C terminus (28). The inhibited complexes were bound to a GSTrap HP column (1 or 5 ml; GE Healthcare, Uppsala, Sweden) and released with 20 mM EDTA (28). The fractions containing the F-ATPase were pooled, dialyzed overnight at 4 °C into buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol (v/v).
Lysine Trimethylation in Rotors of Metazoan ATP Synthases

Solvent Extraction of Hydrophobic Proteins—Some invertebrate mitochondrial membranes (5 mg protein) were washed twice by re-suspension at 4 °C in buffer containing 2 mM Tris-HCl, pH 8.0 and mitochondrial membranes (5 mg protein) were washed twice by re-suspension overnight at 4 °C, 10 min. The pellet was redissolved in a mixture (approximately 40 µl) of 60% (v/v) formic acid, 15% (v/v) trifluoroethanol, and 1% (v/v) hexafluoroisopropanol and 1 mM tris(2-carboxyethyl)phosphine, and applied to a reverse-phase column (75 mm long, 1 mm i.d.) of PLRP-S (polymeric reverse phase made of styrene divinylbenzene copolymer; 5 µm beads, 300 Å pores; Varian, Oxford, UK) equilibrated in solvent A consisting of 50 mM ammonium formate, pH 3.1, 1% (v/v) hexafluoroisopropanol, and 15% (v/v) trifluoroethanol (29). The proteins were eluted with a linear gradient of solvent B consisting of 50 mM ammonium formate, pH 3.1, 70% (v/v) 2-propanol, 20% (v/v) trifluoroethanol, and 1% (v/v) hexafluoroisopropanol (29). The eluate was introduced “on-line” via an electrospray interface into either a Quattro Ultima triple quadrupole instrument (Waters-Micromass, Manchester, UK) or a Q-Trap 4000 mass spectrometer (ABSciex, Phoenix House, Warrington, UK). Both instruments were operated in MS mode and the masses of ions were measured with a single quadrupole. They were calibrated with a mixture of myoglobin and trypsinogen (29). Molecular masses were calculated with MassLynx (Waters, Milford, MA) or Bioanlyst software (ABSciex).

Mass Spectrometric Analysis of Intact Proteins—Samples of purified F-ATPases or of solvent-extracted proteins (20–100 µg) were treated overnight at −20 °C with 20 vols. of cold ethanol, and the precipitated protein was recovered by centrifugation (16,000 × g, 4 °C, 10 min). The pellet was redissolved in a mixture (approximately 40 µl) of 60% (v/v) formic acid, 15% (v/v) trifluoroethanol, and 1% (v/v) hexafluoroisopropanol and 1 mM tris(2-carboxyethyl)phosphine, and applied to a reverse-phase column (75 mm long, 1 mm i.d.) of PLRP-S (polymeric reverse phase made of styrene divinylbenzene copolymer; 5 µm beads, 300 Å pores; Varian, Oxford, UK) equilibrated in solvent A consisting of 50 mM ammonium formate, pH 3.1, 1% (v/v) hexafluoroisopropanol, and 15% (v/v) trifluoroethanol (29). The proteins were eluted with a linear gradient of solvent B consisting of 50 mM ammonium formate, pH 3.1, 70% (v/v) 2-propanol, 20% (v/v) trifluoroethanol, and 1% (v/v) hexafluoroisopropanol (29). The eluate was introduced “on-line” via an electrospray interface into either a Quattro Ultima triple quadrupole instrument (Waters-Micromass, Manchester, UK) or a Q-Trap 4000 mass spectrometer (ABSciex, Phoenix House, Warrington, UK). Both instruments were operated in MS mode and the masses of ions were measured with a single quadrupole. They were calibrated with a mixture of myoglobin and trypsinogen (29). Molecular masses were calculated with MassLynx (Waters, Milford, MA) or Bioanlyst software (ABSciex).

Mass Spectrometric Analysis of Peptides from c-Subunits—The regions of gels containing the c-subunits (immediately above the dye front) were excised, and the proteins were digested “in-gel” with
sequencing grade chymotrypsin (30; 12.5 ng/ml; Roche Applied Science, Burgess Hill, UK) in buffer consisting of 20 mM Tris-HCl, pH 8.0, and 5 mM CaCl₂. Chymotryptic peptides were analyzed by MS and tandem MS with a MALDI-TOF-TOF mass spectrometer (Model 4800 ABSciex) with α-cyano-4-hydroxy-trans-cinnamic acid as the matrix. The instrument was calibrated internally by addition of the autolysis products of trypsin (m/z values 2163.057 and 2273.160) and a calcium-related matrix ion (m/z value, 1060.048). Prominent peptide ions in the mass spectra were fragmented by collision induced dissociation with air and a collision energy of 1 kV, and the fragments were analyzed by tandem MS. Portions of chymotryptic digests were fractionated with an Easy-nLC instrument (Thermo Fisher) on a reverse-phase column (100 mm long, 75 μm i.d.; Nanosparetron, 2421 CA Nieuwkoop, Netherlands) with an acetonitrile gradient in 0.1% (v/v) formic acid, with a flow rate of 300 nl/min. The eluent was analyzed on-line in a LTQ Orbitrap XL electron transfer dissociation (ETD) mass spectrometer (Thermo Fisher, Hemel Hempstead, UK). Peptides were fragmented by collision induced dissociation and electron transfer dissociation ETD. Fragment ion spectra were interpreted manually.

RESULTS

Isolation of Metazoan c-Subunits—F-ATPases purified by affinity chromatography from the mitochondria of vertebrates and five invertebrates, were analyzed by SDS-PAGE (Fig. 1A parts a-q and r-v). From tracks a–q in Fig. 1A, it is evident that the subunit compositions of the vertebrate enzymes are very similar, and it has been confirmed elsewhere, that those of the subunit compositions of the vertebrate enzymes are very similar, and it has been confirmed elsewhere, that those of the vertebrate enzymes are very similar, and it has been confirmed elsewhere, that those of the vertebrate enzymes are very similar, and it has been confirmed elsewhere, that those of the vertebrate enzymes are very similar, and it has been confirmed elsewhere, that those of

Characterization of c-Subunits—The molecular masses of c-subunits (Table I) were determined by liquid chromatography (LC)-ESI-MS by a procedure that included a reverse-phase chromatographic fractionation compatible with membrane proteins (29). A representative mass spectrum of an intact c-subunit is presented in Fig. 2. As the masses of many metazoan c-subunits are known from their sequences, the measured and calculated values were compared (Table I), and in each case the measured mass of the intact protein exceeded the calculated value by 42 ± 1 Da, indicating that either an acetyl group or three methyl groups had been added to the protein post-translationally. The sequences of the c-subunits of the brush tailed possum, T. vulpecula, the spiny dogfish, S. acanthias, and the spiny dogfish, S. acanthias, have not been reported, but their measured masses are also very similar to the values in other vertebrates, suggesting that both their sequences and the protein modification are conserved. No sequences are available currently for the c-subunits from eight other species in Table I, but in four instances the sequences are known in closely related species, and the data suggest that the orthologs have identical sequences and are modified in the same way. Thus, the observed mass of the c-subunit in the

![Figure 2](image-url)
North Atlantic mussel, *M. edulis*, exceeds by 42 Da the calculated value for the Mediterranean mussel, *M. galloprovincialis*, as do the measured masses of the c-subunits of the New Zealand sea urchin (or kina), *E. chloroticus*, in comparison with the calculated value in the Pacific purple sea urchin, *S. purpuratus*, and of the c-subunit from the brown crab, *H. gammarus*, in comparison with the calculated value in the brown crab, *C. pagurus*. Moreover, the measured value of 7684 Da for the c-subunit in the cabbage looper moth, *T. ni*, is 42 Da greater than the calculated values for two other lepidopterans, the fall armyworm, *Spodoptera frugiperda*, and the tobacco hornworm, *Manduca sexta*, indicative of identical sequences and modification of the c-subunits in these three lepidopterans. In contrast, the measured masses of c-subunits in the European lobster, *H. gammarus*, the brown crab, *C. pagurus*, the Australasian sea cucumber, *A. mollis*, and the brine shrimp, *A. salina*, do not correspond to the values in any closely related species. The amounts of c-subunits purified from *B. constrictor* and *C. incrustans* were insufficient to permit the masses of the intact proteins to be measured.

In all of the species where the intact protein mass data show the presence of a post-translational modification with a mass of 42 ± 1 Da, that modification is quantitative, and in no instance were ions observed that correspond to the unmodified, monomethylated or dimethylated c-subunit. Minor ions in some spectra corresponding to the intact c-protein plus 16 mass units have been shown previously to arise from the partial oxidation of the C-terminal methionine residue (17).

In addition to the extensive range of metazoan c-subunits, the c-subunit from the potato mitochondrial F-ATPase, was investigated also. This protein is encoded in mitochondrial DNA, and in common with other mitochondrially encoded proteins, its measured mass of 7617.2 was 28 Da greater than the value calculated from the sequence, arising from the Nα-formylated translational initiation methionine residue (31). There was no evidence that this protein was modified otherwise.

**Localization of Post-translational Modifications** — The post-translational modification of c-subunits was localized to a specific region of the proteins by the MALDI-TOF-MS analysis of chymotryptic digests of the gel bands. In all but the c-subunits from the molluscs, *C. gigas* and *M. edulis*, a peptide with a mass in the range 1343.6–1343.9 Da was observed (Table II), corresponding to residues 37–47 (ARNPSLKQQLF) of almost all known vertebrate sequences (Fig. 3), and in many invertebrate sequences (Fig. 4), plus

**TABLE II**

| Species                | MH+ Calculated | MH+ Observed | Δ         | TM-Lys | Position |
|-----------------------|----------------|--------------|-----------|--------|----------|
| *Homo sapiens*         | 1301.7325      | 1343.7982    | 42.0657   | +      | K43      |
| *Bos taurus*           | 1301.7325      | 1343.7937    | 42.0612   | +      | K43      |
| *Ovis aries*           | 1301.7325      | 1343.7937    | 42.0612   | +      | K43      |
| *Sus scrofa*           | 1301.7325      | 1343.7934    | 42.0609   | +      | K43      |
| *Octolagus cuniculus*  | 1301.7325      | 1343.7952    | 42.0627   | +      | K43      |
| *Mus musculus*         | 1301.7325      | 1343.7943    | 42.0618   | +      | K43      |
| *Rattus norvegicus*    | 1301.7325      | 1343.7931    | 42.0606   | +      | K43      |
| *Cervus elaphus*       | 1301.7325      | 1343.7940    | 42.0615   | +      | K43      |
| *Trichosurus vulpecula*| n.s.           | 1343.7938    | n.s.      | +      | n.s.     |
| *Gallus gallus*        | 1301.7325      | 1343.7946    | 42.0621   | +      | K43      |
| *Anas platyrhynchos*   | 1301.7325      | 1343.7934    | 42.0609   | +      | K43      |
| *Testudo graeca*       | n.s.           | 1343.7949    | n.s.      | +      | n.s.     |
| *Boa constrictor*      | 1301.7325      | 1343.7937    | n.s.      | +      | n.s.     |
| *Xenopus laevis*       | 1301.7325      | 1343.7949    | 42.0624   | +      | K43      |
| *Salmo salar*          | 1301.7325      | 1343.7934    | 42.0609   | +      | K43      |
| *Onchorynchus mykiss*  | 1301.7325      | 1343.7943    | 42.0618   | +      | K43      |
| *Diceranclus labrax*   | n.s.           | 1343.7940    | n.s.      | +      | n.s.     |
| *Squalus acanthis*     | n.s.           | 1343.7949    | n.s.      | +      | n.s.     |
| *Calliphora vomitoria* | 1301.7325      | 1343.7940    | 42.0615   | +      | K43      |
| *Lumbricus terrestris* | 1301.7325⁵     | 1343.7940    | n.s.      | +      | n.s.     |
| *Homarus gammarus*     | n.s.           | 1343.7934    | n.s.      | +      | n.s.     |
| *Cancer pagurus*       | n.s.           | 1343.7934    | n.s.      | +      | n.s.     |
| *Crassostrea gigas*    | 1273.7012      | 1315.7641    | 42.0629   | +      | K43      |
| *Mytilus edulis*       | 1244.7110⁶     | 1286.7657    | 42.0547   | +      | K43      |
| *Trichoplusia ni*      | 1301.7325      | 1343.7934    | n.s.      | +      | n.s.     |
| *Caenorhabditis elegans* | 1301.7325   | 1343.7943    | 42.0618   | +      | K43      |
| *Drosophila melanogaster* | 1301.7325 | 1343.7934    | 42.0609   | +      | K43      |
| *Artemia salina*       | n.s.           | 1343.7940    | n.s.      | +      | n.s.     |
| *Evechinus chloroticus*| 1301.7325⁵     | 1343.7946    | 42.0621   | +      | K43      |
| *Australostichopus mollis* | 1301.7325      | 1343.7940    | n.s.      | +      | n.s.     |
| *Crella incrustans*    | n.s.           | 1343.7949    | n.s.      | +      | n.s.     |

*⁵* accurate mass measurement of triply charged ions in an Orbitrap MS.

*⁶* TM-Lys, trimethyllysine.

*⁷* location of modification; for meaning of *⁵–⁶* see footnotes in Table I.
Fig. 3. Sequences of c-subunits from vertebrate F-ATPases. The secondary structure of the bovine protein is depicted above the aligned sequences. Where sequence data are available a representative species from each vertebrate order is shown. Alanine residues 13, 19, and 23, required for the formation of a c8-ring, trimethylated lysine-43 and glutamate-58, which is essential for proton translocation, and are green, purple, and blue, respectively. Amino acid substitutions are red. The five letter UNIPROT codes for species are on the left; bold codes denote species where lysine-43 has been demonstrated experimentally to be trimethylated. HOMSA, *Homo sapiens*; BOVIN, *Bos taurus* (cow); CANFA, *Canis lupus familiaris* (dog); DASNO, *Dasyus novemcinctus* (armadillo); EQUCA, *Equus caballus* (horse); OVIAR, *Ovis aries* (sheep); SUSSC, *Sus scrofa* (pig); CEREL, *Cervus elaphus* (red deer); MONDE, *Monodelphis domestica* (gray short tailed opossum); MYOLU, *Myotis lucifugus* (bat); ORNAN, *Ornithorhynchus anatinus* (duckbill platypus); TUPCH, *Tupaia chinensis* (Chinese tree shrew); GALVA, *Galeopterus variegatus* (flying lemur); TRIMA, *Trichechus manatus latirostris* (Florida manatee); LOXAF, *Loxodonta africana* (African elephant); ELEED, *Elephantulus edwardii* (Cape elephant shrew); SARHA, *Sarcophilus harrisii* (Tasmanian devil); CHRAS, *Chrysocloris asiatica* (Cape golden mole); MUSMU, *Mus musculus* (mouse); ORYCU, *Oryctolagus cuniculus* (rabbit); RATNO, *Rattus norvegicus* (rat); TURTR, *Tursiops truncates* (bottle nosed dolphin); AMBME, *Ambystoma mexicanum* (axolotl); ANAPL, *Anolis carolinesis* (green anole); PELSI, *Pelodiscus sinensis* (Chinese softshell turtle); PYTBI, *Python bivittatus* (Burmese python); ANOCA, *Anas platyrhynchos* (wild duck); ALGA, *Galbella galga* (chicken); SARC, *Sarcopus coromandus* (emu); CERO, *Cercopithecus aethiops* (chimpanzee); BONUN, *Bos mutus* (bovine); PARSA, *Paraxerus cepapi* (Cape ground squirrel); LOPAN, *Lophopsittacus roseus* (rose-ringed parakeet); ARTH, *Ardea alba* (little egret); MUSO, *Muscicapa striata* (blackcap); NAPBA, *Nasus philippinus* (Cebu crested fireback); SOLPA, *Soliparus fuliginosus* (black mouse lemur); SALIM, *Saimiri sciureus* (squirrel monkey); TUPCH, *Tupaias nigra* (tree shrew); ABOMA, *Atherurus macrourus* (aardvark); MERCO, *Meriones unguiculatus* (striped field mouse); CVIR, *Cricetulus griseus* (striped field mouse); LENF, *Lemur catta* (ring tailed lemur); LAVI, *Lophura erythrophysa* (red crowned ibis); PETH, *Psephotus assimilis* (red-rumped parrot); APTFO, *Aptenodytes forsteri* (emperor penguin); XENLA, *Xenopus laevis* (Western clawed toad); AMBME, *Ambystoma mexicanum* (axolotl); APTFO, *Aptenodytes forsteri* (emperor penguin); XENLA, *Xenopus laevis* (Western clawed toad).
Lysine Trimethylation in Rotors of Metazoan ATP Synthases

42.0606–42.0657 Da). The location of the modified residue in the peptide was obtained by MALDI-TOF analysis of its fragment ions. In a typical example provided by the peptide from the Atlantic salmon, *S. salar* (Fig. 5), the fragment ion spectrum of the 1343.8 Da ion is dominated by a prominent ion with mass of 1284.6 Da. This ion corresponds to the loss of trimethylammonium (59 Da) from the peptide precursor, diagnostic of the presence of a trimethylated lysine (32, 33). In these, and also in other analyses conducted in an Orbitrap mass spectrometer with fragmentation by higher energy collisions (not shown), there was no indication of any immonium ion (126.1 Da), which would arise if the peptide were acetylated. Therefore, in common with the human, bovine, and ovine c-subunits (17), the lysine-43 residues in the c-subunit of the salmon and the other species listed in Table I, are completely trimethylated on their ε-amino groups. In this spectrum, and those arising from the same peptide in other species, the presence of other fragment ions confirmed the sequence ARNPSLKQALF, identical to the sequence in the *M. galloprovincialis* c-subunit, and differing from the vertebrate sequence by the substitution Q44N (Fig. 4). Similarly, the sequence of the *C. gigas* chymotryptic peptide sequence was ARNPSLKQALF, differing from the vertebrate sequence by the substitutions Q44N and Q45N (Fig. 4).

Additional discrimination between trimethylation and acetylation of the lysine residues was provided by consideration of the precise masses of the peptides, as the acetylation of an amino acid in a protein increases its mass by 0.0364 Da more than the parent ions, again providing evidence for trimethylation rather than acetylation of these peptides.

Definitive confirmation of the location and nature of the post-translational modifications was provided by ESI-tandem MS analysis of fragments of the modified chymotryptic peptides produced by ETD. An example is provided by the fragmentation of the triply positively charged chymotryptic peptide (448.6 Da) from the c-subunit of *S. salar* (Fig. 6). A series of c-type fragment ions (c6–c10) and z-type fragment ions (z5–z7, z9, and z10) derived from this peptide, together define most of the sequence of the modified peptide. The mass difference of 170 Da between the c6 and c7 ions and the presence of the z5 ion allowed the modification site to be identified unambiguously as lysine-7 in the peptide (or lysine-43 in the intact c-subunit). The mass spectra of equivalent chymotryptic peptides in c-subunits from other species (see supplemental Table S2) contained similar sets of ion fragments and allowed the modification to be identified and localized in these organisms also.

Similar analyses of chymotryptic peptides with masses of 1287.13 and 1315.76 from the c-subunits of *M. edulis* and *C. gigas*, respectively, also localized the trimethyl modification to lysine-43 (supplemental Figs. S1 and S2). In addition, they showed that the sequence of the *M. edulis* chymotryptic peptide was ARNPSLKQALF, identical to the sequence in the *M. galloprovincialis* c-subunit, and differing from the vertebrate sequence by the substitution Q45A (Fig. 4). Similarly, the sequence of the *C. gigas* chymotryptic peptide sequence was ARNPSLKQALF, differing from the vertebrate sequence by the substitutions Q44N and Q45N (Fig. 4).

**DISCUSSION**

**Conservation of the c8-Ring in Metazoans**—Because of the identity or near identity of the sequences of c-subunits in vertebrates (Fig. 3), the structure of the c-ring in the bovine F1-c8-ring complex can be taken as being representative of the c8-rings that are almost certainly found in the F-ATPases in all of these species. A cross-section of the structures of the bovine c8-rings in the plane of the membrane shows inner and outer concentric rings, each of eight α-helices, corresponding to the N- and C-terminal α-helices, respectively. As these α-helices are not straight, and bend inwards toward the central cavity of the ring, becoming closest at their midpoints, the structure is shaped like an hourglass. Moreover, the requirement for the α-helices to form the c8-ring constrains the amino acid composition of especially the inner ring, where the α-helices are dominated by amino acids with small side chains (glycine, alanine, serine, and cysteine) and at the neck of the hourglass, only alanine residues are found at positions 13, 19, and 23. Their replacement by amino acids with larger side chains would destabilize the c8-ring, and such residues can only be accommodated in the larger c-rings, such as those found in fungi and eubacteria (4). Also, replacement of these alanines by glycines would abolish hydrophobic packing interactions that contribute to the ring’s stability. Thus, these three alanine residues can be considered to be determinants of the capability of the c-subunits to form c8-rings (4).

Among the seventy invertebrate c-sequences shown in Fig. 4, in all but five species the alanines are absolutely conserved.
L. terrestris, T. ni, E. chloroticus, a related species was studied (shown, with the exception of the arthropods and poriferans, where representative species were selected from each class when available. †,

Molecular & Cellular Proteomics 14.4

Fig. 4. Sequences of c-subunits from invertebrate F-ATPases. Where data are available sequences for all known invertebrate species are shown, with the exception of the arthropods and poriferans, where representative species were selected from each class when available. †, a related species was studied (L. terrestris, T. ni, E. chloroticus, and M. edulis, respectively). For the significance of the colors, and the five letter
Lysine Trimethylation in Rotors of Metazoan ATP Synthases

Conservation of Trimethylation of Lysine-43 in Metazoan c-Subunits—Lysine-43 is trimethylated completely in the fifteen vertebrate c-subunits of F-ATPases that were studied. The vertebrate species include representatives of all classes (mammals, reptiles, birds, amphibians, ray-finned, and cartilaginous fishes), except for the sarcopterygii, or lobe-finned fishes (see Fig. 7). With the exception of the c-subunit from an opossum, and an elephant the known sequences of vertebrate c-subunits are identical (Fig. 3); in the opossum sequence the two conservative point substitutions, Ile2Val and Met73Leu, are found at and near the N- and C-terminal ends of the protein, which have no known significant roles in the function of F-ATPases (Fig. 4).

Fig. 5. Tandem-MS analysis of a chymotryptic peptide from the c-subunit of the F-ATPase from Salmo salar. The singly charged chymotryptic peptide (MH+ 1343.69) corresponds to residues 37–47 of the protein. The prominent ion at m/z 1284.63 arises by loss of a trimethylammonium ion and is diagnostic of a trimethylated peptide. The inset contains the MALDI-TOF-MS spectrum of the chymotryptic digest. The residue numbers of the peptides from the salmon c-subunit are given in parentheses. The ion at m/z 1080.0598 is derived from the matrix, and the ion at m/z 1523.8110 is a fragment of chymotrypsin.

and in the exceptional cases either alanine 13 or alanine 23 is replaced either by a serine or a cysteine residue. Hence, it is reasonable to suggest that the ca-ring is likely to be conserved throughout invertebrates. This view is supported by the remarkable general conservation of the sequences of c-subunits from humans to sponges (Figs. 3 and 4). For example, the sequences of c-subunits in humans and the sponge Vaceletia sp. differ in only nine positions. They are all conservative changes, and, with one exception, they occur in the N- and C-terminal ends of the protein, which have no known significant roles in the function of F-ATPases (Fig. 4).
Fig. 6. Tandem MS of a chymotryptic peptide from the c-subunit of \textit{Salmo salar}. The peptide represents residues 37–47 of the protein. A triply charged version (m/z 448.60) was fragmented by ETD, and the fragments were analyzed in an Orbitrap instrument. The upper and lower panels contain ions with m/z values of 100–700 and 700–1370, respectively. In the upper panel, the c- and z-ions are mapped onto the amino acid sequence of the peptide. The mass difference of 170.15 Da between the c6 and c7 ions shows that lysine-7 is trimethylated.

It has been estimated that there are \( \sim 2 \) million invertebrate species on Earth today (34).

The eukaryota are classified in six major groups, opisthokonts, amoebozoa, plantae, chromalveolata, rhizaria, and excavata; the metazoans (animalia), choanoflagellates, ichyosporea, nuclearkard amoebae, and fungi form kingdoms within the opisthokonts (supplemental Fig. S3). The sequences of c-subunits from representatives of other opisthokont kingdoms (choanoflagellates, filastera, ichyosporea, and fungi) show that lysine-43 is conserved except in the fungus, \textit{Pichia angusta}, where an arginine residue is substituted (supplemental Fig. S4). However, in the two cases where the methylation status of the conserved lysine has been investigated, \textit{Saccharomyces cerevisiae} and \textit{Yarrowia lipolytica}, it is not methylated. Also, the three alanines in the N-terminal \( \alpha \)-helix that are conserved are replaced.

Among the prokaryota, where the symmetries of c-subunits have been studied most extensively (11, 12, 14–16) the sequences have diverged even more, and some species have no basic residue in the loop region, and in \textit{Ilyobacter tartari-}
Lysine Trimethylation in Rotors of Metazoan ATP Synthases

Fig. 7. Metazoan tree of life. Part A, the vertebrate tree. Part B, the major metazoan phyla. The red branches contain species where lysine-43 in the c-subunit of mitochondrial F-ATP synthase has been demonstrated to be trimethylated. In part A, the analyzed examples are as follows: Mammalia, Homo sapiens, Bos taurus, Ovis aries, Sus scrofa, Oryctolagus cuniculus, Mus musculus, Rattus norvegicus, Cervus elaphus, Trichosurus vulpecula; Aves, Gallus gallus, Anas platyrhynchos; Reptilia, Testudo graeca, Boa constrictor; Amphibia, Xenopus laevis; Actinopterygii, Salmo salar, Onchorhynchus mykiss, Dicentrachus labrax; Chordichthyes, Squalus acanthias. In part B, the analyzed examples are: Echinodermata, Evechinus chloroticus, Australostichopus mollis; Chordata, see Part A; Annelida, Lumbricus terrestris; Mollusca, Crassostrea gigas, Mytilus edulis; Crustacea, Homarus gammarus, Cancer pagurus, Artemia salina; Hexapoda, Drosophila melanogaster, Trichoplusia ni, Calliphora vomitoria; Nematode worms, Caenorhabditis elegans; Porifera, Crella incrustans.

cus, for example, the conserved lysine is not methylated (supplemental Fig. S5).

Function of Trimethyllysine-43—The trimethyllysine-43 residues in the c-rings of metazoan F-ATP synthases have been proposed to be involved in providing a binding site for the abundant mitochondrial lipid cardiolipin (4). Approximately 75% of cardiolipin in the inner membranes of mitochondria is thought to be a constituent of the inner leaflet of the membrane (35, 36), and bovine F-ATPase is known to require the presence of cardiolipin in the membrane to which it is bound in order to function correctly (37). In the bovine c-ring, the lysine-43 residues are situated close to the inner
surface of the inner mitochondrial membrane in the phospholipid head-group region. Hence, the presence of the trimethyllysine side chains would impede any associations with lipids containing headgroups (4), and that, as cardiolipin has no headgroup and is negatively charged, it would bind preferentially to the ring in this region. The proposed association of phospholipids and cardiolipin with the bovine c-ring is supported by molecular dynamics simulations. (A. Duncan et al., in preparation).

The cardiolipin bound in this way to the c-rings of the F-ATPases could have at least three possible roles: first, to provide the ring with additional stability to help it to survive the rotational torque experienced during catalysis; second, its two negative charges participate in the pathway for protons to exit from the enzyme’s membrane domain into the mitochondrial matrix; third, the cone-shaped cardiolipins could enhance the curvature at the apices of the cristae where the F-ATPases are concentrated in rows of dimers (38, 39).

* Subcellular Site of Methylation of Metazoan c-Subunits—Further studies of the role of methylation of lysine-43 of the c-subunit of F-ATPases would be greatly aided by the identification of the modifying enzyme. Precursors of the human and bovine c-subunits, for example, are each encoded by three nuclear genes (40, 41). In each case, the products differ in the sequences of the N-terminal extensions that direct the proteins to the matrix of the organelle, but removal of the import sequences during the import process produces identical mature c-proteins (40, 41). The subcellular site where the methylation of lysine-43 is carried out is not known, and in principle the modification could be carried out during or after cytoplasmic protein synthesis, but before import of the protein into the mitochondrion, or during or on completion of import of the protein and cleavage of the mitochondrial targeting sequence. Thus, the protein lysine methyltransferase responsible for catalyzing the transfer of methyl groups from S-adenosyl-methionine to the lysine residue could be localized in the cellular cytoplasm, in the intermembrane space or in the matrix of the mitochondrion. Until recently, when the first arginine and lysine methyltransferases were found in the matrix of human mitochondria (42, 43), it was not known whether such enzymes are associated with the matrix of mitochondria. In the case of the c-subunits in porifera, there can be little or no doubt that the methylation of subunit c is an event that takes place in the mitochondrial matrix as, in contrast to other metazoans that have been investigated, where the c-subunit is encoded by nuclear genes, the sponge c-subunits are the products of the mitochondrial genomes (44).

Acknowledgments—We thank Drs K. Jayawardena and M. E. Harbourn for help with MALDI-TOF analyses, and Dr J. Carroll for advice. We thank the donors of animals and animal tissues.

* This work was supported by the intramural programme of the Medical Research Council.
Lysine Trimethylation in Rotors of Metazoan ATP Synthases

Jacobs, H. T., and Holt, I. J. (2002) Biased incorporation of ribonucleotides on the mitochondrial L-strand for apparent strand-asymmetric DNA replication. Cell 111, 495–505

23. Sokolova, I. M. (2004) Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine polikltherm, Crassostrea virginica Gmelin (Bivalvia: Ostreidae). J. Exp. Biol. 207, 2639–2648

24. Wang, Y., Oberley, L. W., Howes, D., Jarvis, D. L., Chauhan, G., and Murhammer, D. W. (2004) Effect of expression of manganese superoxide dismutase in baculovirus-infected insect cells. Appl. Biochem. Biotechnol. 119, 181–193

25. Vallejo, C. G., Sillero, M. A., and Marco, R. (1979) Mitochondrial maturation during Artemia salina embryogenesis. General description of the process. Cell. Mol. Biol. 25, 113–124

26. Grad, L. I., and Lemire, B. D. (2004) Mitochondrial complex I mutations in Caenorhabditis elegans produce cytochrome c oxidase deficiency, oxidative stress, and vitamin-responsive lactic acidosis. Hum. Mol. Genet. 13, 303–314

27. Neuburger, M., Journet, E. P., Bligny, R., Carde, J. P., and Douce, R. (1982) Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. Arch. Biochem. Biophys. 217, 312–323

28. Runswick, M. J., Bason, J. V., Montgomery, M. G., Robinson, G. C., Grad, L. I., and Lemire, B. D. (2004) Mitochondrial complex I mutations in Caenorhabditis elegans produce cytochrome c oxidase deficiency, oxidative stress, and vitamin-responsive lactic acidosis. Hum. Mol. Genet. 13, 303–314

29. Carroll, J., Fearnley, I. M., Wang, Q., and Walker, J. E. (2009) Measurement of the molecular masses of hydrophilic and hydrophobic subunits of ATP synthase and complex I in a single experiment. Anal. Biochem. 395, 249–255

30. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860

31. Bianchetti, R., Lucchini, G., and Sartriana, M. L. (1971) Endogenous synthesis of formyl-methionine peptides in isolated mitochondria and chloroplasts. Biochem. Biophys. Res. Commun. 42, 97–102

32. Zhang, K., Yau, P. M., Chandrasekhar, B., New, R., Kondrat, R., Imai, B. S., and Bradbury, M. E. (2004) Differentiation between peptides containing acetylated or tri-methylated lysines by mass spectrometry: an application for determining lysine 9 acetylation and methylation of histone H3. Proteomics 4, 1–10

33. Hirota, J., Satomi, Y., Yoshikawa, K., and Takao, T. (2003) n-N,N,N-trimethyllysine-specific ions in matrix-assisted laser desorption/ionization-tandem mass spectrometry. Rapid Commun. Mass Spectrom. 17, 371–376

34. Zhang, Z. (2011) Animal biodiversity: an introduction to higher-level classification and taxonomic richness. Zootaxa 3148, 7–12

35. Krebs, J. J., Hauser, H., and Carafoli, E. (1979) Asymmetric distribution of phospholipids in the inner membrane of beef heart mitochondria. J. Biol. Chem. 254, 5308–5316

36. de Kroon, A. I., Dolis, D., Mayer, A., Lill, R., and de Kruijff, B. (1997) Phospholipid composition of highly purified mitochondrial outer membrane of rat liver and Neurospora crassa. Is cardiolipin present in the mitochondrial outer membrane? Biochim. Biophys. Acta 1325, 108–116

37. Eble, K. S., Coleman, W. B., Hantgan, R. R., and Cunningham, C. C. (1990) Tightly associated cardiolipin in the bovine heart mitochondrial ATP synthase as analyzed by 31P nuclear magnetic resonance spectroscopy. J. Biol. Chem. 265, 19434–19440

38. Davies, K. M., Strauss, M., Daum, B., Kief, J. H., Olsiewac, H. D., Rybcovska, A., Zickermann, V., and Kuhlbrandt, W. (2011) Macromolecular organization of ATP synthase and complex I in whole mitochondria. Proc. Natl. Acad. Sci. U.S.A. 108, 14121–14126

39. Davies, K. M., Anselmi, C., Wittig, I., Faraldo-Gomez, J. D., and Kuhlbrandt, W. (2012) Structure of the yeast F1F0-ATP synthase dimer and its role in shaping the mitochondrial cristae. Proc. Natl. Acad. Sci. U.S.A. 109, 13602–13607

40. Gay, N. J., and Walker, J. E. (1985) Two genes encoding the bovine mitochondrial ATP synthase protein specify precursors with different import sequences and are expressed in a tissue-specific manner. EMBO J. 4, 3519–3524

41. Yan, W. L., Lerner, T. J., Haines, J. L., and Gusella, J. F. (1994) Sequence analysis and mapping of a novel human mitochondrial ATP synthase subunit 9 cDNA (ATP5G3). Genomics 24, 375–377

42. Rhein, V. F., Carroll, J., Ding, S., Fearnley, I. M., and Walker, J. E. (2013) NDUFAF7 methylates arginine 85 in the NDUF2 subunit of human complex I. J. Biol. Chem. 288, 33016–33026

43. Rhein, V. F., Carroll, J., He, J., Ding, S., Fearnley, I. M., and Walker, J. E. (2014) Human METTL20 methylates lysine residues adjacent to the recognition loop of the electron transfer flavoprotein in mitochondria. J. Biol. Chem. 289, 24640–24651

44. Osigus, H. J., Eitel, M., Bernt, M., Donath, A., and Schierwater, B. (2013) Mitogenomics at the base of metazoa. Mol. Phylogenet. Evol. 69, 339–351