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

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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 F_1-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.***

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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 F_1 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 F_1 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 F_1-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, ATYP 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 F_1-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 c_8-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 c_8-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. Samples of muscle tissue from rainbow trout (*Onchorhynchus mykiss*), salmon (*Salmo salar*), spiny dogfish (*Squalus acanthias*), duck (*Anas platyrhynchos*), and chicken (*Gallus gallus*), and of liver tissue from sea bass (*Dicentrachus labrax*), and intact specimens of Pacific oysters (*Crassostrea gigas*), mussels (*Mytilus edulis*), brown crabs (*Cancer pagurus*), lobsters (*Homarus gammarus*), and potatoes (*Solanum tuberosum*) were purchased from local food retailers. Gills were excised from oysters and mussels, and the heptopancreas was recovered from crabs and lobsters. Earthworms (*Lumbricus terrestris*), and blowfly larvae (*Calliphora vomitoria*) were obtained from a local fishing supply shop. Eggs of the brine shrimp (*Artemia salina*) were purchased from Reef-Phyto (Bristol, UK) and hatched overnight in sea salts (35 g/L; Sigma, Gillingham, UK) at 20 °C under light. Livers from red deer (*Cervus elaphus*), and of brush tail possum (*Trichosurus vulpecula*) came from Lincoln University, New Zealand. Liver samples from *Boa constrictor* and the Greek tortoise (*Testudo graeca*) were provided by Prof A. Url (Institute of Pathology and Forensic Veterinary Medicine, Vienna, Austria). Ovarian tissue was harvested from female specimens of *Xenopus laevis* donated by Dr J. L. Gallop (Gurdon Institute, Cambridge, UK). Cells of *Trichoplusia ni* (cabbage looper moth) were supplied by Prof. N. J. Gay (Department of Biochemistry, Cambridge, UK). Roundworms (*Caenorhabditis elegans* strain N2) provided by Dr M. De Bono (Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) were grown in liquid culture with *Escherichia coli* strain OP50 as a source of food (20). *Drosophila melanogaster* S2 cells were purchased (Invitrogen, Paisley, UK) and grown in suspension at 20 °C to a cell density of 2×10^7 cells/ml in Express Five serum free media containing 0.05% Pluronic F-68 (1 liter; Invitrogen). Sea urchins (*Evechinus chloroticus*), and sea cucumbers (*Australostichopus mollis*) were a gift from Dr P. Heath (National Institute of Water and Atmospheric Research, Wellington, New Zealand). Reproductive tissue was harvested from sea urchins, and whole specimens of sea cucumbers were used. Samples of *Crella incrustans* were collected from Breaksea Sound (Fiordland, New Zealand) by R. Bishop, K. Schimanski, and K. Blakemore (University of Canterbury, Christchurch, New Zealand).

Live animals were handled according to guidelines provided by the Home Office of the UK Government or in accordance with the Lincoln University Animal Ethics committee and the New Zealand animal welfare act. Where no governmental guidelines exist, invertebrates were killed humanely according to the recommendations of the Royal Society for Prevention of Cruelty to Animals.

Isolation of Mitochondria and Mitochondrial Membranes—Mitochondria were prepared from heart and liver tissue as described previously (21). Mitochondria were recovered by a general procedure 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* (23), *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 Na_2HPO_4, pH 9.2, 100 mM sucrose, and 0.5 mM EDTA, and centrifugation (47,000 $\times$ *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, IF_1, 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).

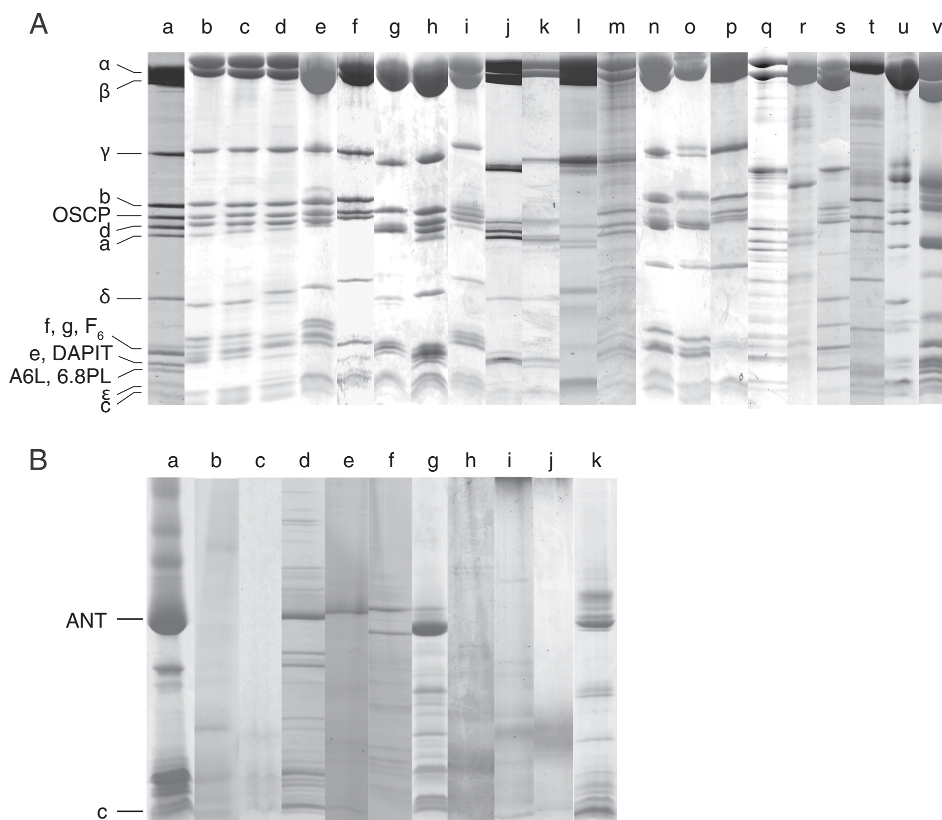

FIG. 1. **Isolation of the c-subunits of F-ATP synthases.** In parts *A* and *B*, respectively, the subunits of F-ATPases isolated by affinity chromatography and the hydrophobic proteins obtained by extraction of mitochondrial membranes with organic solvents were fractionated by SDS-PAGE, and stained with Coomassie blue dye. The tracks in parts *A* and *B* have been assembled from many different gels, as is evident. The c-subunit in each sample was identified by peptide mass fingerprinting of a chymotryptic digest. In part *A*, the positions of the subunits in the human enzyme are indicated on the left. Track a, *Homo sapiens*; b, *Bos taurus*; c, *Ovis aries*; d, *Sus scrofa*; e, *Cervus elaphus*; f, *Mus musculus*; g, *Rattus norvegicus*; h, *Oryctolagus cuniculus*; i, *Gallus gallus*; j, *Anas platyrhynchos*; k, *Testudo graeca*; l, *Boa constrictor*; m, *Xenopus laevis* n, *Salmo salar*; o, *Onchorhynchus mykiss*; p, *Dicentrachus labrax*; q, *Squalus acanthias*; r, *Lumbricus terrestris*; s, *Calliphora vomitoria*; t, *Cancer pagurus*; u, *Homarus gammarus*; v, *Artemia salina.* Part *B*, Analysis by SDS-PAGE of chloroform:methanol extracts from metazoan and plant species. The c-subunit was identified by peptide mass fingerprinting of chymotryptic peptides. The positions of the bovine c-subunit and the adenine nucleotide translocase (ANT) are indicated. Track a, *Bos taurus*; b, *Trichosurus vulpecula* c, *Mytilus edulis*; d, *Crassostrea gigas*; e, *Caenorhabditis elegans*; f, *Drosophila melanogaster*; g, *Trichoplusia ni*; h, *Evechinus chloroticus*; i, *Australostichopus mollis*; j, *Crella incrustans*; k, *Solanum tuberosum*.

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 centrifugation (16,000 × *g*, 10 min, 4 °C). The pellet was suspended by vortexing in nine volumes of a mixture of chloroform/methanol/1 M ammonium formate, pH 3.7 (66.7:31.3:2, by vol.), and then the phases were separated by centrifugation (16,000 × *g*, 10 min, 4 °C). The upper phase, and a precipitate at the interface were removed, and the proteins in the lower phase were precipitated overnight at −20 °C with four vols. of diethyl ether. The precipitate was redissolved in the chloroform/methanol/ammonium formate mixture containing 0.2% (w/v) SDS, and evaporated to dryness *in vacuo*. The residue was redissolved in 2% (w/v) lithium dodecyl sulfate and analyzed by SDS-PAGE.

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) and Bioanalyst (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 $CaCl_2$. 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.; Nanoseparations, 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. 1*A* parts a-q and r-v). From tracks a–q in Fig. 1*A*, it is evident that the subunit compositions of the vertebrate enzymes are very similar, and it has been confirmed elsewhere, that those of human, bovine, porcine, and ovine enzymes are identical (28). Attempts to purify the F-ATPases from some other invertebrate mitochondria were unsuccessful, mainly because the available material was insufficient to allow appropriate variation of the experimental parameters. In these instances, the c-subunit and other proteolipids were extracted from mitochondrial membranes with an organic solvent and fractionated by SDS-PAGE (Fig. 1*B*). The c-subunit was identified in both the purified F-ATPases and the solvent extracts by mass spectrometric analysis as a diffuse band running at the foot of the gels.

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 $\pm$ 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 spur-thighed tortoise, *T. graeca,* the European sea bass, *D. labrax*, 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

TABLE I
Molecular masses of intact c-subunits from metazoan F-ATPases

Species	Molecular mass (Da)		
	Calculated[a,b]	Observed[c]	Δ
Homo sapiens	7608.0	7650.4	42.4
Bos taurus	7608.0	7650.5	42.5
Ovis aries	7608.0	7650.3	42.3
Sus scrofa	7608.0	7650.2	42.2
Oryctolagus cuniculus	7608.0	7650.0	42.0
Mus musculus	7608.0	7650.3	42.3
Rattus norvegicus	7608.0	7650.3	42.3
Cervus elaphus	7608.0	7650.4	42.4
Trichosurus vulpecula	n.s.	7649.7	
Gallus gallus	7608.0	7650.2	42.2
Anas platyrhynchos	7608.0	7650.0	42.0
Testudo graeca	n.s.	7650.1	
Xenopus laevis	7608.0	7650.2	42.2
Salmo salar	7608.0	7650.5	42.5
Onchorhynchus mykiss	7608.0	7650.3	42.3
Dicentrachus labrax	n.s.	7650.3	
Squalus acanthias	n.s.	7649.0	
Calliphora vomitoria	7642.0	7684.4	42.4
Lumbricus terrestris	7724.1[d]	7766.4	42.3
Homarus gammarus	n.s.	7641.4	
Cancer pagurus	n.s.	7670.0	
Crassostrea gigas	7494.7	7537.3	42.6
Mytilus edulis	7534.9[e]	7576.9	42.0
Trichoplusia ni	7642.0[f]	7684.0	42.0
Caenorhabditis elegans	7580.9	7623.1	42.2
Drosophila melanogaster	7642.0	7684.0	42.0
Artemia salina	n.s.	7670.0	
Evechinus chloroticus	7620.0[g]	7662.0	42.0
Australostichopus mollis	n.s.	7709.0	

[a] calculated from the sequence of the c-subunit.
[b] n.s., sequence not known.
[c] measured by LC-ESI-MS.
[d–g] calculated from sequences of c-subunits in *Lumbricus rubellus*, *Mytilus galloprovincalis*, *Manduca sexta*, and *Strongylocentrotus purpuratus,* respectively.

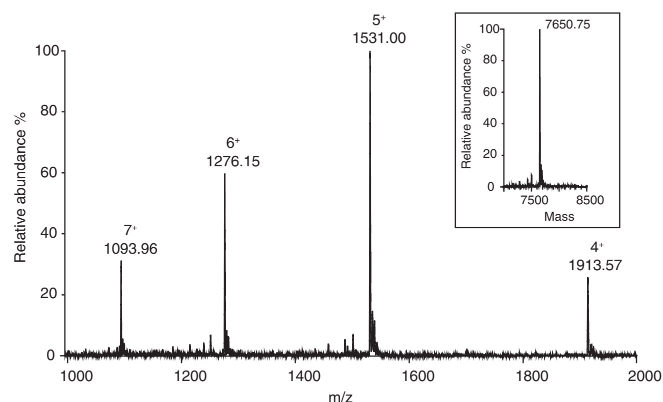

FIG. 2. **Mass spectral analysis of the intact c-subunit of the F-ATPase from *Salmo salar* by ESI-MS.** A series of multiply charged ions from the c-subunit are indicated. The insert contains a mathematical transformation of these data to a molecular mass scale.

TABLE II
The site of trimethylation in c-subunits of F-ATPases from metazoans

Species	Modified chymotryptic peptide				
	MH$^+$ Calculated	MH$^+$ Observed[a]	Δ	TM-Lys[b]	Position[c]
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
Oryctolagus 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	n.s.	1343.7937	n.s.	+	n.s.
Xenopus laevis	1301.7325	1343.7949	42.0624	+	K43
Salmo salar	1301.7325	1343.7934	42.0609	+	K43
Onchorhynchus mykiss	1301.7325	1343.7943	42.0618	+	K43
Dicentrachus labrax	n.s.	1343.7940	n.s.	+	n.s.
Squalus acanthias	n.s.	1343.7949	n.s.	+	n.s.
Calliphora vomitoria	1301.7325	1343.7940	42.0615	+	K43
Lumbricus terrestris	1301.7325[d]	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	1244.7110[e]	1286.7657	42.0547	+	K43
Trichoplusia ni	1301.7325[f]	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	1301.7325[g]	1343.7946	42.0621	+	K43
Australostichopus mollis	n.s.	1343.7940	n.s.	+	n.s.
Crella incrustans	n.s.	1343.7949	n.s.	+	n.s.

[a] accurate mass measurement of triply charged ions in an OrbiTrap MS.
[b] TM-Lys, trimethyllysine.
[c] location of modification; for meaning of [d–g] see footnotes in Table I.

North Atlantic mussel, *M. edulis*, exceeds by 42 Da the calculated value for the Mediterranean mussel, *M. galloprovincalis,* 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, *Stronglyocentrotus purpuratus*, and of the c-subunit from the earthworm, *L. terrestis*, in comparison with the calculated value from the red earthworm, *L. rubellus*. 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 frugipera*, 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. pagarus*, 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

Helix A — Helix B

Species	10 20 30 40 50 60 70	Class
HOMSA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
BOVIN	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
CANFA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
DASNO	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
EQUCA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
OVIAR	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
SUSSC	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
CEREL	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
MONDE	DVDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAL	mammalia
MYOLU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
ORNAN	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
TUPCI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
GALVA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
TRIMA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
LOXAF	DVDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
ELEED	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
SARHA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
CHRAS	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
MUSMU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
ORYCU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
RATNO	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
TURTR	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	mammalia
ANOCA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	reptilia
PELSI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	reptilia
PYTBI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	reptilia
ANAPL	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
GALGA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
TAEGU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
CALAN	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
FALPE	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
MELUN	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
COLLI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
APTFO	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	aves
XENLA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	amphibia
AMBME	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	amphibia
DANRE	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ONCMY	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
SALSA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
TAKRU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
CYNSE	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ORYLA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
POERE	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ANOFI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ORENI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
PERFL	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
OSMMO	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ESOLU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ASTME	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
CYPCA	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
ICTPU	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
LEPOC	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	actinopterygii
LATCH	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	sarcopterygii
CALMI	DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM	chondrichthyes

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 c_8-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, *Dasypus 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, *Chrysochloris asiatica* (Cape golden mole); MUSMU, *Mus musculus* (mouse); ORYCU, *Oryctolagus cuniculus* (rabbit); RATNO, *Rattus norvegicus* (rat); TURTR, *Tursiops truncates* (bottle nosed dolphin); ANOCA, *Anolis carolinesis* (green anole); PELSI, *Pelodiscus sinensis* (Chinese softshell turtle); PYTBI, *Python bivittatus* (Burmese python); ANAPL, *Anas platyrhynchus* (wild duck); GALGA, *Gallus gallus* (chicken); TAEGU, *Taenio guttat* (zebrafinch); CALAN, *Calypte anna* (Anna's hummingbird); FALPE, *Falco peregrinus* (peregrine falcon); MELUN, *Melopsittacus undulatus* (budgerigar); COLLI, *Columba livia* (rock pigeon); APTFO, *Aptenodytes forsteri* (emperor penguin); XENLA, *Xenopus laevis* (West African clawed toad); AMBME, *Ambystoma mexicanum*

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 ARNPSLKQQLF, particularly in the N-terminal region, but usually these spectra did not contain sufficient information to allow the modification to be localized definitively, and therefore other analyses were conducted, as described below. Peptides with masses of 1315.75 and 1286.76 Da, were observed in the chymotryptic digests of the c-subunits from *C. gigas* and *M. edulis*, respectively, and their fragment ion spectra (supplemental Fig. S1) also contained abundant ions with masses 59 Da less than the parent ions, again providing evidence for trimethylation rather than acetylation of these peptides.

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 trimethylation (33). The masses of all of the modified chymotryptic peptides were significantly closer to the theoretical masses of the trimethylated peptide (supplemental Table S1).

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. galloprovincalis* 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 ARNPSLKNNLF, differing from the vertebrate sequence by the substitutions Q44N and Q45N (Fig. 4).

DISCUSSION

Conservation of the c_8-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 F_1-c_8-ring complex can be taken as being representative of the c_8-rings that are almost certainly found in the F-ATPases in all of these species. A cross-section of the structures of the bovine c_8-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 c_8-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 c_8-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 c_8-rings (4). Among the seventy invertebrate c-sequences shown in Fig. 4, in all but five species the alanines are absolutely conserved,

(axolotl); DANRE, *Danio rerio* (zebrafish); ONCMY, *Onchorhynchus mykiss* (rainbow trout); SALSA, *Salmo salar* (salmon); TAKRU, *Takifugu rubripes* (pufferfish); CYNSE, *Cynoglossus semilaevis* (tongue sole); ORYLA, *Oryzias latipes* (Japanese medaka); POERE, *Poecilia reticulata* (guppy); ANOFI, *Anoplopoma fimbria* (sablefish); ORENI, *Oreochromis niloticus* (Nile tilapia); PERFL, *Perca flavescens* (yellow perch); OSSMO, *Osmerus mordax* (rainbow smelt); ESOLU, *Esox lucius* (northern pike); ASTME, *Astyanax mexicanus* (Mexican tetra); CYPCA, *Cyprinus carpio* (common carp); ICTPU, *Ictalurus punctatus* (channel catfish); LEPOC, *Lepisosteus oculatus* (spotted gar); LATCH, *Latimeria chalumnae* (coelocanth); CALMI, *Callorhinchus milii* (elephant shark).

Helix A — Helix B

	Species	10 20 30 40 50 60 70	Phylum
	BRABE	DIDTAAKFIGAGAATVGAAGSGAGIGTVFGSLCIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFVILFAM	chordata
	CIOIN	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAL	chordata
	CIOSA	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFTYAILGFALSEAMGLFCLMVAFLILFAL	chordata
†	STRPU	DVEAAAKFIGAGAATVGLAGSGAGIGTVFGSLIIGYARNPSLKQQLFTYAILGFALSEAMGLFCLMMAFLILFAL	echinodermata
	HELRO	DIDQAAKYIGAGAATVGVAGSGAGIGSVFGSLVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAF	annelida
	GLYTR	DIDQAAKYIGAGAATVGVAGSGAGIGSVFGSLVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAF	annelida
	PLADU	DIDQAAKYIGAGAATVGVAGSGAGIGSVFGSLVIGYARNPSLKQQLFSYAILGFALSEA----------------	annelida
	CAPTE	DIDQAAKYIGAGAATIGAGGGSGAGIGSVFGSLVIGFARNPSIKGQLFSYTILGFALSEAMGLFCLMMAFLILFAF	annelida
	HYDEL	DIDQAAKYIGAGCATAGVAGSGAGIGTVFGSLMISVARNPSMKAQLFSYAILGFALSEAMGLFCLMIAFLFAVRY	annelida
	RIFPA	--------IGAGAATVGVAGSGAGIGSVFGSLVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAF	annelida
†	LUMRU	DIDQAAKYIGAGAATVGVAGSGAGIGSVFGSLVMGYARHPVLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAF	annelida
	ACYPI	DIDSAAKFIGAGAATVGIAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	APIME	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIVGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	CULEX	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	DROME	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	CALVO	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	GLOMM	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	IXOSC	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	LITVA	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
†	MANSE	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	NASVI	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	OPICA	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSPIIGYARYPSLIQQLFSYAILGFALSEAMGLFCLMVFLLLFAF	arthropoda
	STRMA	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	PEDHU	DIDSAAKYIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMSFLLLFAF	arthropoda
	PENJP	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	ACAPA	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	SIMVI	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
†	SPOFR	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	STEMI	DIDSAAKFIGAGAATVGVAGSGAGIGSVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	STOCA	DIDSAAKFTGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	TRICA	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAF	arthropoda
	APLCA	DIDQAAKYIGAGAATVGVAGSGAGIGSVFGSLVIGYARNPSLKGQLFSFAILGFALSEAMGLFCLMMAFLLLFAF	mollusca
	HALDI	DIDQAAKYIGAGAATVGVAGSGAGIGSVFGSLVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLLLFAL	mollusca
	LOTGA	DIDQAAKYIGAGAATAGAAGSGAGIGSVFGSLVLAFARNPSLKQQLFSYAILGFALSEAMGLFCIMMAFLFLFAL	mollusca
	SINCO	DIDQAARFIGAGAATVGVAGSGAGIGSIFGSLCIAYARNPSLKQQLFTYAVLGFALAEAMGLFCLMMAFMIVYIL	mollusca
†	MYTGA	DIDQAAKYIGAGAATVGAAGSGAGIGNVFGALVVGYARNPSLKQALFSYAILGFALSEAMGLFCIMVAFMILFAL	mollusca
	CRAGI	DIDQAAKYIGAGAAAVGAAGSGSGIGTVFGSLIIGYARNPSLKNNLFTYAVLGFALSEAMGLFCFLLAAGILFAF	mollusca
	ECHGR	DIDQAAKYIGAGAATIGVAGSGAGIGSVFGNLVMGYARNPGLKQQLFSYAILGFALSEAMGLFCLMMAFLILYAF	platyhelminthes
	OPIVI	DIDQAAKYIGAGAATVGAAGSGAGIGSVFGNLVIGYARNPGLKQQLFSYAILGFALSEAMGLFCLMMAFLILYAF	platyhelminthes
	CLOSI	DIDQAAKYIGAGAATVGAAGSGAGIGSVFGNLVIGYARNPGLKQQLFSYAILGFALSEAMGLFCLMMAFLILYAF	platyhelminthes
	HYMMI	DIDQAAKYIGAGAATIGVAGSGAGIGSVFGNLVMGYARNPGLKQQLFSYAILGFALSEAMGLFCLMMAFLILYAF	platyhelminthes
	SCHMA	DIDQAAKYIGAGAATVGCAGSGAGIGSVFGSLTLAYARNPGLKQQLFTYAILGFALSEAMGLFCLVMAFLILYVF	platyhelminthes
	BRUMA	DIDSAAKYVGAGAATVGVAGSGAGIGNVFGSLVIGYARNPSAKNQLFSYAILGFALSEAMGLFCLCMGFCILFAF	nematoda
	CAEEL	DIDSAAKYIGAGAATVGVAGSGAGIGNVFGALVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLTMGFMILFAL	nematoda
	MELHA	DIDSAAKYIGAGAATAGVAGSGAGIGNVFGSLLIGFARNPSLKQQLFSYAILGFALSEAMGLFCLTMGFLILFAL	nematoda
	NECAM	DIDSAAKYIGAGAATVGVAGSGAGIGNVFGALVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLTMGFMILFAL	nematoda
	HAECO	DIDSAAKYIGAGAATVGVAGSGAGIGNVFGALVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLTMGFMILFAL	nematoda
	ANCCE	DIDSAAKYIGAGAATVGVAGSGAGIGNVFGALVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLTMGFMILFAL	nematoda
	LOALO	DIDSAAKYVGAGAATVGVAGSGAGIGNVFGSLVIGYARNPSAKNQLFSYAILGFALSEAMGLFCLTMGFCILFAF	nematoda
	ASCSU	DIDSAAKYIGAGAATVGVAGSGAGIGNVFGALVIGYARNPSLKAQLFSYAILGFALSEAMGLFCLTMGFMILFAL	nematoda
	TRIAD	DIDSAAKFIGAGAATVGVAGSGAGIGTVFGSLVIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAL	placozoa
	CARBA	DIETAAKFIGAGAATVGAAGSGAGIGTVFGSLVMGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAL	cnidaria
	NEMVE	DVDSAAKFIGAGAATVGAAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMMAFLILFAL	cnidaria
	HYDMA	DIEQAAKFIGAGAATVGCAGSGAGIGTVFGSLIIGYARNPSLKPQLFSYAILGFALSEAMGLFSLMMSFLILFGL	cnidaria
	PLEBA	DESLEQAAKFIGAGCATVGVAGSGAGIGTVFGSLVEGYARNPSLKTQLFSYCVLGFALSEAMGLFCLMIAFMILFAL	ctenophora
	MNELE	DESLEQAAKFIGAGCATVGVAGSGAGIGTVFGSLVEGYARNPSLKTQLFSYCVLGFALSEAMGLFCLMIAFMILFAL	ctenophora
	AGESC	MAAEILTAAKFVGAGAASIGAAGSGAGIGTVFGNLIIGYARNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLILFGL	porifera
	APHVA	MRDNLTFCAKLIGAGAATIGVAGRGAGIGTVFGNLIIGYARNPKLKQQLFTYAILGFAISEAMGLFCLMMAFLILYGI	porifera
	APLFU	MTVEILSAAKFVGAGAATIGAAGSGAGIGSVFGNLIIGYARNPSLKQQLFTYAILGFALSEAMGLFCLMMAFLILFAL	porifera
	ECTFE	MATEILTAAKYVGAGAASIGAAGSGAGIGTVFGNLIIGYSRNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLLLFGL	porifera
	HALDU	MTPEILSAAKFVGAGAATIGAAGSGAGIGTVFGNLIIGYSRNPSLKQQLFTYAILGFALSEAMGLFCLMMAFLLLYAF	porifera
	HIPLA	MSELMDAARYIGAGAATIGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLLLFAL	porifera
	IGENO	MTTEILSASKFIGAGAATIGVAGSGAGIGTVFGNLIIGYARNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLILFAL	porifera
	IRCST	MSELMDAARYIGAGAATIGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLLLFAL	porifera
	OSCCA	MTELS--AKFIGAGAATVGAAGSGAGIGTVFGSLVIGYARNPSLKQQLFTYAILGFALSEAMGLFCLMMAFLILFGL	porifera
	SUBDO	MATEILTGAKFVGAGAASIGAAGSGAGIGTVFGNLIIGYARNPSLKQQLFAYAILGFAISEAMGLFCLMIAFLILFGL	porifera
	TOPOP	MATEILTAAKYVGAGAASIGAAGSGAGIGTVFGNLIIGYSRNPSLKQQLFTYCILGFAISEAMGLFCLMMAFLILFGL	porifera
	CLACL	TETLLKCGKFIGAGAATIGVSGSGAGIGFVFGNYLIAMSRNPALSGQMFNYALLGFALSEAMALFALMIAFLILFGL	porifera
	VACSP	MSELMDAARYIGAGAATIGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLLLFAL	porifera
	AXICO	MATEILTAAKFVGAGAASIGAAGSGAGIGTVFGNLIIGYARNPSLKQQLFTYAILGFALSEVMGLFCLMMAFLILFAL	porifera
	GEONE	MATEILTAAKFVGAGAASIGAAGSGAGIGTVFGNLIIGYARNPSLKQQLFTYAILGFAISEAMGLFCLMMAFLILFAL	porifera

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

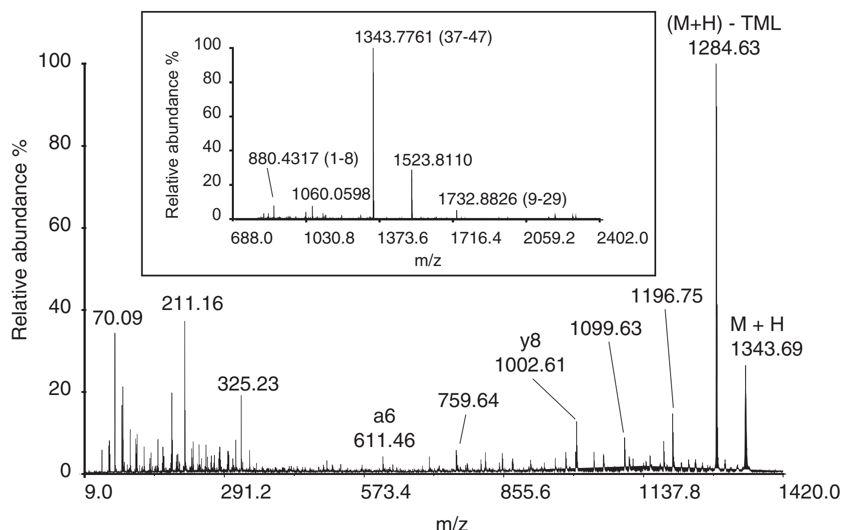

FIG. 5. **Tandem-MS analysis of a chymotryptic peptide from the c-subunit of the F-ATPase from *Salmo salar*.** The singly charged chymotryptic peptide (MH^+ 1343.69) corresponds to residues 37–47 of the protein. The prominent ion at *m/z* 1284.63 arises by loss of a trimethylammonium ion and is diagnostic 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* 1060.0598 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 c_8-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).

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 termini of

codes on the left, see the legend to Fig. 6. BRABE, *Branchiostoma belcheri* (Japanese lancelet); CIOIN, *Ciona intestinalis* (vase tunicate); CIOSA, *Ciona savignyi* (solitary sea squirt); STRPU, *Stronglyocentrotus purpuratus* (purple sea urchin); HELRO, *Helobdella robusta* (Californian leech); GLYTR, *Glycera tridactyla*; PLADU, *Platynereis dumerilii* (Dumeril's clam worm); CAPTE, *Capitella teleta*; HYDEL, *Hydroides elegans*; RIFPA, *Riftia pachyptila* (giant tube worm); LUMRU, *Lumbricus rubellus* (red earthworm); ACYPI, *Acyrthosiphon pisum* (pea aphid); APIME, *Apis mellifera* (honeybee); CULEX, *Culex pipiens* (common house mosquito) DROME, *Drosophila melanogaster* (fruit fly); CALVO, *Calliphora vomitoria* (blue bottle fly); GLOMM, *Glossina morsitans* (Savannah tsetse fly); IXOSC, *Ixodes scapularis* (black legged tick); LITVA, *Litopenaeus vannamei* (white leg shrimp); MANSE, *Manduca sexta* (tobacco hawkmoth); NASVI, *Nasonia vitripennis* (jewel wasp); OPICA, *Opisthacanthus cayaporum* (South American scorpion); STRMA, *Strigamia maritima* (European centipede); PEDHU, *Pediculus humanus* (head louse); PENJP, *Peneus japonica* (Kuruma prawn); APACA, *Acartia pacifica* (copepod); SIMVI, *Simulium vittatum* (black fly); SPOFR, *Spodoptera frugiperda* (fall armyworm); STEMI, *Stegodyphus mimosarum* (social spider); STOCA, *Stomoxys calcitrans* (stable fly); TRICA, *Tribolium castaneum* (red flour beetle); APLCA, *Aplysia californica* (California sea hare); HALDI, *Haliotis diversicolor* (variously colored abalone); LOTGA, *Lottia gigantea* (owl limpet); SINCO, *Sinonovacula constricta* (Chinese razor clam); MYTGA, *Mytilus galloprovincalis* (Mediterranean mussel); CRAGI, *Crassostrea gigas* (Pacific oyster); ECHGR, *Echinococcus granulosus* (hydatid worm); OPIVI, *Opisthorchis viverrini* (Southeast Asian liver fluke); CLOSI, *Clonorchis sinensis* (Chinese liver fluke); HYMMI, *Hymenolepis microstoma* (rodent tapeworm); SCHMA, *Schistosoma mansoni* (blood fluke); BRUMA, *Brugia malayi*; CAEEL, *Caenorhabditis elegans*; MELHA, *Meloidogyne hapla* (northern root knot nematode); NECAM, *Necator americanus* (New World hookworm); HAECO, *Hemonchus contortus* (barber pole worm); ANCCE, *Ancylostoma ceylanicum* (hookworm); LOALO, *Loa loa* (eye worm); ASCSU, *Ascaris suum* (pig roundworm); TRIAD, *Trichoplax adhaerans* ; CARBA, *Carukia barnesi* (Irukandji jellyfish); NEMVE, *Nematostella vectensis* (starlet sea anenome); HYDMA, *Hydra magnipapillata* (hydra); PLEBA, *Pleurobrachia bachei* (sea gooseberry); MNELE, *Mnemiopsis leidyi* (sea walnut); AGESC, *Agelas schmitdi* (brown tubular sponge); APHVA, *Aphrocallistes vastus* (cloud sponge); APLFU, *Aplysina fulva* (rope sponge); ECTFE, *Ectyoplasia ferox* (brown encrusting octopus sponge); HALDU, *Halisarca dujardini*; HIPLA, *Hippospongia lachne* (sheepswool sponge); IGENO, *Igernella notablis*; IRCST, *Ircinia strobilina* (black ball sponge); OSCCA, *Oscarella carmela*; SUBDO, *Suberites domuncula*; TOPOP, *Topsentia ophiraphidites*; CLACL, *Clathrina clathrus* (Mediterranean sponge); VALSP. *Vaceletia sp.*; AXICO, *Axinella corrugate* (marine sponge); GEONE, *Geodia neptuni* (leathery barrel sponge).

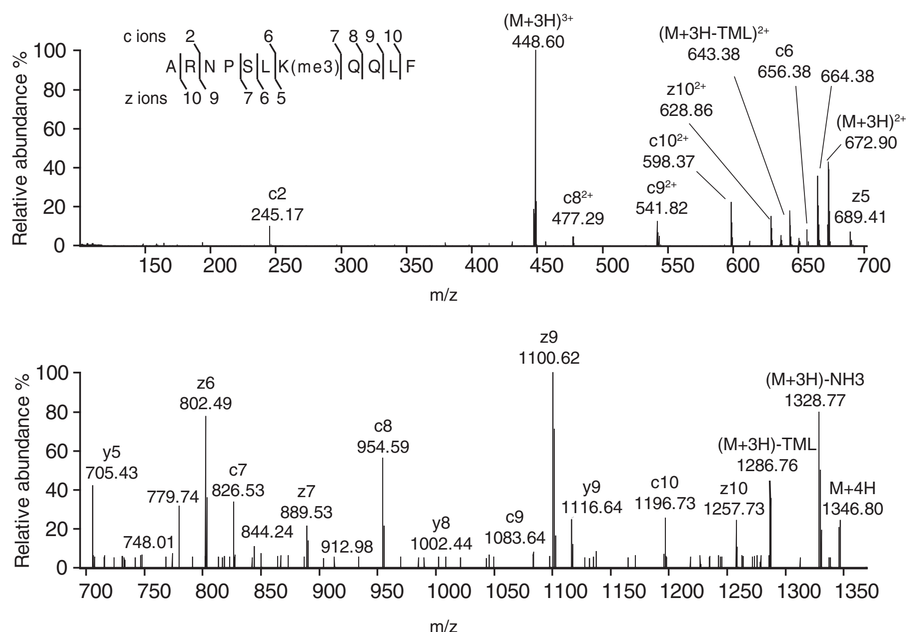

FIG. 6. **Tandem MS of a chymotryptic peptide from the c-subunit of *Salmo salar*.** The peptide represents residues 37–47 of the protein. A triply charged version (*m/z* 448.60) was fragmented by ETD, and the fragments were analyzed in an OrbiTrap instrument. The upper and lower panels contain ions 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.

protein, respectively, as is Ile2Val in the elephant. Therefore, it is reasonable to conclude from the identity or near identity of the sequences of c-subunits that all of the vertebrate F-ATPases will not only contain c_8-rings, but that on the basis of the current investigations, that all vertebrate c-proteins will contain a fully trimethylated lysine-43. It has been estimated that there are ~50,000 vertebrate species on Earth today (34).

Likewise, lysine-43 of the c-subunit of the F-ATPase is trimethylated completely in the 12 invertebrates that were examined. The species are derived from six major phyla, namely echinoderms, annelid worms, molluscs, arthropods, nematode worms, and sponges (see Fig. 7). Moreover, the lysine residue is absolutely conserved in almost all invertebrate c-subunits of known sequence (Fig. 4), with the exception of the South American scorpion, *Opisthacanthus cayaporum*, where it is evidently replaced by an isoleucine residue, and the Mediterranean sponge, *Clathrina clathrus*, where the lysine is replaced with a serine residue. Given the otherwise absolute conservation, it seems likely that the presence of isoleucine and serine at this position has arisen from DNA sequencing errors, and that in reality the lysine is also conserved in *O. cayaporum* and *C. clathrus*. Therefore, it is not unreasonable to suggest that lysine-43 of c-subunits will be trimethylated completely in F-ATPases in these phyla, and possibly also in the invertebrate phyla that are not represented in the sequence alignments (Nemertea, Onychophora, Tardigrada, Gastrotricha, Priapulida, Loricifera, Rotifera, Kinorhyncha, Gnathostomulida, Entoprocta, Cycliophora, Phoronida, Brachiopoda, Bryozoa, and Chaetognatha). It has been estimated that there are ~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, icthyosporea, nuclearid amoebae, and fungi form kingdoms within the opisthokonts (supplemental Fig. S3). The sequences of c-subunits from representatives of other opisthokont kingdoms (choanoflagellates, filasterea, icthyosporea, and fungi) show that lysine-43 is conserved except in the fungus, *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, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, it is not methylated. Also, the three alanines in the N-terminal α-helix that are conserved in metazoans are frequently mutated to amino acids with large side chains. It is known that the c-ring in *S. cerevisiae* has 10-fold symmetry (7), and is likely that all of these species form c-rings with symmetries greater than eight. These trends are found also in other groups in supplemental Fig. S4, and in the mitochondria from the potato, *S. tuberosum*, the lysine occurs one residue later in the sequence, and it is unmethylated. The chromoalveolates have no basic residues at all within the vicinity of the loop between the two α-helices of subunit c.

Among the prokarya, 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 *Ilyobacter tartari-*

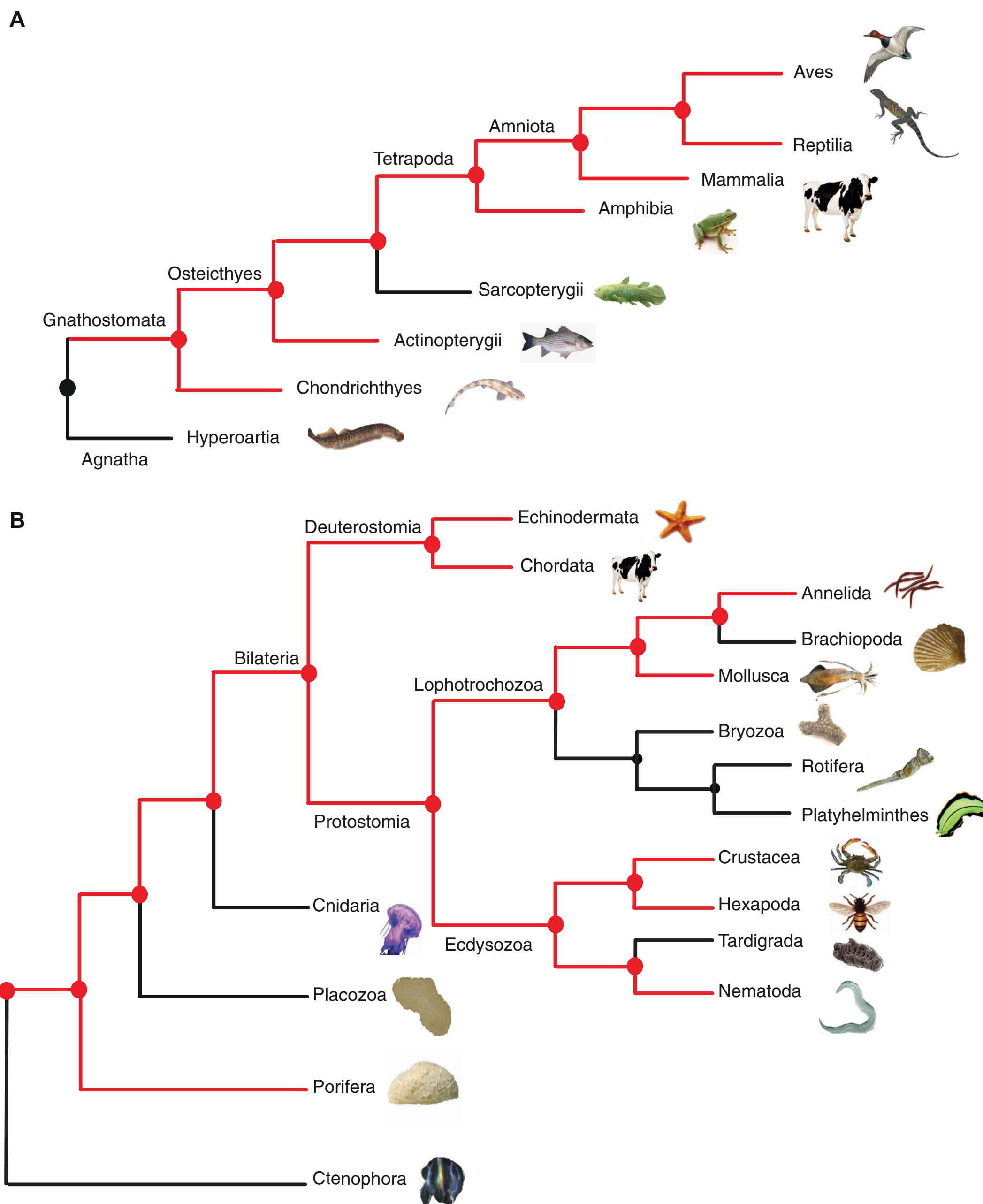

FIG. 7. **Metazoan tree of life.** Part *A*, the vertebrate tree. Part *B*, the major metazoan phyla. The red branches contain species where lysine-43 in the c-subunit of mitochondrial F-ATP synthase has been demonstrated to be trimethylated. In part *A*, the analyzed examples are as follows: Mammalia, *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 gracea*, *Boa constrictor*; Amphibia, *Xenopus laevis*; Actinopterygii, *Salmo salar*, *Onchorhynchus mykiss*, *Dicentrachus labrax*; Chondrichthyes, *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 in 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).

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S This article contains supplemental Figs. S1 to S5 and Tables S1 and S2.

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