Characterisation of the c-subunit of F-ATP synthase in metazoans

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

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by

Huibing Jiang

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This evolutionary bioenergetics study was shared between the Mitochondrial Biology Unit (MBU) Cambridge UK and Lincoln University. The whole project has been published in advance of submission of this thesis for examination as Thomas B. Walpole, David N. Palmer, **Huibing Jiang**, Shujing Ding, Ian M. Fernley, and John E. Walker in *Molecular and Cellular Proteomics*, 14: 828-840 (2015). This paper has been reprinted at the end of this thesis, Appendix 1 page 62

There have also been a number of conference presentations of all or parts of this work including: Gorden Research Conference (2011, New Hampshire), Graduate Student Poster Conference (2012, Christchurch).

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science.

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The mitochondrial F-ATP synthase synthesises ATP molecules, the energy currency of life, by utilising the proton transmembrane gradient force generated from the oxidation of nutrients in the electron transport chain. The bioenergetic cost (in protons) to make one ATP molecule is proportional to the size of the c-ring (i.e. number of the c-subunits) of the protein. Traditionally, this was thought to be 3.3-5 protons, until the recent discovery that it is more efficient in bovine ATP synthase. Compared with the c_{10-15} F-ATP synthase driving rings in yeast, eubacterial, and chloroplast ATP synthases, the bovine molecular machine has a c_8-ring instead. Studies indicated that trimethylation of lysine 43 of the c-subunit is critical for the formation of the c_8-ring. Since the amino acid sequences of c-subunits are identical in almost all vertebrates and are highly conserved across other Metazoans, it is suggested that all vertebrates and probably all, or most, invertebrates would contain c_8-rings and the bioerengetic cost to produce one ATP molecule is 2.7 protons in all animals. In the present project, this hypothesis was examined by isolation of whole F-ATP synthase complex or c-subunits alone purified from selected animals by applying a GST tagged ligand purification method or by iodixanol gradient purification of mitochondria followed by chloroform/methanol extraction of the c-subunits. C-subunits were then separated on SDS-PAGE and several mass spectrometric techniques used to characterise any modifications of the c-subunits. C-subunits from possum, kina, sea cucumbers and sponge were isolated and characterised in this thesis and a total of eighteen vertebrate and fifteen invertebrate organisms were surveyed in the overall study. In every case, a high intensity peak of mass 1343 was detected when c-subunit chymotryptic digests were analysed by MALDI-TOF MS. Furthermore, peptides from chymotryptic digests of c-subunits analysed by ETD-MS with Orbitrap MS confirmed lysine 43 as the trimethylated residue in all species surveyed in these experiments. We conclude that all metazoan c subunits are trimethylated and their F-ATP synthases are driven by c_8-rings. This evolutionary bioenergetics study was shared between Lincoln University and the Mitochondrial Biology Unit (MBU) Cambridge UK.

Keywords: ATP synthase, ATPase, c_8-ring, c-subunit, subunit c, lysine trimethylation.

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List of Abbreviations

2ME	2-mercaptoethanol
µ-	micro-
^{o}C	degrees celcius
Å	Ångstrom
AAC	ADP/ATP carrier
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATP9	F-ATP synthase c-subunit gene
BCA	bicinchoninic acid
b.p.	base pair
BSA	bovine serum albumin
Da	Dalton
DAPIT	diabetes-associated protein in insulin-sensitive tissue
DCCD	dicyclohexylcarbodiimide
DDM	dodecylmaltoside
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESI-MS	electrospray ionization mass spectrometry
ETC	electron transport chain
ETD-MS	electron transfer dissociation mass spectrometry
F_1	ATP synthase fraction 1
F_6	coupling factor 6
F_o	ATP synthase oligomycin binding fraction
FADH	reduced flavin adenine dinucleotide
fMet-tRNA	N-formylmethionyl-tRNA
g	grams
GSH	reduced glutathione
GST	glutathionse-S-transferase
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPLC	high-performance liquid chromatography
I_{1-60}	residues 1-60 of the inhibitor protein of F_1F_o-ATP synthase

IF_1	inhibitor protein of F_1F_o-ATP synthase
IMM	inner mitochondrial membrane
IMS	inter-membrane space
k-	kilo-
l	litre
LC-ESI-MS	liquid-chromatography-electrospray-ionization-tandem mass spectrometry
LCMS	liquid chromatography mass spectrometry
m-	milli-
M	molar
M-	mega-
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MBU	Mitochondrial Biology Unit
min	minutes
MS	mass spectrometry
mtDNA	mitochondrial DNA
NADH	reduced nicotinamide adenine dinucleotide
NCLs	neuronal ceroid lipofuscinoses
OMM	outer mitochondrial membrane
OSCP	oligomycin sensitivity conferring protein
PAGE	polyacrylamide gel electrophoresis
pH	$-\log[H^+]$
p.m.f.	proton motive force
P_i	inorganic phosphate
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
TCA	tricarboxylic acid
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TOF	time of flight
tRNA	transfer RNA
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
V	voltage
v/v	volume to volume
w/v	weight to volume

1 Introduction

1.1 The mitochondrion

The mitochondrion is a double membrane-bound organelle which is found in most eukaryotic cells. Each mitochondrion contains two membranes, a smooth outer mitochondrial membrane (OMM) and an extensively invaginated inner mitochondrial membrane (IMM). The invaginations are called cristae. The space between the two membranes is referred as the inter-membrane space (IMS), and the space encapsulated by the IMS is termed the matrix. *Figure 1-1* shows the overall structure of a mitochondrion.

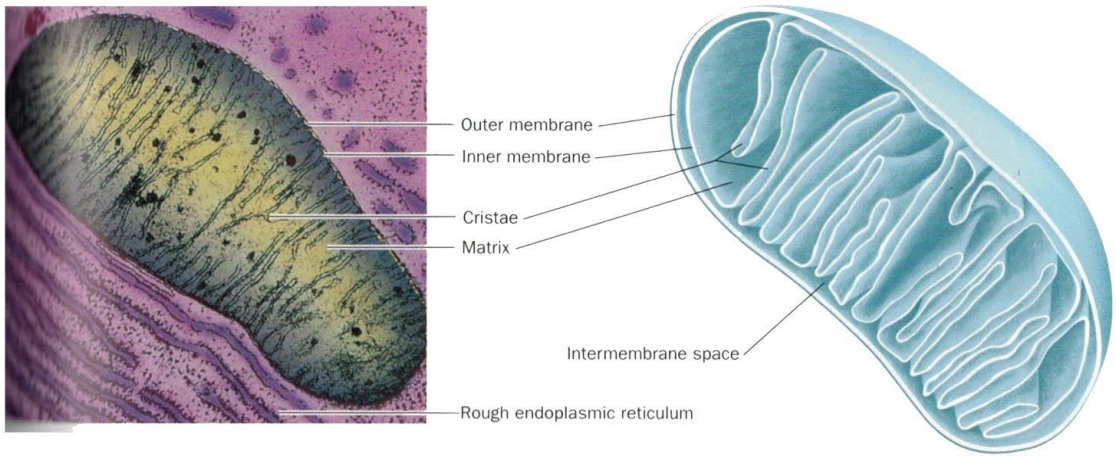


Figure 1-1 The mitochondrion

Left: An electron micrograph of an animal mitochondrion. Right: A cutaway diagram of a mitochondrion (Figure adapted from Voet et al., 2006).

Mitochondria are thought to have arisen by a fusion between a bacterium and an archaeon; this called the endosymbiotic theory. It is supported by the presence of a small circular genome of bacterial origin within the mitochondria (Anderson et al., 1981; Martin & Muller, 1998; Sagan, 1967). During its evolution, the endosymbiont has transferred most of its mitochondrial genes into the cellular nucleus, and only a few are retained in the mitochondrial DNA (mt DNA) (section 1.1.2). The mitochondrial DNA encodes a bacteria-like protein synthesis machinery, including ribosomal RNAs and tRNAs as well as a number of mitochondrial proteins, the number diminishing the more recently species were established. One reason the synthesis of these proteins may be retained in the mitochondrion is that their highly hydrophobic properties make them difficult to import.

Mitochondria supply cells with the metabolic energy required to maintain life, in the form of adenosine triphosphate (ATP) synthesised through the process of oxidative phosphorylation. Oxidative phosphorylation utilises the products generated by the oxidation of nutrients to generate an electrochemical gradient across the inner mitochondrial membrane. Figure 1-2 shows the metabolic process of breaking one molecule of glucose to generate 10 molecules of NADH and two molecules of $FADH_2$.

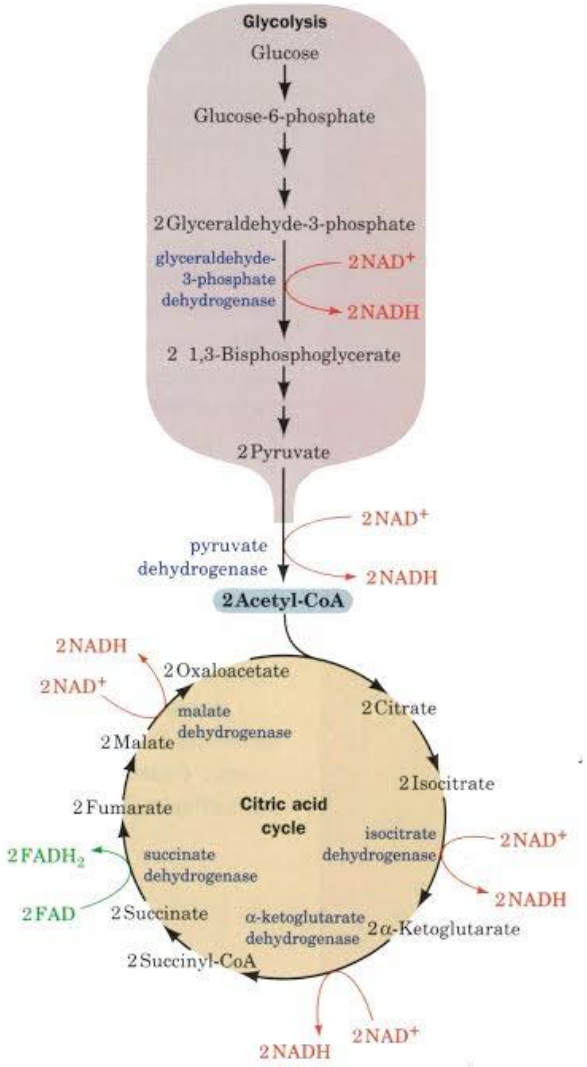


Figure 1-2 Glycolysis and the citric acid cycle generate NADH and $FADH_2$ to fuel the oxidative phosphorylation

1 mole of glucose generates 10 moles of NADH and 2 moles of $FADH_2$. NADH is indicated in red and $FADH_2$ is in green (Figure adapted from Voet et al., 2006).

1.1.1 The electron transport chain

The series of enzymes involved in oxidative phosphorylation are called the electron transport chain (ETC). This consists of protein complexes embedded in the inner mitochondrial membrane, namely complex I (NADH:ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (cytochrome bc_1 complex), complex IV (cytochrome c oxidase) and complex V (ATP synthase) (For a review see: Jacoby et al., 2012). Electrons captured from donor molecules (i.e. NADH and $FADH_2$) are transferred through these complexes, generating an electrical potential across the membrane ($\Delta\Psi$), by the pumping of hydrogen ions to generate a difference in the proton concentration on each side of the membrane (ΔpH), referred to as the proton motive force (p.m.f.). The p.m.f. drives ATP synthesis by complex V (ATP synthase).

For every two electrons from NADH that pass through the ETC in mammalian mitochondria, a total of ten protons are translocated across the IMS, four from complex I, four from complex III, and two from complex IV respectively (Figure 1-3). Complex II is the only respiratory complex that does not pump protons. It oxidises succinate to fumarate via $FADH_2$, and provides two ubiquinols which in turn translocate six protons into the IMS, through complex III and IV.

As mentioned above (section 1.1), 1 molecule of glucose generates 10 molecules (i.e. 10 pairs of electrons) of NADH and 2 molecules (i.e. 2 pairs of electrons) of $FADH_2$, and each of them results in 10 and 6 protons translocated across the IMM respectively. Hence there are 112 protons translocated (10x10 + 2x6) per glucose consumed.

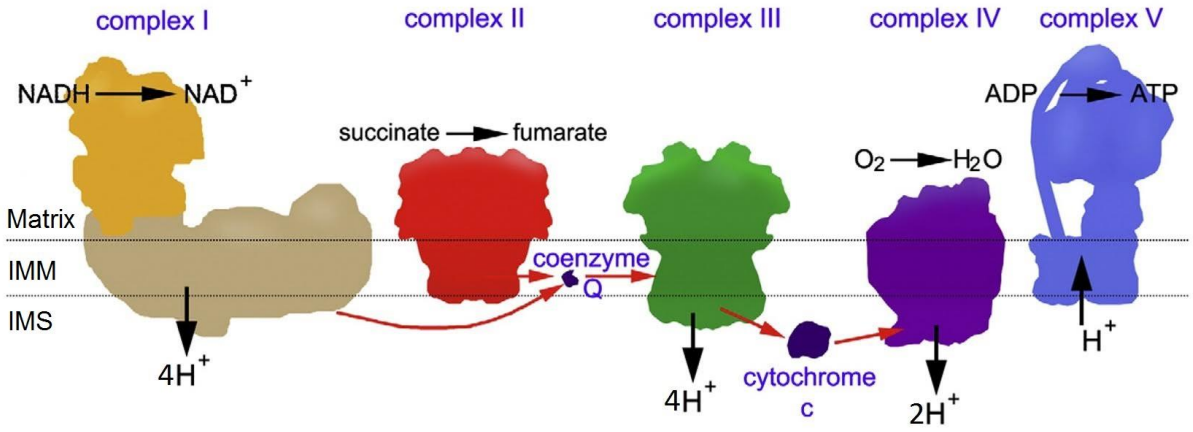


Figure 1-3 A schematic representation of the electron transport chain

NADH and succinate are provided by the citric acid cycle. Complexes I, III and IV pump protons across the membrane to generate a p.m.f. which in turn triggers the production of ATP by complex V. Complex II oxidises succinate to fumarate. It does not pump any protons. The pathway of electrons is indicated by red arrows, whilst translocations of protons are indicated by black arrows. The positions of the matrix, inner mitochondrial membrane IMM), and inter membrane space (IMS) are indicated (Figure adapted from Dudkina et al., 2010).

1.1.2 The mitochondrial genome

Human mitochondrial DNA (mtDNA) is 16.6 kbp in length and encodes a total of 37 genes, 13 protein subunits of the ETC, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (Anderson et al., 1981). The 13 protein genes are genes coding for cytochrome c oxidase subunit subunits 1, 2 and 3, NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5 and 6, cytochrome b, as well as ATP synthase subunits a and A6L (Anderson et al., 1981).

Other metazoan mtDNA encodes the same 37 genes in many cases, but more have also been reported in some species (Helfenbein et al., 2004; Jeyaprakash & Hoy, 2007; Lavrov et al., 2005; Osigus et al., 2013). In general the c-subunit of F-ATP synthase is nuclearly encoded, but in the ancestral metazoan, *Porifera*, it is still encoded in the mtDNA. The differences between nuclear and mitochondrial encoded F-ATP synthase c-subunits will be described in greater detail in section 1.3.1.

1.2 The F-ATP synthases

F-ATP synthases are multi-subunit enzymes that catalyse the synthesis of adenosine triphosphate (ATP), the “energy currency of life”, from adenosine diphosphate (ADP) and inorganic phosphate (P_i). Under anaerobic conditions, some eubacteria ATP synthases can run in reverse, using the energy from the hydrolysis of ATP to pump protons out of the cell to balance up the thermodynamic gradient, hence these are also called ATPases.

There are different types of ATPases, which differ in structure and function (For a review see: Müller & Grüber, 2003). For instance, A-ATPases are found in archea, the V-ATPases are found in eukaryotic vacuoles and the F-ATPases found in eubacteria, chloroplasts and mitochondria. The mitochondrial F-ATP synthase is the subject of the current project.

1.2.1 Structure of F-ATP synthase

In the 1960s, Efraim Racker and colleagues discovered that the mitochondrial F-ATP synthase is composed of two functional units, F_1 and F_o. The F_1 fraction derives its name from the term "fraction 1" as it is a necessary coupling factor for ATP synthesis. The F_o fraction is named for providing the F-ATP synthase with its sensitivity to oligomycin, oligomycin being an inhibitor of ATP synthase. It binds to the F_o fraction and blocks proton translocation (Kagawa & Racker, 1966). Therefore F-ATP synthase is often written as F_1F_o-ATP synthase.

The structure of some F-ATP synthases have been solved mainly by X-ray crystallography. It consists of eight different subunits in bacteria and chloroplasts, and 18 in animal mitochondria, some of the subunits occurring as multiple copies (Chen et al., 2007; Walker, 2013). Figure 1-4 and Table 1 show organization of protein subunits in various F-ATP synthases.

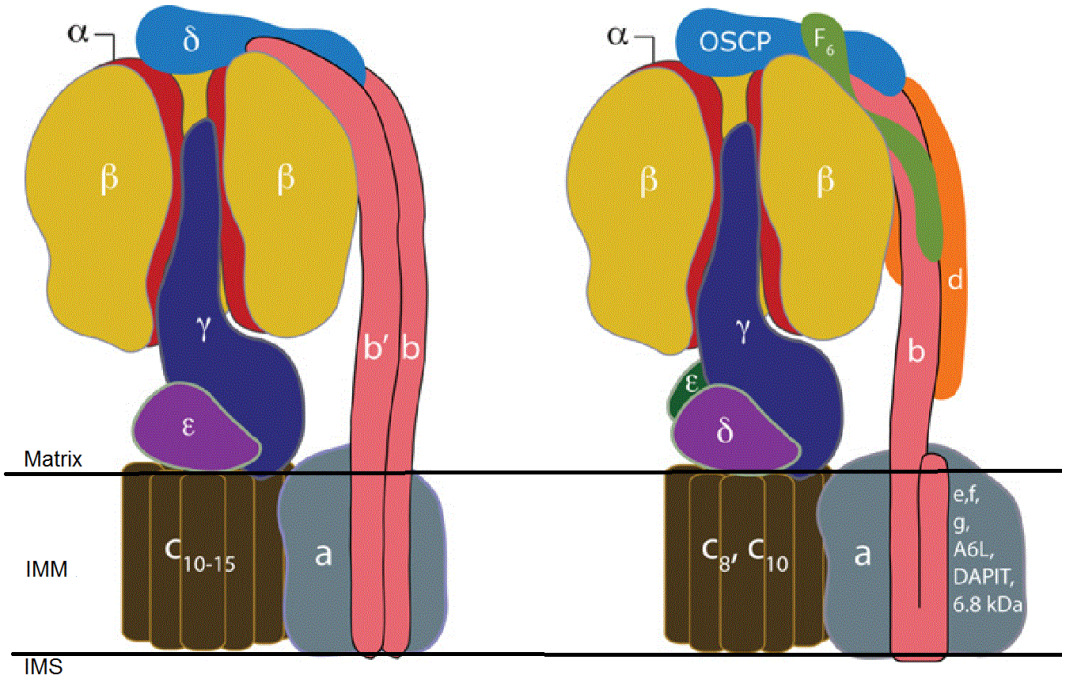


Figure 1-4 Organization of protein subunits in F-ATP synthases

The bacterial and chloroplast F-ATP synthases are shown on the left, and the more complex mitochondrial F-ATP synthase is shown on the right. In both models one of the α-subunits is removed to expose the elongated γ-subunit (Figure adapted from Walker, 2013).

Table 1 The subunit composition of well characterised F-ATP synthases

Escherichia coli	*Saccharomyces cerevisiae*	*Bos taurus*
α	α	α
β	β	β
γ	γ	γ
ε	δ	δ
--	ε	ε
δ	OSCP	OSCP
a	a	a
b	b	b
c	c	c
--	d	d
--	e	e
--	f	f
--	g	g
--	h	F6
--	i/j	--
--	k	--
--	ATP8	A6L
--	IF_1	IF_1
--	--	DAPIT
--	--	6.8 kDa

Homologous subunits are shown on the same line. Dashes indicate where no homologous subunit exists (Walker, 2013) (Table adapted from Walpole 2015).

The best characterised metazoan F-ATP synthase is the bovine enzyme from *Bos taurus*. The subunits comprising the F_1 domain are the α, β, γ, δ, and ε-subunits present in the stoichiometry of 3:3:1:1:1 (Abrahams et al., 1994). The α and β-subunits are alternatively arranged in a hexameric ring with a large central cavity filled by the long asymmetric coiled-coil γ-subunit, which connects the F_o and F_1 domains together. It penetrates the F_1 domain and protrudes beneath it, interacting with the c-ring (Gibbons et al., 2000). The subunit ε appears to stabilize the foot of the central stalk, where ε, γ, and δ-subunit all interact extensively.The F_1 domain can also contain an inhibitor protein, IF_1, which functions to prevent hydrolysis of ATP when the p.m.f. is weak (Gledhill et al., 2007; Pullman & Monroy, 1963).

The peripheral stalk contains the b, d, F6, and OSCP-subunits, all present in single copies (Collinson et al., 1996). Together they hold the two domains together to prevent futile rotation of the F_1 domain without catalysis.

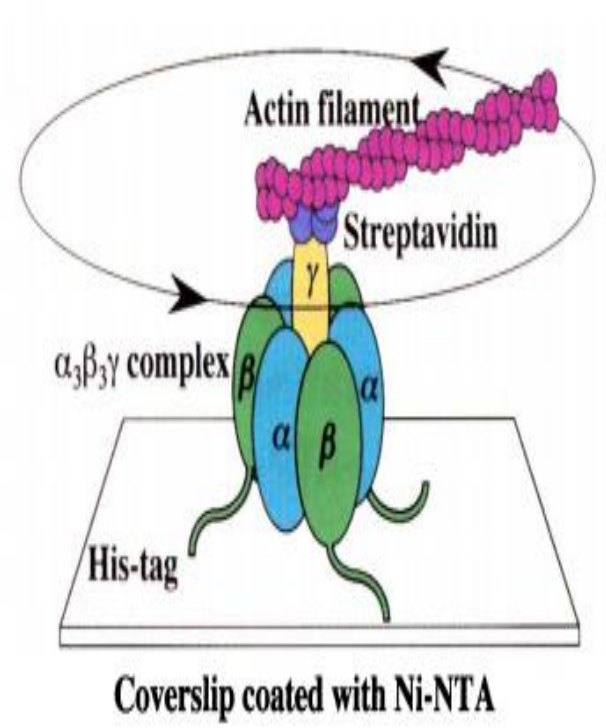
The F_o domain is embedded in the inner mitochondrial membrane and is the least characterised portion of the F-ATP synthase. In the bovine enzyme it is comprised of subunits a, c, e, f, g, A6L, DAPIT, and the 6.8 kDa proteolipid (Carroll et al., 2009; Chen et al., 2007; Collinson et al., 1994; Fearnley & Walker, 1986; Walker et al., 1991). Only the stoichiometries of the a and c-subunits are known, 1 and 8 respectively. Subunits e, f, g, A6L, DAPIT, and the 6.8 kDa proteolipid are thought to have no direct role in ATP synthase and are termed the supernumerary or minor subunits. The c-subunit (formerly also known as subunit 9, the lipid-binding protein or the dicyclohexylcarbodiimide (DCCD) reactive proteolipid) is a membrane protein that folds into a hairpin structure of two transmembrane α helices connected by a cytoplasmic loop (Girvin et al., 1998; Stenton-Dozey & Heath, 2009). Eight such subunits are arranged into a ring structure, called the c-ring (Watt et al., 2010). The c-ring is in contact with the a-subunit, and together they allow the translocation of protons across the membrane.

1.2.2 Mechanism of F-ATP synthase

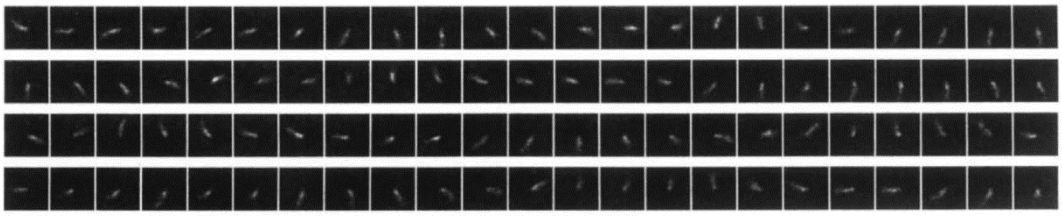
The F-ATP synthase uses the p.m.f. to synthesise ATP. During ATP synthesis, the translocation of protons through the c-ring and the a-subunit is coupled with the rotation of the γ-subunit of the F_1 domain. Rotation of the asymmetric γ-subunit causes conformational changes in the catalytic nucleotide-binding sites on the three β-subunits resulting in the synthesis of ATP molecules.

Each β-subunit conformation has a different nucleotide binding affinity. In the crystal structure of the F_1 domain two of the β subunits, $\beta_{DP,}$ and β_{TP} adopt similar conformations, while the third conformation, β_E, is significantly more open (Abrahams et al., 1994). These are also referred as the 'tight' (leading to ATP synthesis), 'open' (release of ATP), and 'loose' states (ready to bind substrates) (Abrahams et al., 1994). The β_E conformation contains no bound nucleotide as its nucleotide-binding domain and C-terminal domain have been pushed outwards by the curvature of the γ-subunit. Each 360° rotation of the γ-subunit takes each β-subunit through each of the three states and produces 3 ATP molecules.

Rotational catalysis was demonstrated in biophysical experiments using an actin filament attached to the γ-subunit (Noji et al., 1997). N-terminal His tags were engineered into β-subunits and used to anchor $\alpha_3\beta_3\gamma$ subcomplexes on to Ni^{2+} covered coverslips with the γ-subunits protruding upwards (Figure 1-5 A). Upon the addition of ATP, rotation of the actin filament in 120° steps was observed using a fluorescent microscope (Figure 1-5 B). This was powered by the hydrolysis of ATP causing the $\alpha_3\beta_3\gamma$ subcomplex to run in reverse. Later experiments showed that this 120° rotation in an intact F-ATPase complex was achieved by two sub-steps, 90° and 30° (Yasuda et al., 2001).



A



B

Figure 1-5 Demonstration of the rotary mechanism of F_1-ATP synthase

Part A, the system used for observation of the rotation of the γ-subunit in the $\alpha_3\beta_3\gamma$ subcomplex. Ni-NTA stands for Ni^{2+}-nitriylotriacetic acid. Part B, sequential images of a rotating actin filament observed from the top showing rotation of the protein (Figures adapted from Noji et al., 1997).

1.2.3 Regulation of F-ATP synthase

A small endogenous protein regulates the hydrolytic activity of mitochondrial F-ATPase (Pullman & Monroy, 1963). This protein, IF_1, is thought to conserve ATP during a decline in the p.m.f. caused by an imbalance between proton pumping and the demand for ATP. IF_1 binds to the ATP synthase in the F_1 domain at a pH of 6.7 or lower. When the p.m.f. is restored the inhibitor is ejected. The binding site for IF_1 is shown in Figure 1-6. It involves interactions with β_{DP}, β_E, α_{DP}, α_{TP}, and γ-subunits (Bason et al., 2011; Cabezón et al., 2003; Gledhill et al., 2007). In this way the wasteful hydrolysis of ATP in mitochondria is prevented. No functional significance has been reported for the nucleotide-phosphate binding to the α-subunits.

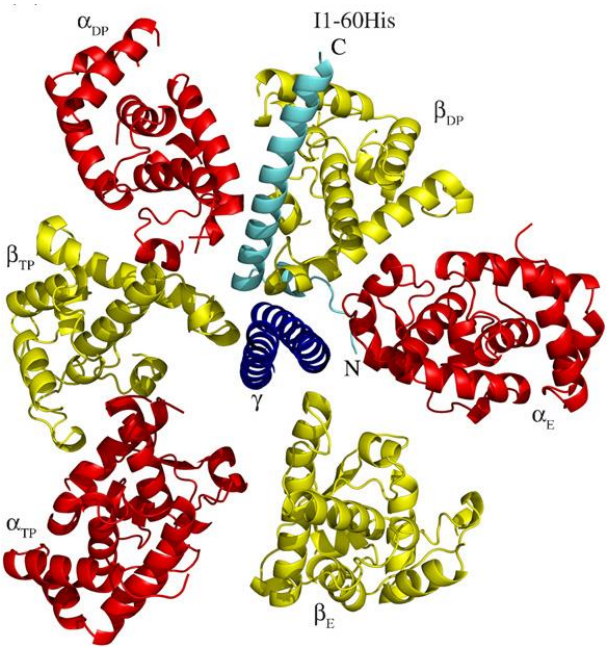


Figure 1-6 Cross-sectional view of the C-terminal domains of the α- and β-subunits looking up along the axis of the γ-subunit showing interactions of the inhibitor protein with the subunits of F-ATPase

The α, β and γ-subunits are shown in ribbon representations in red, yellow and dark blue, respectively. The inhibitor protein is light blue (Figure adapted from Bason et al., 2011).

1.2.4 Bioenergetic cost

According to the structure of the F-ATP synthase as described previously, the number of protons translocated for each 360° rotation is the same as the number of c-subunits in the c-ring and every 360° rotation of the enzyme produces 3 ATP molecules. The c-ring of *S. cerevisiae* consists of 10 c-subunits (Stock et al., 1999), and so 10 protons are translocated and three ATP molecules produced per 360° rotation. Therefore, the bioenergetic cost (i.e. H^+/ATP ratio) of *S. cerevisiae* ATP synthase is 10/3=3.3 protons per ATP synthesized (i.e. number of c-subunit in the c-ring / number of ATP produced).

However, the number of c-subunits that make up the c-ring is different in different life forms, ranging between 10 to 15 in yeast, eubacterial, and plant chloroplast ATP synthases (Matthies et al., 2009; Meier et al., 2005; Pogoryelov et al., 2012; Pogoryelov et al., 2009; Vollmar et al., 2009); therefore,the bioenergetic cost of these F-ATP synthases ranges between 3.3 and 5 protons per ATP synthesized.

The bioenergetic cost of making an ATP in mammalian F-ATP synthase was unknown until recently when Watt and colleagues determined the c-ring size in bovine F-ATP synthase (Watt et al., 2010). Previously it was speculated to be 9 or 12 which would make the bioenergetic cost of making one ATP 3 or 4. However, cross-sections of the structure of the bovine c-ring complex showed that there are only eight c-subunits in the ring (Figure 1-7). This means that the bioenergetic cost of the bovine enzyme is only 2.7 protons to make one ATP, making the bovine enzyme the highest ATP production gearing discovered so far.

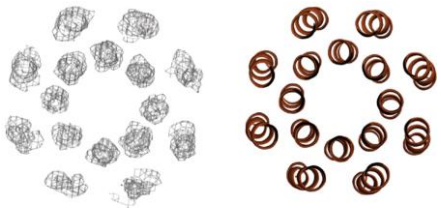


Figure 1-7 Cross-section of the c-ring of bovine F-ATP synthase

The cross-section was taken at the midpoint of the α-helices, with the eight N-terminal α-helices in the inner ring, and the eight C-terminal α-helices in the outer ring. Left is the electron density, and the structural interpretation of the density is shown on right (Figure adapted from Watt et al., 2010).

1.2.5 The c_8-ring

By looking into how the construction of a c_8-ring is possible, Watt and colleagues (2010) used the structural model of the yeast c_{10} complex to construct models of c_8 and c_9-rings with the bovine diameter. In both models there were serious side chain clashes of residues Ile13, Leu19, and Ile23 of the yeast c-subunit. In the bovine c-subunit, these amino acids are all alanine residues, and a bovine c_8-ring has no side chain clashes. Therefore, the presence of alanine residues at positions 13, 19, and 23 were thought to be essential for the formation of c_8-ring (Watt et al., 2010). In the same study, Watt and colleagues also imply that the trimethylation of c-subunit lysine 43 (Figure 1-8) was critical for the formation of the c_8-ring.

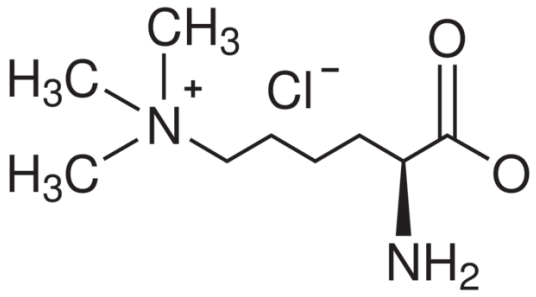


Figure 1-8 Molecular structure of trimethylated lysine

The lysine 43 residue is located at the beginning of the C-terminal α helix and its quaternary amino group is exposed to the phospholipid bilayer in the head-group region. It would clash with the head major group of the bilayer phospholipids and impede their binding to the ring (Watt et al., 2010). However when it interacts with cardiolipin, an abundant phospholipid with no head group, found almost exclusively in the inner mitochondrial membrane where it is essential for the optimal function of ATP synthase, the exposed amino groups of the lysine 43 residues could become the cardiolipin binding sites (Watt et al., 2010). Therefore, the lysine 43 residues were proposed to bind to cardiolipins to avoid a clash with the phospholipid bilayer head groups (Figure 1-9).

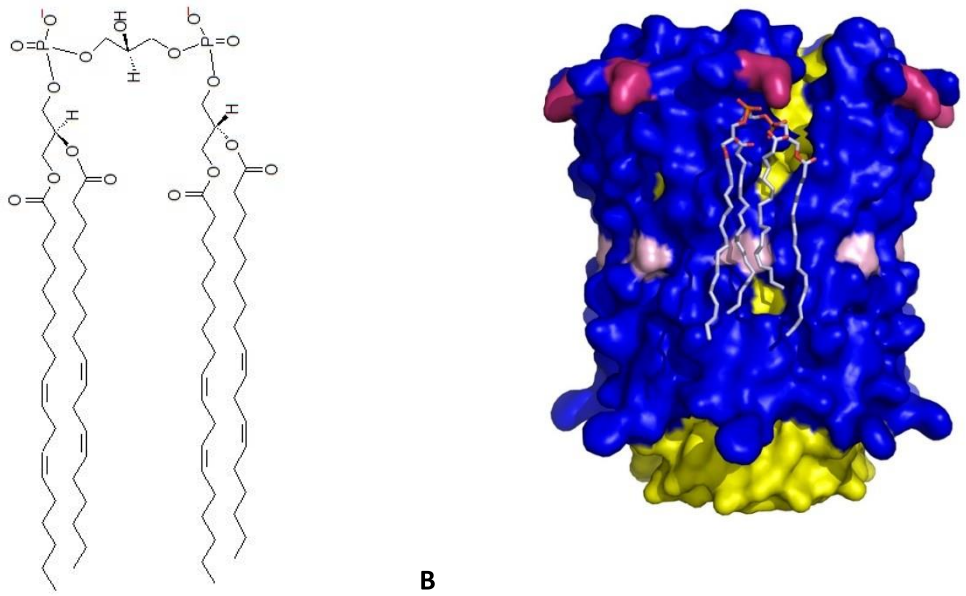


Figure 1-9 Structure of cardiolipin and its binding to the bovine c_8-ring

Part A, molecular structures of cardiolipin. Cardiolipin is made up of two phosphatidyl groups connected by a glycerol, therefore cardiolipin bears four acyl chains, and has no head group. Part B, the proposed structure of cardiolipin binding to the bovine c_8-ring. Lysine 43 is highlighted in purple and glutamate in pink. Here one cardiolipin molecule is shown in association with a lysine 43 residue (Figures adapted from Walker, 2013).

In addition, unlike the tightly packed c_{15}-ring, a c_8-ring would have gaps between C-terminal α-helices, exposing the inner ring to lipid bilayer (Figure 1-10) (Watt et al., 2010). It is proposed that these gaps are occupied by the acyl groups of cardiolipins, which stabilize the c_8-ring within the phospholipid bilayer (Watt et al., 2010).

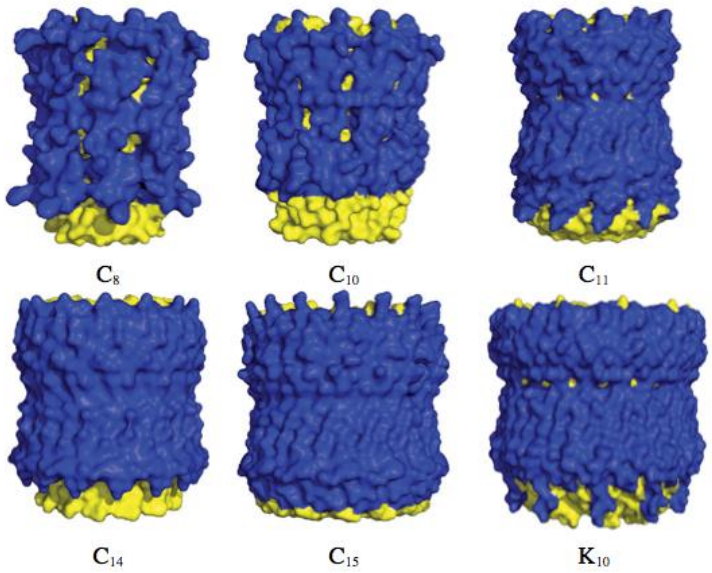
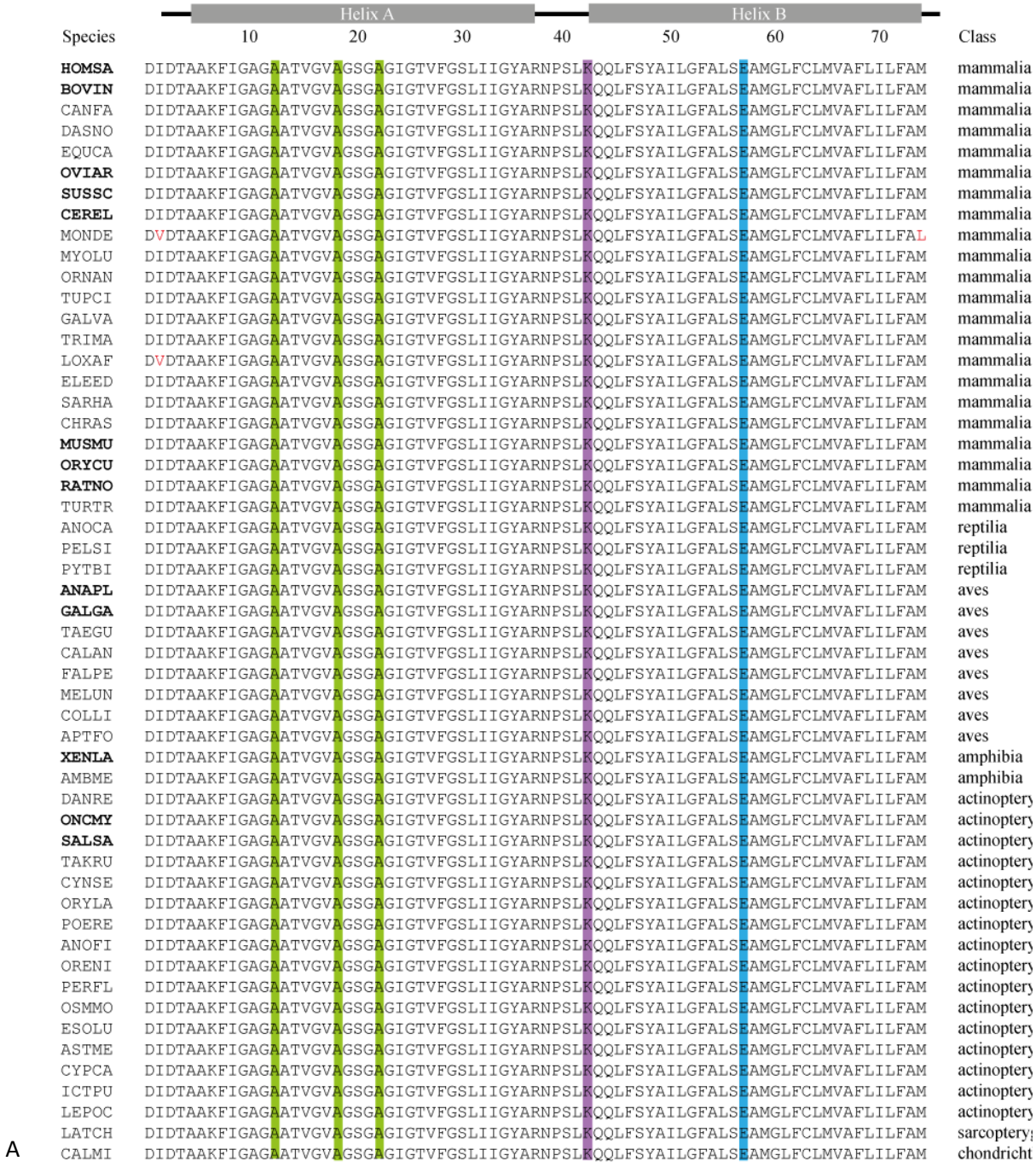


Figure 1-10 The c-rings from various organisms

Unlike the tightly packed c_{15}-ring, a c_8-ring has gaps between the eight outer C-terminal α-helices and thus the inner ring is exposed to the lipid bilayer (Figure adapted from Watt et al., 2010).

C-subunit amino acid sequences from ATP synthase from vertebrates and invertebrates were compared, shown as Figure 1-11 (Watt et al., 2010). The c-subunit sequence is identical in almost all vertebrates and is highly conserved across other animalia phyla. With only one exception, the proton binding Glu 58 and alanine residues at positions 13, 19 and 23, are all conserved throughout the aligned vertebrate and invertebrate sequences. Therefore, it was proposed that all vertebrates and possibly all or most invertebrates contain c_8-rings and the bioenergetic cost for all these enzymes to produce an ATP molecule is also 2.7 protons (Watt et al., 2010).


Helix A
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Species
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LOXAF DVDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM mammalia
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SARHA DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM mammalia
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ANOCA DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM reptilia
PELSI DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM reptilia
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CALAN DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM aves
FALPE DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM aves
MELUN DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM aves
COLLI DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM aves
APTFO DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM aves
XENLA DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM amphibia
AMBME DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM amphibia
DANRE DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
ONCMY DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
SALSA DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
TAKRU DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
CYNSE DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
ORYLA DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
POERE DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
ANOFI DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
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ESOLU DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
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ICTPU DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
LEPOC DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM actinoptery
LATCH DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM sarcoptery
CALMI DIDTAAKFIGAGAATVGVAGSGAGIGTVFGSLIIGYARNPSLKQQLFSYAILGFALSEAMGLFCLMVAFLILFAM chondricht

A

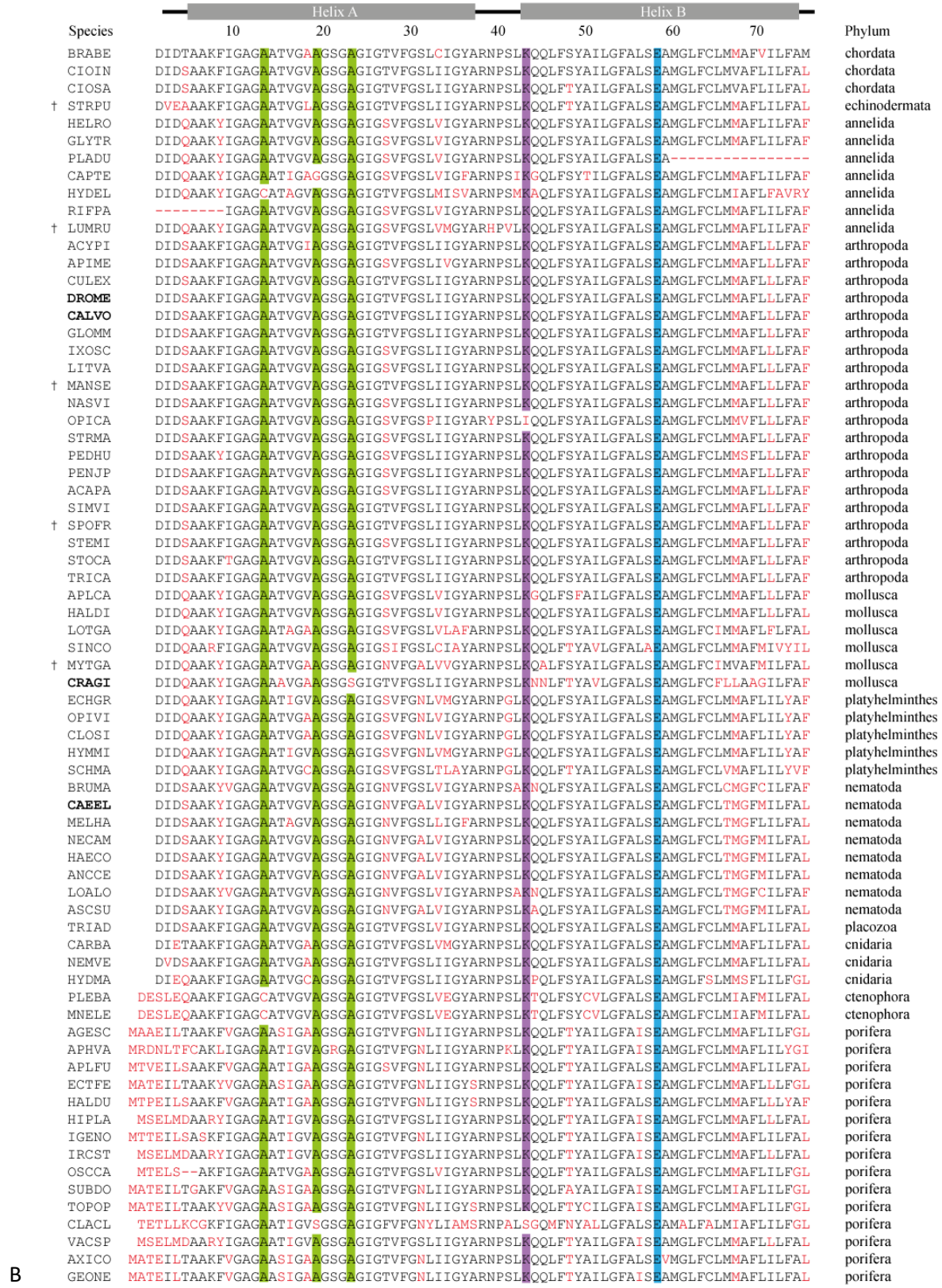


Figure 1-11 Sequence alignment of ATP synthase c-subunit in vertebrates and invertebrates, compared with the human sequence

Part A, sequence alignment of ATP synthase c-subunit in vertebrates. Part B, sequence alignment of ATP synthase c-subunit in invertebrates. The green boxes indicate alanines 13, 19, and 23 required for the formation of the c_8-ring. The purple box and blue boxes show, respectively, the positions of the lysine residue that is known to be trimethylated in the human, bovine, ovine, porcine, and rabbit enzymes and of the glutamate residue that is involved in proton translocation through the inner membranes of mitochondria (Figures adapted from Walpole et al., 2015).

1.3 The c-subunit

1.3.1 Genetic components of the c-subunit

Mammalian c-subunit is a nuclear gene product, synthesized on cytoplasmic ribosomes. It is encoded by three different genes (ATG5G1, ATG5G2, and ATG5G3), which are expressed in different ratio in various tissues and are translated into three different protein isoforms, P1, P2, and P3 (Dyer et al., 1989; Dyer & Walker, 1993; Gay & Walker, 1985; Medd et al., 1993; Yan et al., 1994). These three isoforms consist of different N-terminal mitochondrial import sequences that are cleaved off by matrix peptidases during entry to mitochondria (Schatz & Butow, 1983). More interestingly, these three genes contain an identical C-terminal mature protein c-subunit, which consists of 75 amino acids (Figure 1-12).

Figure 1-12 Amino acid composition of the Homo sapien (human) mature c-subunit protein

The alanine residues required for the formation of a c_8-ring are indicated in green. The proposed binding site for cardiolipin is indicated in purple. The essential residue for proton translocation through the membrane is indicated in blue.

In contrast to the mature protein, the N-terminal mitochondrial import sequences of P1, P2 and P3 are different, as shown in Figure 1-13 (Dyer et al., 1989; Dyer & Walker, 1993; Gay & Walker, 1985; Medd et al., 1993; Yan et al., 1994).

```
                10          20          30          40          50          60          70
P1        MQTA GALFISPALI RCCTRGLIRP VSASFLMSPV MSSKQPSYSM FPLQVARREF QTSVVSRDID
P2  MFACSKFVST PSLVKSTSQL LSRPLSAVVL KRRPEILTDE SLSSLAVSCP LTSLVSSRSF QTSAISRDID
P3  MFACAKLACT PSLIRAGSRV AYRPISASVL SRPEASRTGE GSTVFNGAQN GVSQLIQREF QTSAISRDID

                80          90          100         110         120         130         140
P1 TAAKFIGAGA ATVGVAGSAA GIGTVFGSLI IGYARMPSLK QQLPSYAILG FALSEAMGLF CLMVAFLILF AM
P2 TAAKFIGAGA ATVGVAGSAA GIGTVFGSLI IGYARMPSLK QQLPSYAILG FALSEAMGLF CLMVAFLILF AM
P3 TAAKFIGAGA ATVGVAGSAA GIGTVFGSLI IGYARMPSLK QQLPSYAILG FALSEAMGLF CLMVAFLILF AM
```

Figure 1-13 The amino acid sequences for the P1, P2, and P3 precursors

The identical mature protein sequences are in bold (Yan et al., 1994).

Sponges are part of the earliest divergence within the metazoans and their c-subunit DNA (ATP9) has been discovered to be encoded in the mitochondrial genome in all major lineages (Lavrov et al., 2005; Osigus et al., 2013). Compared with the mature c-subunit sequences of the human c-subunit, sponges have an extra 2-3 N-terminal amino acids, and no mitochondrial import sequences (Figure 1-14).

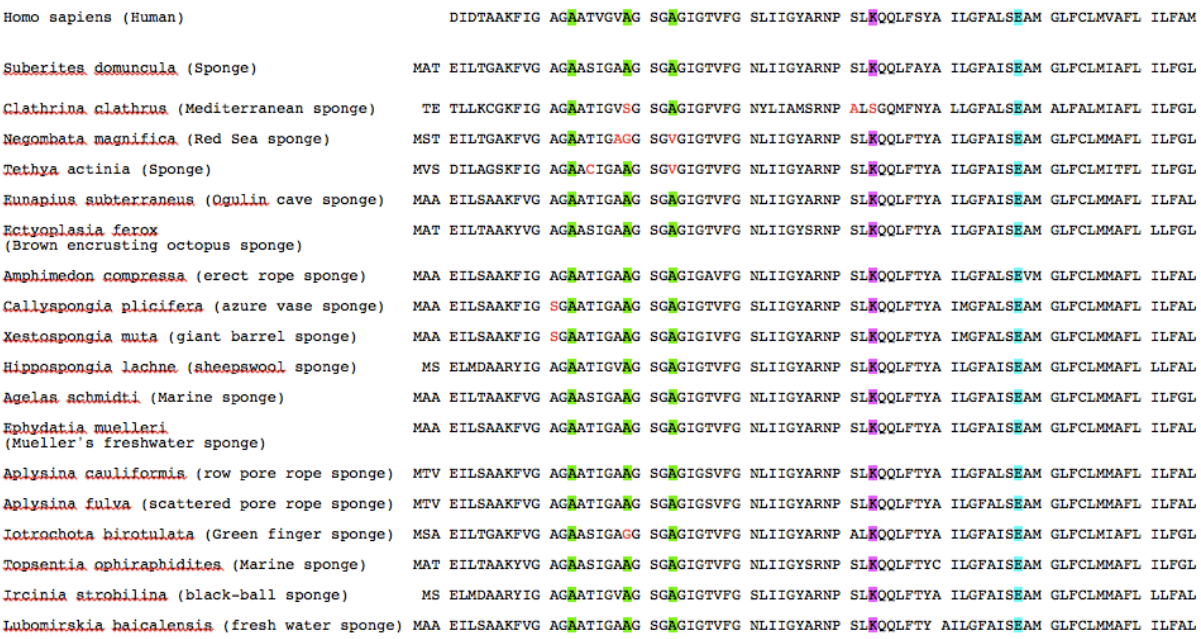


Figure 1-14 Alignment of c-subunit of sponges against the human sequence

The alanine residues required for the formation of a c_8-ring are indicated in green. The proposed binding site for cardiolipin is indicated in purple. The essential residue for proton translocation through the membrane is indicated in blue.

As presented in Figure 1-14, all sponge c-subunit genes start with methionine (M). Unlike the nuclear machinery, mitochondria use an N-formylmethionyl-tRNA (fMet-tRNA) as the initiator of protein synthesis (Epler et al., 1970; Galper & Darnell, 1969; Osawa et al., 1992; Taanman, 1999). In mitochondria, fMet-tRNA is coded for by the same codons as methionine, AUG and AUA. When the codons are used for initiation, fMet-tRNA is used and forms the first amino acid of the nascent peptide chain. All subsequent AUG or AUA translate into methionine. Hence all mitochondrial encoded genes start with the code for methionine (Anderson et al., 1981).

Coincidentally, the c-subunit gene is differently distributed between the nuclear and mitochondrial genomes in different filamentous fungi species, but ATP9 gene distribution in these fungi are more complicated. They could have up to 3 ATP9 copy numbers and five different distributions: 1) one single gene in the mitochondrial genome; 2) one single gene in the nuclear genome; 3) one copy in each of the mitochondrial and nuclear genome; 4) two copies in the nuclear genome; 5) two copies in the nuclear genome and one in the mitochondrial genome (De´quard-Chablat et al., 2011). This diversity is indicative of an active process of ATP9 gene transfer from the mitochondria to the nucleus during the evolution of filamentous fungi (De´quard-Chablat et al., 2011). Additionally, *S. cerevisiae* has only one ATP9 gene in the mitochondrial genome and its c-subunit is not trimethylated (Liu et al., 2015).

1.3.2 C-subunit and Batten disease

Mature c-subunits, with no mitochondrial import sequences, are found to abnormally accumulate in storage bodies of Batten disease (Palmer, 2015; Palmer et al., 1992). Batten disease (neuronal ceroid lipofuscinoses, NCLs) is a group of recessively inherited neurodegenerative lysosomal storage diseases. A collective incidence of 1 in 12,500 live births world-wide has been suggested for Batten disease (Rider & Rider, 1988). Affected children start life normally but develop behavioural changes, visual failure, and progressive mental and motor deterioration. They live to between 7 years old and early adulthood. Adult onset cases have also been reported. There are many forms of NCLs arising from different mutations in different genes. At least 8 genetically distinct human NCLs are known (Kousi et al., 2012), and a total of 13 suggested (NCLs mutation database: http://www.ucl.ac.uk/NCLs). Almost all forms of disease share two definitive hallmarks, massive brain atrophy and the accumulation of fluorescent storage bodies in neurons and most other cells throughout the body.

Early studies found that approximately two-thirds of the stored material in ovine NCLs is protein and the major protein is identical to the c-subunit of mitochondrial ATP synthase (Fearnley et al., 1990; Palmer et al., 1986; Palmer et al., 1992; Palmer et al., 1989). The mature c-subunit contains two lysine residues, at positions 7 and 43 (Figure 1-12). Later analyses of the c-subunit stored in the canine form of the disease suggested that 1 of 2 lysine residues in this protein was trimethylated; Edman sequencing of the N-terminal region determined that lysine 7 was not methylated and so it was assumed that the trimethylated residue was at position 43 (Katz et al., 1994). A trimethylation-containing protein is the precursor of the carnitine biosynthetic pathway, therefore accumulation of trimethylated c-subunit in lysosomal storages bodies was proposed to cause carnitine deficiency in NCLs affected subjects (Katz et al., 1994). It was also speculated to be involved in the etiology of the disease (Katz et al., 1994; Katz et al., 1995; Katz & Rodrigues, 1991; Katz et al., 1997). However dietary carnitine was supplied to patients without any benefit.

However connections between the etiology of NCLs, trimethylation of c-subunits and the application of carnitine as cure for NCLs remained controversial until the discovery that normal bovine mitochondrial c-subunits all contained post-translationally modified lysine 43 resulting in an intact molecular mass of 7650 Da, 42 Da greater than its cDNA calculated value (Chen et al., 2004).

Even today, the biochemical origin(s) of the disease remain unknown and little is understood about c-subunit turnover. Although it has been long known that c-subunits are accumulated in NCLs, studies of why c-subunit accumulates have not yielded the answer.

2 Experimental Rationale

The c-ring of the bovine ATP synthase is made of eight c-subunits. A feature of all the bovine c-subunits is that their lysine 43 are completely trimethylated, which is not a common lysine modification. Alignments of metazoan c-subunits suggested that they are highly conserved, as is lysine 43, leading to the proposal that all metazoan c-subunit would be trimethylated in this position.

A collaborative project between MBU Cambridge and Lincoln University aimed to investigate the conservation of the trimethylation modification across a wide variety of metazoan phyla using a GST tagged inhibitor protein which allowed the rapid purification of pure F-ATP synthases from isolated mitochondria. C-subunits were then separated on SDS gels and several mass spectrometric techniques used to characterise the c-subunits fully. Firstly the molecular masses of the c-subunits were determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS), and compared to the calculated protein sequences to indicate any post-translational modifications. Secondly the c-subunits were enzymatically digested with chymotrypsin and the peptides analysed by MALDI-TOF and ESI-Orbitrap mass spectrometry. This multi-faceted analysis allowed a comprehensive and thorough characterisation of each c-subunit, determining both the nature and location of any modification.

The purpose of the current thesis was to complete the tree of metazoans by investigating animals most easily available in New Zealand, and attention was paid to examples where there was a deviation from the conserved core c-subunit sequence, such as *porifera* where the c-subunit is mitochondrially encoded. The common brushtail possum (*Trichosurus vulpeculac*), kina (*Evechinus chloroticus*), sea cucumber (*Australosrochopus mollis)*, and sponges (*Crella incrustans*) were chosen.

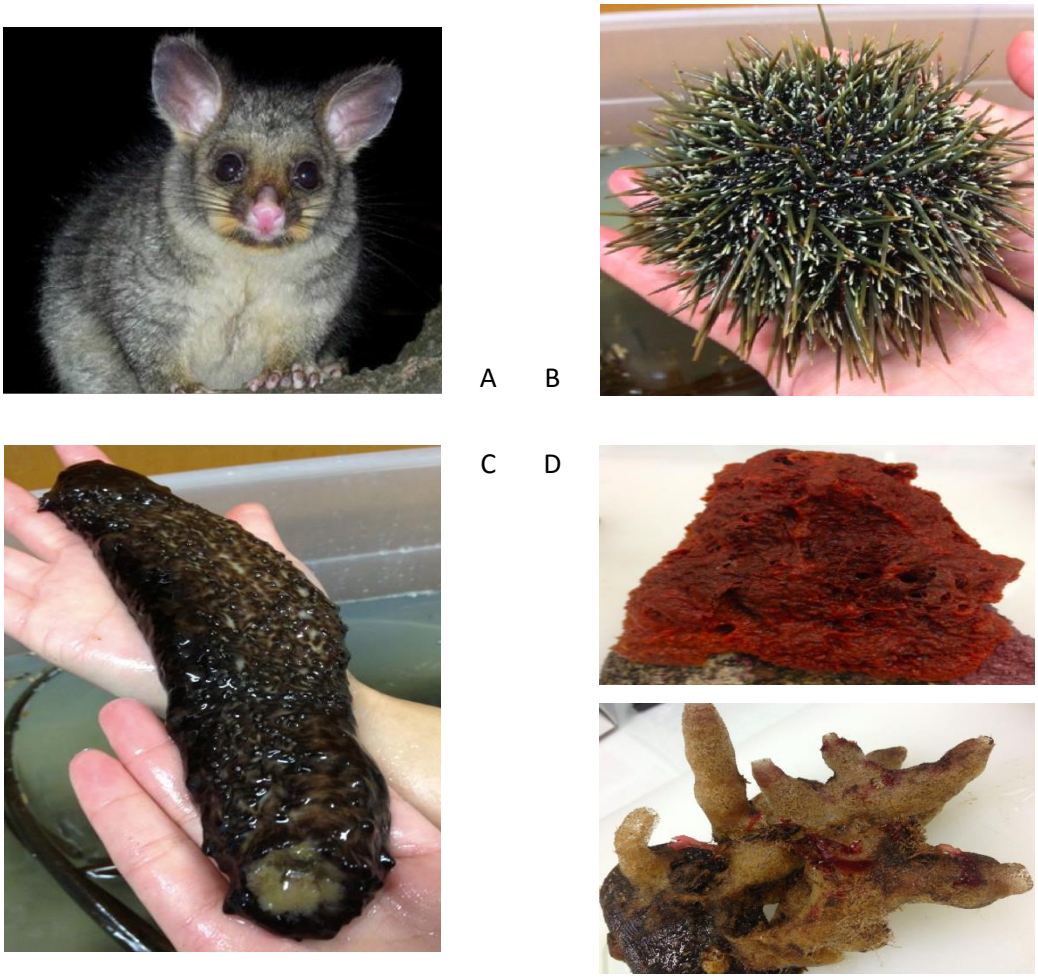


Figure 2-1 Animals studied in this project at Lincoln University

Part A, possum. Part B, kina. Part C, sea cucumber. Part D, sponges.

The common brushtail possum (*Trichosurus vulpeculac*) is an arboreal marsupial introduced from Australia to establish a fur industry to New Zealand. Numbers in New Zealand have risen to the point where it is a pest. The echinoderm kina (*Evechinus chloroticus*) is a sea urchin endemic to New Zealand and commercially fished in small quantities in restricted areas around New Zealand (Lawrence, 2007). The sea cucumber (*Australosrochopus mollis)* is an echinoderm that plentiful in New Zealand coastal waters and is often found in large numbers under green-lip mussel (*Perna canaliculus*) farms (Stenton-Dozey & Heath, 2009). Sponges represent the basal metazoan phylum in the evolutionary tree of life. They are easily found in New Zealand waters where there are about 700 known species and the real number may be twice that (www.teara.govt.nz). All these animals were chosen because of their special availability in New Zealand, and to fill in phyla required for a complete investigation of the status of lysine 43 trimethylation throughout the metazoan tree (Figure 2-2).

C-subunits were also isolated and investigated from bovine (*Bos taurus*), pig (*Sus scrofa*), ovine (*Ovis aries*), sheep (*Oryctolagus cuniculus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), red deer (*Cervus elaphus*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), Greek tortoise (*Testudo graeca*), snake (*Boa constrictor*), frog (*Xenopus laevis*), salmon (*Salmo salar*), rainbow trout (*Onchorhynchus mykiss*), seabass (*Dicentrachus labrax*), spiny dogfish (*Squalus acanthisa*), earth worm (*Lumbricus terrestris*), Pacific oyster (*Crassostrea gigas*), razor clams, mussels (*Mytilus edulis),* lobster (*Homarus gammarus*), brown crab (*Cancer paurus*), brine shrimp (*Artemia salina*), cells of the cabbage looper moth (*Tricholusia ni*), brown fly larvae (*Calliphora vomitoria*), nematode worms (*Caenorhabditis elegans*) and potatoes (*Solanum tuberosum*) by Thomas Walpole, Prof David Palmer, and myself in the MBU laboratory (Figure 2-2) (Walpole et al., 2015).

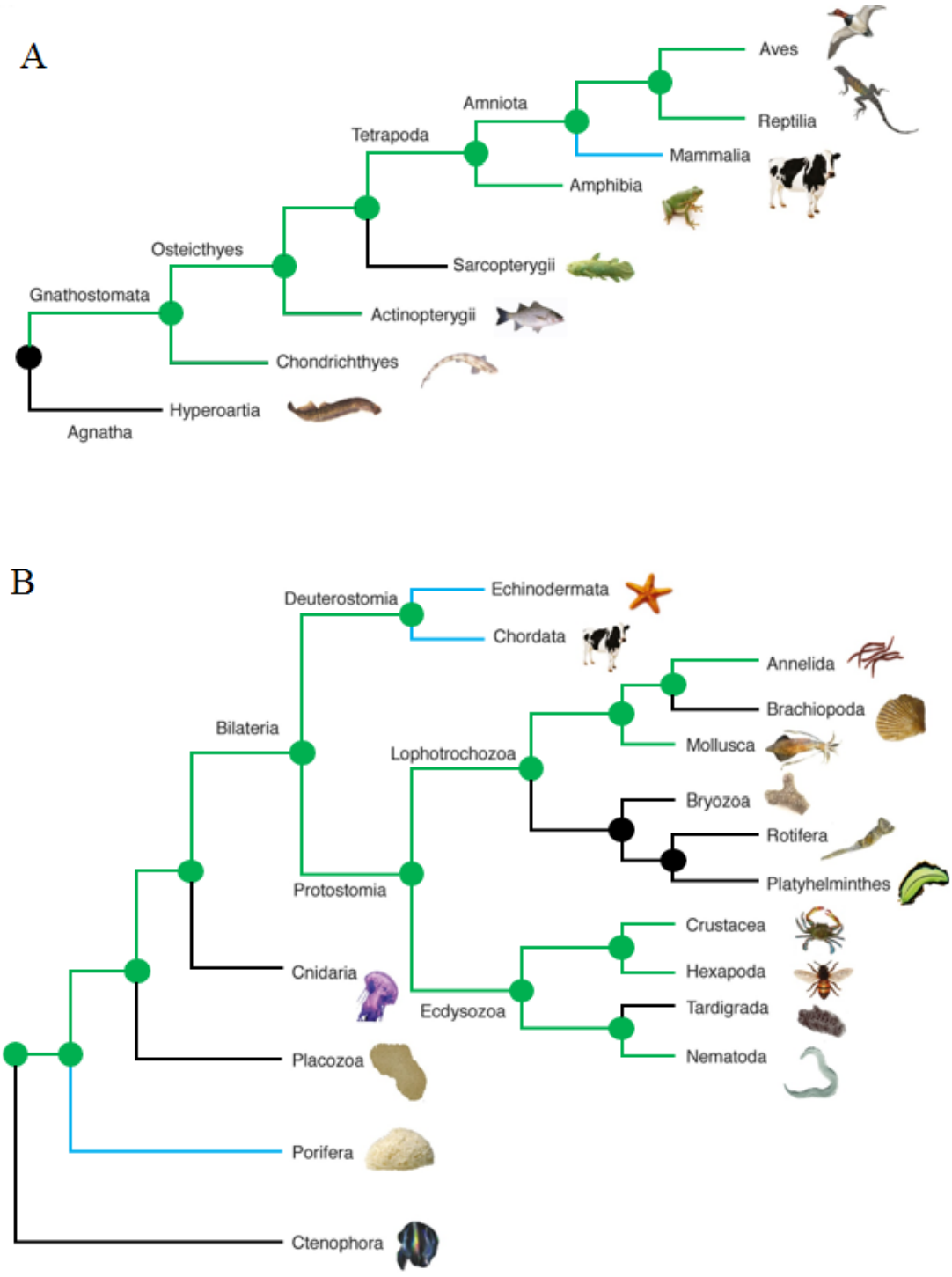


Figure 2-2 Metazoan tree of life

Part A, the vertebrate tree. Part B, the major metazoan phyla. Part A, analysed examples were as follows: Mammalia - human, bovine, ovine, porcine, rabbit, mouse, rat, red deer, possum; Aves - chicken, duck; Reptilia - tortoise, snake; Amphibia - frog; Actinopterygii - salmon, trout, seabass; Chonrichthyes - spiny dogfish. Part B, analysed examples were as follows: Echinodermata - kina, sea cucumber; Chordata - see Part A; Annelida - earth worm; Mollusca - Pacific oyster, razor clam, mussel; Crustacea - lobster, brown crab, brine shrimp; Hexapoda - fruit fly, cabbage looper, brown fly; Nematode - Caenothabditis elegans; Porifera - sponge.

The blue branches denote experiments that were performed at Lincoln University. The green branches denote experiments that were performed at MBU Cambridge (including chordata). The black branches denote species that were not investigated in this project (Figure adapted from Walpole et al., 2015).

3 Materials and Methods

3.1 Sources of animals

Possums were provided by Martin Ridgway (Lincoln University, Christchurch). Sea cucumbers and kinas were gifted by Philip Health (NIWA, Mahanga Bay Aquaculture Facility, Wellington). They were sent to Lincoln University by fast courier and kept in a 60 litre aerated tank with red seaweed for feed. Sponges were collected from Pigeon bay, Dusky Sound and Breaksea Sound by Renny Bishop, Kate Schimanski, and Kath Blackemore (University of Canterbury, Christchurch). They were kept in sea water and transported back to Lincoln University and stored at 4^{o}C until sacrificed.

C-subunits were also isolated from bovine (*Bos Taurus*), pig (*Sus crofa*), sheep (*Ovis aries*), rabbit (*Oryctolagus cuniculus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*) , chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), red deer (*Cervus elaphus*), earth worm (*Lumbricus terrestris*), brownfly larvae (*Calliphora vomitoria*), salmon (*Salmo salar*), rainbow trout (*Onchorhynchus mykiss*), spiny dogfish (*Squalus acanthisa*), seabass (*Dicentrachus labrax*), shark, Greek tortoise (*Testudo graeca*), snake (*Boa constrictor*), frog (*Xenopus laevis*), brown crab (*Cancer paurus*), lobster (*Homarus gammarus*), Pacific oyster (*Crassostrea gigas*), mussels (*Mytilus edulis),* brine shrimp (*Artemia salina*), cells of the cabbage looper moth (*Tricholusia ni*) and potatoes (*Solanum tuberosum*) by Thomas Walpole, Prof David Palmer, and myself in the MBU laboratory (Walpole et al., 2015).

Both fresh and frozen animal tissues could be used for these experiments, however fresh tissues were preferred as they contain more active mitochondria, and hence provide higher yields of isolated ATP synthase. To keep the mitochondria active, all mitochondria preparations were performed at 4^{o}C unless otherwise stated and buffers were kept on ice. Live animals were handled according to the Lincoln University Animal Ethics committee and the New Zealand Animal Welfare Act, 1999, and amendments. Where no documentary guidance exists, invertebrates were killed humanely.

3.2 Possum

3.2.1 Isolation of crude mitochondria

Fresh livers were dissected from recently executed possums, cut into small pieces and washed with cold homogenization buffer (225 mM sorbitol, 75 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% bovine serum albumin (BSA), 1 mM EDTA, and 0.4 mM dithiothreitol (DTT), pH 7.6) to remove blood.

Tissues were homogenized using a Potter-Elvehjem homogenizer in 10x the volume of homogenization buffer, until the homogenate had a smooth consistency, and spun at 900 g, 10 min, 4°C, to remove unbroken cells and nuclei. The supernatants were collected into separate tubes, the pellets re-suspended in homogenization buffer by 5-10 gentle strokes of a pestle of a loose-fitting Dounce homogenizer, and spun again, 900 g, 10 min, 4°C. The combined supernatants were then centrifuged 10,000 g, 10 min, 4°C. The crude mitochondrial pelleted fractions were re-suspended in homogenization buffer and the slow and fast centrifugations repeated.

3.2.2 Preparation of phosphate washed mitochondria

After isolation, the crude mitochondria were re-suspended in ice cold phosphate washing buffer (50 mM Na_2HPO_4, 100 mM sucrose, and 0.5 mM EDTA, pH 9.12) and kept in ice, 30 min, to wash out endogenous binding inhibitors. The mitochondria were then spun at 48,000 g, 30 min, 4°C, the pellet re-suspended in phosphate washing buffer, and spun, 48,000 g, 30 min. Protein concentrations of phosphate washed mitochondria were estimated by the BCA assay and adjusted to 10 mg of protein per ml in 10% glycerol, 20mM Tris-HCl, pH 8.0.

3.2.3 Isolation of F-ATP synthase

3.2.3.1 Preparation of inhibited F-ATP synthase

A 10% aqueous solution of n-dodecyl β-D-maltopyranoside (DDM, Glycon Biochemicals, Luckenwalde, Germany) was added to the 10 mg/ml mitochondrial suspensions to yield a final concentration of 1% DDM. This was incubated at RT, 10 min, and spun, 48,000 g, 10 min, 4°C. An aliquot of the supernatant was kept as a pre-inhibition sample and recombinant bovine F-ATPase inhibitor protein, $IF_{1\text{-}60}$-GST-HIS, consisting of residues 1-60 with a glutathione-S-transferase domain and six histidine residues attached to its C-terminus (Runswick et al., 2013) added to the rest of the supernatant at a concentration of 5 µl per ml of solubilised membranes. Then Mg/ATP buffer (200 mM $MgSO_4$ and 400 mM ATP) was added, 15 µl per ml, 5 min, 37°C, repeated 3 times. The binding mechanism between F-ATP synthase and $IF_{1\text{-}60}$-GST-HIS is shown in Figure 3-1.

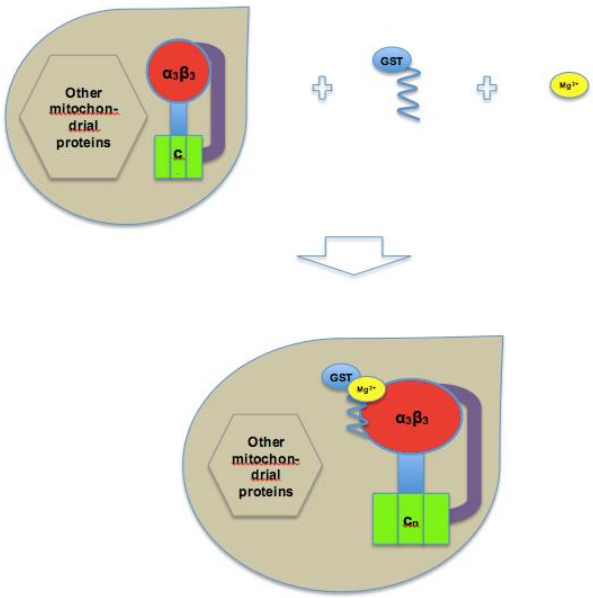


Figure 3-1 Cartoon of the binding between F-ATP synthase and IF_{1-60}-GST-HIS

In the presence of magnesium, IF_{1-60}-GST-HIS binds to the β subunit of F-ATP synthase to inhibit the F-ATP synthase and form an IF_{1-60}-GST-HIS-F-ATP synthase super complex, which can be separated from the other mitochondrial proteins by GST affinity chromatography.

3.2.3.2 Application to the GST affinity column

The inhibited sample solutions were spun, 48,000 g, 20 min, 4°C to remove any precipitate, NaCl added to a final concentration of 150 mM and the samples filtered (0.2 μm pore size) to remove any remaining particles.

Before loading the filtered samples on to the FPLC (Bio-RAD Biologic Duoflow, USA), a 1 ml Hi-Trap GST affinity column (GE Healthcare, Sweden) was equilibrated with running buffer (20 mM Tris, 10% glycerol, 0.15 M NaCl, 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Soltec Ventures, USA) and 0.05% DDM, pH 7.2) to get a steady baseline. The column was loaded with 10 mg of protein at a flow rate of 0.5 ml/min, to bind the IF_{1-60}-GST-HIS with the attached inhibited ATP synthase to the column (Fig 3-3 (A)). The ATP synthase was eluted with EDTA buffer (25 mM EDTA, 20 mM Tris-HCl, 10% glycerol, 0.15 M NaCl, 5 mM TCEP and 0.05%DDM, pH 7.2) which scavenged the magnesium ions binding the inhibitor and the ATP synthase together (Fig 3-3 (B)). When the conductivity reached a peak, indicating that the elution buffer had reached the detector, the pump was stopped and left overnight to allow time for dissociation of the ATP synthase and the IF_{1-60} fragment to take place. Next day the pump was started and the ATP synthase eluted immediately as a sharp peak. The inhibitor still bound to the column was subsequently eluted with 40 mM GSH buffer (Figure 3-3 (C)).

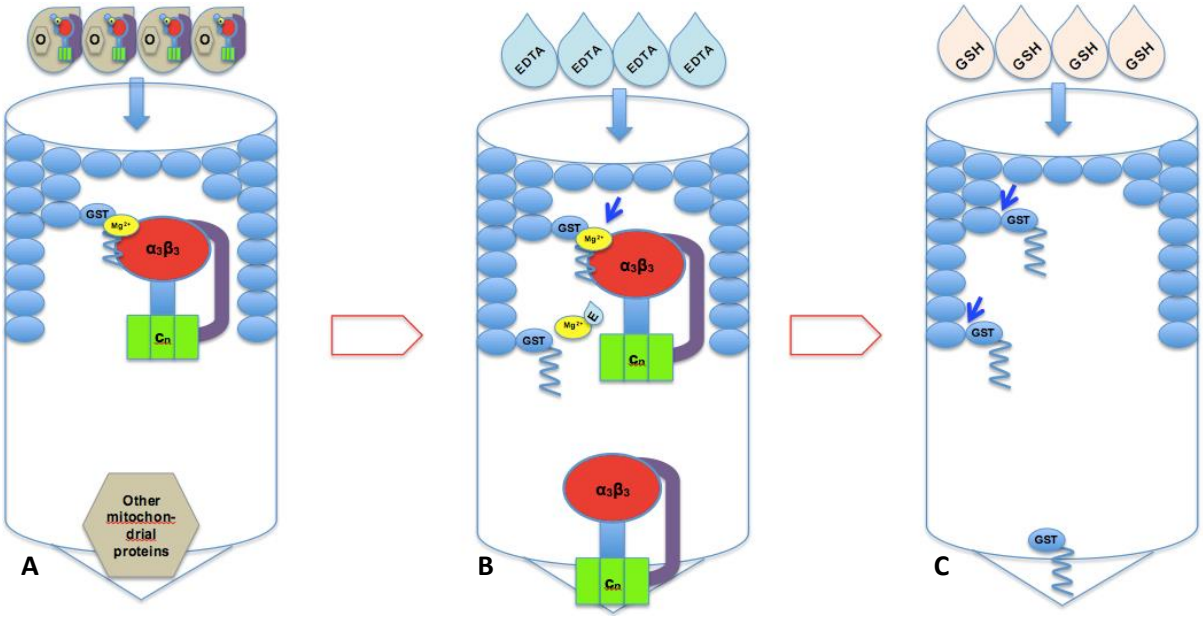


Figure 3-2 The F-ATP synthase purification using the Hi-Trap GST affinity column

Part A, the GST tag on the IF_{1-60} inhibitor binds to the GST tagged beads on the column and any unbound mitochondrial proteins wash through. Part B, the EDTA scavenges magnesium ions to break the binding between the inhibitor and the ATP synthase which frees the ATP synthase. Part C, the inhibitors are washed off with GSH buffer.

3.2.4 Isolation of c-subunits

Fractions containing ATP synthase, according to the elution profile, were loaded onto SDS-PAGE to separate all 16 subunits. Purified bovine ATP synthase was used as a marker using 4-20% Biorad gradient gels and 15% gels. Gels were run at fixed voltages of 80 V to let sample migrate through the stacking gel and then 120 V for subunit separation. Gels were stained with Coomassie blue. When gels were fully destained, sample bands that migrated at the same position as the bovine c-subunit marker were excised and cut into small pieces, placed into 1.5 ml Eppendorf tubes, washed with 100 µl of deionised water, 60 min, then 2x with 100 µl of 20 mM Trish-HCl, pH 8.0, 60 min. The gel pieces with covered with 100 µl of 50% acetonitrile and 20 mM Tris-HCl, pH 8.0, until they turned white (~30 min) then dried at 37°C in a CentriVap concentrator (Labconco, Kansas city, USA), sealed and stored at -20°C until posting to the MBU for mass spectrometric analysis.

3.2.5 Mass spectrometric analysis

On receipt at the MBU, the dried gel pieces were rehydrated and subjected to LC-ESI MS and MALDI-TOF MS for c-subunit characterization.

3.2.5.1 LC-ESI MS of ATP synthase/c-subunit

The molecular masses of the c-subunits were measured as described by Carroll et al. (2009). Firstly 20-100 μg of purified F-ATP synthase or solvent extracted proteins were precipitated overnight with 20 volumes of ice-cold ethanol. Proteins were recovered by centrifugation 16,000 g, 4°C, 10 min, pellets were redissolved in 30 μl of a solution of 60% formic acid, 15% trifluoroethanol, 1% hecafluoropropan-2-ol, and 24% water. Samples were loaded onto a reverse-phase column (75 mm, 1 mm i.d.) of PLRP-S (polymeric reverse phase made of styrene divinylbenzene copolymer on 5 μm beads with 300 Å pores; Varian, Oxford, UK), equilibrated in buffer containing 50 mM ammonium formate, pH 3.1, 1% hexafluoroisopropanol and 15% trifluoroethanol. Proteins were eluted with a linear gradient of buffer containing 50 mM ammonium formate, pH 3.1, 70% 2-propanol, 20% trifluoroethanol, and 1% hexafluoroisopropanol. The elute was introduced "on-line" into either an Quattro Ultima triple quadeopole mass spectrometer (Waters-Micromass, Manchester, UK) or a Q-Trap 4000 mass spectrometer (ABSciex, Phoenix House, Warrington, UK). Molecular masses were calculated using MassLynx software (Waters, Milford, MA) and Bioanalyst (ABSciex, Phoenix House, Warrington, UK).

3.2.5.2 MALDI-TOF and OrbiTrap MS of chymotrypsin digests of c-subunits

C-subunit characterizations were achieved by MALDI-TOF and OrbiTrap MS analyses as described by Shevchenko et al. (2006). The rehydrated c-subunit gel pieces were digested overnight, 37°C, with 12.5 ng/ml chymotrypsin (Roche Applied Science, Burgess Hill, UK) in solution containing 20 mM Tris-HCl, pH 8.0, and 5 mM $CaCl_2$. 18 hours later, peptides were extracted from the digests with 5% formic acid, 1 h with vortexing, followed with 60% acetonitrile in 4% formic acid, 1 h with vortexing. Peptides were loaded onto the target plate and analysed by MS and tandem MS with a MALDI-TOF-TOF mass spectrometer (Model 4800 ABScie) with α-cyano-4-hydroxy-trans-cinnamic acid as the matrix. Peptide masses and partial sequences were compared against the NCBI and internal databases. Tandem ESI-MS of the intact peptide ions generated MS/MS data, which were used to determine the location and nature of any modification.

3.3 Kina, sea cucumber and sponges

Fresh kina were cut open, the yellow eggs collected and washed with cold homogenization buffer (300 mM sucrose, 50 mM KCl, 50 mM NaCl, 30 mM HEPES, 10 mM EDTA, and 2 mM DTT, pH 7.4) to remove sea water.

Fresh sea cucumbers were cut into small pieces, washed with cold homogenization buffer as above, minced with homogenization buffer and homogenized in a Polytron (PT 3100, Kinematica Ag, Littau, Switzerland), 2x 10 sec.

Any stones and mussel shells were removed from fresh sponges which were washed as above, cut into small pieces with scissors then homogenized in a Polytron, 4x 10 sec.

3.3.1 Isolation of crude mitochondria

All three tissues were homogenized further to a smooth consistency using a Potter-Elvehjem homogenizer (1500 rpm) in 10x volume of homogenization buffer. The homogenates were spun, 900 g, 10 min, to remove unbroken cells and nuclei and the supernatants collected into separate tubes. The pellets were re-suspended in homogenization buffer and spun again, 900 g, 10 min. Then the combined supernatants were centrifuged at 10,000 g for 15 min to give a crude mitochondrial fraction that was re-suspended in homogenization buffer and the slow and fast spins repeated.

3.3.2 Self-generated iodixanol gradient purifications

After isolation, the crude mitochondrial pellets were resuspended in buffer containing 250 mM sucrose, 1 mM EDTA, and 20 mM HEPES, pH 7.4. Two parts of the resuspended mitochondria samples were mixed in one part of an OptiPrep solution (containing 60% v/v of iodixanol, Sigma-Aldrich, Missouri, USA) to yield a final iodixanol concentration of 20%. Samples were transferred to Ti70 ultracentrifuge tubes (Beckman, California, USA) to fill about 80% of the tube volume and then 60% sucrose (w/v) layered at the bottom of the tubes so as to make sure tubes were fully filled and balanced within 0.01g. Tubes were spun, 270,000 g (60,575 rpm, Beckman XL-90 ultracentrifuge) overnight in a 70Ti rotor, decelerated from 4000 rpm without braking to avoid disturbing fraction layers. The brown mitochondria-like fractions were harvested by careful pipetting and combined with 20 volumes of homogenization buffer to achieve a final iodixanol concentration of less than 1%. Protein concentrations were determined (BCA assays) then adjusted to 10 mg/ml with glycerol/Tris solution.

3.3.3 Chloroform/methanol extraction

Mitochondria were pelleted from glycerol/Tris suspensions containing 10 mg of protein at 4^{o}C and resuspended in 0.5 ml of 2 mM Tris solution, pH 8.0, washed 2x with 2 mM Tris solution and 9 volumes of chloroform/methanol/ammonium formate pH 3.7 solution (66.7:31.3:2, v/v) added, immediately mixed, vortexed, 5 min and spun 10 min, 4^{o}C, to separate the phases. Insoluble proteins

formed a brown disc between the two phases. The upper methanol phase was removed and the lower phase collected and 4 volumes of diethyl ether added to precipitate the proteins at -20°C overnight. The diethyl ether was removed and the precipitated protein redissolved in a small amount of the chloroform/methanol mixture. The dissolved sample was separated into two tubes and 2 μl of 20% SDS was added to one. The solvents were dried and the tube without SDS sealed with parafilm and sent to the MBU for LC-ESI MS analysis. The proteins in the other tube were dissolved in 2% SDS dye followed by SDS-PAGE analysis as described in section 3.2.3.

There was no formation of two phases in the sponge mitochondrial preparations when this procedure was followed. A chloroform/methanol/saline solution (2:1:1) extraction was used instead. The upper milky methanol/saline phase was removed and 4 volumes of diethyl ether added to the lower phase to precipitate the proteins as described above.

3.3.4 Mass spectrometric analysis

3.3.4.1 LC-ESI MS of ATP synthase c-subunit

The dried ether precipitate pellets without SDS added were sent to the MBU for analysis as described in section 3.2.5.1.

3.3.4.2 MALDI-TOF MS and OrbiTrap MS of chymotrypsin digests of c subunit

Bands that co-migrated with the bovine c-subunit marker were excised from gels and analysed as described in section 3.2.5.2.

4 Results

4.1 Isolation of crude mitochondria

Crude mitochondria were isolated from possum, kina, sea cucumber and sponges by differential centrifugation using the methods described in sections 3.2.1 and 3.3.1. Crude mitochondrial pellets were usually brownish in colour, but those from kina eggs were yellowish, the same colour as the tissue of origin. Possum liver yielded 9.2 mg of crude mitochondrial protein per g of tissue. Yields from sea cucumber, kina, and sponge were 3.7, 3.8, 2.6, and 2.2 mg per g of tissue respectively.

Table 2 Purification of animal mitochondria

Animal	**Tissue used**	**Initial Weight (g)**	**Crude mitochondria (mg)**
Possum	Liver	60	550
Sea cucumber	Whole animal	600	2236
Kina	Eggs	200	766
Sponge	Whole animal	45	118

4.2 Purification of possum and bovine F-ATP synthase by GST affinity chromatography

After binding of the $IF_{1\text{-}60}$-GST-HIS as described in section 3.2.3.1, inhibited possum and bovine F-ATP synthases were loaded onto the GST affinity column, followed by overnight incubation in EDTA solution to break the binding between the inhibitor and the F-ATP synthase by scavenging the Mg^{2+} ions, in order to free the F-ATP synthases. The inhibitor molecules still bound to the GST column were then eluted with GSH solution. Following this method, 1.43 mg of possum ATP synthase per 10 mg of possum crude mitochondria were recovered (Figure 4-1, Figure 4-2).

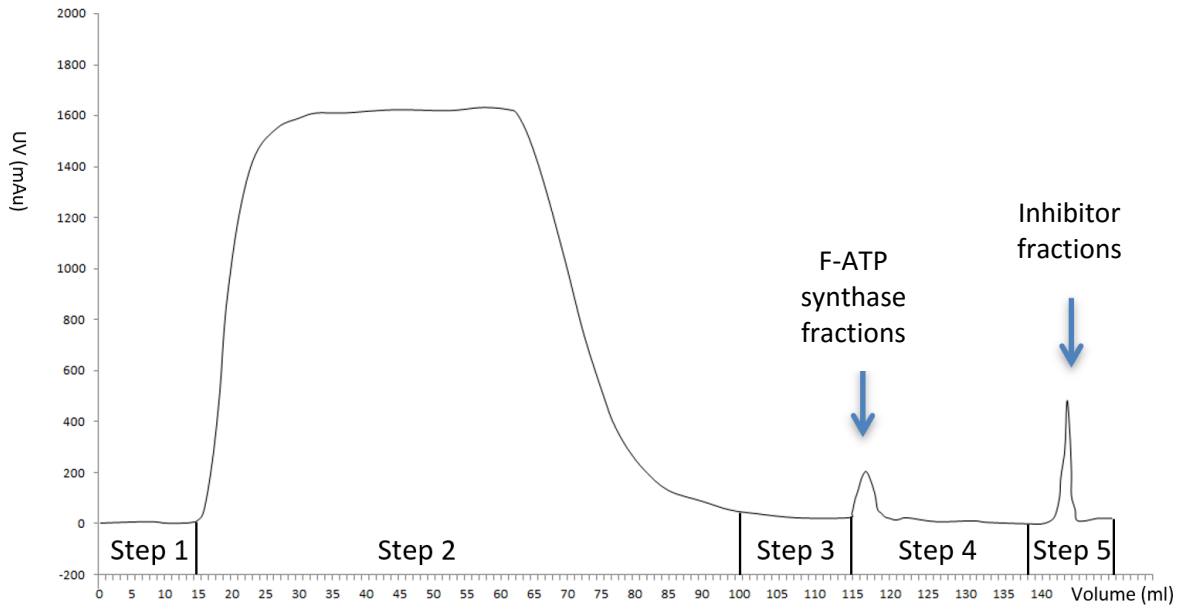


Figure 4-1 Purification of bovine F-ATP synthase

The elution profile recorded at 280nm. Step 1, the GST affinity column equilibrated with running buffer to get a steady baseline. Step 2, all the other mitochondrial components except the dissolved inhibited F-ATP synthase washed off with running buffer, completed when the UV trace returned to baseline. Step 3, EDTA solution was pumped onto the column and when conductivity peaked elution was paused overnight. Step 4, next day elution was resumed and the ATP synthase eluted immediately as a sharp peak. Step 5, the bound inhibitors were then eluted with GSH buffer.

During the possum experiment, the 280nm filter failed, and the elution trace was obscure. The 254nm filter was used instead. This resulted in a higher background (Figure 4-2), but detectable separation of F-ATP synthase was achieved.

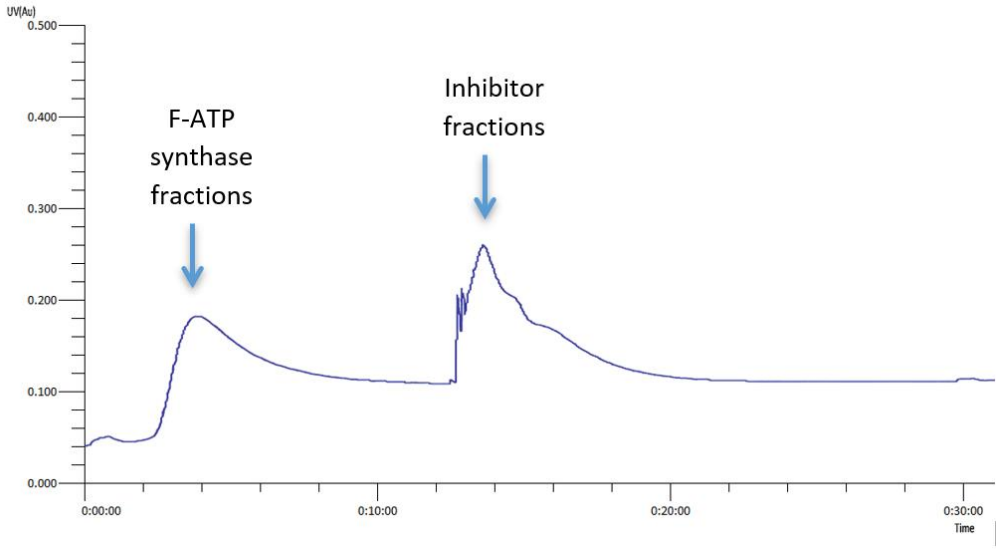


Figure 4-2 Separation of possum F-ATP synthase

4.3 Purification of kina, sea cucumber and sponge c-subunits by using iodixanol gradients and chloroform/methanol extractions

For all the vertebrate organisms tested, intact ATP synthases were purified successfully by standard mitochondrial isolation followed by inhibition with bovine $IF_{1\text{-}60}$-GST-HIS. However the bovine inhibitor protein failed to inhibit the lower organism ATP synthases. A new protocol was developed involving further purification of crude mitochondria using self-generating iodixanol gradients (Figure 4-3).

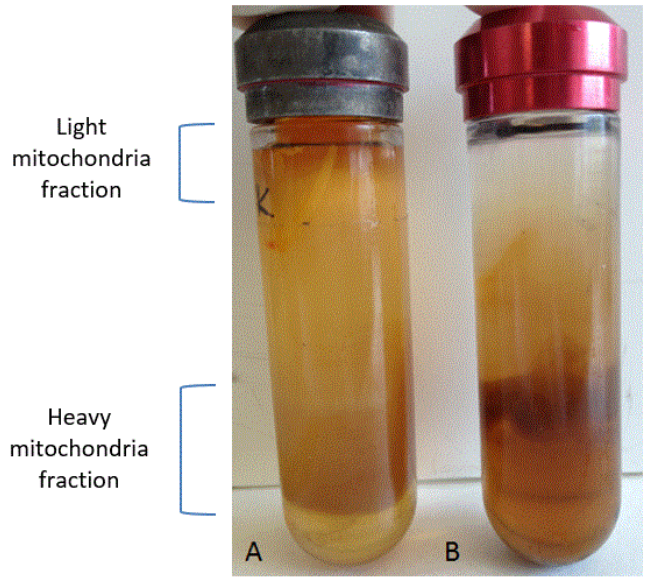
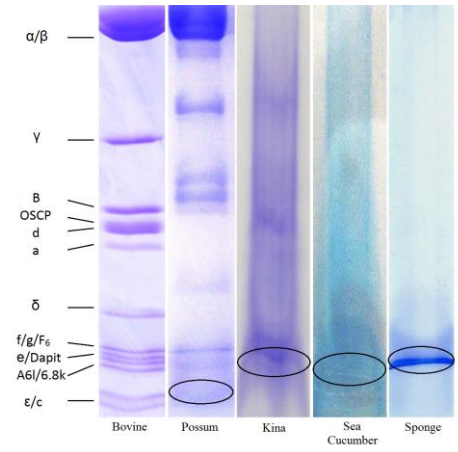


Figure 4-3 Purification of mitochondria from kina and sea cucumber in self-generated iodixanol gradients.

Part A, purification of mitochondria from kina; Part B, purification of mitochondria from sea cucumber. This process separated the heavy and light mitochondria into two fraction bands. Both fractions were collected separately.

The purified possum F-ATP synthase, and kina, sea cucumber, and sponge chloroform/methanol extraction products were analysed on 15% or 4-20% SDS-PAGE (Figure 4-4 A). Purified bovine F-ATP synthase was used as the marker. A composite gel of the ATP synthases from all species analysed in the project is also included (Figure 4-4 B).

A



B

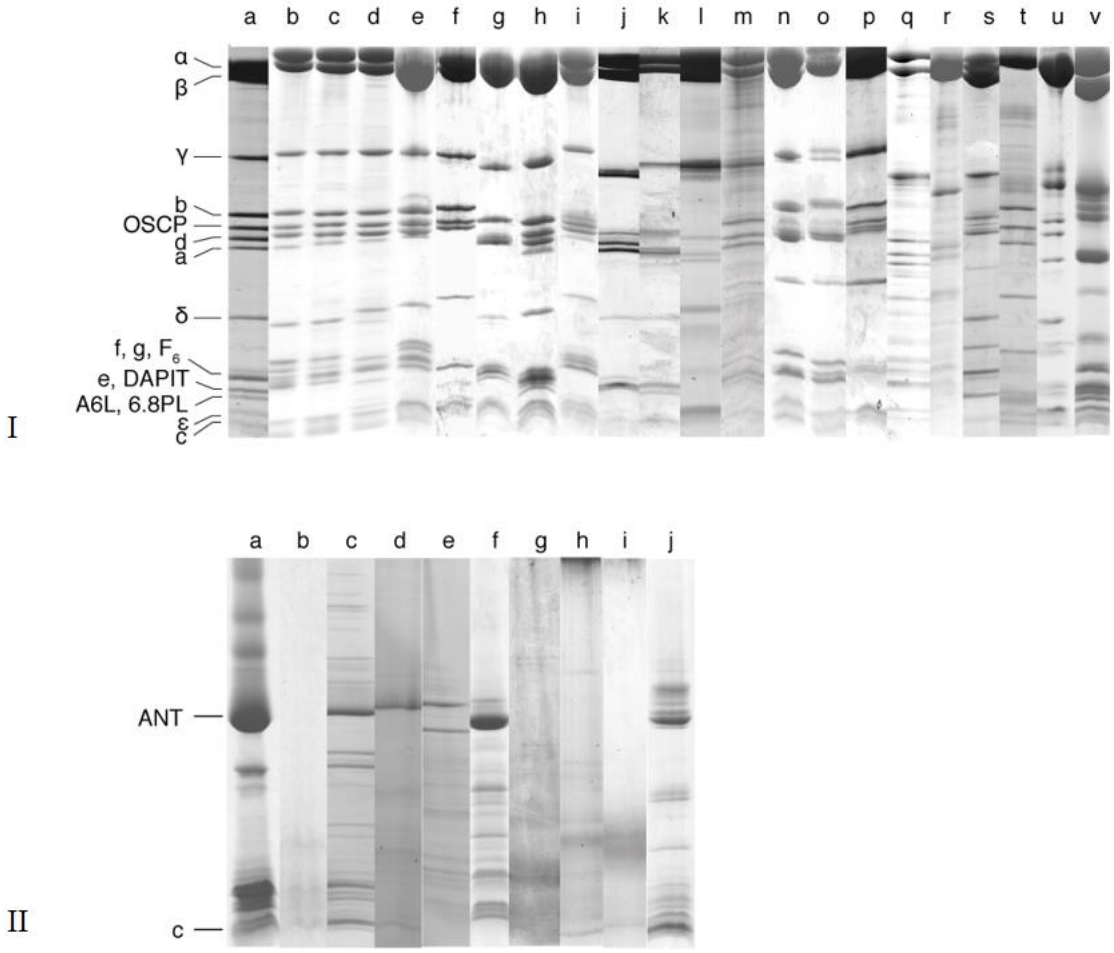


Figure 4-4 SDS-PAGE

Part A, the subunits of possum F-ATPases isolated by affinity chromatography and the hydrophobic proteins of kina, sea cucumber and sponge obtained by extraction of mitochondrial membranes with organic solvents were fractionated by SDS-PAGE, and stained with Coomassie blue dye. Possum F-ATP synthase (15% gel); kina (4-20% Bio-RAD gradient gel); sea cucumber (4-20% Bio-RAD gradient gel); and sponge (15% gel). Part B, SDS-PAGE from entire project. i and ii, 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. Part B I, 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 II, a, Bos taurus; b, Mytilus edulis; c, Crassostrea gigas; d, Caenorhabditis elegans; e, Drosophila melanogaster; f, Trichoplusia ni; g, Evenchinus chloroticus; h, Australostichopus mollis; i, Crella incrustans; j, Solanum tuberosum. Adenine nucleotide translocase (ANT) are indicated (Figure adapted from Walpole et al., 2015).

4.4 LC-ESI MS

F-ATP synthase samples were fractionated by reverse-phase HPLC and then introduced "on-line" into either a Quattro Ultima triple quadeopole mass spectrometer or a Q-Trap 4000 mass spectrometer, where molecular masses were calculated using MassLynx software and Bioanalyst by Ian Fearnley, Shujing Ding, and Kamburpola Jayawardena at the MBU. The result of the salmon F-ATP synthase analysis is shown as an example (Figure 4-5). The observed masses were compared with the theoretical calculated masses based on available genome sequences.

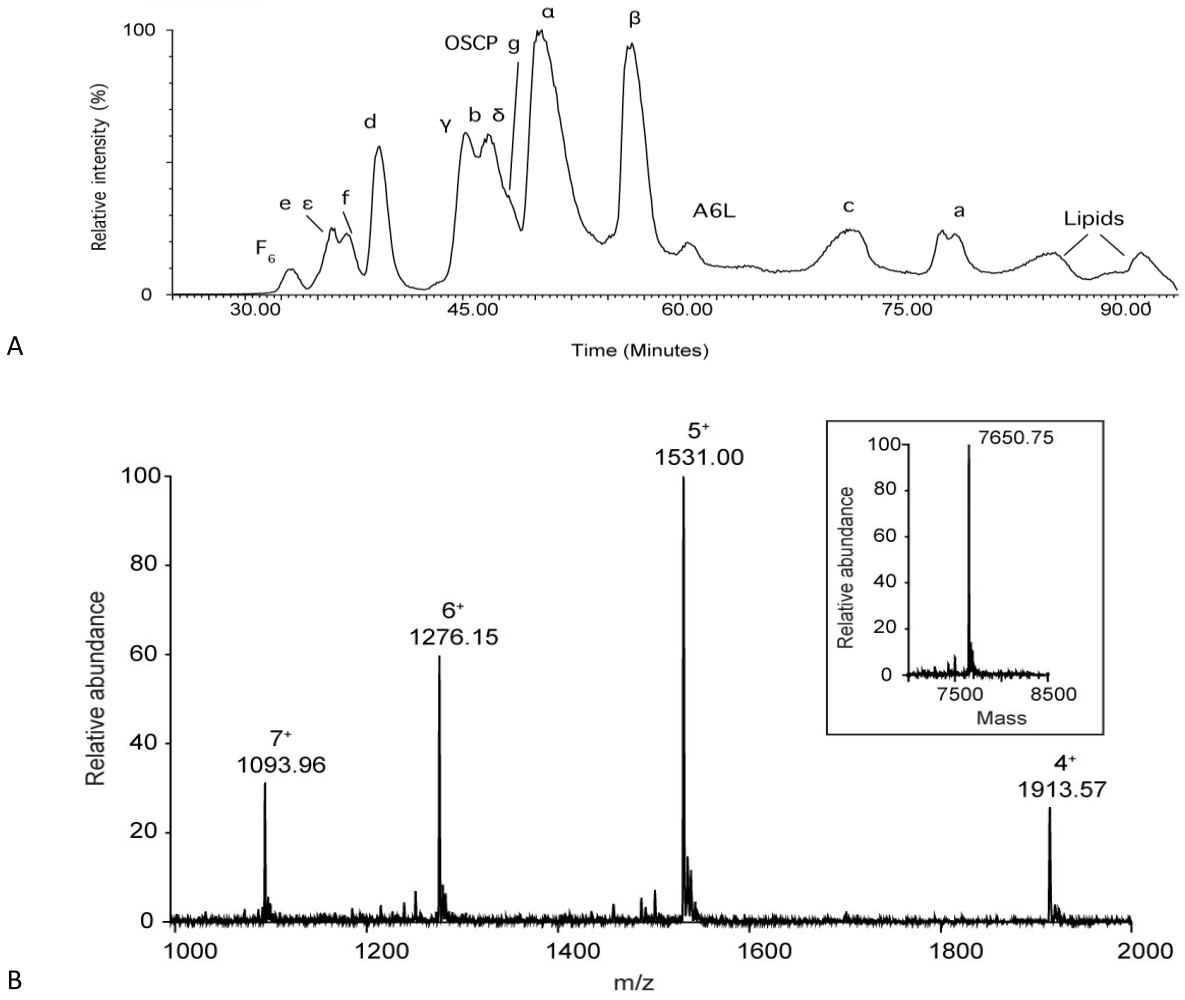


Figure 4-5 Mass spectral analysis of the intact ATP synthase and c-subunit from salmon

Part A, total ion current chromatogram of F-ATP synthase. All subunits are separated and subunit identities are based on the comparisons of observed and calculated masses. Part B, molecular mass measurement of c-subunit by ESI-MS. The insert contains the mathematical transformation of the spectrum to a molecular mass scale (Figures adapted from Walpole, 2015; Walpole et al., 2015).

All observed c-subunit molecular masses from metazoan ATP synthase are shown in Table 3.

Table 3 Molecular masses of the intact c-subunits from metazoan ATP synthases isolated in this thesis (possum, kina, sea cucumber and sponge) compared with data from the rest of the project

Species	Calculated Mass (Da)	Observed Mass (Da)	Δ
Possum	n.s.	7649.7	-
Kina	7620.0	7662.0	42.0
Sea cucumber	n.s.	7709.0	-
Sponge	n.s.	n.a.	-
Human	7608.0	7650.4	42.4
Bovine	7608.0	7650.5	42.5
Sheep	7608.0	7650.3	42.3
Pig	7608.0	7650.2	42.2
Rabbit	7608.0	7650.0	42.0
Mouse	7608.0	7650.3	42.3
Rat	7608.0	7650.3	42.3
Red deer	7608.0	7650.4	42.4
Chicken	7608.0	7650.3	42.3
Duck	7608.0	7650.3	42.3
Greek tortoise	n.s.	7650.1	-
Frog	7608.0	7650.2	42.2
Salmon	7608.0	7650.8	42.8
Rainbow trout	7608.0	7650.3	42.3
Seabass	n.s.	7650.3	-
Spiny dogfish	n.s.	7649.0	-
Brownfly larvae	7642.0	7684.4	42.4
Earth worm	7724.1	7766.4	42.3
Lobster	n.s.	7641.4	-
Brown crab	n.s.	7670.0	-
Brine shrimp	n.s.	7670.0	-
Pacific oyster	7494.7	7537.3	42.6
Mussel	7534.9	7576.9	42.0
Cabbage looper moth	7642.0	7684.0	42.0
Fruit fly	7642.0	7684.0	42.0
Nematode worms	7580.9	7623.1	42.2

Intact masses of metazoan c-subunit were measured by LC-ESI-MS. Theoretical mass calculated from the genome sequence of the mature c-subunit. n.s. no genome sequence available; n.a. no intact mass of c-subunit available (Table adapted from Walpole et al., 2015).

4.5 MALDI-TOF MS

SDS-PAGE gel bands containing c-subunit were excised then digested with chrymotrypsin for MALDI-TOF MS analysis. Chymotrypsin cleaves amine bonds where the carboxyl side of the amide bond is a large hydrophobic amino acid; one of tyrosine (Y), tryptophan (W), or phenylalanine (F). Cleavage sites in c-subunit are indicated in Figure 4-6, as well as the calculated masses for each resulting peptide.

Figure 4-6 Chrymotrypsin digestion sites of the c-subunit peptide and the masses of the resulting peptides

The chrymotrypsin digestion sites are indicated in yellow. The alanine residues required for the formation of a c_8-ring are indicated in green. The proposed binding site for cardiolipin is in purple. The essential residue for proton translocation through the membrane, glutamic acid (E), is indicated in blue.

The digested samples were mixed in a matrix that absorbs UV light so that a small part of the matrix heats rapidly and is vaporized together with the sample, giving the peptides positive charges. A detector attracts the positively charged peptides and the smaller the peptide the sooner it reaches the detector as the charge to mass ratio determines the time of fight. Consequently, the time of peptide flight can be converted to the mass of the peptide and compared to the calculated value of a peptide. Differences between calculated and measured values indicate posttranslational modification to specific peptides. An intensive peak of mass 1343 was detected in all samples (Figure 4-7 insert portion, Figure 4-8 insert portion, Figure 4-9 insert portion, and Figure 4-10 insert portion), corresponding to residues 37-47 (ARNPSLKQQLF) with an additional 42 Da characteristic of either trimethylation or N-acetylation of a lysine residue.

This peptide was fragmented further by MALDI-TOF-TOF MS, which generated MS/MS data (Figure 4-7 main portion, Figure 4-8 main portion, Figure 4-9 main portion, and Figure 4-10 main portion). A peptide fragment of mass 1284 was detected in all samples, corresponding to residues 37-47 (ARNPSLKQQLF) with a loss of 59 Da. The mass of the trimethyl-ammonium ion is 59 Da, and no mass of 129 Da corresponding to the loss of acetylated lysine was observed in any spectra. Therefore, the 42 Da modification is concluded to be trimethylation within residues 37-47 of the c-subunits.

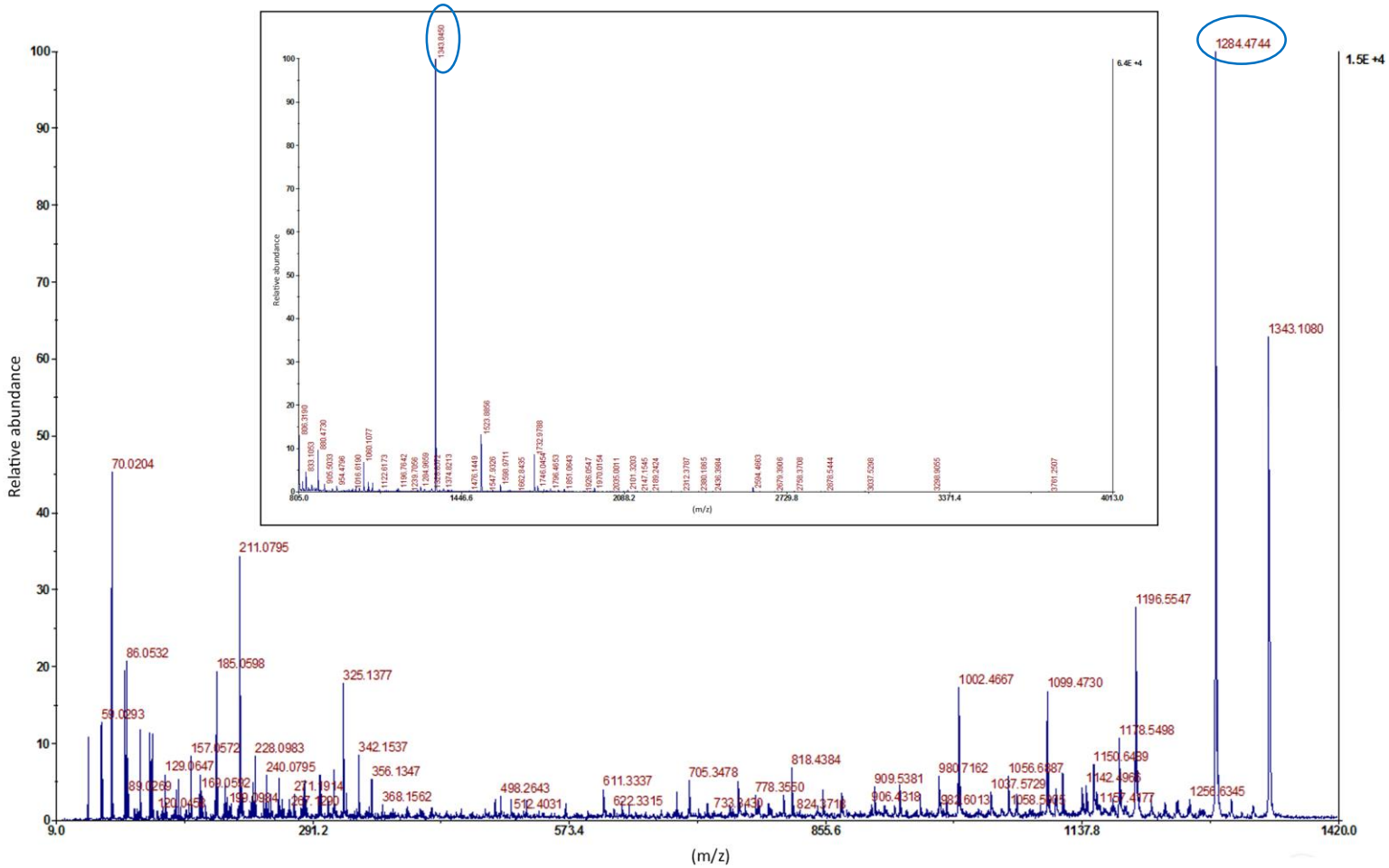


Figure 4-7 Mass spectral analysis of a chymotryptic digest of the c-subunit of the F-ATP synthase from possum

The main part of the figure shows the tandem MS analysis of a chymotryptic peptide (1343.8 m/z, the insert portion), corresponding to residues 37-47 of the c-subunit. The prominent ion at m/z 1284.5 corresponds to the loss of a trimethyl-ammonium ion.

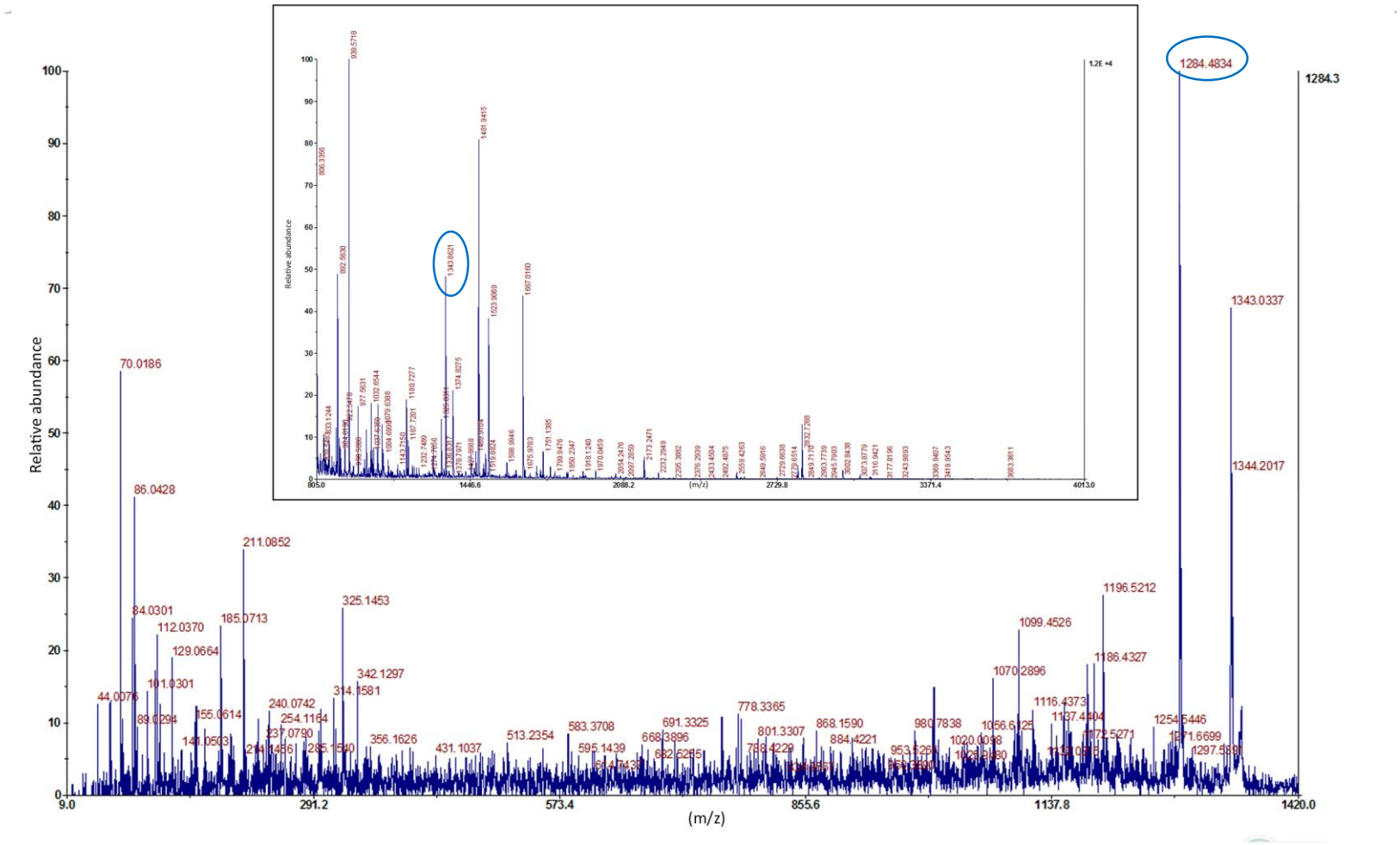


Figure 4-8 Mass spectral analysis of a chymotryptic digest of the c-subunit of the F-ATP synthase from kina

The main part of the figure shows the tandem MS analysis of a chymotryptic peptide (1343.8 m/z, the insert portion), corresponding to residues 37-47 of the c-subunit. The prominent ion at m/z 1284.5 corresponds to the loss of a trimethyl-ammonium ion.

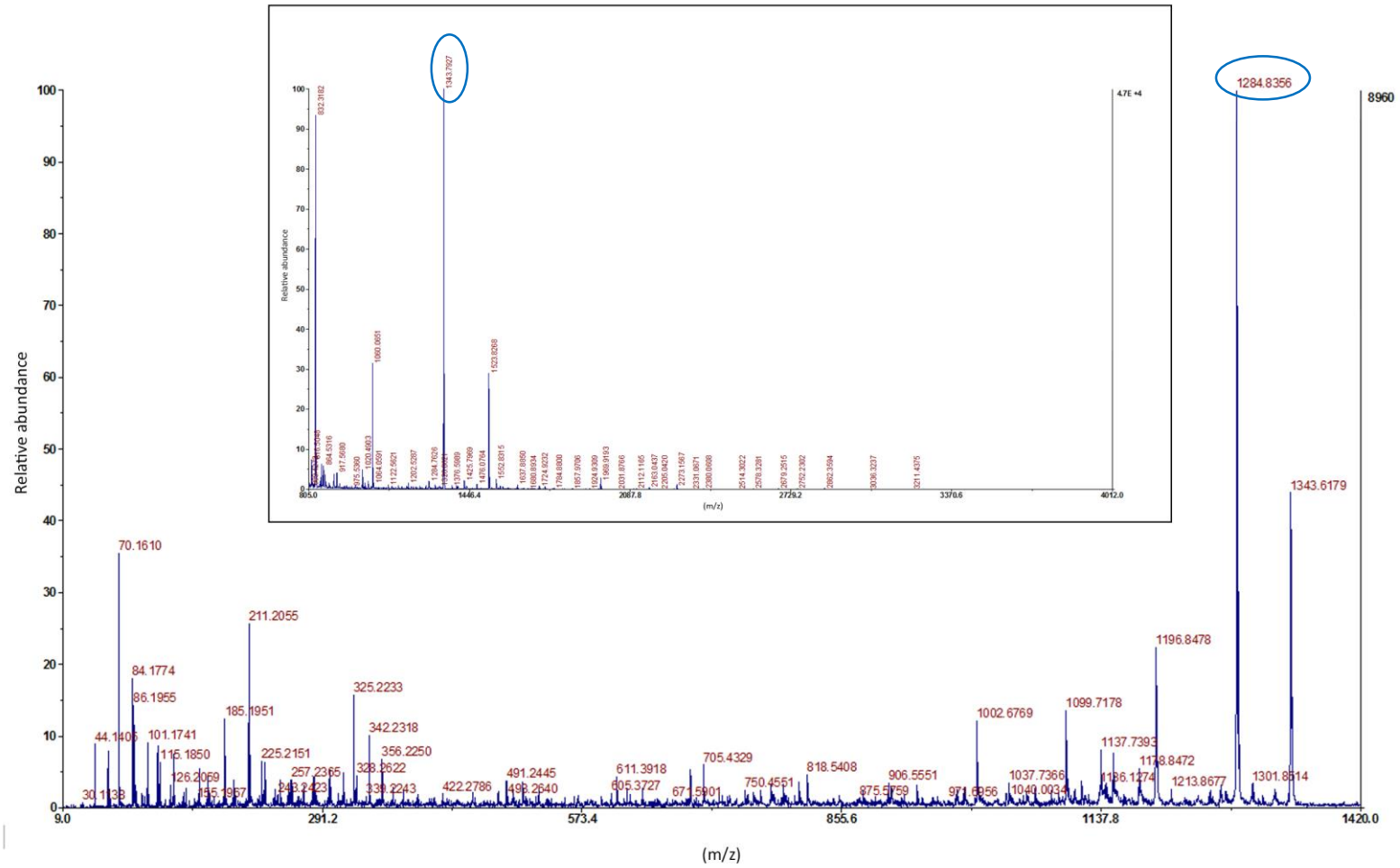


Figure 4-9 Mass spectral analysis of a chymotryptic digest of the c-subunit of the F-ATP synthase from sea cucumber

The main part of the figure shows the tandem MS analysis of a chymotryptic peptide (1343.8 m/z, the insert portion), corresponding to residues 37-47 of the c-subunit. The prominent ion at m/z 1284.8 corresponds to the loss of a trimethyl-ammonium ion.

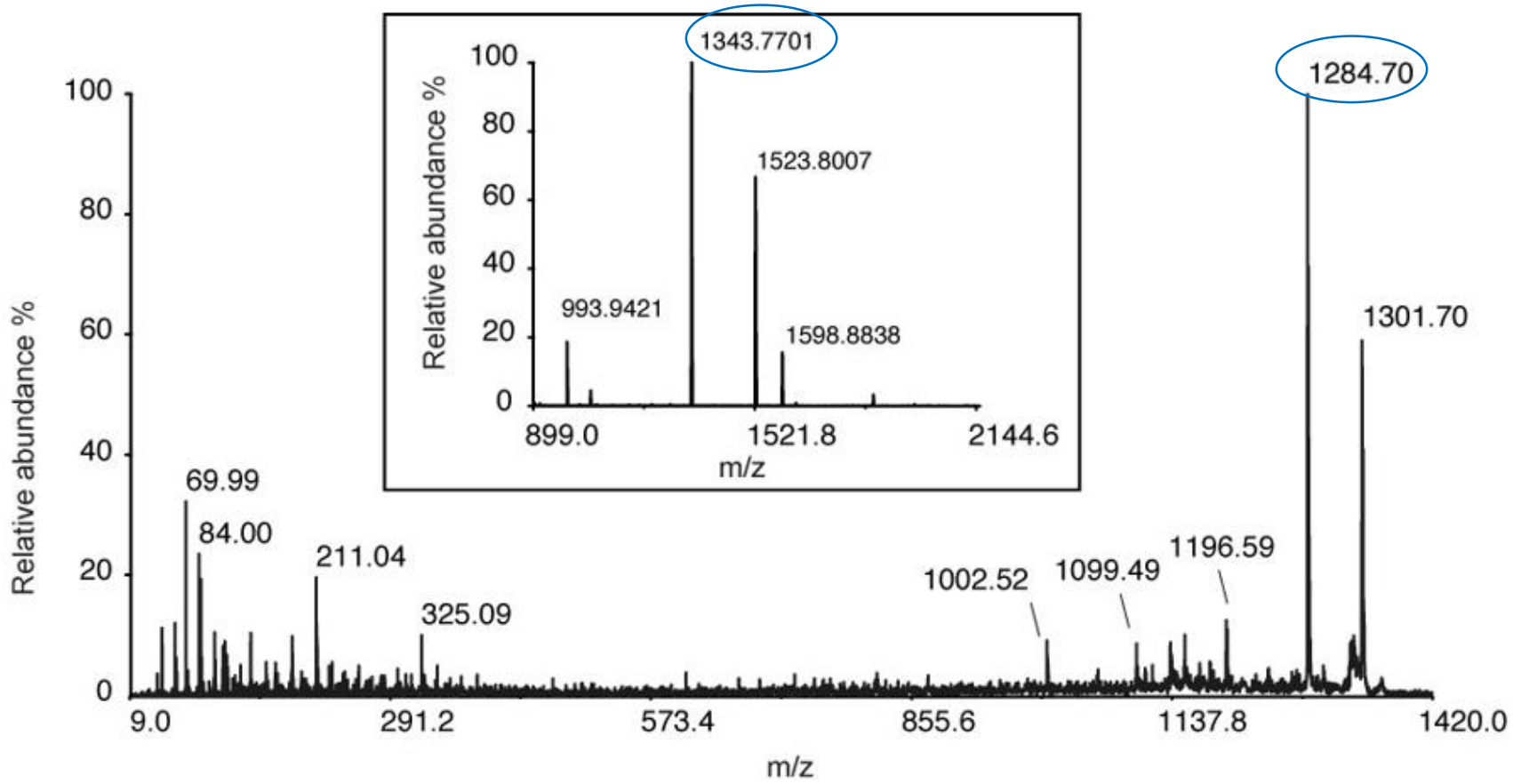


Figure 4-10 Mass spectral analysis of a chymotryptic digest of the c-subunit of the F-ATP synthase from sponge

The main part of the figure shows the tandem MS analysis of a chymotryptic peptide (1343.8 m/z, the insert portion), corresponding to residues 37-47 of the c-subunit. The prominent ion at m/z 1284.7 corresponds to the loss of a trimethyl-ammonium ion (Figure adapted from Walpole, 2015).

4.6 ETD-MS with Orbitrap MS

Although the MALDI-TOF MS/MS data had showed that the modification of the c-subunit was trimethylation within residues 37-47 of the protein, it did not provide enough information to localise the modification to one specific residue in the peptide. Using ETD-MS with Orbitrap MS allowed the sequence of the modified peptide to be deduced. In all cases, peaks labelled c6 and c7 correspond to the sequence ARNPSL and ARNPSLK respectively. In the event of no modification, c6 and c7 should be 656 Da and 784 Da. However ETD-MS with Orbitrap MS data (Figure 4-11) indicated c7 was 826 Da, i.e. it contained an extra 42 Da, which means that the extra 42 Da is on lysine 7 of the peptide residues 37-47 (i.e. lysine 43 of the c-subunit).

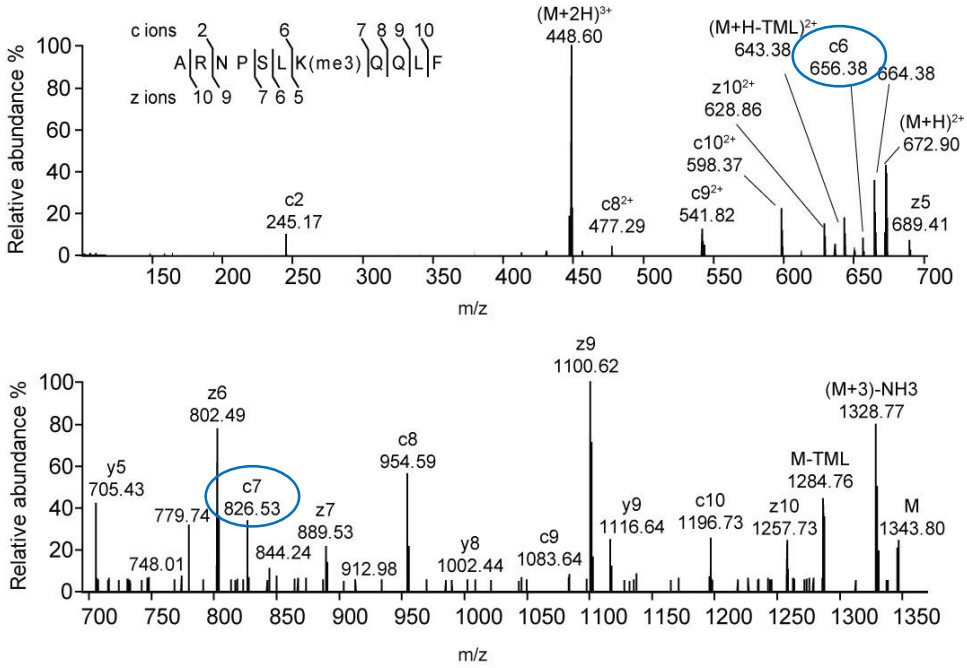


Figure 4-11 Analysis by tandem MS of a chymotryptic peptide containing residues 37-47 from the c-subunit of salmon

The peptide was fragmented by ETD and the fragments were analysed in an Orbitrap XL instrument. In the upper and lower panels, spectra ranging from 100-700 m/z and 700-1370 m/z are shown, respectively. The c and z type ions are mapped onto the amino acid sequence. The mass difference of 170.15 Da between the c6 and c7 ions corresponds to the mass of lysine 7 plus an additional 42 Da, showing that lysine 7 of the peptide residues 37-47 (i.e. lysine 43 of the c-subunit) is trimethylated (Figure adapted from Walpole et al., 2015).

Table 4 summarised results from the mass spectrum analyses of the chymotriptic peptides containing the trimethylation site in the c-subunit of metazoan species studied in this project. They all contain a trimethylated lysine 43.

Table 4 Analysis of the chymotriptic peptides containing the trimethylation site in the c-subunits of metazoan species

Species	Calculated	Observed	Δ	Trimethylated	Position
Possum	n.s.	1343.7938	n.a	Yes	K43
Kina	1301.7325	1343.7946	42.0621	Yes	K43
Sea cucumber	n.s.	1343.7940	n.a	Yes	K43
Sponge	n.s.	1343.7949	n.a	Yes	K43
Human	1301.7325	1343.7982	42.0657	Yes	K43
Bovine	1301.7325	1343.7937	42.0612	Yes	K43
Sheep	1301.7325	1343.7937	42.0162	Yes	K43
Pig	1301.7325	1343.7934	42.0609	Yes	K43
Rabbit	1301.7325	1343.7952	42.0627	Yes	K43
Mouse	1301.7325	1343.7943	42.0618	Yes	K43
Rat	1301.7325	1343.7931	42.0606	Yes	K43
Red deer	1301.7325	1343.7940	42.0615	Yes	K43
Chicken	1301.7325	1343.7946	42.0621	Yes	K43
Duck	1301.7325	1343.7934	42.0609	Yes	K43
Greek tortoise	n.s.	1343.7949	n.a	Yes	K43
Snake	n.s.	1343.7937	n.a	Yes	K43
Frog	1301.7325	1343.7949	42.0624	Yes	K43
Salmon	1301.7325	1343.7934	42.0609	Yes	K43
Rainbow trout	1301.7325	1343.7943	42.0618	Yes	K43
Seabass	n.s.	1343.7940	n.a	Yes	K43
Spiny dogfish	n.s.	1343.7949	n.a	Yes	K43
Brown fly larvae	1301.7325	1343.9740	42.0615	Yes	K43
Earth worm	1301.7325[6]	1343.7940	n.a	Yes	K43
Lobster	n.s.	1343.7930	n.a	Yes	K43
Brown crab	n.s.	1343.7934	n.a	Yes	K43
Pacific oyster	1273.7012	1315.7641	42.0625	Yes	K43
Mussel	1244.7110	1286.7657	42.0547	Yes	K43
Cabbage looper moth cells	1301.7325	1343.7934	n.a	Yes	K43
Nematode worms	1301.7325	1343.7943	42.0618	Yes	K43
Fruit fly	1301.7325	1343.7934	42.0609	Yes	K43
Brine shrimp	n.s.	1343.7940	n.a	Yes	K43

n.s. no genome sequence available; n.a. no intact mass difference available (Table adapted from Walpole et al., 2015).

5 Discussion and Conclusion

Recent studies indicated that higher animals synthesise ATP more efficiently than other life forms, which is possible because trimethylation of lysine 43 of the c-subunit allows higher gearing of the ATP synthase complex (Watt et al., 2010). This project was to determine the extent of this modification in the metazoan phyla.

5.1 Purification of metazoan c-subunits

Mitochondria are small vesicles. In order to preserve them, animal tissues were homogenised using a Potter-Elvehjem homogenizer. Where animal tissues were too tough for the Potter-Elvehjem homogenizer, minimal disruption with a Polytron was used.

Crude mitochondria were purified from possum liver at a yield of 9.2mg crude mitochondrial protein per gram tissue (Table 4-1), consistent with the yields obtained from other vertebrate species determined by Walpole et al. (2015). However the yields from lower animal species were a lot lower (Table 4-1). This is predictable when the whole animal is used as the source and these lower animals do not require as much energy as mammals and have fewer mitochondria.

The extraordinary specificity of bovine $IF_{1\text{-}60}$ allowed active possum F-ATP synthase to be purified from a crude detergent solubilised fraction in a single step. $IF_{1\text{-}60}$-GST-HIS prepared according to Runswick et al. (2013) bound at the catalytic interface between the α- and β-subunits of the enzyme to form a F_1F_0-$I_{1\text{-}60}$-GST-HIS complex. This $IF_{1\text{-}60}$-GST-HIS complex was then bound to a GSTrap HP column, and the active F-ATP synthase recovered by breaking the magnesium dependent bond between the enzyme and the inhibitor with EDTA.

However the bovine $IF_{1\text{-}60}$ did not bind to the F-ATP synthases of lower metazoan species, and F-ATP synthase could not be isolated in this way from Pacific oyster, mussel, razor clam, sea cucumber, kina, or sponge. This is not surprising since there are significant differences between the bovine and lower order animal inhibitors (Figure 5-1).

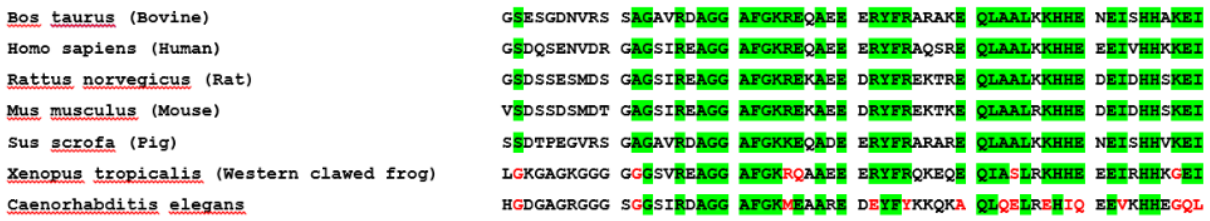


Figure 5-1 Alignment of the sequences of residues 1-60 of bovine IF_1, with the same regions from other vertebrate species, and the equivalent region of Caenorhabditis elegans ATP synthase inhibitor

Conserved residues are in green.

The *Caenorhabditis elegans* ATP synthase inhibitor was then prepared as it has been completely characterised, but unfortunately this did not work any better than the bovine inhibitor for these lower order animals (Walpole et al, 2014, unpublished result). Subsequently c-subunits were isolated from gradient purified mitochondria by Folch chloroform/methanol extraction (Folch et al., 1957).

5.2 Trimethylation of metazoan F-ATP synthase c-subunit

A total of eighteen vertebrate organisms were surveyed in this study. They covered at least one example of each class except the *sarcopteryfians* and the *agnathans* (Figure 5.2). In every case, a high intensity peak of mass 1343 was detected when c-subunit chymotryptic digests were analysed by MALDI-TOF MS. This mass corresponds to residues 37-47 of the c-subunit with an extra mass of 42 Da. No fragments of mass 1301 were detected in any experiments. In other words, all vertebrates surveyed in this experiment contain trimethylated c-subunits and no unmodified c-subunits (Figure 5-2). Furthermore, chymotryptic digests of vertebrate c-subunit peptide analysed by ETD-MS with Orbitrap MS confirmed lysine 43 as the trimethylated residue in all vertebrate species surveyed in these experiments. Since sequence data reveals that the c-subunit is absolutely conserved throughout vertebrate species (Figure 1-11), it is speculated that all vertebrate c-subunits are trimethylated at lysine 43.

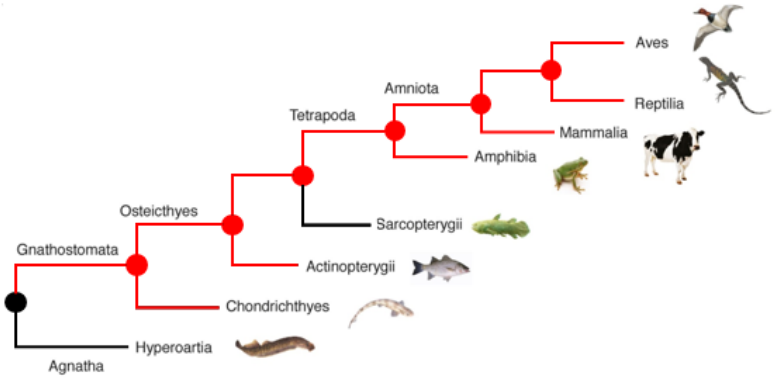


Figure 5-2 The vertebrate tree of life

The red branches denote species where lysine 43 in the c-subunit of F-ATP synthase has been demonstrated to be trimethylated (Figure adapted from Walpole et al., 2015).

Lysine 43 of c-subunit is also trimethylated in the fifteen invertebrate organisms surveyed in this study. The species cover six major phyla, namely *Echinodermata, Annelida, Mollusca, Arthropoda, Nematoda, and Porifera* (Figure 5-3). Since sequence data reveal that the c-subunit is highly conserved throughout invertebrate species (Figure 1-11), it is speculated that all invertebrates c-subunits are also trimethylated at lysine 43. Together, we conclude that all metazoan c-subunits contain trimethyllysine-43.

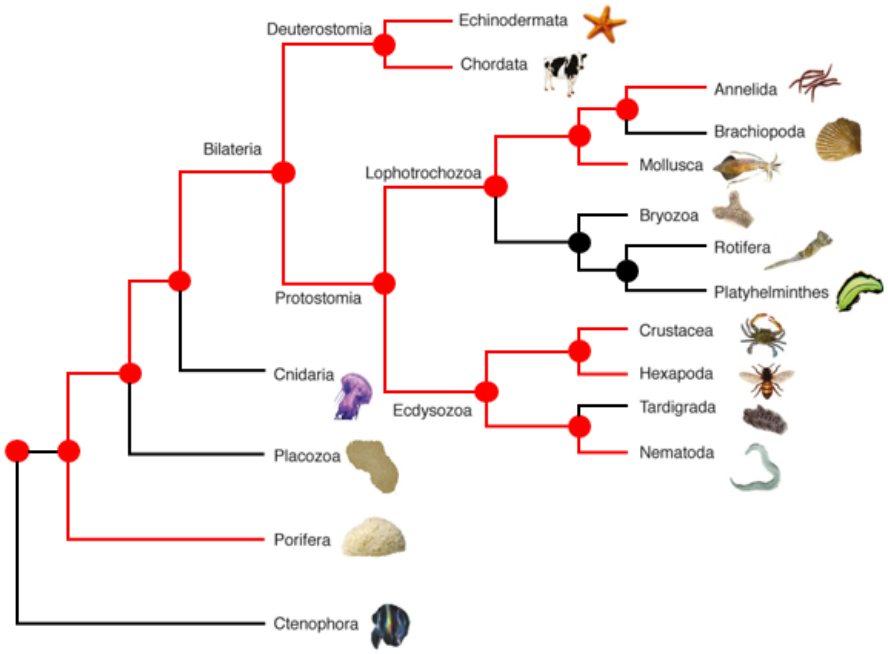


Figure 5-3 The invertebrate tree of life

The red branches denote species where lysine 43 in the c-subunit of F-ATP synthase have been demonstrated to be trimethylated (Figure adapted from Walpole et al., 2015).

5.2.1 Sponge c-subunit

Sponges represent one of the earliest divergence within metazoans (Figure 5-2), and as described in section 1.1.2, sponge c-subunit DNA (ATP9) is encoded on the mitochondrial genome. This study demonstrates that sponge c-subunit is trimethylated in the same way as the other metazoans. This indicates that in early metazoans the c-subunit gene modification has not migrated from the mitochondrial genome to the nuclear one, in turn indicating that c-subunit trimethylation happens inside mitochondria.

Both nuclear and mitochondrial encoded protein syntheses occur via a similar translation process but nuclear encoded protein synthesis occurs in the cytosol or rough endoplasmic reticulum, while the protein synthesis location in mitochondria is not clear. It will be interesting to find out if there is some region of the inner mitochondrial membrane that acts as a rough endoplasmic reticulum surrogate for mitochondrial protein synthesis.

As described in section 1.3.1, three different nuclear encoded mammalian c-subunit genes are translated into three different isoforms, P1, P2, and P3. Although these three isoforms bear different N-terminal mitochondrial import sequences, they produce one identical mature protein which consist of 75 amino acids (Figure 1-13). However, even within the same gene across different species, amino acid sequences of the mitochondrial import sequences vary, in contrast to their mature protein sequences which are absolutely conserved (Figure 5-4). In contrast, mitochondrial encoded c-subunits lack such N-terminal mitochondrial import sequences (Figure 1-14). This indicates the enormous selective pressure on the mature c-subunit sequence. It is so probably because then they can pack into a c_8-ring which requires the trimethylation of lysine 43.

```
Human    MQTAGALFIS PALIRCCTRG LIRPVSASFL NSPVNSSKQP SYSNFPLQVA RREFQTSVVS RDIDTAAKFI
Bovine   MQTTGALLIS PALIRSCTRG LIRPVSASFL SRPEIQSVQP SYSSGPLQVA RREFQTSVVS RDIDTAAKFI
Sheep    MQTTGALLIS PALIRSCTRG LIRPVSASFL SRPEIPSVQP SYSSGPLQVA RREFQTSVVS RDIDTAAKFI
Pig      MQTTGALLIS PALLRSCTRG LIRPVSASFL SRPEIPSEQP PCSSVPLQVA RREFQTSVVS RDIDTAAKFI
Mouse    MQTTKALLIS PALIRSCTRG LIRPVSASLL SRPEAPSKQP SCSSSPLQVA RREFQTSVIS RDIDTAAKFI
Rat      MQTTKALLIS PVLIRSCTRG LIRPVSASLL SRPEAPSKKP SCCSSPLQVA RREFQTSVIS RDIDTAAKFI

Human    GAGAATVGVA GSGAGIGTVF GSLIIGYARN PSLKQQLFSY AILGFALSEA MGLFCLMVAF LILFAM
Bovine   GAGAATVGVA GSGAGIGTVF GSLIIGYARN PSLKQQLFSY AILGFALSEA MGLFCLMVAF LILFAM
Sheep    GAGAATVGVA GSGAGIGTVF GSLIIGYARN PSLKQQLFSY AILGFALSEA MGLFCLMVAF LILFAM
Pig      GAGAATVGVA GSGAGIGTVF GSLIIGYARN PSLKQQLFSY AILGFALFEA MGLFCLMVAF LILFAM
Mouse    GAGAATVGVA GSGAGIGTVF GSLIIGYARN PSLKQQLFSY AILGFALSEA MGLFCLMVAF LILFAM
Rat      GAGAATVGVA GSGAGIGTVF GSLIIGYARN PSLKQQLFSY AILGFALSEA MGLFCLMVAF LILFAM
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Figure 5-4 Alignments of P1 genes (ATP5G1) of various species

The identical mature protein sequences are in bold.

Coincidentally, the ATP9 gene is differently distributed between the nuclear and mitochondrial genomes in different filamentous fungi species, but ATP9 gene distribution in these fungi is more complicated. They have up to three ATP9 copy numbers in five different distributions: 1) one single gene in the mitochondrial genome; 2) one single gene in the nuclear genome; 3) one copy in each of the mitochondrial and nuclear genomes; 4) two copies in the nuclear genome; 5) two copies in the nuclear genome and one in the mitochondrial genome (De´quard-Chablat et al., 2011). This diversity of ATP9 gene distribution is indicative of an active process of gene transfer from the mitochondria to the nucleus during the evolution of filamentous fungi (De´quard-Chablat et al., 2011). Among these all, *Saccharomyces cerevisiae* has only one ATP9 gene in the mitochondrial genome, and it is not trimethylated. Therefore, trimethylation is not dependent on the location of the genome.

5.3 Non-metazoan eukaryotic F-ATP synthase c-subunits

In this study, the mitochondrial F-ATP synthase c-subunit was also isolated from a plant, the potato (*Solanum tuberosum*). Unlike animals, plants have chloroplasts which also contain ATP synthase. In order to eliminate the chloroplast ATP synthase, mitochondria were isolated from potato tubers. Mass spectrum results indicated that the potato mitochondrial c-subunit is not trimethylated (Walpole et al., 2015). Alignment of c-subunit sequence of plant species against human (Figure 5-5) reveals that although plants have a lysine residue at the similar position (i.e. K44), they lack of the three alanine residues also required to form a c_8-ring (Watt et al., 2010).

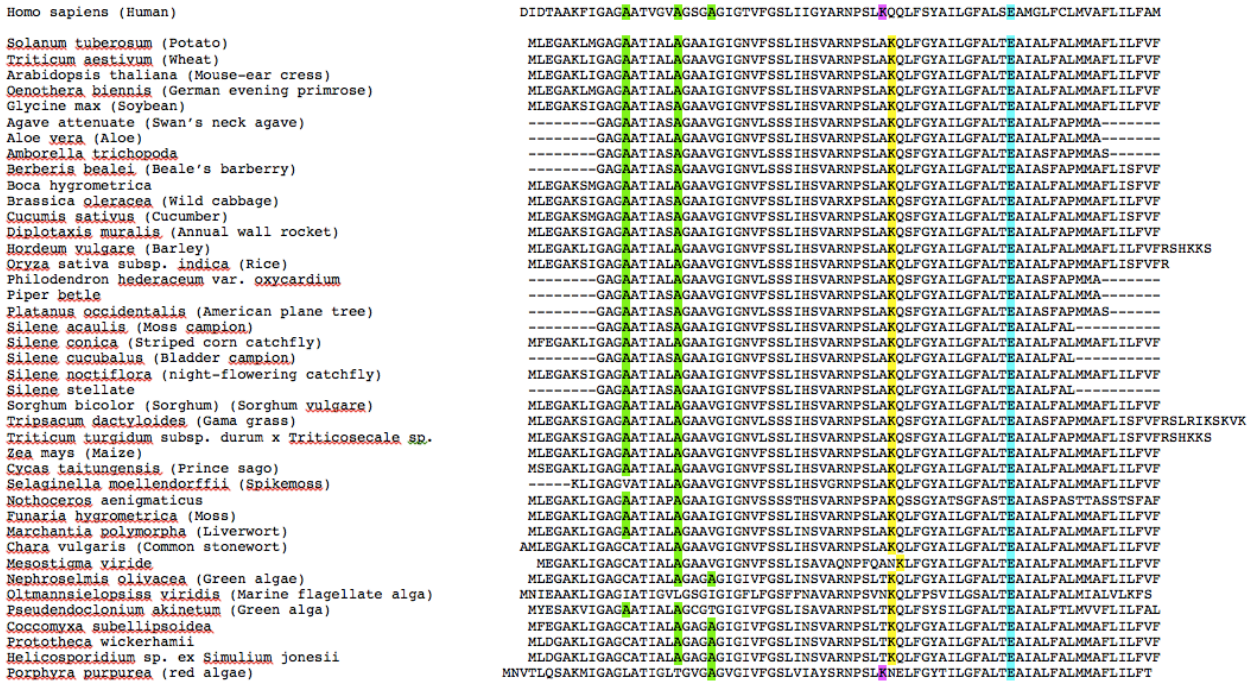

Figure 5-5 Alignment of c-subunits sequence of plant species against the human sequence

The alanine residues required for the formation of a c_8-ring are indicated in green. The proposed binding site for cardiolipin is indicated in purple. The essential residue for proton translocation through the membrane is indicated in blue.

The fungal species *Saccharomyces cerevisiae* and *Yarrowia lipolytica* c-subunits contain an unmodified lysine residue in an equivalent position to lysine 43 in the metazoans (Thomas Charlesworth, MBU, Cambridge, personal communication). However *Saccharomyces cerevisiae* has a c_{10}-ring (Stock et al., 1999), and the same for the fungal species *Pichia angusta* (Sidong Liu, MBU, Cambridge, personal communication). Alignment of c-subunit sequences of the fungal species mentioned above against the human sequence (Figure 5-5) reveals that like the potato, they lack the three critical alanine residues.

Figure 5-6 Alignment of c-subunit sequences of fungal species against the human sequence

The alanine residues required for the formation of a c_8-ring are indicated in green. The proposed binding site for cardiolipin is indicated in purple. The essential residue for proton translocation through the membrane is indicated in blue.

5.4 Trimethylation and the c_8-ring

Trimethylation of lysine 43 is confirmed in all the metazoan c-subunits analysed in these experiments. Sequence data reveals all metazoan species contain the three alanine residues that are required to form c_8-ring (Figure 1-9). These residues are not conserved in other c-rings which have ten to fifteen c-subunits (Figure 5-6). Thus trimethyllysine-43 of c-subunit appears to be a hallmark of a c_8-ring.

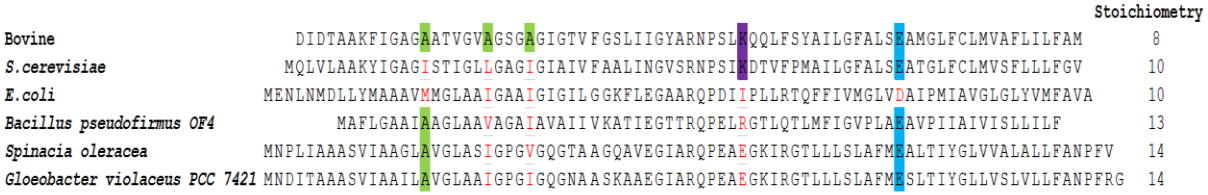

Figure 5-7 Alignment of c-subunit sequences from ATP synthases with known stoichiometry

The alanine residues required for the formation of a c_8-ring are indicated in green. The proposed binding site for cardiolipin is indicated in purple. The essential residue for proton translocation through the membrane is indicated in blue.

A direct observation of the c_8-ring has been achieved on the bovine molecule but remains to be established for other animals (Zhou et al., 2015). Based on the above, we conclude that all metazoans (including bovines) contain c_8-rings and that their bioenergetic cost of making one ATP is 2.7 protons.

The stoichiometry of the potato mitochondrial c-ring is unknown, however it is speculated it will not be a c_8-ring due to its lack of the three alanine residues and the lack of trimethylation of lysine 43. The same can be said about other plants, and probably all non-metazoan eukaryotes.

5.5 Possible function of trimethyllysine-43 of the c-subunit

Protein methylation is a very energetically expensive modification for a cell. Because each methylation event costs 12 molecules of ATP (Atkinson, 1977), the trimethylation of a single c-subunit requires 36 molecules of ATP, and one c_8-ring requires a total of 288 molecules of ATP. This is a huge investment for a cell, and such a modification would have been removed during evolution if it unadvantageous. Hence the conservation of trimethyllysine-43 of c-subunit across metazoan is highly likely to have specific function.

A c_8-ring appears to be less stable than rings with more subunits. Fungal c-rings resistant to denaturation by SDS and are observed to migrate as oligomers on SDS-PAGE (Runswick et al., 2013), whilst metazoan c-rings are readily denatured and migrate at the foot of SDS-PAGE as monomers (Figure 4-4). The bovine F-ATP synthase was demonstrated to be tightly associated with cardiolipin (Eble et al., 1990). The crystal structure of the bovine c_8-ring (Figure 1-10) reveals that the outer ring formed by the C-terminal α-helix had large gaps between each subunit, which exposes the N-terminal α-helix inner ring. These gaps are thought to be occupied by the acyl groups of cardiolipin, which lacks a head-group but now interacts with the trimethylation modification in order to stabilize the c-ring within the mitochondrial membrane (Watt et al., 2010).

5.6 Methyltransferases

Since the sponge c-subunit is encoded in the mitochondrial genome and it is trimethylated in the same way as the other metazoan c-subunits, the methyltransferase(s) involved was speculated to be located within mitochondria. Attempts were made to identify this mitochondrial methyltransferase by Thomas Walpole in the MBU laboratory. The expression of candidate proteins were supressed and the methylation status of the c-subunit were studied by western blotting and mass spectrometry. However none of the candidate proteins screened had any effect on the methylation status of the c-subunit (Walpole, 2015). Identification of the methyltransferase responsible for the methylation of the c-subunit would allow further investigation into the function of the modification.

Furthermore, c-subunit is not the only mitochondrial protein that is trimethylated, the ADP/ATP carrier (AAC) being the other major trimethylated protein identified in mitochondria (Rey et al., 2010), along with citrate synthase (Bloxham et al., 1981; Bloxham et al., 1982) and the ETF β subunit

(Rhein et al., 2014). It appears likely that there are other proteins containing trimethylation, but no comprehensive study of all TML proteins has been presented.

While c-subunits accumulate in most cells in many forms of NCLs (Palmer, 2015), it has not been found to do so in various homologous yeast knockout models. One possibility is that demethylation may be a necessary step in turnover of metazoan c-subunit and that interruption of this process is caused by the mutations underlying different forms of Batten disease.

5.7 Conclusion

The c-subunit of the F-ATP synthase is trimethylated in every metazoan species examined in this study. Since the amino acid sequences of the c-subunits of metazoans are very highly conserved, it is suggested that trimethylation of lysine 43 will be conserved in the c-subunits of all metazoan organisms. Trimethylation of the c-subunit in metazoans appears to be a hallmark of the c_8-ring. Despite a huge diversity in morphology and metabolic function amongst all metazoan species from sponges to humans, it is very likely that they all contain a c_8-ring in their F-ATP synthases and their bioenergetic cost of making an ATP molecule is 2.7 protons.

6 Appendix

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

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 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.***

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).

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⌘ *Author's Choice*—Final version full access.

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Author contributions: J.E.W. designed research; T.B.W., D.N.P., H.J., S.D., and I.M.F. performed research; T.B.W., S.D., I.M.F., and J.E.W. analyzed data; T.B.W., I.M.F., and J.E.W. wrote the paper; J.E.W. supervised the project.

[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 ReefPhyto (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).

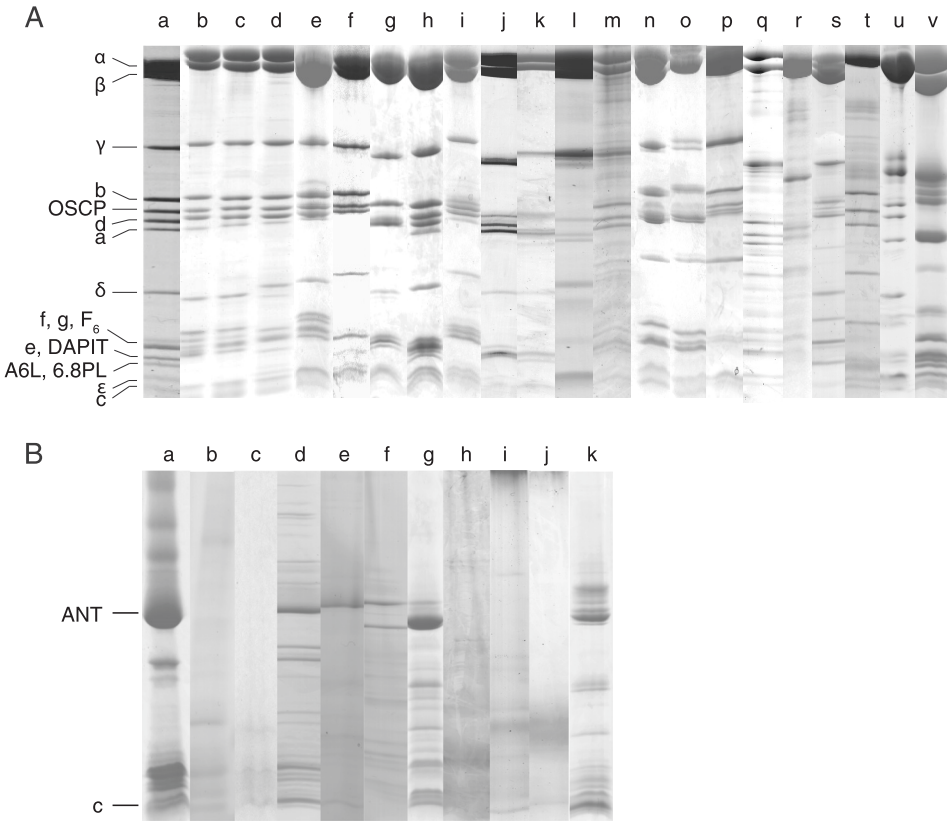

FIG. 1. **Isolation of the c-subunits of F-ATP synthases.** In parts *A* and *B*, respectively, the subunits of F-ATPases isolated by affinity chromatography and the hydrophobic proteins obtained by extraction of mitochondrial membranes with organic solvents were fractionated by SDS-PAGE, and stained 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 resuspension 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 ± 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.

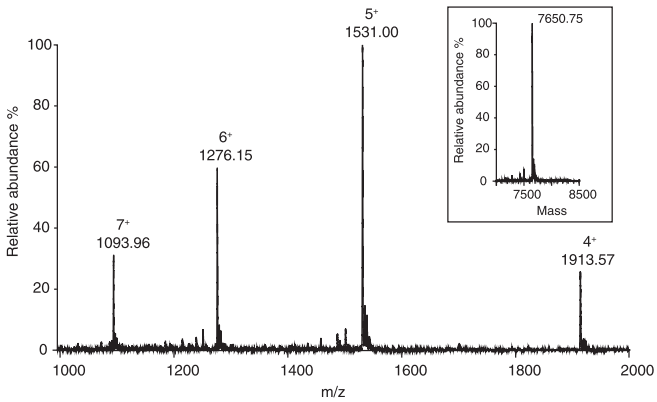

FIG. 2. **Mass spectral analysis of the intact c-subunit of the F-ATPase from *Salmo salar* by ESI-MS.** A series of multiply charged ions from the c-subunit are indicated. The insert contains a mathematical transformation of these data to a molecular mass scale.

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

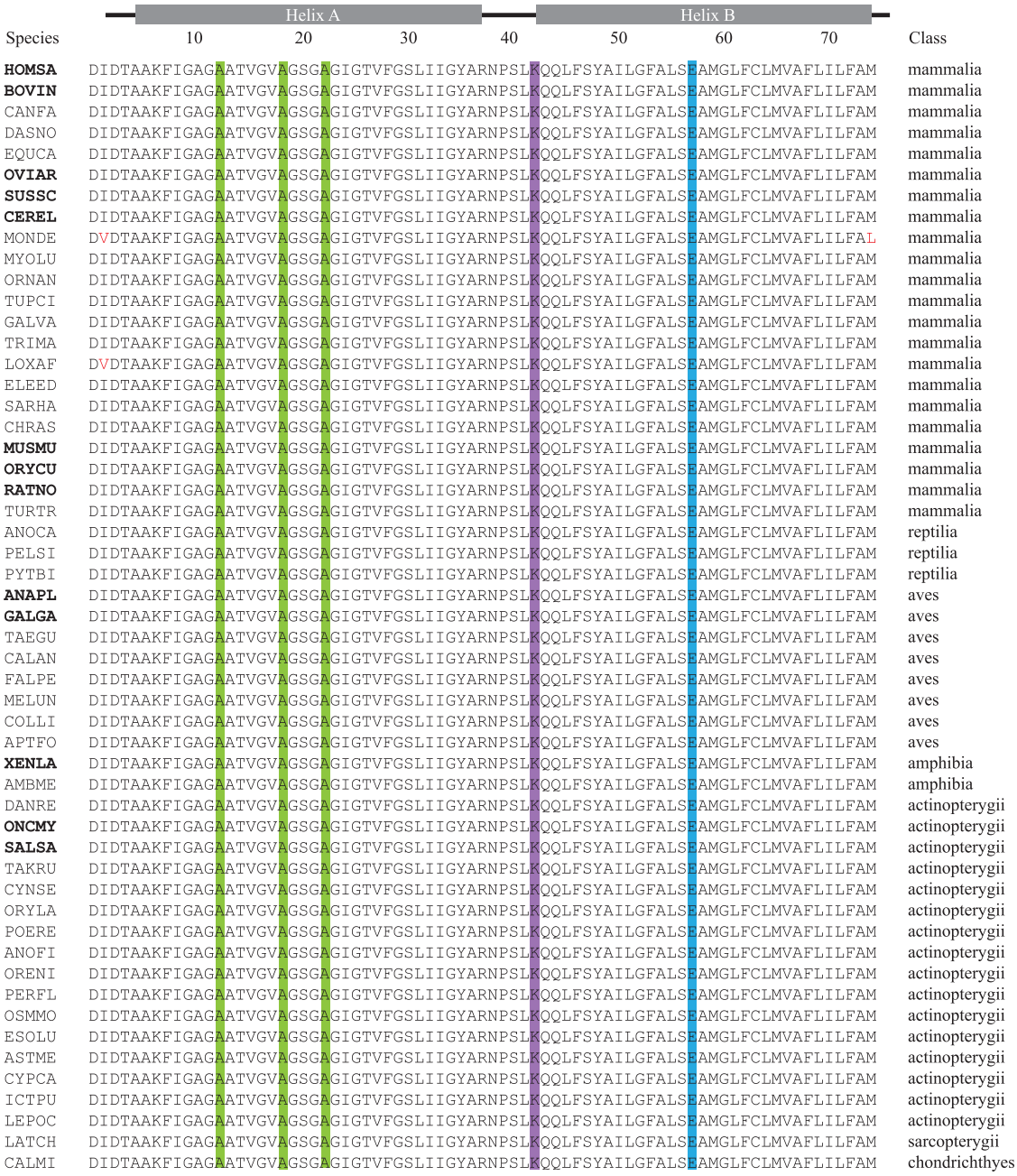

FIG. 3. **Sequences of c-subunits from vertebrate F-ATPases.** The secondary structure of the bovine protein is depicted above the aligned sequences. Where sequence data are available a representative species from each vertebrate order is shown. Alanine residues 13, 19, and 23, required for the formation 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).

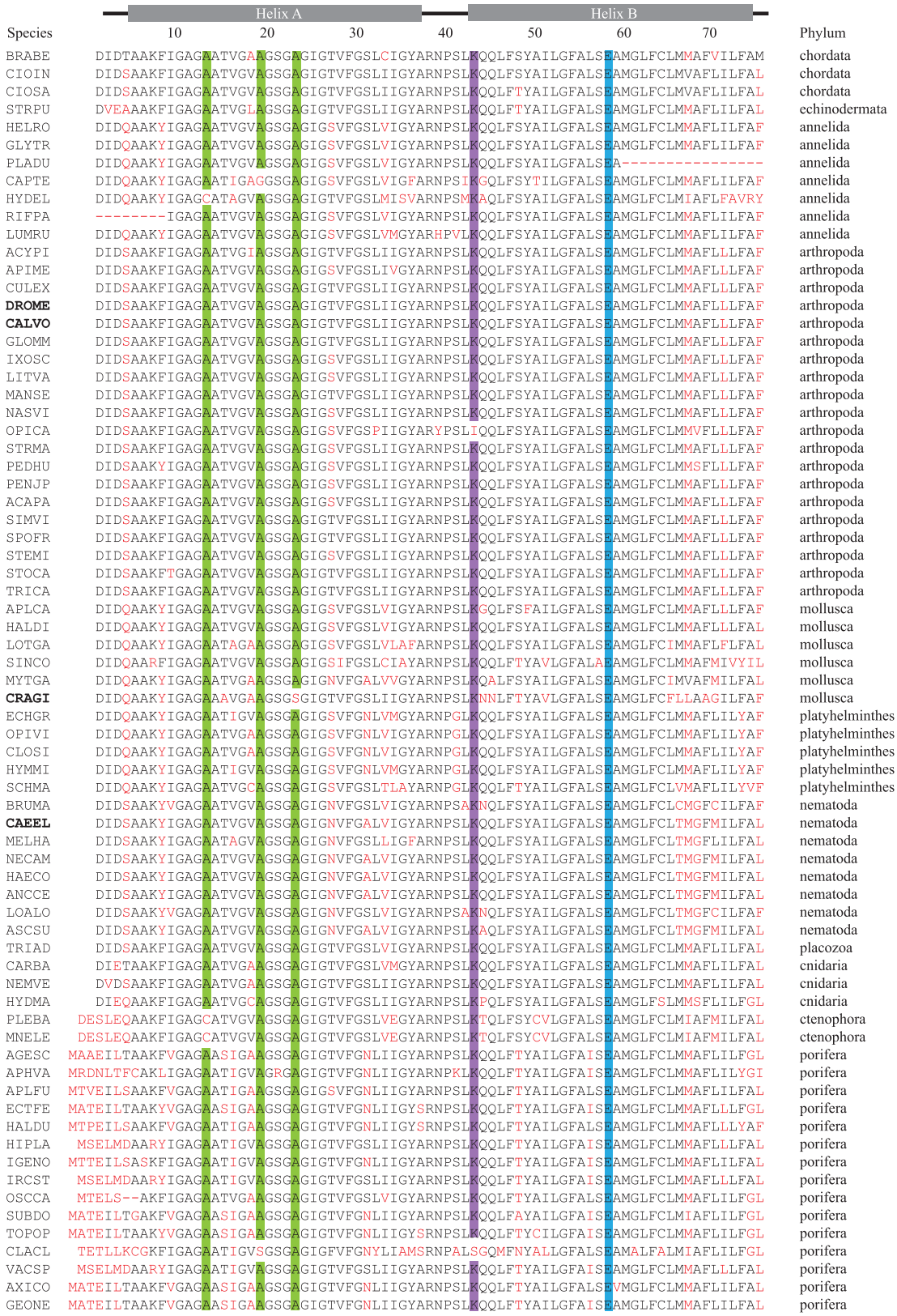

FIG. 4. **Sequences of c-subunits from invertebrate F-ATPases.** Where data are available sequences for all known invertebrate species are shown, with the exception of the arthropods and poriferans, where representative species were selected from each class when available. †, a related species was studied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

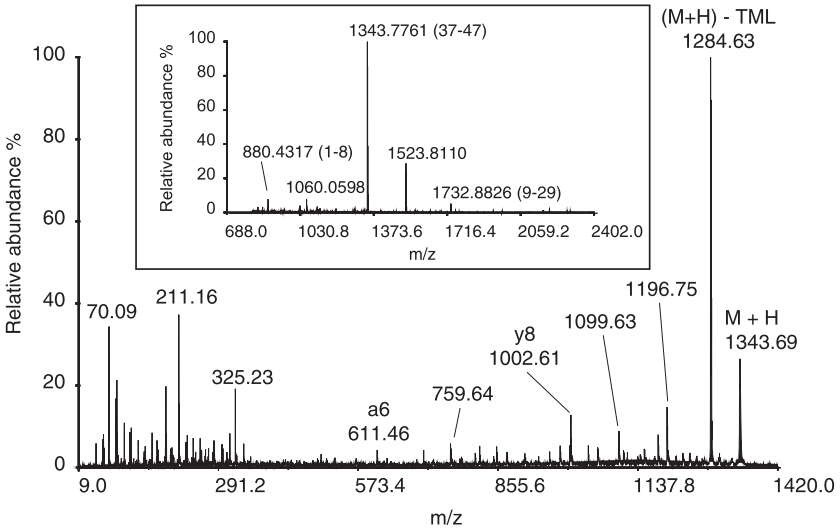

FIG. 5. **Tandem-MS analysis of a chymotryptic peptide from the c-subunit of the F-ATPase from *Salmo salar*.** The singly charged chymotryptic peptide (MH^+ 1343.69) corresponds to residues 37–47 of the protein. The prominent ion at *m/z* 1284.63 arises by loss of a trimethylammonium ion and is diagnostic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

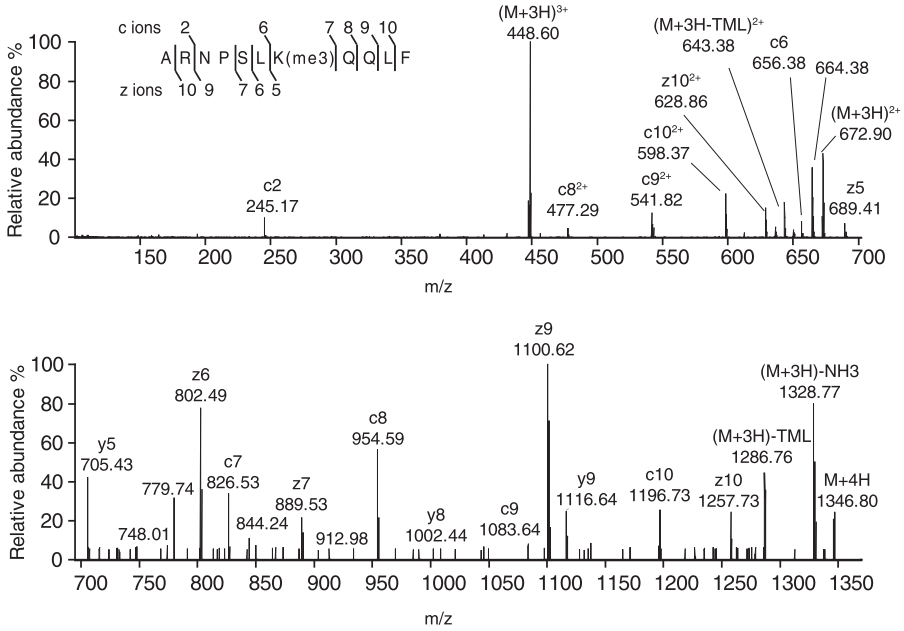

FIG. 6. **Tandem MS of a chymotryptic peptide from the c-subunit of *Salmo salar*.** The peptide represents residues 37–47 of the protein. A triply charged version (*m/z* 448.60) was fragmented by ETD, and the fragments were analyzed in an OrbiTrap instrument. The upper and lower panels contain ions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

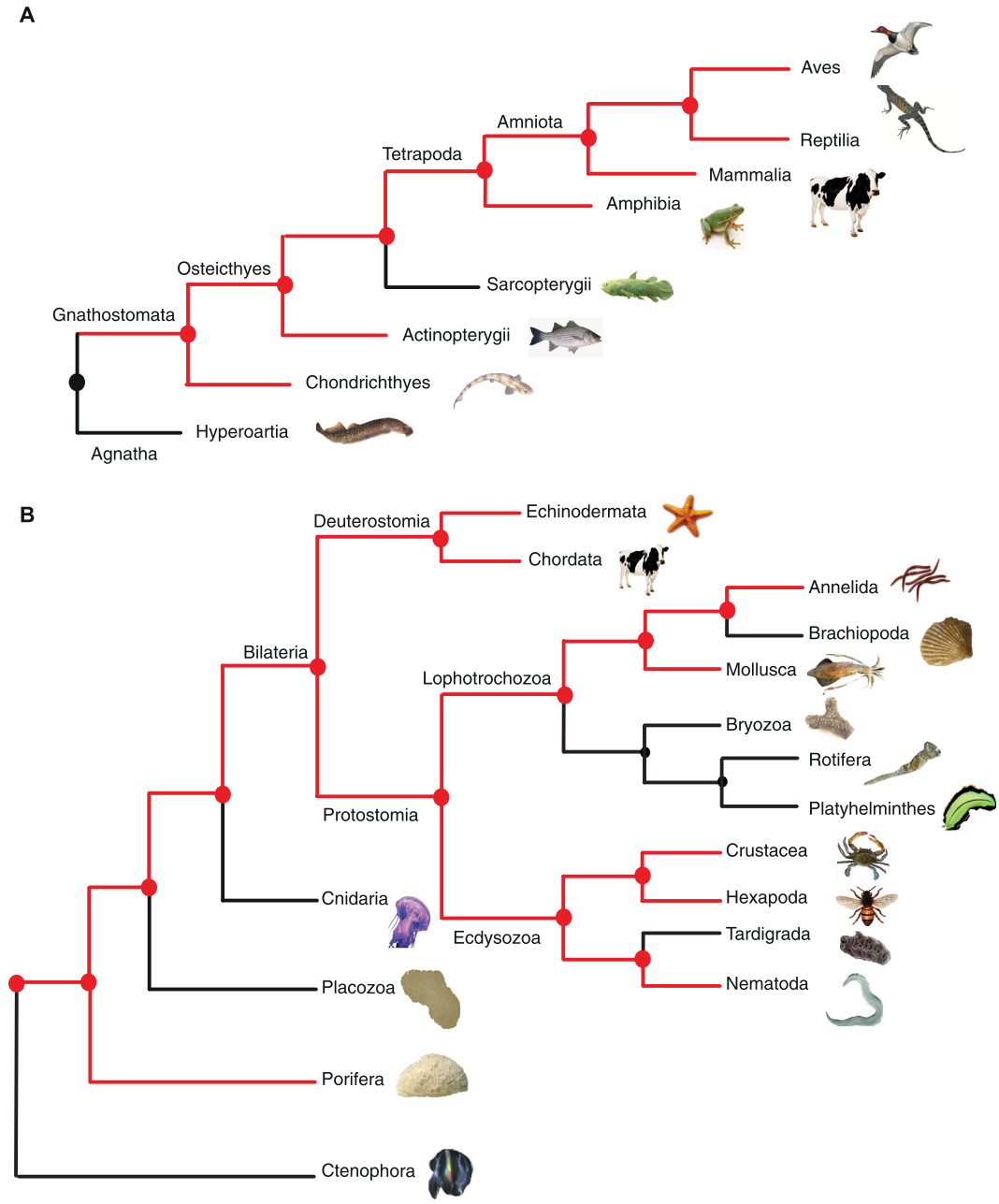

FIG. 7. **Metazoan tree of life.** Part *A*, the vertebrate tree. Part *B*, the major metazoan phyla. The red branches contain species where lysine-43 in the c-subunit of mitochondrial F-ATP synthase has been demonstrated to be trimethylated. In part *A*, the analyzed examples are as follows: Mammalia, *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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