Lysine 43 Is Trimethylated in Subunit c from Bovine Mitochondrial ATP Synthase and in Storage Bodies Associated with Batten Disease*†

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The hydrophobic membrane protein, subunit c, has been isolated from ATP synthase purified from bovine heart mitochondria. It has also been obtained from lysosomal storage bodies associated with ceroid lipofuscinosis from ovine liver and from human brain tissue of a victim of Batten disease. It is likely that the lysosomal protein has originated from the mitochondrion. These samples have been characterized by mass spectrometric methods. Irrespective of its source, subunit c has an intact molecular mass of 7650 Da, 42 Da greater than the value calculated from the amino acid sequence, and the protein has been modified post-translationally. In all three samples, the modification is associated with lysine 43, which lies in a polar loop region linking the two transmembrane α-helices of the protein. This residue is conserved throughout vertebrate sequences. The additional mass arises from trimethylation and not acetylation at the ε-N-position of the residue. These experiments show that the post-translational modification of subunit c is not, as has been suggested, an abnormal phenomenon associated with the etiology of Batten disease and ceroid lipofuscinosis. Evidently, it occurs either before or during import of the protein into mitochondria or at a mitochondrial location after completion of the import process. The function of the trimethyllysine residue in the assembled ATP synthase complex is obscure. The residue and the modification are not conserved in all ATP synthases, and their role in the assembly and (or) functioning of the enzyme appear to be confined to higher organisms.

Subunit c is a small hydrophobic protein that forms oligomeric rings in the membrane domain of ATP synthases from mitochondria, chloroplasts, and eubacteria (1–3). Together with the central stalk of the enzyme (subunits γ, δ, and ε) (4) with which it is intimately associated (1), it forms the rotary element of the enzyme that couples the transmembrane proton-motive force to the synthesis of ATP from ADP and phosphate in its catalytic F1 domain (5). In the mitochondria of vertebrates, subunit c is composed of 75 amino acids and, as far as is known, its sequence is absolutely conserved in mammals and the only known substitutions in vertebrates are Ile70-Leu in carp and Met60-Val in flounders. In Saccharomyces cerevisiae and Escherichia coli, the sequence of subunit c is 60 and 20% conserved, respectively, relative to the mammalian protein. More than 25 years ago, the mitochondrial protein was proposed to be folded into two transmembrane α-helices linked by a polar loop structure containing the absolutely conserved sequence Arg-Asn-Pro (residues 38–40 in the bovine protein) (6, 7). This proposal has been borne out by subsequent extensive genetic and biochemical studies, especially of the E. coli protein, by solution NMR studies in organic solvents of the E. coli protein (8, 9), and by x-ray crystallographic analysis of the F1-c10 complex of the enzyme from S. cerevisiae (1). The studies of the bacterial protein have also confirmed the absolute requirement of the carboxylate group of Asp61 (Glu58 in the bovine protein) in the second transmembrane helix for active proton translocation through the membrane (10).

Subunit c has also been found in large amounts in fluorescent storage bodies associated with most forms of a group of neurodegenerative diseases called Batten disease in man or the (neuronal) ceroid lipofuscinoses. Each form is caused by mutations in specific genes. A form in sheep is syntenic with the human CLN61 (early juvenile or late infantile variant) disease (11–13). Specific lysosomal storage of subunit c has been demonstrated by protein sequencing of bodies from these sheep and of human samples associated with the CLN2 (late infantile), CLN3 (juvenile), (14–16), CLN5, and CLN8 human forms (17, 18). They are not associated with the CLN1 (infantile) form (19). The stored protein and the normal mitochondrial subunit c were found to be identical by protein sequencing (15, 16). By amino acid analysis of acid hydrolysates of storage bodies, one mole of trimethyllysine was detected per mole of subunit c and it was proposed that the modification of lysine 43 was associated with the etiology of the disease (20–23). An unmodified lysine residue was detected at lysine 7 by Edman sequencing of the intact stored protein, and therefore, the only other lysine residue in the protein at position 43 was assumed to be the modified residue (14, 16, 22, 23). Consistent with these proposals, molecular mass measurements by electrospray mass spectrometry of intact subunit c isolated from sheep storage bodies showed the presence in the protein of an additional mass of 42 Da but neither the location nor the chemical nature of the modification has been determined directly (24). Other post-translational modifications, including S-methyl methionine, have also been proposed to be associated with stored subunit c.

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The on-line version of this article (available at http://www.jbc.org) contains Supplemental Tables I–III and Figs. 1–4.

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‡ The abbreviations used are: CLN, neuronal ceroid lipofuscinosis gene; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Q-TOF, quadrupole time-of-flight.
from different forms of Batten disease at different times, and consequently, accounts of the modification status of the stored proteins are contradictory (25, 26).

As reported here, samples of subunit c isolated from bovine mitochondrial ATP synthase and from human and ovine storage bodies have been analyzed by mass spectrometry. Their molecular masses are identical, and they all contain an additional mass of 42 Da. In the bovine, human, and ovine proteins, this mass is associated with lysine 43 and arises from trimethylation of the ε-amine group of this residue. As the modification is present in both the mitochondrial protein and in storage bodies, the etiology of the ceroid lipofuscinoses (Batten disease) is not associated with the post-translational modification of lysine 43.

EXPERIMENTAL PROCEDURES

Purification of ATP Synthase—ATP synthase was purified from bovine heart mitochondria (27).

Purification of Subunit c for Molecular Mass Analysis—Samples of subunit c were extracted from bovine heart mitochondrial membranes in mixtures of chloroform methanol (28, 29). The crude proteolipid extract from mitochondrial membranes was fractionated by gel filtration on Toyopearl HW-55 in a mixture of chloroform, methanol, and water (46:46:8 by volume) containing 60 mM sodium acetate, pH 7.0. Fractions containing subunit c were desalted before analysis by electrospray ionization mass spectrometry (ESI-MS). Chloroform and water were added to fractions (final composition of chloroform:methanol:water 8:4:3 by volume), causing phase separation of the upper aqueous methanol layer from a mostly chloroform layer. The lower layer was removed. The proteins in it were concentrated in a stream of nitrogen and then precipitated with diethyl ether (4 volumes) at −20 °C. Subunit c was extracted from purified storage bodies with chloroform:methanol (16).

Isolation of Storage Bodies—Storage bodies were isolated from livers of sheep affected with ceroid lipofuscinosis and from a sample of frozen brain tissue of a deceased patient affected by the late infantile disease (15, 30).

Polyacrylamide Gel Electrophoresis—The subunits of bovine mitochondrial ATP synthase and samples of human and ovine storage bodies isolated from Batten tissues were dissolved in 5% lithium dodecyl sulfate containing 10 mM dithiothreitol and analyzed by SDS-PAGE on an acrylamide gradient (10–22%) (31). Proteins were visualized by staining with Coomassie Blue R250 dye (British Drug Houses, Poole, United Kingdom).

Protein Characterization by Peptide Mass Fingerprinting—Protein bands were excised and cleaved “in-gel” at 37 °C with chymotrypsin (12.5 ng/μl; 20 μl Tris-HCl, pH 8.0) without prior reduction and alkylation of cysteine residues (32). Sequencing grade chymotrypsin was purchased from Roche Applied Science. Peptides were analyzed by peptide mass fingerprinting in a MALDI-TOF mass spectrometer (Tosispec, Micromass, Altrincham, United Kingdom) or by tandem MS peptide sequencing in a Q-TOF instrument (Micromass, Altrincham, United Kingdom) as described previously (33). Intact protein molecular masses were measured in chloroform:methanol by ESI-MS using a Sciex API III′ triple quadrupole mass spectrometer (34).

RESULTS AND DISCUSSION

Molecular Mass Measurements of Subunit c—Samples of subunit c from mitochondria and storage bodies were analyzed by ESI-MS. The average molecular mass of the bovine mitochondrial subunit c was 7650 ± 1 Da, 42 Da greater than the value of 7608 calculated from its amino acid sequence (Table I). Essentially, the same value was obtained with the samples of subunit c from storage bodies. Thus, all of the samples, irrespective of their source, contain a post-translational modification with a mass of 42 Da (Table I).

In both samples, masses of 7666 and 7683–7685 were also observed (see Supplementary Figs. S1, A–C). These masses arise from partial oxidation of one and two methionines, respectively, in the protein (see below). The abundance of these species indicates that 30–45% of the total protein is oxidized in this way. Other than this oxidation, which is likely to be an in vitro artifact (see below), the samples from bovine mitochondria and ovine storage bodies were homogenous and no other masses relating to subunit c were observed by ESI-MS. The spectra of subunit c from human storage bodies contain additional masses corresponding to the partial loss of one and two amino acids from the C terminus of the protein (see Supplementary Fig. S1B). No masses corresponding to unmodified subunit c were observed, and so the addition of 42 mass units appears to be quantitative. Subunit c purified from bovine mitochondria is only partially resolved from subunit A6L of ATP synthase in these experiments, and trace amounts of this protein with mass of 7965.4 were observed in the spectrum.

Location and Identification of the Modification—The site of modification was investigated by peptide mass mapping by MALDI-TOF and tandem ESI-MS of fragments produced by chymotryptic digestion of samples purified by SDS-PAGE (Fig. 1). The MALDI-TOF spectrum of the chymotryptic digest of subunit c from ATP synthase contained three peptides with m/z values of 880.4338, 1343.7844, and 1732.9155 (see Figs. 2 and Fig. S4 and Supplementary material). One peptide with a mass of 1343.7844 corresponded to chymotryptic peptide Ch-4 (residues 37–47) with an additional mass of 42 Da. The two other peptides corre-
sponded to Ch-1 (residues 1–8) and Ch-2 (residues 9–29) (see Fig. 2). These data show that the region from 37–47 contains the modification. They confirm that lysine 7 is unmodified and that the site of modification is not in residues 1–29.

All four protein bands examined from gels of storage body proteins contained only subunit c. Fragments of no other protein were identified (15, 16). The 3.5-kDa band represents the monomeric subunit c, and the bands migrating with higher molecular sizes are aggregates of it. In the chymotryptic digests of both human and ovine storage bodies, the same three fragments, Ch-1, Ch-2, and Ch-4, were observed in MALDI-TOF MS analyses of the 3.5-kDa bands (Figs. 3, B and C, respectively). As in the bovine protein, the modification lay in chymotryptic fragment Ch-4.

The site and nature of the modification were determined by tandem MS analysis of chymotryptic fragment Ch-4. A doubly charged ion from Ch-4 from mitochondria with an m/z of 672.3 [M+H]^{2+} was fragmented by collision-induced dissociation in a Q-TOF mass spectrometer. From the product ion spectrum (Fig. 4), a partial amino acid sequence was deduced from both y- and b-fragment ions. They confirm the peptide as ARNPSLKQQLF, where the underlined sequence was deduced from the data. A mass difference of 170.15 Da observed between the b_{7} and b_{6} ions corresponds to the modified lysine 43 (Fig. 4). This mass corresponds to a lysine (128.17 Da) plus an additional mass of 42 Da, thus confirming the location of the modified residue. The spectrum also contains fragment ions arising by the partial loss of trimethylamine-N(CH\text{3})	ext{3}, (mass = 59 Da) from the fragment ions y_{9}, y_{8}, and b_{7} (Fig. 4), which are diagnostic of peptides containing trimethyllysine (35). In addition, the product ion spectrum contains an immonium ion (m/z 84) also diagnostic for trimethyllysine, and there was no evidence for an immonium ion (m/z 126) arising from an acetylated lysine residue (35). The corresponding Ch-4 peptides from

![Fig. 2. Amino acid sequence of mammalian mitochondrial subunit c. The location of the modified lysine is marked with an asterisk, and the chymotryptic fragments characterized by mass spectrometry are indicated. The sequences of five chymotryptic peptides characterized from the mitochondrial ATP synthase subunit c are underlined and denoted Ch-1–Ch-5. Three fragments were characterized from subunit c isolated from either human or ovine storage bodies (lower portion of figure).](image-url)

![Fig. 3. MALDI-TOF MS analysis of chymotryptic digests from subunit c of mitochondrial ATP synthase and from storage bodies associated with Batten disease. Protein bands of 3.5 kDa were excised from SDS-PAGE gels (Fig. 1) digested in-gel with chymotrypsin, and a portion of the digests were analyzed by MALDI-TOF MS. A, subunit c from bovine mitochondrial ATP synthase. B, stored subunit c in the human brain storage bodies. C, stored subunit c from ovine liver storage bodies. Peaks marked Ch correspond to the predicted chymotryptic fragments of subunit c, and the numbers denote the order of the peptide in the sequence. The spectra shown were calibrated with Ch-1 (residues 1–8) and Ch-2 (residues 9–29) as internal calibration standards. In A, the peak marked with an asterisk is a fragment of chymotrypsin.](image-url)
stored human and ovine subunits c were characterized by tandem MS in a similar way. These spectra were qualitatively similar to those of subunit c from ATP synthase (see Supplementary Figs. S2 and S3). As in the mitochondrial protein, they define the modification of the stored proteins as N-trimethylation of lysine 43.

Additional evidence for trimethylation as opposed to N-acetylation (mass difference between the modifications of 0.0364 Da) was obtained from the MALDI-TOF spectrum of Ch-4 after recalibration with fragments Ch-1 and Ch-2 (m/z values 880.4338 and 1732.9155, respectively) as internal standards. The masses of the modified chymotryptic peptide from subunits c from bovine ATP synthase and from human and ovine storage bodies (1343.7844, 1343.7949, and 1343.7800, respectively) give mass values for the modifying group of 42.0592, 42.0697, and 42.0548 Da and support modification by trimethylation rather than acetylation.

Five additional chymotryptic fragments of bovine subunit c were identified by tandem MS analysis of digests of the bovine mitochondrial protein. They cover residues 1–47 and 70–73, and altogether, the tandem MS data cover 84% of the sequence of mitochondrial subunit c (see Supplemental Table SI). They indicate minor N-terminal heterogeneity in the protein involving the loss of residues 1–2 and 1–3. Similar heterogeneities have been noted in stored versions of subunit c.

Intact protein ions in the ESI-MS spectra of subunit c from the [M+H+16]⁺ species with a mass of 7666 were also analyzed by tandem MS. A series of multiply charged fragment ions localized the additional 16 Da to the C-terminal methionine residue, consistent with oxidation to methionine sulfoxide (see data in Supplementary Fig. S4). These [M+H+16]⁺ masses were observed in ESI-MS of mitochondrial and stored forms of subunit c (Table I). The intensity of the [M+H+16]⁺ species differed from experiment to experiment with the same samples, suggesting that oxidation arose during either the sample preparation in chloroform and methanol solutions or during electrospray ionization (36). No methionine-containing peptides were isolated in these experiments, and so it was not possible to examine the state of methionine oxidation by MALDI mass spectrometry.

**Biological Significance**—The finding that lysine 43 is modified by trimethylation in both the bovine mitochondrial protein assembled into ATP synthase and in the version that is stored in ovine and human lysosomal bodies in ceroid lipofuscinoses has potential implications both for the etiology of Batten disease and also for the function of the protein in ATP synthesis.

Several pieces of evidence point to the stored material having a mitochondrial origin. Among the most persuasive is the correct and rather precise removal of the mitochondrial import sequence in identical fashion from both the stored and mitochondrial protein (15). The presence of the trimethyllysine at residue 43 in both forms adds further support. However, it should be remembered that without investigation of subunit c from ovine or human ATP synthase at present it is not absolutely certain that the modification by trimethylation of the stored material takes place before the formation of storage.
bodies, although it is highly likely to do so. If so, the modification does not lead to nor is it a consequence of ceroid lipofuscinoses. The ceroid lipofuscinoses are an example of a lysosomal storage disease where the defect is likely to lie at the level of lysosomal function (37). Several human genes associated with the disease have been identified. Two of them, CLN1 (infantile) and CLN2 (classic late infantile), encode soluble lysosomal enzymes, a cholesterase and serine protease, respectively (38, 39). Four others, CLN3 (juvenile), CLN5 (Finnish variant late infantile), CLN6 (early juvenile), and CLN8 (Northern epilepsy or progressive epilepBy with mental retardation), encode proteins of unknown function predicted to be transmembrane proteins (12, 13, 40–42).

As yet, the biochemical origin(s) of the disease are unknown but any dysfunction associated with methylation of lysine 43 of subunit c can now be eliminated. Any possible modification of the stored subunit c by a fluorophore is also excluded, and the cause of fluorescence of storage bodies in situ is unconnected to lysine methylation (43).

The significance of the modification of lysine 43 of the subunit c assembled into ATP synthase is also obscure. This residue is adjacent to the membrane surface loop containing the conserved sequence Arg-Asn-Pro (residues 38–40) that connects its two transmembrane a-helices (7). The c-ring of the membrane domain of the bovine ATP synthase by analogy with the c-ring in the enzyme in mitochondria in S. cerevisiae probably contains 10 protomers (44). In contrast, the yeast subunit appears to be unmodified.2 This might suggest that the significance of the trimethylation is confined to a narrower range of species and that it reflects an association with one of the so-called “minor” subunits that is either absent from the yeast enzyme or is poorly conserved between the yeast and bovine enzymes. Trimethylation is not present in any of the other subunits of bovine ATP synthase (34, 45).

Finally, it is worth noting that trimethyllysine derived from protein breakdown, in diet for example, provides the precursor for carnitine biosynthesis, which is itself in part a mitochondrial process (21). Whether the breakdown of the relatively abundant subunit c contributes significantly to this process remains to be established.

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