The sequence of the major protein stored in ovine ceroid lipofuscinosis is identical with that of the dicyclohexylcarbodi-imide-reactive proteolipid of mitochondrial ATP synthase

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The ceroid lipofuscinoses are a group of neurodegenerative lysosomal storage diseases of children and animals that are recessively inherited. In diseased individuals fluorescent storage bodies accumulate in a wide variety of cells, including neurons. Previous studies of these bodies isolated from tissues of affected sheep confirmed that the storage occurs in lysosomes, and showed that the storage body is mostly made of a single protein with an apparent molecular mass of 3500 Da with an N-terminal amino acid sequence that is the same as residues 1–40 of the c-subunit (or dicyclohexyl-carbodi-imide-reactive proteolipid) of mitochondrial ATP synthase. In the present work we have shown by direct analysis that the stored protein is identical in sequence with the entire c-subunit of mitochondrial ATP synthase, a very hydrophobic protein of 75 amino acid residues. As far as can be detected by the Edman degradation, the stored protein appears not to have been subject to any post-translational modification other than the correct removal of the mitochondrial import sequences that have been shown in other experiments to be present at the N-terminal of its two different precursors. No other protein accumulates in the storage bodies to any significant extent. Taken with studies of the cDNAs for the c-subunit in normal and diseased sheep, these results indicate that the material that is stored in lysosomes of diseased animals has probably entered mitochondria and has been subjected to the proteolytic processing that is associated with mitochondrial import. This implies that the defect that leads to the lysosomal accumulation concerns the degradative pathway of the c-subunit of ATP synthase. An alternative, but less likely, hypothesis is that for some unknown reason the precursors of subunit c are being directly mis-targeted to lysosomes, where they become processed to yield a protein identical with the protein that is normally found in the mitochondrial ATP synthase assembly, and which then accumulates.

INTRODUCTION

The human neuronal ceroid lipofuscinoses are a group of recessively inherited lysosomal storage diseases of children that lead to blindness, seizures, dementia and premature death. Characteristic features are retinal and brain atrophy and the accumulation of fluorescent bodies in a wide variety of cells, including neurons. In the light microscope these bodies resemble the lipopigments ceroid and lipofuscin, and it is from this similarity that the name of the disease derives (Zeman & Dyken, 1969). However, the biochemical defect underlying the disease remains unknown.

Ceroid lipofuscinoses also occur in animals. In particular, a flock of affected South Hampshire sheep has been maintained and studied as a model for the human diseases (Jolly et al., 1980, 1982, 1988, 1989; Graydon & Jolly, 1984; Mayhew et al., 1985; Palmer et al., 1986a,b, 1988, 1989). Storage bodies isolated from tissues of these sheep were analysed in order to determine the nature of the stored component, in the expectation that it might provide clues to the nature of the defect. They have been shown to contain normal lysosomal lipids (Palmer et al., 1986a), and have a metal content that is consistent with storage occurring in lysosomes (Palmer et al., 1988). At least two-thirds of the mass of the bodies is protein, mainly a component with an apparent molecular mass of about 3500 Da that stains poorly with Coomassie Blue dye. This protein is soluble in organic solvents and so can be classified as a proteolipid. [The term 'proteolipid'

was first applied by Folch & Lees (1951) to describe proteins that are soluble in chloroform/methanol mixtures and so have lipid-like properties. It does not necessarily imply that the protein has covalently attached lipid.] It is not a normal lysosomal component (Palmer et al., 1986b). The sequence of the N-terminal 40 amino acid residues of this protein was found to be identical with that of the highly hydrophobic DCCD-reactive proteolipid, or subunit c, of bovine and human mitochondrial ATP synthase. Furthermore, the stored protein accounted for at least 40 % of the protein present in the storage bodies. It was concluded from these studies that ovine ceroid lipofuscinosis is a proteo-lipid proteinosis, specifically a lysosomal-mitochondrial-ATP-synthase-subunit proteinosis (Palmer et al., 1989).

In the present work we have shown that the major protein in the storage bodies is the intact and unmodified c-subunit of ATP synthase. Its complete sequence is identical with that of subunit c from human and bovine mitochondrial ATP synthase (Gay & Walker, 1985; Dyer & Walker, 1990) and also to subunit c from normal sheep (S. M. Medd & J. E. Walker, unpublished work).

MATERIALS AND METHODS

Isolation of storage bodies and mitochondrial inner-membrane vesicles

Storage bodies were isolated from tissues of sheep affected with ceroid lipofuscinosis (Palmer et al., 1988). Mitochondria

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were prepared from affected and control tissues by the method of Nedergard & Cannon (1979). Submitochondrial particles (mitochondrial inner-membrane vesicles) were obtained by sonication for 2 min in a Soniprep 150 instrument with 30 μm setting of a suspension of mitochondria in 0.28 M-sucrose/ 0.01 M-Tris/HCl buffer, pH 7.4, at a concentration of 5–20 mg of protein/ml (Wehrle et al., 1978).

Electron microscopy

Isolated storage bodies were fixed first in 2% (v/v) glutar-aldehyde and 3% (w/v) paraformaldehyde in 0.1 M-phosphate buffer, pH 7.2, and then in OsO_4. They were embedded in epoxy resin, and thin sections were stained with uranyl acetate and lead citrate. Mitochondrial inner-membrane vesicles and storage bodies were negatively stained on grids coated with plastic (Formvar) and carbon. The grids were floated on a solution of 1% (w/v) BSA for 10 s at 20 °C. Then excess BSA was blotted off and the grids were transferred to sample suspensions (0.4 mg of protein/ml) for 60 s at 20 °C, washed firstly with distilled water for 30 s and secondly with a solution of 1% (w/v) phosphotungstic acid for 10 s, after which excess stain was removed, and the grids were dried.

Extraction of proteolipids from storage bodies

Proteolipids were extracted from storage bodies by the method of Fillingame (1976) with 20 vol. of chloroform/methanol (2:1, v/v) containing 100 mM-ammonium acetate, pH 7.2. After addition of 0.2 vol. of distilled water, the solution was shaken and left to stand at 4 °C until the aqueous and organic phases separated. Proteins that had formed a precipitate at the interface were removed by filtration and retained. The lower organic phase was separated off, diluted with 1 vol. of chloroform and its volume reduced to one-fifth by rotary evaporation at 30 °C. Proteolipids were precipitated from this extract with 10 vol. of diethyl ether at −70 °C for 1 h. The protein from the solvent interface and the ether-precipitated proteolipids were washed sequentially with methanol and water, and dissolved in 1% (w/v) lithium dodecyl sulphate in preparation for PAGE. At no stage were the proteins allowed to become dry.

PAGE

This was carried out in the presence of lithium dodecyl sulphate as described previously (Palmer et al., 1988). After electro-phoresis, proteins were fixed for 60 min in 12% (w/v) trichloroacetic acid (200 ml). Then the gels were washed for 60 min with 40% (v/v) methanol containing 10% (v/v) acetic acid (800 ml), twice for 30 min with 10% (v/v) ethanol containing 5% (v/v) acetic acid (400 ml) and finally for 10 min with a solution of 3.4 mM-$K_2Cr_2O_7$ containing 3.2 mM-HNO_3 (200 ml). After removal of all of the oxidizing agent from the gels by washing with water, they were stained for 10 min with 12 mM-$AgNO_3$ (200 ml), rinsed for 2 min with distilled water and developed by successive additions of portions of a solution of 0.28 M-Na_2CO_3 containing 6.33 mM-formaldehyde (0.019%). The first development was for 1 min, the second for 5 min and the third was continued until the desired staining intensity was reached. Development was stopped by washing the gels for 5 min with 5% (v/v) acetic acid (400 ml). All steps after the addition of the $AgNO_3$ solution were carried out under a photographic safe-light (Kodak, Wratten series filter OB). Gels were then de-stained for 2 min with Farmer's reducer [200 ml; this is a solution of 30 mM-$K_3Fe(CN)_6$ and 65 mM-$Na_2S_2O_3$]. After complete re-moval of this reagent by washing with distilled water, the gels were stained again with 12 mM-$AgNO_3$, and developed as above. Gels were stored in distilled water. Dilute Farmer's reducer was also used on occasion to clear background staining of gels, and

to reduce the staining intensity of gels. In one experiment the gel was stained with silver by the procedure of Ansorge (1985).

Electrophoretic transfer of proteins to membranes

After fractionation by electrophoresis of samples of total storage bodies from sheep pancreas, proteins were transferred by electrophoresis to poly(vinylidene difluoride) membranes (Matsudaira, 1987). Regions of the membranes on which proteins had been adsorbed were located by staining with Coomassie Blue dye.

Amino acid analyses

Amino acid analyses were carried out with a Beckman 119BL amino acid analyser after hydrolysis of samples in vacuo in 6 M-HCl for 24 h at 110 °C. Cysteine contents were determined by analysis of cysteic acid after oxidation with performic acid (Hirs, 1967).

Protein cleavages

Two procedures were employed for cleavage of the proteins with CNBr. In the first, proteolipids (0.5–1 mg) dissolved in chloroform/methanol (2:1, v/v) containing 100 mM-ammonium acetate, pH 7.2, were precipitated with diethyl ether at −70 °C, and then dissolved in distilled 98% (v/v) formic acid (1.0 ml) containing CNBr (100 mg). This solution was left under N_2 in the dark at 25 °C for 24 h. In the second procedure, samples of storage bodies or of chloroform/methanol extracts of the storage bodies were dissolved in 98% (v/v) formic acid and then the acid was diluted to 70%. After the addition of a single white crystal of CNBr (approx. 1 mg), the mixture was left for 4 h at room temperature under N_2 and in the absence of light. In both procedures the excess reagents and formic acid were evaporated off in vacuo.

Cleavage with N-bromosuccinimide was performed at 55 °C for 2 h by the addition of a 100–200 fold molar excess of reagent over the tyrosine content to the protein solubilized in 70% (v/v) formic acid solution (Wachter & Werhahn, 1979). Then the solvent was evaporated off in vacuo.

M.s.

Mass spectra were obtained from a VG70-250S double-focusing magnetic-sector mass spectrometer (VG Analytical, Manchester, U.K.) fitted with a VG liquid secondary-ion source and associated Cs+-ion gun. CNBr digests of proteins isolated from storage bodies obtained from pancreas were dissolved in acidified glycerol and loaded on to the stainless-steel target of the sample-insertion probe. Samples were bombarded with 35 keV Cs+ ions (ion current 1–2 μA). The secondary-ion beam was accelerated from the source at 8 keV. The magnet was scanned linearly between 890 and 1250 Da over 15 s. Each analysis consisted of four to six scans of CsI dissolved in water (reference ions 912.3352 and 1172.1451 Da) followed by four to six scans of the sample.

Attachment of peptides to glass supports

Peptides with C-terminal homoserine or spirolactone derivatives, arising from digestion with CNBr or N-bromosuccinimide respectively, were covalently coupled to 3-aminopropyl-glass supports (5 mg) by acylation of its amino groups (Horn & Laursen, 1973; Wachter & Werhahn, 1979). After coupling, the peptides immobilized on the glass were washed with methanol containing 0.025% (v/v) trifluoroacetic acid. The isolated proteolipid or total storage-body protein was coupled by its amino groups to p-phenylene di-isothiocyanato glass (5–6 mg; Wachter et al., 1973). The reaction was performed either in 0.1 M-$NaHCO_3$ containing 1% (w/v) lithium dodecyl

sulphate or in chloroform/methanol (2:1, v/v) containing 0.5%
(v/v) triethylamine.

Protein sequence analysis

Proteins were sequenced by automated Edman degradation
with the aid of either an Applied Biosystems model 470 gas-
phase sequencer or a model 477 pulsed-liquid-phase protein
sequencer, both instruments being equipped with on-line detec-
tion of PTH derivatives by reverse-phase h.p.l.c. Filter discs
were impregnated with Polybrene (3 mg) and NaCl (200 μg) and
precycled for three cycles. Samples of storage bodies, of extracted
proteolipids and of digests were solubilized in formic acid and
loaded onto these pretreated discs. In the case of peptides
covalently coupled to glass supports, the dried glass (approx.
5 mg) was loaded on to a cartridge filter that had been dampened
with either 25 % (v/v) trifluoroacetic acid or methanol containing
0.1 % (v/v) triethylamine. Then the upper half of the reaction
cartridge block, holding both the glass-fibre disc and the glass
beads, was inverted on to the lower block in the cartridge holder
and sealed as usual with a Zitex gasket.

Protein samples that had been transferred to poly(vinylidene
difluoride) membranes were sequenced in the model 470 gas-
phase sequencer. Pieces of membrane with adsorbed protein
were excised and placed in the reaction chamber of the instrument
in the presence of a Teflon seal only. Neither Polybrene nor a
glass-fibre filter disc was employed in these experiments.

S-Pyridylethylation of cysteine residues

Cysteine residues were reduced and alkylated in the presence
of the vapours of tri-(n-butyl)phosphine and 4-vinylpyridine as
described by Amons (1987), except that the reactions were
performed on protein covalently bound to glass supports. The
glass beads were retained in a small sintered column, which was
placed in a re-sealable vial under vacuum. Then the beads were
washed with acidified methanol to remove contaminants and
reaction by-products, thereby avoiding the need for extra washing
routines in the protein sequencer programs (Amons, 1987). The
PTH derivative of S-pyridylethylcysteine was eluted between the
valine PTH derivative and diphenylthiourea in the h.p.l.c.
analytical separation system used.

Reaction of amino groups with phenyl isocyanate

The protein was solubilized in a solution of either 0.1 M-
NaHCO$_3$ and 1% (w/v) lithium dodecyl sulphate or
chloroform/methanol (2:1, v/v) containing 0.5% triethylamine.
Phenyl isocyanate (Fluka AG, Buchs, Switzerland) was added to
final concentration of 5% and the solution was incubated at
45 °C for 2 h. The modified protein was precipitated with 4 vol. of
diethyl ether by leaving it to stand for at least 1 h at −20 °C, and
collected by centrifugation.

RESULTS

Electron microscopy of isolated bodies

Storage bodies isolated from pancreas, kidney and liver of
sheep affected with ceroid lipofuscinosis were not contaminated
with other subcellular organelles or particles derived from them,
including mitochondrial inner-membrane vesicles, which have
a distinctive ultrastructure (Fig. 1). Thin sections of isolated
pancreatic storage bodies showed a preponderance of curvilinear
structures, but also contained lamellar regions (Fig. 1a). Negative
staining of the isolated mitochondrial inner-membrane vesicles
revealed the presence of F$_1$-ATPase, part of the ATP synthase
complex, as extramembranous protrusions attached to the outer
surface of these inverted vesicles (Fig. 1c). Similar structures
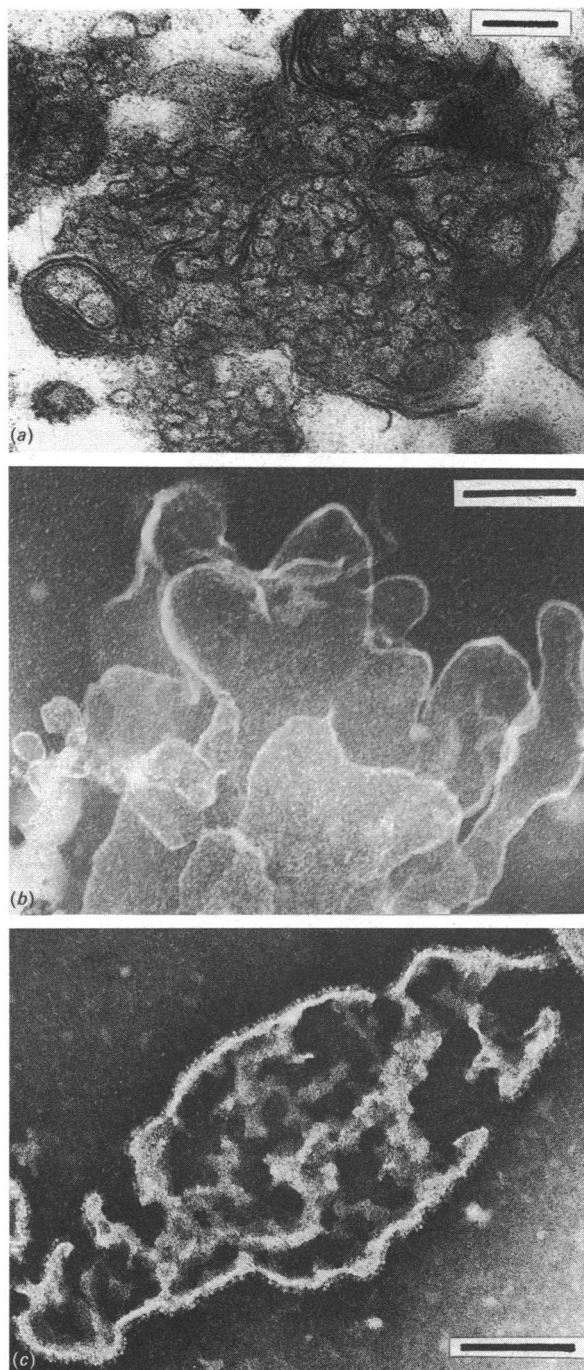

Fig. 1. Electron micrographs of isolated storage bodies and mitochondrial
inner-membrane vesicles

(a) Thin section of isolated pancreatic storage bodies (magnification
× 46000). (b) Isolated pancreatic storage bodies (magnification
× 72000). (c) Mitochondrial inner-membrane vesicles stuck to a
Formvar-coated grid and negatively stained with phosphotungstic
acid (magnification × 72 000). The bars represent 200 nm.

were not seen on the surface of the negatively stained isolated
storage bodies, which was relatively smooth (Fig. 1b).

Analysis of the storage bodies by PAGE

Many hydrophobic proteins, including the DCCD-reactive
proteolipid subunit of ATP synthase, stain poorly with
Coomassie Blue dye. Therefore various silver staining, destaining
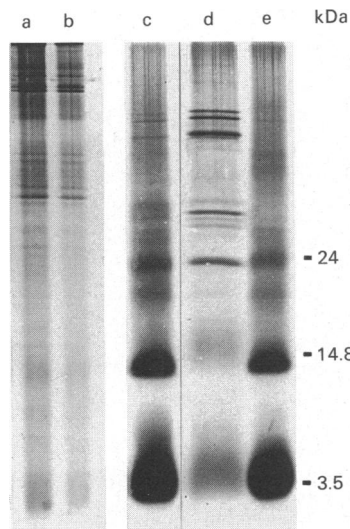and overstaining techniques were explored. The preferred

Fig. 2. Silver-stained PAGE-gel analyses of sheep mitochondrial inner-membrane vesicles and of pancreatic storage bodies

Lanes a and b, mitochondrial inner-membrane vesicles (20 μg of protein/lane) from sheep liver of normal and diseased animals respectively; a 20 % polyacrylamide gel was used. Lane c, pancreatic storage bodies; lane d, the chloroform/methanol-insoluble residue, and lane e, the chloroform/methanol/ammonium acetate-soluble proteolipids; in lanes c–e a 15 % gel was employed and 15 μg of protein was analysed in each case. See the Materials and methods section for further experimental details. The numbers on the right-hand side indicate apparent molecular masses calculated from the migration of molecular-mass markers.

Fig. 3. Comparison by PAGE of bovine ATP synthase and sheep storage bodies associated with ceroid lipofuscinosis

The proteins were revealed by silver staining by the procedure of Ansorge (1985). Track a, storage bodies; track b, ATP synthase. The positions of subunits of ATP synthase, including subunit c, are indicated at the right-hand side of the Figure.

Table 1. Proteolipid content of sheep storage bodies

The proportion of proteins recovered as proteolipid by diethyl ether precipitation of chloroform/methanol/ammonium acetate extracts is expressed as mol/100 mol of the total amino acid residues present in storage bodies. The values for pancreas, liver and kidney are the means ± S.E.M. for seven, four and four determinations respectively.

Tissue	Proteolipid extracted (% of total protein)
Pancreas	69.4 ± 0.95
Liver	69.9 ± 1.77
Kidney	68.7 ± 1.60

method, which is described, detected the greatest number of components, and many of them were not revealed by staining with Coomassie Blue dye. Sheep mitochondrial inner-membrane vesicles contain a large number of proteins (see Fig. 2, lanes a and b), but little subunit c of ATP synthase was detected in the region where it is known to migrate (Walker et al., 1990) in the submitochondrial particles prepared from tissues from either affected or normal animals. In contrast, the purified storage bodies contain a relatively small number of proteins, the dominant band seen on polyacrylamide gels having an apparent molecular mass of about 3500 Da, as does the c-subunit of mitochondrial ATP synthase (see Fig. 3). Other proteins were also observed, particularly bands with apparent molecular masses of 14800 and 24000 Da. These latter bands were also detected in bodies isolated from other tissues, but they were most clearly seen in storage bodies from pancreas, although their relative intensities varied from one preparation to another, and from gel to gel. All three components were soluble in chloroform/methanol/ammonium acetate (see Fig. 2), and so they can be classified as proteolipids (Fillingame, 1976; Lees et al., 1979). Quantification by amino acid analysis showed that almost 70 % of total proteins present in the storage bodies was extracted and recovered in this way (Table 1), and on average 26.7 % of the proteins was not extracted. This residual material consisted mainly of a number of minor higher-molecular-mass components, but probably also contained non-extracted proteolipid (see Fig. 2).

The amino acid compositions of total storage bodies have high contents of alanine, glycine and leucine and contain little histidine, and are very similar to the amino acid composition of the c-subunit of mitochondrial ATP synthase. This suggests that subunit c is the overwhelmingly preponderant component in the storage bodies (Table 2). The compositions of the extracted proteolipids are even closer to that of mitochondrial subunit c,
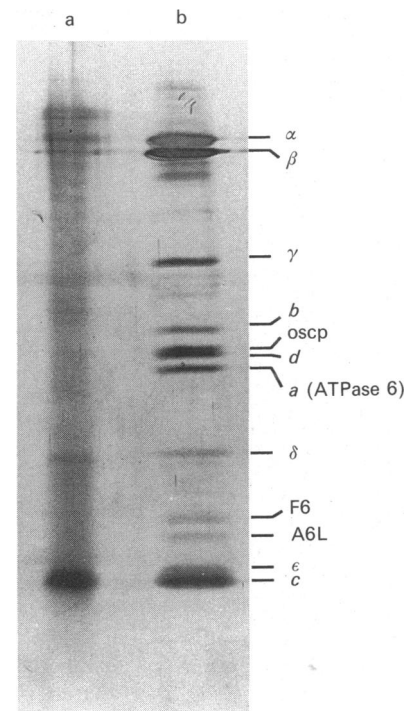
and it seems likely either that the proteins of higher molecular mass seen on polyacrylamide gels of extracts of storage bodies (Fig. 2) are aggregates of mitochondrial subunit c, or, alternatively, that they possess similar amino acid compositions to it.

In order to try and identify the various bands that were detected on polyacrylamide gels, they were transferred to poly(vinylidene difluoride) membranes. Sequence analysis confirmed that the N-terminal sequence of residues 1–36 of the 3.5 kDa band is identical with that of the c-subunit of mitochondrial ATP synthase. The less abundant protein components of the storage bodies were examined in the same manner, and the same sequence was also obtained from both the 14.8 kDa and the 24 kDa bands, indicating that they are both oligomers of the 3.5 kDa protein. The initial yields of the 3.5 kDa, 14.8 kDa and 24 kDa proteins in this experiment were 21.8, 1.7 and 2.6 pmol respectively. The tendency of the c-subunit

Table 2. Amino acid composition of sheep storage-body proteins

The values for pancreas, liver and kidney are the means for seven, four and four determinations respectively. Abbreviation: N.D., not determined. Values for ATP synthase subunit c are deduced from the nucleotide sequence of the cDNA encoding the sheep protein (S. M. Medd & J. E. Walker, unpublished work).

	Amino acid composition (mol of residue/mol)						
	Total storage body			Chloroform/methanol-extracted proteolipid			ATP synthase subunit c
Amino acid	Pancreas	Kidney	Liver	Pancreas	Kidney	Liver	
Aspartic acid	4.0	4.2	3.9	3.6	3.6	3.8	3
Threonine	4.7	4.6	3.8	3.9	3.8	3.5	3
Serine	4.0	4.5	4.5	4.5	4.5	4.4	5
Glutamic acid	3.8	3.7	4.5	4.0	4.1	3.5	3
Proline	2.0	1.5	3.1	1.4	1.4	1.4	1
Cysteine	0.4	N.D.	N.D.	0.9	1.4	1.4	1
Glycine	8.9	8.3	8.2	9.2	10.1	9.5	11
Alanine	9.2	9.2	9.2	10.9	11.0	10.8	13
Valine	6.2	5.6	4.3	6.2	5.3	5.5	4
Methionine	2.0	2.0	2.5	1.7	2.2	2.6	3
Isoleucine	6.4	6.2	4.8	6.6	6.5	6.2	7
Leucine	9.1	9.2	10.1	8.7	9.5	9.9	9
Tyrosine	3.2	3.8	2.7	2.9	2.1	2.3	2
Phenylalanine	5.9	6.2	6.3	5.8	5.8	6.1	7
Histidine	0.9	0.8	1.1	0.5	0.7	0.6	0
Lysine	2.3	2.6	3.1	2.3	2.0	2.3	2
Arginine	2.1	2.9	3.1	2.0	1.2	1.4	1
Tryptophan	N.D.	N.D.	N.D.	N.D.	N.D.	N.D	0
Total	–	–	–	–	–	–	75

to form such oligomers has been noted before (summarized in Walker *et al.*, 1990). So it appears that almost all of the protein that accumulates in the storage bodies is a single polypeptide. Previously the minor sequence APEYXSXXAMV was detected by direct sequencing of proteins extracted from storage bodies (Palmer *et al.*, 1989), and we have also detected this sequence in similar experiments at a level corresponding to about 6% of the mitochondrial c-subunit. It is related to residues 7–17 of the sequence of a proteolipid associated with a vacuolar H⁺-translocating ATPase, which has the *N*-terminal sequence MSEAKNGPEYASFFAVM... (Mandel *et al.*, 1988), and also to an *N*-terminal sequence reported by Walker *et al.* (1986) of a protein isolated from preparations of mouse gap junctions by Dr. J. D. Pitts and Dr. J. Shuttleworth. At present it is unclear whether this protein is an intrinsic component of storage bodies or whether it represents some low level of contamination either by an organelle that contains a vacuolar ATPase or possibly by gap junctions.

Protein sequence analysis of storage-body proteolipid

N-Terminal sequence. In the previous experiments the sequence of residues 1–40 was determined, but no sequence was detected beyond residue 40 (Palmer *et al.*, 1989). In the present work attempts have been made to extend the *N*-terminal sequence data beyond the 40th residue. *N*-Terminal degradation has been repeated with much larger quantities of protein, and we have tried to improve the quality of the sequence data by treating the sample with *o*-phthalaldehyde at proline residue 40 to try to 'silence' signals arising from background noise (Machleidt & Hofner, 1982; Brauer *et al.*, 1984). Also, an extended cleavage reaction has been carried out at proline residue 40 (Brandt *et al.*, 1976). None of these experiments extended the *N*-terminal sequence significantly. This is possibly because the positive charge of the single arginine residue at residue 38 helps to anchor the

protein to the Polybrene-coated disc in the sequencer, and once this amino acid has been removed in the course of the Edman degradation the sample dissolves in solvents and is lost. In principle, covalent attachment of protein samples to glass, as used in solid-phase protein sequence analysis, prevents such sample loss. Therefore storage-body protein was coupled to *p*-phenylene di-isothiocyanato-glass, and the sequence obtained in this experiment extended to, but not beyond, residue 42. This position is followed by the lysine residue closest to the *C*-terminus of the polypeptide chain of the mitochondrial c-subunit, and so once this position has been passed the remaining protein becomes detached from the glass and is lost.

C-Terminal region. In order to examine the sequence in the *C*-terminal region of the storage-body proteolipid, the protein was digested separately with CNBr and *N*-bromosuccinimide, and the mixtures of the peptides so produced were sequenced as described below. If the storage-body proteolipid were identical with the mitochondrial c-subunit over its entire length, this would be expected to produce three fragments from both cleavages (see Fig. 4).

Initial indications that the CNBr digest contained fragments that were the same as those from the *C*-terminal region of the c-subunit were obtained by m.s. The products of CNBr digestion of extracted pancreatic storage bodies were analysed by liquid secondary ion m.s., using Cs⁺-ion-bombardment excitation. Two major peaks with masses of 976.5708 and 994.5977 Da were observed in the region between 890 and 1250 Da. These masses are consistent with those expected for the homoserine lactone and homoserine forms of the *C*-terminal fragment of mitochondrial c-subunit, calculated as 976.5871 and 994.5977 Da for $C_{51}H_{78}N_9O_{10}$ and $C_{51}H_{80}N_9O_{11}$ respectively (Fig. 4), the homoserine being formed from methionine-75 by reaction with CNBr.

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                10                      20
D I D T A A K F I G A G A A T V G V A G S G A G I
├───────────────────────────CB1─────────────────────
├─────────────────NBS1────────────────────


                30                      40                      50
G T V F G S L I I G Y A R N P S L K Q Q L F S Y A

─────────────────────CB1─────────────────────

─────────── NBS1 ───────────┤ ├───────────NBS2─────────────┤ ├─


                60                      70
I L G F A L S E A M G L F C L M V A F L I L F A M
───────────CB1───────────┤ ├───CB2───┤ ├───────CB3───────┤
─────────────────NBS3─────────────
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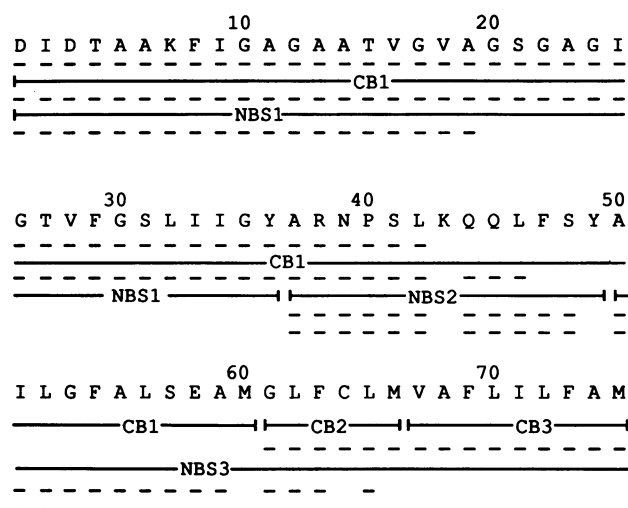
Fig. 4. Amino acid sequence of the major proteolipid in storage bodies associated with ceroid lipofuscinosis

The fragments arising from digestion with CNBr (CB) and N-bromosuccinimide (NBS) are indicated by continuous bars. Dashed lines represent the sequences determined by Edman degradation. Residues 1–42 were determined on the intact protein.

Further confirmation was obtained by automated Edman degradation of the unfractionated CNBr digest of storage-body proteolipid. Three sequences were observed in approximately equimolar amounts. They are consistent with the known N-terminal sequence of the storage-body proteolipid, and with those of two fragments, named CB2 and CB3 (Fig. 4), arising by cleavage after methionine residues. These sequences are identical with those expected from the mitochondrial c-subunit. The PTH derivative of homoserine is co-eluted with the threonine PTH derivative and was detected in cycles 6 and 9 of the analysis. It is reasonable to conclude that it arose in each case from the C-terminal methionine residues of peptides CB2 and CB3. Thus after nine cycles of Edman degradation only one sequence remained, that corresponding to the N-terminal sequence of peptide CB1. Further degradation of this peptide allowed almost all of the first 46 residues of this peptide to be identified unambiguously. In an additional experiment confirmation of cysteine-63 in peptide CB3 was obtained by sequencing protein that had reacted with 4-vinylpyridine.

In order to simplify the sequence analysis of the mixture of CNBr-cleavage peptides, the intact protein was treated with phenyl isocyanate before cleavage, thereby blocking the N-terminus of the protein. Subsequent CNBr cleavage, immobilization of the products to aminopropyl-glass and sequence analysis yielded the sequences of peptides CB2 and CB3 only. In cycle 3 of this degradation only the phenylalanine PTH derivative was detected, and its yield was approximately that expected from simultaneous release of two phenylalanine residues (residues 63 and 69). These experiments with the CNBr-cleavage peptides generated the sequence of the N-terminal 46 amino acid residues of the protein and those of peptides CB2 and CB3. They support the view that the storage-body proteolipid is identical with the authentic mitochondrial c-subunit from residues 1–46 and from residue 60 to the C-terminal methionine-75.

Protein sequence analysis of the mixture of products of cleavage of storage-body proteolipid with N-bromosuccinimide generated three sequences. These originated from the N-terminus of the protein and from peptides resulting from cleavages after tyrosine-36 and tyrosine-49. These peptides are named NBS1, NBS2 and NBS3 respectively. The sequences correspond exactly

to those that would be produced by N-bromosuccinimide cleavage of the mitochondrial c-subunit. It was surprising to find the N-terminal sequence of peptide NBS3 in this experiment, since it has no C-terminal spirolactone, and so it would not be expected to couple to aminopropyl-glass. However, it is evident from the position of 'silent' residues during the sequence analysis that this peptide is coupled at positions that correspond to methionine residues. In most experiments peptide NBS3 was coupled at a single site corresponding to methionine-60, and no sequence was obtained after this residue. In other experiments it was noted that peptide NBS3 was also coupled via methionine-66, and so it was possible to confirm the overlap between peptides NBS3 and CB2.

As in the analysis of the products of CNBr cleavage, the analysis of the N-bromosuccinimide-cleavage products was simplified by treating the protein with phenyl isocyanate before cleavage. Under these conditions two sequences corresponding to peptides NBS2 and NBS3 were detected, and the experiment provided unambiguous sequence for the whole of peptide NBS2 and for residues 50–63 of peptide NBS3. In common with earlier observations made during the sequence analysis of the bovine mitochondrial proteolipid (Wachter & Werhahn, 1979), serine-48 was detected in low yield. As expected, no signals were observed from the C-terminal tyrosine residues 36 and 49 of peptides NBS1 and NBS2 respectively. These tyrosine residues are converted into spirolactone derivatives by N-bromo-succinimide digestion. They acylate the amino groups of the glass derivative and remain attached to the glass support during sequence analysis, no identifiable derivative being released. However, tyrosine-36 was identified directly during the N-terminal sequence analysis of the intact protein, but no direct sequence evidence has been obtained for residue 49, although in the absence of histidine and tryptophan in the protein its presence can be inferred from the specificity of N-bromosuccinimide cleavage, and is supported by the attachment of peptide NBS2 to aminopropyl-glass.

Most cleavage reactions conducted on storage-body proteolipid with N-bromosuccinimide were complete, since products of partial digestion were not detected. However, in a minority of digests incomplete cleavage was observed and analysis of these digests gave the sequence from alanine-37 to serine-57, and so produced the overlap between peptides NBS2 and NBS3. (This experiment was conducted on protein that had been treated with phenyl isocyanate before cleavage, as described above.) In other experiments with N-bromosuccinimide, longer cleavage times (up to 12 h) were employed and other minor cleavage products were detected by sequencing. All of these sequences are consistent with the final sequence of the stored protein. Additional cleavages were observed at the Gly–Thr bond at residues 20–21 and of Gly–Ser bonds at residues 26–27 and 30–31, and at an Asp–Thr bond (residues 3–4). Almost certainly these were caused by acid hydrolysis, since they were also observed by incubation of the storage protein in 70% formic acid alone at 55 °C for 12 h.

Amino acid residue 43 of the storage-body proteolipid has not been identified in any of the experiments described above. In the c-subunit of mitochondrial ATP synthase this is a lysine residue, and the amino acid composition of the stored protein indicated two lysine residues per molecule (Table 2). Identification of this residue in N-bromosuccinimide digests was prevented by the coincident release of the lysine PTH derivative from residue 7 of peptide NBS1. In subsequent experiments the N-terminal sequence of the intact protein was first blocked by reaction with phenyl isocyanate before digestion with N-bromosuccinimide. However, this reaction also modifies the ε-amino groups and the compound derived from ε-phenylcarbamoyl-lysine (rather than PTH derived from the usual ε-phenylthiocarbamoyl-lysine) is co-

eluted with the leucine PTH derivative in the h.p.l.c. analysis of amino acid PTH derivatives, and so it could not be distinguished from the leucine PTH derivative arising from overlap of the preceding leucine-42. Immobilization of the storage-body proteolipid by reaction with p-phenylene di-isothiocyanato-glass occurs via residues 1, 7 and 43, no amino acid PTH derivative being observed at the first two of these positions in Edman degradation. However, the degradation proceeds beyond residue 7 up to residue 42, indicating the presence of an attachment site beyond residue 7, and consistent with the presence of a lysine residue at position 43.

The sequences generated by the analysis of CNBr and N-bromosuccinimide digests cover the entire sequence of the storage-body proteolipid isolated from sheep pancreas, except for a single overlap between peptides CB2 and CB3. The overlap between these two peptides, the presence of lysine-43 and indeed the sequence of the entire sequence of the sheep mitochondrial c-subunit have been confirmed independently by analysis of cDNAs from normal sheep and from animals affected with ceroid lipofuscinosis (S. M. Medd & J. E. Walker, unpublished work). Together with the amino acid composition of storage-body protein (Table 2), these data show that the sequence of the proteolipid found in storage bodies from tissues of sheep with ovine ceroid lipofuscinosis is identical with that of the entire c-subunit of ATP synthase rather than being an N-terminal fragment of the protein. There is no evidence that the protein has been modified post-translationally (for example by ubiquitination), although these experiments do not (and cannot) completely exclude the possibility of modifications to a minority of the protein molecules (or of modifications that are not stable under the conditions used during protein sequence analysis).

DISCUSSION

It has been shown by direct protein sequence analysis that the sequence of the major protein stored in lipopigmented storage bodies in tissues of sheep affected with ceroid lipofuscinosis is identical with that of the complete DCCD-reactive proteolipid (subunit c) of human, bovine and ovine mitochondrial ATP synthase (Sebald et al., 1979; Gay & Walker, 1985; Farrell & Nagley, 1987; Dyer & Walker, 1990; S. M. Medd & J. E. Walker, unpublished work). Its amino acid composition also supports this conclusion. There is no evidence for the presence in the bodies of fragments of this protein truncated at either the N- or the C-terminus. Nor is there evidence of modification of the protein that has accumulated in the storage bodies, although on the basis of the sequencing experiments the possibility that a minority of the protein chains have been modified post-translationally cannot be excluded. In this respect it should be borne in mind that the fluorescence of the storage bodies remains unexplained. Mechanisms by which proteins can be modified to form fluorophores have been proposed, although there is no evidence that they are the basis of the fluorescence of the storage bodies. These include peroxidation reactions leading to the formation of malonaldehyde, which can react with the protein to form a fluorescent Schiff base product (Chio et al., 1969; Koobs et al., 1978), and the glycation of lysine residues by glucose to produce heterocyclic fluorescent compounds (Pongor et al., 1984).

We have conducted experiments to exclude the possibility that the presence of the c-subunit in preparations of bodies arises during isolation of the bodies, and is caused by contamination with membranes of mitochondrial origin. Firstly, by electron microscopy of preparations of bodies we were unable to detect the presence of the 'lollipops' of the F_1 extrinsic membrane region of ATP synthase. These structures are characteristic of mitochondrial inner membranes and are visible in samples of mitochondrial vesicles from normal and diseased animals (Fig. 1). Secondly by using PAGE we have compared the complexity of the protein components of mitochondrial inner membranes with that of storage bodies. Subunit c was barely visible in gels of the mitochondrial inner-membrane vesicles, despite the use of the specialized silver staining developed to detect storage-body proteins (Fig. 2), whereas it is the dominant component of storage bodies, and other higher-molecular-mass bands appear to be oligomers of it. Therefore contamination of bodies by mitochondrial membranes would tend to diminish the concentration of subunit c in the storage body rather than be the source of it. Lack of contamination of the storage bodies by mitochondria is also supported by analysis of their lipid contents. The distinctive mitochondrial phospholipid cardiolipin was not found in lipids extracted from storage bodies, and the lipid composition was characteristic of lysosomes (Palmer et al., 1986a,b, 1988).

Further evidence for the lack of contamination of storage bodies by mitochondria comes from the sequence analysis of chloroform/methanol extracts of storage bodies. The only major sequence observed is that of the DCCD-reactive proteolipid, whereas it has been demonstrated before that chloroform/methanol extracts of mitochondria contain a wide range of different proteolipids (Fearnley & Walker, 1986, 1987). None of these was detected in storage-body material. The only other minor sequence detected, and solely in sheep storage bodies from pancreas, is related to the N-terminal sequence of a 17 kDa protein isolated from mouse gap-junction preparations by Dr. J. Shuttleworth and Dr. J. D. Pitts and to residues 7–17 of the proteolipid isolated from bovine vacuolar (chromaffin-granule) H^+-translocating ATPase (Walker et al., 1986; Mandel et al., 1988). Whether this minor sequence represents an intrinsic component of storage bodies is unclear at present. Its presence in preparations of storage bodies could be due either to minor contamination by membranes containing gap junctions or to the presence of a vacuolar H^+-translocating ATPase. Lysosomes themselves contain an H^+-translocating ATPase that is related in its subunit structure to other vacuolar H^+-translocating ATPases and also to the mitochondrial ATP synthase, and it has been suggested that they contain in their membrane sectors a subunit that is related to, but is not identical with, the mitochondrial DCCD-reactive proteolipid (E. J. Bowman et al., 1988; B. J. Bowman et al., 1988; Manolson et al., 1988; Zimniak et al., 1988), and so the protein that has accumulated in storage bodies is not part of the lysosomal enzyme.

Other experiments conducted in the present work confirm that the storage bodies are composed largely of the c-subunit of mitochondrial ATPase. On the basis of previous experiments it can be calculated that the c-subunit accounts for a minimum of 73 % of the protein mass in the storage bodies (Palmer et al., 1989), and this value is consistent with the proportion of the proteins that was recovered in chloroform/methanol extracts of bodies. This value is probably an underestimate, as it is based upon the initial yield in the N-terminal sequencing, which varies from protein to protein and is never quantitative. In addition, the extraction of subunit c and its aggregates was incomplete.

These results indicate that the genetic lesion in ovine ceroid lipofuscinosis causes the specific accumulation in lysosomes of the complete and unmodified subunit c of mitochondrial ATP synthase to such an extent that it accounts for over 73 % of their protein. Minimally two-thirds of the mass of the bodies is protein, and so subunit c alone accounts for at least half of the total mass of storage bodies. Subunit c of mitochondrial ATP synthase has previously only been detected in mitochondria, as part of the F_0 portion of the oligomeric ATP synthase complex

(Senior, 1979; Sebald & Hoppe, 1981; Walker et al., 1990). In man, cattle and sheep it has two expressed nuclear genes called P1 and P2 (Gay & Walker, 1985; Dyer et al., 1989; Dyer & Walker, 1990; S. M. Medd & J. E. Walker, unpublished work). The P1 and P2 genes encode identical mature c-subunits of ATP synthase, but they differ extensively in their N-terminal precursor sequences. These N-terminal extensions direct the cytoplasmically synthesized precursor to the mitochondrion, and are removed during entry into the organelle by specific proteolytic processing. The finding in the present work that the c-subunit that is stored in lysosomes has the same N-terminus as the c-subunit found in mitochondria strongly suggests that the stored material at some stage has entered mitochondria and has been subjected to the proteolytic processing associated with entry into this organelle. It follows from this argument that the defect associated with lipofuscinosis that leads to the accumulation there of the c-subunit is at a later stage in the life of the c-subunit. However, little or nothing is known about the degradation and turnover of the mature subunit c or of other subunits of ATP synthase. An alternative explanation that we have considered is that the stored protein has been misdirected to lysosomes without being routed through mitochondria. This hypothesis implies that a second proteolytic processing machinery to produce the mature c-subunit from its precursors is also present in lysosomes, and perhaps this is less likely than the alternative explanation outlined above, which we prefer. Studies of the sheep genes have shown that the two precursor sequences for the c-subunit are the same in normal and diseased animals, as are the mRNA concentrations, suggesting that regulation of subunit c expression is also unaffected (S. M. Medd & J. E. Walker, unpublished work). These findings now have an increased significance, since we have shown recently that the storage bodies associated with the juvenile and late-infantile human forms of the Batten's disease contain large amounts of intact and unmodified subunit c of mitochondrial ATP synthase (D. N. Palmer, I. M. Fearnley & J. E. Walker, unpublished work).

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