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Lysine Methylation of Mitochondrial ATP Synthase Subunit c Stored in Tissues of Dogs with Hereditary Ceroid Lipofuscinosis*

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Certain forms of ceroid lipofuscinosis, a hereditary degenerative disease, are characterized by accumulation of large amounts of subunit c of mitochondrial ATP synthase in lysosomal storage bodies of numerous tissues. The subunit c protein appears to constitute a major fraction of the total storage body protein. In previous studies it was demonstrated that hydrolysates of total storage body protein from affected humans and sheep contain significant amounts of ε-*N*-trimethyllysine (TML). This finding suggested that one or both of the two lysine residues of subunit c might be methylated in the stored form of the protein. The normal subunit c protein from mitochondria does not appear to be methylated. Using a putative canine model for the juvenile form of ceroid lipofuscinosis, analyses were conducted to determine whether lysosomal storage of subunit c was accompanied by lysine methylation of this protein. In affected dogs, as in humans and sheep with hereditary ceroid lipofuscinosis, the storage bodies were found to contain large amounts of subunit c protein, as indicated by polyacrylamide gel electrophoresis and partial amino acid sequence analysis. The subunit c protein partially purified from isolated storage bodies was found to contain lysine and TML in an almost equimolar ratio. Normal subunit c contains 2 lysine residues, one at position 7 and the other at position 43. Removal of the first 7 residues of the partially purified protein through sequential Edman degradation resulted in a dramatic increase in the TML to lysine ratio in the residual protein. This suggests that lysine residue 43 is methylated. Confirmation that residue 43 of the stored protein is TML was obtained by amino acid sequence analysis after cleavage of the protein with trypsin. This finding strongly suggests that specific methylation of lysine residue 43 of mitochondrial ATP synthase plays a central role in the lysosomal storage of this protein.

The hereditary ceroid lipofuscinoses are a group of inherited storage diseases characterized by the intracellular deposition of large numbers of autofluorescent inclusions in many tissues. Clinical symptoms in human subjects with these diseases include visual loss, seizures, psychomotor and cognitive deterioration, and early death (1–3). The ceroid lipofuscinoses can be divided into four major forms, commonly designated as infantile, late infantile, juvenile, and adult types (1–3). In the late infantile and juvenile forms, there is evidence that the storage material contains large amounts of the subunit c protein of mitochondrial ATP synthase (4, 5). Animal models for juvenile ceroid lipofuscinosis have been identified in sheep, (6, 7), dogs (8, 9), and recently in mice (10). It appears that at least in the sheep and mouse models of the disease, there is also specific storage of the subunit c protein (5, 10, 11). The metabolic basis for the accumulation of this protein is currently not known.

Amino acid compositional analysis of storage body proteins from affected sheep and from humans with the juvenile form of ceroid lipofuscinosis revealed that they are associated with substantial amounts of the modified amino acid ε-*N*-trimethyllysine (TML)[1] (12–14). This indicates that at least in some forms of ceroid lipofuscinosis, there is specific storage of a protein or proteins containing ε-*N*-trimethyllysine. Storage body preparations appear to contain a number of proteins in addition to ATP synthase subunit c (4, 11, 15). The methylated lysine may have arisen from any of these proteins. However, it appears that subunit c is the only protein common to storage bodies from all sources, so it seems likely that the modified amino acid is derived from this protein. To evaluate this possibility, studies were undertaken to determine whether storage bodies isolated from English setters with hereditary ceroid lipofuscinosis contained the subunit c protein in which one of the lysine residues was methylated.

EXPERIMENTAL PROCEDURES

Dogs and Tissues—Tissues from English setters with hereditary ceroid lipofuscinosis were obtained from two colonies established from dogs first described as having this disease by Koppang (9). Brains and kidneys were dissected at necropsy from affected dogs between 20 and 24 months of age. The same tissues were obtained from unrelated age-matched English setters that did not have the disease. All tissues were frozen and stored at −70 °C until used for storage body preparation. The presence of cellular inclusions characteristic of ceroid lipofuscinosis in dog tissues was assessed using fluorescence and electron microscopy (12, 13, 16).

Storage Body Isolation—Storage bodies were isolated from the tissues using adaptations of the techniques developed for sheep with hereditary ceroid lipofuscinosis (17). Samples of cerebral cortex gray matter were dissected from brains and weighed. Approximately 5.0 g of tissue was diced and placed in 25 ml of 0.4 mM Tris-HCl, pH 7.42. The tissue was homogenized on ice in a glass sleeve with a motor-driven Teflon pestle. The homogenate was filtered through gauze and then sonicated for 2 min. The resulting suspension was filtered through glass wool and centrifuged at 5900 × g_{max} for 10 min at 4 °C. The supernatant was decanted, and the pellet was resuspended in 20 ml of CsCl in the 0.4 mM Tris buffer, with the CsCl concentration adjusted to give a specific gravity of 1.170 ± 0.005. The suspension was subjected to cen-

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[1] The abbreviations used are: TML, ε-*N*-trimethyllysine; PAGE, polyacrylamide gel electrophoresis.

trifugation for 40 min at 12,000 × *g* max. The supernatant was discarded and the pellet was resuspended in a solution of CsCl in 0.4 mM Tris with the CsCl concentration adjusted to give a specific gravity of 1.125 ± 0.003. The suspension was centrifuged for 60 min at 12,000 × g_{max}. The resulting pellet was washed once with 150 mM NaCl and twice with deionized water by resuspension in 2.0 ml of liquid in a preweighed vial and centrifugation at 5000 × g_{max} for 30 min.

The procedure for storage body isolation from kidney was similar to that used for brain. Specimens of tissue, each of approximately 4.5 g, were diced and then homogenized in 25 ml of 0.4 mM Tris-HCl, pH 7.4. The homogenates were filtered through gauze and then sonicated on ice for 1 min. After sonication the samples were subjected to centrifugation at 70 × g_{max} for 5 min. The supernatants were filtered through glass wool and then centrifuged at 1400 × g_{max} for 20 min. The resulting pellets were resuspended in 20 ml of the Tris buffer and centrifuged once more at 1400 × g_{max} for 20 min. After removing the supernatants, the pellets were then resuspended in 25 ml of deionized water and subjected to centrifugation at 19,000 × g_{max} for 25 min. The pellets from two preparations were then pooled and suspended in 12 ml of 17% CsCl in 0.4 mM Tris (specific gravity 1.146 ± 0.003), and the suspensions were centrifuged at 14,000 × g_{max} for 40 min. The resulting pellets were each suspended in 2.0 ml of deionized water and were pooled in one preweighed glass vial. The vial was centrifuged at 5000 × g_{max} for 30 min, and the supernatant was removed and discarded. The pellet was washed by resuspension in 2.0 ml of deionized water and centrifugation at 5000 × g_{max} for 30 min. Aliquots of these storage body preparations were examined with fluorescence and electron microscopy to assess the effectiveness of the isolation procedures (12, 16).

Protein Extraction from Storage Bodies—The isolated storage bodies from both brain and kidney were subjected to extraction using a method that has been reported to yield a fraction from sheep storage bodies that is highly enriched in the mitochondrial subunit c protein (11). Aliquots of the storage bodies were sonicated in 10 volumes of chloroform/methanol (2:1, v:v) containing 100 mM ammonium acetate. After addition of 2 volumes of water, the solutions were sonicated again and then allowed to stand at 4 °C until the aqueous and organic phases separated. The samples were then subjected to centrifugation at 1000 × *g* for 10 min, which caused insoluble material to collect at the interface between the organic and aqueous phases. The lower organic phases were transferred to preweighed vials. The remainder of each sample, consisting of the aqueous phase and insoluble interface, was washed with 2.0 ml of chloroform/methanol (2:1) containing 100 mM ammonium acetate. The organic phase from this wash step was removed and pooled with the previously collected organic phase. This solution was then diluted with 1 volume of chloroform and evaporated under argon to 20% of the starting volume. Proteins were precipitated from the latter solution by the addition of 10 volumes of diethyl ether and incubation at −70 °C for 1 h. The precipitate was washed consecutively with methanol and water. The aqueous phases and interfacial material were collected separately. Thus, each storage body preparation yielded three fractions: the organic extract, the aqueous phase, and the interfacial material.

Gel Electrophoresis—Crude storage body preparations and organic extracts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) to assess their protein compositions. Samples were solubilized in a buffer consisting of 2% SDS, 10 mM Tris-HCl, and 0.5 mM Na_2EDTA, pH 8.00. The samples were incubated at 60 °C for 1 h with occasional vigorous mixing. Prior to being applied to the gels, the samples were centrifuged to pellet any material that had not dissolved. Aliquots of the supernatants were subjected to electrophoresis on precast high density polyacrylamide gels (PhastGel high density, Pharmacia LKB Biotechnology Inc.). The gels were fixed with 12.5% glutaraldehyde and then stained with silver using a modification of the technique described by Fearnley and colleagues (11). The gels were incubated with 0.5% $AgNO_3$, developed with 2.5% Na_2CO_3 containing 0.04% formaldehyde, partially destained in 65 mM $Na_2S_2O_3$, restained with 0.5% $AgNO_3$, and developed again. After the second development, the gels were washed with 5% acetic acid, incubated in 10% acetic acid, 10% glycerol for 5 min, and then were destained with Kodak Kodafix diluted 1:1 with water. The gels were destained until optimum contrast between the protein bands and the background was obtained.

Amino Acid Composition and Sequence Analyses—Amino acid compositions were determined for the crude storage body preparations, the extracts prepared from them, and for peptides obtained by partial Edman degradation of the extracted proteins. The samples were lyophilized, weighed, and then suspended in 6 N HCl at a concentration of approximately 2.0 mg/ml. The samples were then purged with argon, tightly capped and heated at 110 °C for 24 h. Aliquots of the digests were analyzed for the presence of ε-*N*-trimethyllysine using thin layer chromatography (12, 13). The amino acid compositions of the digests were determined with a Beckman model 6300 amino acid analyzer. Quantification was based on the analysis of amino acid standards, including ε-*N*-trimethyllysine. The identity of TML was confirmed as described previously (12).

The storage body extracts expected to be enriched in the subunit c protein were subjected to amino acid sequence analyses. An aliquot of each extract was solubilized by incubation in 2% SDS at room temperature for 60 min at a protein concentration of approximately 2 μg/ml. The resulting solution was subjected to centrifugation at 10,000 × *g* for 15 min, and 100 μl of the supernatant was spotted onto either a Sequelon (Millipore) or ProBlot polyvinylidene difluoride (Applied BioSystems) membrane. The proteins that bound to the membranes were then subjected to amino acid sequence analysis with an Applied Biosystems 470 gas-phase sequencer equipped with a model 120 phenylthiohydantoin analyzer.

Sequence analysis of the whole protein isolated from storage bodies terminated after residues 18 to 24, apparently because the peptide remaining after removal of the amino-terminal region of the protein no longer adhered to the membranes. Thus, additional analyses were undertaken to determine whether the lysine residue at position 43 of the stored subunit c protein was methylated. The ether-precipitated protein was solubilized in 2% SDS, bound to a ProSpin membrane, and subjected to seven cycles of Edman degradation to remove the 7 terminal amino acids. The remainder of the protein, which was still bound to the membrane, was subjected to acid hydrolysis in 6 N HCl, and the amino acid compositions of the hydrolysates were determined.

In order to obtain a more definitive determination of whether the second lysine residue of the storage body subunit c was methylated, subunit c protein preparations from storage bodies were subjected to proteolysis with trypsin. Normal mitochondrial subunit c protein has three potential trypsin cleavage sites (18, 19), one each at the lysine residues at positions 7 and 43, and one at the arginine residue at position 38 (see Fig. 8). The subunit c preparations from brain and kidney were solubilized in 2% SDS and then bound to a ProSpin membrane (Applied Biosystems, Foster City, CA). Approximately 3.5 nmol of subunit c protein was bound to each membrane. The membrane was then incubated in 0.5% polyvinylpyrollidine-40 in 100 mM acetic acid at 37 °C for 30 min. Excess polyvinylpyrollidine-40 was washed from the membrane with three brief rinses in deionized water, followed by two washes in 50 mM Tris, 5 mM $CaCl_2$, pH 8.10 (Tris buffer). Following these rinses, the membrane was incubated with 0.2 nmol trypsin in 400 μl of the Tris buffer for 7 h at 37 °C. An additional 0.15 nmol of trypsin was then added, and the incubation was continued at 37 °C for an additional 14 h. Finally, the membrane was washed three times with the Tris buffer and twice with water, and the protein fragments remaining bound to the membrane were then subjected to amino acid sequence analysis as described earlier.

RESULTS

Both fluorescence and electron microscopy demonstrated an abundant accumulation of disease-specific storage bodies in kidney and cerebral cortex of affected dogs. The storage body isolation procedure yielded preparations from both kidney and brain that were highly enriched in these inclusions, as determined by fluorescence and electron microscopy. Material was obtained from normal dog tissues using the storage body isolation procedure. The material isolated from normal dog brain did not fluoresce and was composed primarily of membrane fragments. A weak fluorescence was present in the material isolated from control dog kidneys. This material was composed largely of connective tissue fragments such as basement membranes, as well as membrane fragments.

The average yields of material in the storage body fractions from brain and kidney tissues of affected dogs are shown in Table I. The yields were somewhat variable between preparations, apparently due to uneven tissue distributions of the storage bodies and differences in the ages of the dogs used. An average of slightly over 1.0 mg dry weight of material/g of tissue was obtained in the storage body fractions from both brain and kidney of affected animals. Approximately the same amount of material was obtained from the tissues of normal dogs using the same isolation procedure (Table I).

SDS-PAGE indicated that the most prominent protein in the

TABLE I
Characteristics of storage body fraction from normal and affected dogs

	Yield[a]	Protein
	mg/g	%
Affected brain	1.05 ± 0.04[b]	62 ± 4
Normal brain	1.44 ± 0.43	39 ± 2
Affected kidney	1.18 ± 0.39	62 ± 14
Normal kidney	0.91 ± 0.21	58 ± 18

[a] mg of dry weight/wet weight of tissue.
[b] All values are the means and standard errors of data from three to six storage body preparations.

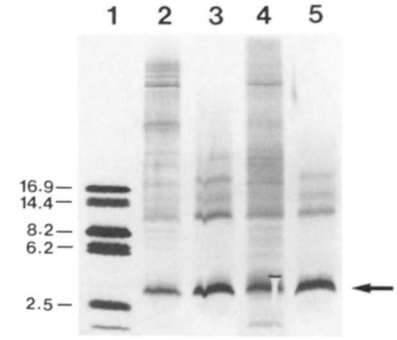

FIG. 1. **SDS-PAGE analysis of proteins from storage bodies isolated from kidney and brain of dogs with ceroid lipofuscinosis.** The storage bodies were isolated as described under "Experimental Procedures." Aliquots of the isolated storage bodies were subjected to chloroform/methanol extraction, and subunit c protein was precipitated from these extracts with ether. Both the crude storage bodies and extracted and precipitated proteins were solubilized with SDS and analyzed with SDS-PAGE. *Lane 1*, molecular mass standards (cyanogen bromide cleavage products of horse heart myoglobin having the molecular masses (in kDa) given at the *left* of the figure); *lane 2*, crude storage body preparation from kidney; *lane 3*, "subunit c" preparation from kidney storage bodies; *lane 4*, crude storage body preparation from brain; *lane 5*, subunit c preparation from brain storage bodies. Approximately 100 ng of each molecular mass standard and 200 ng of total protein in each of the other preparations was analyzed. The band with the apparent molecular mass of 3.5 kDa (indicated by *arrow*) was the most prominent component in the crude storage body preparations and was greatly enriched in the subunit c preparations. The bands in *lanes 3* and *5* with higher apparent molecular mass probably represent oligomeric aggregates of subunit c formed during the isolation procedure (11).

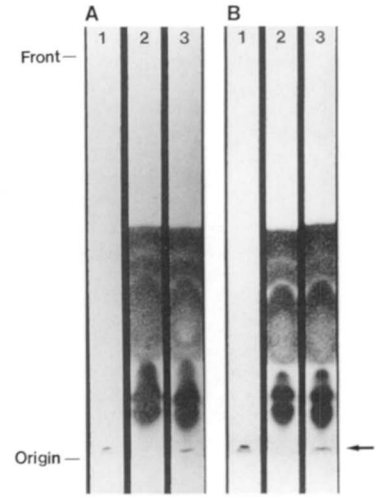

FIG. 2. **Thin layer chromatograms of HCl digests of crude storage body preparations from affected and control tissues.** Chromatograms of samples from brain (*A*) and kidney (*B*). The stationary phase consisted of silica gel G, and the mobile phase was butanol/acetic acid/water (4:1:1). The samples were chromatographed for 100 min and the plates were treated with ninhydrin. *Lanes 1*, TML standard; *lanes 2*, digests of samples from normal control dogs; *lanes 3*, digests of samples from affected dogs. Storage bodies were isolated from affected dog tissues and equivalent fractions were isolated from control dog tissues as described in the text. Aliquots of each digest corresponding to 40 μg of protein were chromatographed. The standard lanes contain 0.50 (*A*) and 0.75 (*B*) μg of TML. The *arrow* indicates the location of TML in the chromatograms.

crude storage body preparations from both tissues had a mobility consistent with an apparent molecular mass of 3.5 kDa, as does the subunit c protein from mitochondrial ATP synthase (11) (Fig. 1). There were also a number of higher molecular weight bands in the gels of proteins from the storage body preparations. The crude storage bodies were extracted with chloroform/methanol, and proteins in the resulting chloroform-rich phases were precipitated with ether. SDS-PAGE analysis of the precipitates indicated that they were greatly enriched in the protein with an apparent molecular mass of 3.5 kDa (Fig. 1). Several higher molecular weight bands could usually be seen in these preparations. These bands have been attributed to subunit c aggregation (5, 11, 15).

Crude storage body preparations from affected dog tissues and equivalent fractions from normal dog tissues were subjected to acid hydrolysis, and the products were analyzed with thin layer chromatography (TLC). The hydrolysates of the storage bodies contained a ninhydrin-reactive component with a low chromatographic mobility identical to that of a TML standard (Fig. 2). This component was not detectable in hydrolysates of equivalent preparations from normal dog tissues (Fig. 2). A component with the same chromatographic mobility as TML was greatly enriched in the chloroform-extracted ether-precipitated proteins of the storage bodies, but was not detected in the hydrolysates of proteins that did not extract from the storage bodies with chloroform (Fig. 3). The identity of this component was confirmed using the same criteria previously used to demonstrate the presence of TML in the storage body protein of sheep with hereditary ceroid lipofuscinosis (12): it co-migrated with TML on TLC with three different mobile phases; its isobutyl ester co-migrated on TLC with the isobutyl ester of a TML standard; it had the same retention time as TML on ion exchange chromatography; and it produced the same molecular ion as TML upon fast atom bombardment-mass spectrometry analysis. Quantitative amino acid analysis indicated that the protein contents of the storage body preparations from brain and kidney were both approximately 62% protein. The mean protein contents of the chloroform extracts of the storage body fractions from these tissues were approximately 33 and 52% for brain and kidney respectively. The material from control tissues that was obtained by the same procedure used to isolate the storage bodies was also subjected to chloroform extraction and ether precipitation. The yield of material from control tissues using this procedure was less than 2% of that obtained from affected dog tissues.

The overall amino acid compositions of the crude storage body fractions from brain and kidney of affected dogs differed significantly from the amino acid composition one would predict if subunit c were the only protein present (Tables II and III). In the crude storage body preparations from affected dogs, the fractions of the total lysines that were trimethylated were an average of 13% in brain and 6% in kidney (Tables II and III). The chloroform extraction and ether precipitation procedure to which the crude storage bodies were subjected yielded four fractions: an aqueous phase, an organic phase, material at the interface of the aqueous and organic phases, and material that was precipitated from the organic phase with ether. Amino acid composition analysis was performed on each of these fractions except the organic phase. The amino acid compositions of the proteins in the aqueous phases of the extracts and at the aqueous-organic solvent interfaces were less similar to that one

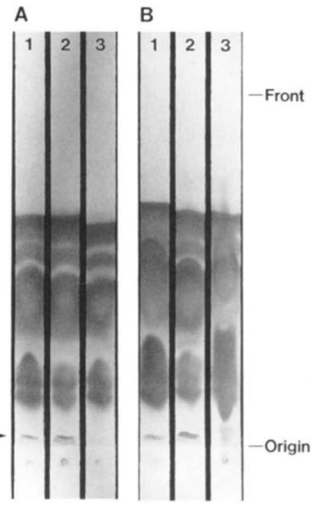

FIG. 3. **Thin layer chromatograms of HCl digests of fractions obtained by extraction from kidney and brain storage bodies.** Chromatograms of samples from brain (*A*) and kidney (*B*) of affected dogs. Chromatography was performed as described in the legend to Fig. 2. *Lanes 1*, crude storage body hydrolysates; *lanes 2*, digests of storage body proteins that extracted into chloroform and precipitated with ether; *lanes 3*, hydrolysates of storage body components that remained at the interface between the aqueous and organic phases after chloroform/methanol extraction. Storage bodies were isolated from affected dog tissues, extracted, and hydrolyzed as described in the text. Aliquots of each digest corresponding to approximately 40 μg of protein were chromatographed. The location of TML in the chromatograms is indicated by the *arrow*. TML was present the crude storage body preparations, was enriched in the fractions that extracted into chloroform and precipitated with ether, and was greatly diminished in the chloroform-insoluble proteins.

TABLE II
Amino acid compositions of storage body preparations from brains of dogs with hereditary ceroid lipofuscinosis

Amino acid	Crude	Aqueous	Interface	Precipitate[a]	Subunit c[b]
Asx	7.0[c]	10.7	8.0	4.8	4.0
Thr	4.2	4.6	6.3	6.0	4.0
Ser	6.5	5.9	6.0	7.3	6.7
Glx	7.8	14.7	9.8	4.6	4.0
Pro	4.5	6.8	5.4	3.2	1.3
Gly	10.2	7.1	9.0	12.5	14.7
Ala	11.1	7.3	9.0	14.2	17.3
Cys	1.2	2.9	1.8	1.0	1.3
Val	7.5	6.7	7.2	7.3	5.3
Met	0.7	1.5	0.7	1.2	4.0
Ile	6.7	4.5	5.6	7.8	9.3
Leu	13.1	9.1	11.6	13.6	12.0
Tyr	2.4	1.1	2.0	2.0	2.7
Phe	7.2	3.7	5.8	8.1	9.3
His	1.5	1.9	2.2	0.6	0.0
Lys	3.9	8.0	5.1	1.9	2.7
Arg	4.0	3.5	4.5	2.5	1.3
TML	0.6	0.3	0.2	1.6	0.0

[a] Methods used to fractionate the crude storage bodies into aqueous, interface, and precipitate fractions are described in the text.
[b] Values for subunit c are those predicted based on the reported amino acid sequence (18, 19).
[c] Values are mole percents. Each experimental value is the mean of three determinations.

would expect from subunit c than were the amino acid compositions of total proteins in the crude storage body preparations (Tables II and III). The proteins precipitated from the chloroform extracts with ether, on the other hand, had amino acid compositions similar to that expected from subunit c. The ether-precipitated fractions were greatly enriched in TML, whereas the TML contents of the proteins in the other fractions were less than in the crude storage bodies (Tables II and III). In the ether-precipitated protein from brain, TML represented an average of 46% of the total lysine (Table II). In the same fraction from kidney, an average of 52% of the lysines were trimethylated (Table III). Thus, the same fraction of the crude storage bodies that was enriched in subunit c (as indicated by both gel electrophoresis and amino acid composition analysis) was also enriched in TML.

TABLE III
Amino acid compositions of storage body preparations from kidneys of dogs with hereditary ceroid lipofuscinosis

Amino acid	Crude	Aqueous	Interface	Precipitate[a]	Subunit c[b]
Asx	8.5[c]	10.8	8.6	4.5	4.0
Thr	5.3	5.3	5.4	4.7	4.0
Ser	6.0	5.6	5.9	7.4	6.7
Glx	9.8	13.7	10.2	4.8	4.0
Pro	5.8	6.7	6.1	3.5	1.3
Gly	10.4	7.2	9.1	15.0	14.7
Ala	9.5	7.2	9.1	16.5	17.3
Cys	1.5	1.2	1.4	0.4	1.3
Val	6.7	5.5	6.6	5.5	5.3
Met	1.4	1.9	2.1	2.9	4.0
Ile	5.0	4.0	4.7	7.5	9.3
Leu	10.4	8.0	9.8	11.8	12.0
Tyr	1.7	2.4	2.4	1.5	2.7
Phe	4.7	4.2	4.6	7.1	9.3
His	2.3	2.4	2.4	0.4	0.0
Lys	5.9	9.2	6.4	2.1	2.7
Arg	4.7	4.4	5.0	2.1	1.3
TML	0.4	0.3	0.2	2.3	0.0

[a] Methods used to fractionate the crude storage bodies into aqueous, interface, and precipitate fractions are described in the text.
[b] Values for subunit c are those predicted based on the reported amino acid sequence (18, 19).
[c] Values are mole percents. Each experimental value is the mean of three determinations.

Amino acid sequence analyses were performed to confirm that the ether-precipitated protein from the kidney and brain storage bodies was primarily subunit c. A single dominant sequence and variable minor sequences were obtained in analyses of material from each of the tissues. Partial sequences obtained through residue 18 of the protein from brain and residue 23 of the protein from kidney were identical to that of the amino-terminal portion of mitochondrial subunit c protein (Fig. 4). Several minor amino acid peaks were present during many early cycles of the sequence analysis, indicating that the subunit c was not completely pure. Subunit c has 2 lysine residues, 1 at position 7, and 1 at position 43 (19). Residue number 7 was found to be unmodified lysine, as it is in the mitochondrial subunit c protein. The amino acid composition analyses suggested that the protein isolated from the storage bodies was subunit c in which one of the lysine residues was trimethylated. Because the methylation was not found to be present at residue 7, it appeared likely that lysine residue 43 of the storage body form of subunit c was trimethylated. To evaluate this possibility, the ether-precipitated proteins were subjected to seven cycles of Edman degradation to remove the 7 amino-terminal amino acids. The remaining peptides were subjected to amino acid composition analysis. Removal of the amino-terminal amino acids resulted in an increase in the ratio of TML to lysine from approximately 1:1 in the subunit c preparations from both brain and kidney to almost 7:1 in brain and almost 3:1 in kidney (Table IV). In the samples from kidney, the mole percent of TML was essentially what would be expected if subunit c containing TML instead of lysine at residue 43 were subjected to the same analysis (Table IV). The mole percent of TML in the preparations from brain actually exceeded that which would be expected from subunit c with TML at residue 43 (Table IV). The small amounts of residual lysine in samples from both tissues were probably due to the presence of minor protein contaminants of subunit c, as indicated by the fact that the amino acid compositions did not exactly match the composition predicted if only subunit c were

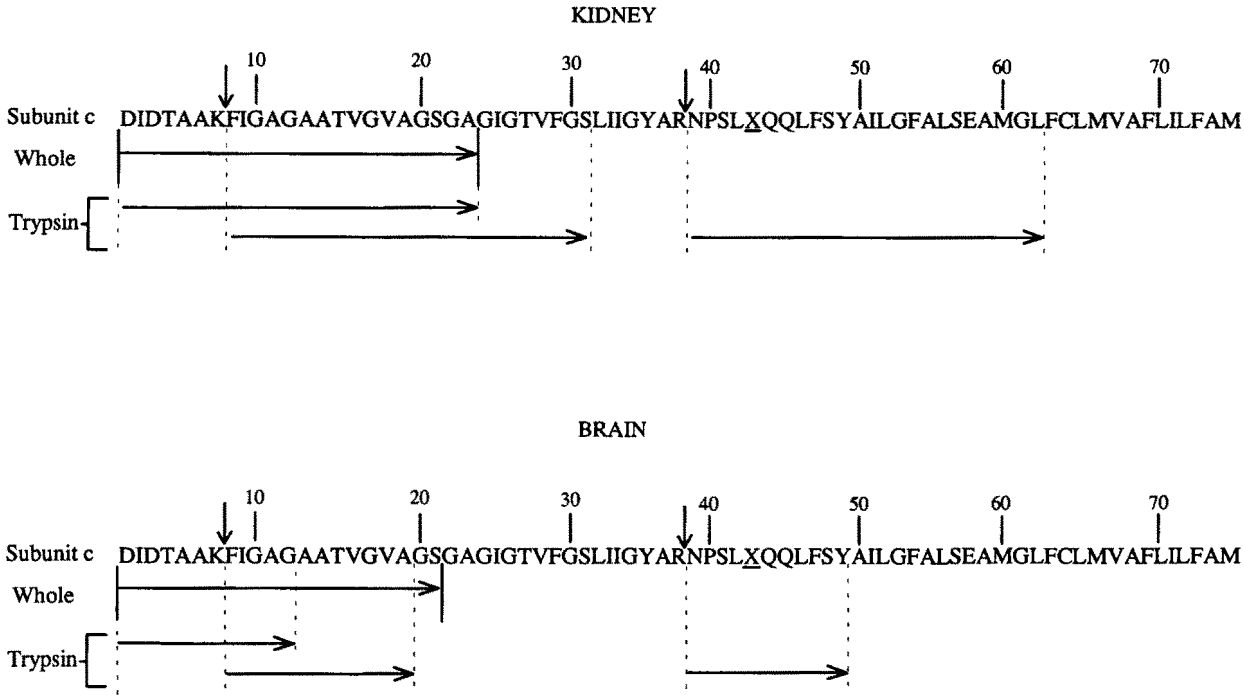

FIG. 4. **Comparison between amino acid sequences of chloroform-extracted proteins from dog brain and kidney storage bodies and the reported amino acid sequence of the mitochondrial subunit c protein.** The amino acid sequence shown is that reported for bovine mitochondrial ATP synthase subunit c (18). Residue 43, indicated by the "X," was reported to be lysine in the normal mitochondrial protein. The *vertical arrows* above the sequence indicate the trypsin cleavage sites. The *horizontal arrows* below the sequence delineated by *solid lines* indicate the sequence determined from the whole protein isolated from storage bodies. The *horizontal arrows* delineated by *dotted lines* indicate the sequences determined after cleavage of the storage body proteins with trypsin. In the sequences obtained from the storage body proteins from both tissues "X" was identified as TML by the chromatographic retention of its phenylthiohydantoin-derivative on the amino acid analyzer.

TABLE IV
Amino acid compositions of storage body proteins after seven cycles of Edman degradation

Amino acid	Brain	Kidney	Subunit c[a]
Asx	3.3[b]	3.3	1.5
Thr	5.0	4.4	2.9
Ser	7.5	7.6	7.4
Glx	4.8	5.3	4.4
Pro	2.6	4.0	1.5
Gly	14.1	14.4	16.2
Ala	15.1	15.8	16.2
Cys	0.8	1.1	1.5
Val	6.3	7.0	5.9
Met	1.2	1.2	4.4
Ile	7.7	8.4	8.8
Leu	14.3	12.3	13.2
Tyr	2.9	1.7	2.9
Phe	8.6	8.0	10.3
His	0.4	0.6	0.0
Lys	0.4	0.6	1.5
Arg	2.3	2.7	1.5
TML	2.7	1.6	0.0

[a] Values for subunit c are those predicted based on the reported amino acid sequence (18, 19).
[b] Values are mole percents. Each experimental value is the mean of two determinations.

present (Table IV). A more definitive demonstration that the origin of the storage body TML was residue 43 of subunit c came from amino acid sequence analysis after cleavage of the ether-precipitated proteins with trypsin. Amino acid sequence analysis of the cleavage products of the protein from kidney produced three sequences, one corresponding to the first 23 amino acids at the amino-terminal end of subunit c, one corresponding to residues 8–31 of the protein, and another corresponding to residues 39–62 (Fig. 4). The primary phenylthiohydantoin-derivatives obtained from the fifth cycle of Edman degradation of the trypsin cleavage products were those of alanine, glycine, and TML. No nonmethylated lysine was detected on the fifth sequencing cycle, indicating that residue 43 of the protein was completely methylated. Similar results were obtained from analysis of the subunit c preparation from brain (Fig. 4). Based on the previous sequence analysis of the whole protein, the alanine residue could be assigned to residue 5 of subunit c, and the glycine residue could be assigned to residue 12. Thus, the data strongly suggest that lysine residue 43 of the storage body protein is trimethylated.

DISCUSSION

Subunit c of mitochondrial ATP synthase has been shown to be a major constituent of the lysosomal storage material that accumulates in tissues of humans with two forms of hereditary ceroid lipofuscinosis (4, 15, 20). This protein is also present in large amounts in the storage bodies from sheep with an inherited disease that is apparently analogous to the human juvenile form of ceroid lipofuscinosis (11). Recently, it was reported that subunit c also accumulates in tissues of mice with a similar disease (10). A canine form of ceroid lipofuscinosis has been studied in English setters for over 30 years (9). In the present study we have established that mitochondrial ATP synthase subunit c is abundant in the lysosomal storage bodies from tissues of affected English setters.

Amino acid sequence data from work by Fearnley and colleagues have suggested that the subunit c protein that accumulates in storage bodies of both affected humans and animals is normal and contains no post-translation modifications (11). However, the latter investigators did not identify the amino acid residue at position 43 of the storage body protein (11). Previous investigations in our laboratory have indicated that the total storage body protein from both affected sheep and humans with the juvenile form of ceroid lipofuscinosis is rich in the modified amino acid TML (12, 13). Based on the abundance of TML in total storage body protein digests, it appeared likely

that this methylated form of lysine arose from the stored subunit c protein. Palmer and colleagues (14), however, proposed that the TML that co-extracts with the stored subunit c may not be part of this protein. The amino acid sequence data from the present study rule out the latter interpretation, at least in the canine form of the disease. Normal subunit c contains 2 lysine residues, one at position 7 and the other at position 43 (18, 19) (see Fig. 4). In the form of the protein present in the storage bodies of affected dogs, our data indicate that the ϵ-amino group of the lysine at position 43 is trimethylated.

The discovery of this specific modification in the stored form of subunit c provides an important clue in the search for the biochemical and genetic defect underlying hereditary ceroid lipofuscinosis. Because the form of ATP synthase subunit c found in mitochondria is not normally methylated, it appears likely that the methylation of lysine residue 43 is closely associated with the primary defect in this disease. Any mechanisms proposed to explain the accumulation of this specific methylated protein must be consistent with the fact that ceroid lipofuscinosis shows an autosomal recessive pattern of inheritance. The mode of inheritance of this disease makes it unlikely that abnormal methylation of subunit c results from a mutation in a gene coding for a protein-lysine-methylating enzyme (21, 22). Such a mutation could alter the substrate specificity of a methylase such that subunit c would be inappropriately methylated. However, this type of mutation would be expected to show a dominant pattern of inheritance or at least to have some effect on carriers. Heterozygous individuals, whether they be humans, sheep, or dogs, have not been reported to show clinical symptoms of the disease. Thus, it appears much more likely that the subunit c protein is normally methylated at some point in its life cycle and that the defect responsible for the disease involves the cellular processing of the methylated form of the protein.

A normal fate for trimethylated lysine residues from proteins is metabolic conversion to carnitine, an important co-factor in mitochondrial fatty acid metabolism (23). The first step in carnitine biosynthesis is methylation of protein lysine residues (24). Mammals do not appear to be capable of methylating free lysine (23, 24). The methylated protein is then degraded resulting in the release of free TML. TML is metabolized through a series of enzymatic steps to form carnitine (25). It is possible that subunit c protein is normally methylated at lysine residue 43 at some point in its life cycle and that it serves as a major source of TML for carnitine biosynthesis. If this is the case, a defect in the carnitine biosynthetic pathway could account for the accumulation of the methylated form of subunit c. Impairment of carnitine biosynthesis could cause carnitine precursors, including TML-containing subunit c, to accumulate. Preliminary data suggest that plasma carnitine levels are significantly depressed in dogs with ceroid lipofuscinosis,[2] which is consistent with the possibility that these animals have a defect in the carnitine biosynthetic pathway.

The normal pathway for turnover of subunit c and other mitochondrial proteins is not well understood. It is possible that subunit c is normally degraded via a nonlysosomal pathway. When the methylated form accumulates in large amounts, it clearly appears in the lysosomal system (5). Because of its hydrophobic nature, trimethylated subunit c may be resistant to proteolytic degradation when present at high concentrations inside secondary lysosomes. Further studies will be required to define the role that methylation of one of its lysine residues plays in the lysosomal storage of this protein.

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