Transglutaminase Activity Is Present in Highly Purified Nonsynaptosomal Mouse Brain and Liver Mitochondria†

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Received January 14, 2005; Revised Manuscript Received March 29, 2005

ABSTRACT: Several active transglutaminase (TGase) isoforms are known to be present in human and rodent tissues, at least three of which, namely, TGase 1, TGase 2 (tissue transglutaminase), and TGase 3, are present in the brain. TGase activity is known to be present in the cytosolic, nuclear, and extracellular compartments of the brain. Here, we show that highly purified mouse brain nonsynaptosomal mitochondria and mouse liver mitochondria and mitoplast fractions derived from these preparations possess TGase activity. Western blotting and experiments with TGase 2 knock-out (KO) mice ruled out the possibility that most of the mitochondrial/mitoplast TGase activity is due to TGase 2, the TGase isoform responsible for the majority of the activity ([^{14}C]putrescine-binding assay) in whole brain and liver homogenates. The identity of the mitochondrial/mitoplast TGase(s) is not yet known. Possibly, the activity may be due to one of the other TGase isoforms or perhaps to a protein that does not belong to the classical TGase family. This activity may play a role in regulation of mitochondrial function both in normal physiology and in disease. Its nature and regulation deserve further study.

Human and rodent tissues contain eight active transglutaminases (TGases;[1] EC 2.3.2.13) that catalyze the Ca^{2+}-dependent covalent linkage of the carboxamide moiety of a Q residue (acyl donor) in a protein/peptide substrate to the ϵ-amino group of a K residue (acyl acceptor) in a protein/peptide cosubstrate. The reaction results in the formation of an N^{ϵ}-(γ-L-glutamyl)-L-lysine (GGEL) cross-link and ammonia (*1*). TGases can also utilize amines, diamines, and polyamines as acyl acceptors (*2*). Human brain (*3*) and rat brain (*4*) possess at least three TGase proteins, namely, TGase 1, 2 (tissue transglutaminase, tTGase), and 3. In addition, the human brain also contains mRNAs for TGases 5, 6, and 7 (*5*). Increased brain TGase activity and increased GGEL immunoreactivity have been reported for Alzheimer disease (AD) (*6*, *7*), Huntington disease (HD) (*8*−*13*), Parkinson disease (PD) (*14*), and progressive supranuclear palsy (*15*).

Considerable evidence suggests that increased protein cross-linking occurs in diseased brain. For example, free GGEL is increased in HD CSF and brain (*11*, *16*) and in AD CSF (*17*). Free GGEL is also increased about 30-fold in digests of protein obtained from AD brain (*3*, *18*). While TGase activity is not causative of neurodegeneration, it is possible that TGase(s) contribute(s) to the disease progression (e.g., refs *19* and *20*). Circumstantial evidence showing that the *in vitro* TGase inhibitor cystamine (*9*, *11*) increases the life expectancy of HD transgenic mice and more direct evidence from experiments showing the prolongation of life expectancy in HD transgenic mice exhibiting a TGase 2 KO (*21*) are consistent with this possibility. Under standard assay conditions ([^{14}C]putrescine-binding assay), TGase 2 is the most active isoform in this tissue (*21*). However, the

† This work was supported in part by the National Institutes of Health Grants PO1 AG14930 and NS38741.

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[1] Abbreviations: AD, Alzheimer disease; AspAT, aspartate aminotransferase; BSA, bovine serum albumin; CSF, cerebrospinal fluid; cyt, cytosolic; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; GDH, glutamate dehydrogenase; GGEL, N^{ϵ}-(γ-L-glutamyl)-L-lysine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HD, Huntington disease; K, lysine; IM, inner mitochondrial membrane; KGDHC, α-ketoglutarate dehydrogenase complex; KO, knock out; LDH, lactate dehydrogenase; mit, mitochondrial; OM, outer mitochondrial membrane; PD, Parkinson disease; PDI, protein disulfide isomerase; Q, glutamine; Q_n, polyglutamine domain; RFU, relative fluorescence intensity; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TGase, transglutaminase (nonspecified isotype); TGase 1, transglutaminase 1 or keratinocyte transglutaminase (TGase K); TGase 2, transglutaminase 2 or tissue transglutaminase (tTGase or TGase C); TGase 3, skin or epidermal transglutaminase (TGase E); Topo I, topoisomerase I; WT, wild type.

10.1021/bi0500877 CCC: $30.25
Published on Web 05/05/2005

mechanism by which TGase 2 (and other TGases) contributes to brain dysfunction is unknown.

Many human neurodegenerative diseases are characterized in part by decreased activities of key mitochondrial enzymes of energy metabolism in the brain (*22*−*25*). For example, α-ketoglutarate dehydrogenase complex (KGDHC) activity is reduced in both damaged and undamaged regions of AD brain (*26* and references quoted therein). Complex II/III activity is reduced in HD brain in the most severely affected regions (caudate and putamen), and complex IV activity is reduced in HD putamen (*27*, *28*). Aconitase activity is reduced in affected regions of HD brain (*27*, *28*), and NADH CoQ1 reductase (complex I) deficiency occurs in the substantia nigra of PD patients (*29*, *30*). KGDHC is inactivated in the presence of TGase 2 and a Q donor (*31*). Aconitase and KGDHC are inactivated in mouse brain mitochondria incubated with TGase 2 (*32*). Therefore, it is possible that the deficits in KGDHC and aconitase activities in diseased brain are due at least in part to aberrant TGase activity.

Indirect evidence suggests that aberrant TGase activity and mitochondrial abnormalities are linked in models of neurodegenerative diseases. For example, 3-nitropropionic acid [a potent irreversible inhibitor of succinate dehydrogenase (*22*)] increases *in situ* TGase activity in SH-SY5Y cells stably overexpressing human TGase 2 (*33*). Lesort et al. (*33*) suggested that lowered levels of ATP and GTP (TGase 2 inhibitors) resulting from mitochondrial dysfunction in the 3-nitropropionic acid-treated cells cause *dis*inhibition of TGase 2 activity. Mitochondrial-mediated apoptosis is induced by staurosporine in neuronal SK-N-BE cells and 3T3 fibroblast cells expressing high levels of TGase 2 protein (*34*). The mitochondria in these cells exhibit gross morphological changes, are hyperpolarized, produce high levels of reactive oxygen species, and rapidly lose membrane potential.

Despite a link between aberrant TGase activity and mitochondrial dysfunction, no previous studies indicate whether TGase activity is present in mitochondria. To address this issue, we purified nonsynaptosomal mitochondria from mouse brain and determined whether TGase activity is present in these organelles and within the matrix/inner membranes (i.e., mitoplasts). Among mammalian organs, TGase-specific activity is generally highest in liver, and the predominant liver TGase (TGase 2) has been extensively studied (e.g., ref *2*). Moreover, liver mitochondria serve as a "gold standard". Thus, for comparative purposes, we also determined whether mouse liver mitochondria and mitoplasts can catalyze TGase reactions. Here, we report that highly purified mouse brain nonsynaptosomal mitochondria/mitoplasts and mouse liver mitochondria/mitoplasts possess TGase activity that is unlikely to be due to cytosolic contamination. The implications of these findings are discussed.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies. Semipurified guinea pig liver TGase 2 [~4.5 units/mg of protein, where one unit is the amount that catalyzes the formation of 1 μmol of hydroxamate per minute from a standard reaction mixture containing carbobenzoxyglutaminylglycine and hydroxylamine (*2*)], sucrose (ultrapure), HEPES (ultrapure), EDTA, EGTA, *N*,*N*-dimethylcasein, digitonin, sodium α-ketoglutarate, sodium pyruvate, ammonium acetate, L-aspartate, bovine serum albumin (BSA, fatty acid free), ADP, NADH, Ficoll 400-DL (ultrapure), trichloroacetic acid (TCA), protease inhibitor cocktail (category number P-8340), and GTP-agarose beads, were obtained from Sigma (St. Louis, MO). Succinylated casein was from ICN (Irvine, CA). [1,4-^{14}C]Putrescine (118 Ci/mol) was obtained from Life Sciences Products, Inc. (Wilmington, DE). Fluorescein cadaverine [5-((5-aminopentyl)thioureidyl)fluorescien dihydrobromide] was from Molecular Probes (Eugene, OR). Laemmli buffer was from Bio-Rad (Hercules, CA). Three sources of TGase 2 antibodies were used: Polyclonal rabbit anti-guinea pig primary TGase 2 antibodies from CovalAb (category number 997-PTG) (Lyon, France) (*35*), polyclonal rabbit anti-human primary TGase 2 Ab-4 antibodies from Lab Vision-NeoMarkers (category number RB-060-P) (Fremont, CA) (*36*), and polyclonal rabbit anti-human primary TGase 2 antibodies prepared by the method of Kim et al. (*3*). Anti-GRP78 (ER marker) rabbit antibodies were from ABR (Golden, CO); anti-oxidative phosphorylation complex I, 39-kDa subunit IV (complex I; inner mitochondrial membrane marker) monoclonal mouse antibodies were from Molecular Probes (Eugene, OR); and anti-topoisomerase I (Topo I, nuclear marker) mouse monoclonal antibodies were from Topogen, Inc. (Columbus, OH). In some cases, antibodies to complex I, Topo I, and GRP78 obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) were also used. Secondary anti-mouse IgG (H + L) alkaline phosphatase (AP) conjugated antibodies were from Promega (Madison, WI), and anti-rabbit IgG (H + L) AP conjugated antibodies were from Bio-Rad (Hercules, CA). The AP conjugated substrate kit was obtained from Bio-Rad (Hercules, CA). Secondary anti-mouse and anti-rabbit IgG (H + L) horseradish peroxidase (HRP) conjugated antibodies, enhanced chemiluminescence (ECL) Western blotting detection reagents, and films were from Amersham (Piscataway, NJ).

Animals and Tissues. All experimental procedures were approved by the Weill Medical College of Cornell University Institutional Animal Care and Use Committee (protocol 0304-113A). In one set of experiments, male C57BL/6N (Harlan Sprague Dawley, Indianapolis, IN) or B6/CBA51/J (Jackson Laboratories, Bar Harbor, ME) mice were used. In another set of experiments, male TGase $2^{-/-}$ (KO) mice and their controls (TGase $2^{+/+}$) were used. Both the TGase $2^{-/-}$ and their TGase $2^{+/+}$ controls were on the same mixed SVJ129-C57BL/6 background (cf. ref *37*). Brain and liver samples from these animals were shipped on dry ice from the Victor Chang Cardiac Research Institute, Darlinghurst, New South Wales, Australia, to the Burke Medical Research Institute, White Plains, NY, where the actual biochemical measurements on these specimens were performed. The mice used in both sets of experiments were fed *ad libitum* and had full access to water.

Preparation of Brain Mitochondria. Nonsynaptosomal brain mitochondria were obtained from 6-month-old B6/CBA51/J and 14-month-old C57BL/6N male mice by a modification of the method of Lai and Clark (*38*). The main subcellular components of each fraction are listed in Table 1. All steps were carried out at 0−4 °C unless otherwise stated. The mice were sacrificed by decapitation. Forebrains from 15 animals (~5 g total) (except where indicated) were

Table 1: Main Components of the Mouse Brain Fractions

fraction	main components
H	homogenate containing all cellular components
P1	nuclei, ER, cell-membrane fragments, and blood cells
S1	crude cytosol containing small nuclear fragments, some ER components, small cell-membrane fragments, and most subcellular organelles
P2	nuclei, ER, cell-membrane fragments
S2	crude cytosol containing most subcellular organelles
P3	crude mitochondria plus synaptosomes
S3	cytosolic components including ER
P4	semipurified nonsynaptosomal mitochondria
S4A	buffer-soluble cytosolic biomolecules including proteins
S4B	damaged organelles, myelin, large protein complexes, and associated lipids
S4C	synaptosomes, microsomes, peroxisomes
P5	purified nonsynaptosomal mitochondria
S5	wash of P5
P6	highly purified nonsynaptosomal mitochondria
S6	wash of P6
P7	mitoplasts prepared in the presence of protease inhibitors
S7	wash of P7
P7*	mitochondria treated with digitonin in the presence of protease inhibitors
S7*	wash of P7*

used for each preparation of nonsynaptosomal mitochondria. The brains were homogenized in 40 mL of buffer I (320 mM sucrose, 10 mM HEPES, and 0.5 mM EGTA at pH 7.4) using a Dounce homogenizer. The homogenate (H) was centrifuged for 3 min at 1300*g*. The pellet (P1) was suspended in 40 mL of buffer I and saved. The supernatant (S1) was centrifuged at 1300*g* for 3 min, and the pellet (P2) was saved. The supernatant (S2) was centrifuged at 17000*g* for 8 min, and the supernatant (S3) was saved. The pellet (P3) was suspended in 10 mL of buffer I and applied to the top of 7 mL of 10% (w/v) Ficoll overlaid by 7 mL of 7.5% (w/v) Ficoll. After centrifugation at 99000*g* for 20 min, aliquots were removed consecutively from the top down, namely, a clear layer (S4A), a white myelin-containing layer (S4B), and a light yellow synaptosomal mitochondria-containing layer (S4C). The nonsynaptosomal mitochondria-containing pellet (P4) at the bottom of the tube was resuspended in 15 mL of buffer I and centrifuged at 12000*g* for 8 min. The supernatant (S5) was saved. The semipurified nonsynaptosomal mitochondria-containing pellet (P5) was resuspended in 7.5 mL of buffer I supplemented with 0.5 mg/mL BSA. After centrifugation at 12000*g* for 8 min, the supernatant (S6) was saved. The highly purified nonsynaptosomal mitochondria-containing pellet (P6) was resuspended in 0.2 mL of buffer II (320 mM sucrose, 10 mM HEPES, and 0.02 mM EGTA at pH 7.4).

Preparation of Brain Mitoplasts. An aliquot (50 μL) of the nonsynaptosomal mouse brain mitochondrial pellet (P6) suspension in buffer II was subjected to osmotic shock by addition of 900 μL of 3 mM HEPES (pH 7.4) containing 50 μL of protease inhibitor cocktail. After incubation on ice for 15 min, the suspension was centrifuged at 17000*g* for 10 min to yield the mitoplast pellet (P7) and supernatant (S7). The mitoplast fraction was suspended in 50 μL of buffer II.

Treatment of Nonsynaptosomal Brain Mitochondria with Digitonin. A portion of the P6 pellet suspension (50 μL) was treated with 950 μL of 0.015% (w/v) digitonin. After incubation at 0 °C for 15 min, the suspension was centrifuged at 17000*g* for 10 min. The pellet and supernatant were designated P7* and 7S*, respectively. The pellet P7* was suspended in 50 μL of buffer II. Digitonin removes cholesterol, a major lipid component of the outer mitochondrial membrane (OM), resulting in the loss of OM integrity. The inner mitochondrial membrane (IM) remains intact after digitonin treatment (*39*).

Preparation of Liver Mitochondria. Liver mitochondria were prepared from 6-month-old B6/CBA51/J male mice (4 or 5 livers in each preparation) by the procedure of Cooper et al. (*40*). The mitochondria were suspended in 1 mL of buffer II. When highly purified rat liver mitochondria, prepared in the absence of Ficoll, were centrifuged through a Ficoll gradient, no changes occurred in the specific activities of the mitochondrial marker enzymes glutamate dehydrogenase (GDH) and mitochondrial aspartate aminotransferase (mitAspAT) (data not shown).

Preparation of Liver Mitoplasts. Mouse liver mitoplasts were prepared from the highly purified mitochondria by the same procedure used to obtain brain mitoplasts.

Treatment of Liver Mitochondria with Digitonin. Mouse liver mitochondria were treated with digitonin by the same protocol used for the brain samples.

Sample Collection and Storage. Samples collected at each stage of the mitochondria isolation procedure, mitoplasts, and mitochondria exposed to digitonin were treated with protease inhibitor cocktail such that the cocktail was diluted 50-fold. Aliquots of 50−200 μL of each fraction were transferred into Eppendorf tubes and frozen immediately at −80 °C.

Preparation of Mitochondria from Frozen Tissues. In some experiments, mitochondria were prepared from previously frozen brains and livers of TGase $2^{-/-}$ mice and their controls (TGase $2^{+/+}$). The frozen tissue was ground in dry ice to a powder before homogenizing in isolation buffer at 0 °C (method 1) or was thawed and then homogenized in isolation buffer at 0 °C (method 2). The procedures for obtaining mitochondria from these homogenates were the same as those described above for the isolation of mitochondria from nonfrozen tissues.

Enzyme Assays. Total TGase activity was determined by measuring the incorporation of [1,4-^{14}C]putrescine into succinylated casein in the presence of Ca^{2+} and DTT (*3*). Generally, about 1 mg of protein from each subcellular fraction was used in each assay. After incubation for 1 h, the protein in the reaction mixture was precipitated with an equal volume of 10% (w/v) TCA; the precipitate was collected on a filter, washed, and counted (*3*). In most cases, the blank consisted of the complete reaction mixture (lacking the enzyme source and EDTA) incubated for 1 h before addition of TCA (blank 1). Mitochondria (1 mg of protein) sonicated in the complete reaction mixture containing 10 mM EDTA (to bind Ca^{2+}) and then incubated for 1 h before addition of TCA served as an additional blank (blank 2). Blank 2 takes into account any possible cross-linking that is not Ca^{2+}-dependent. Samples with low counts were counted for 1 h with a maximal error rate in the counts of ∼10%. The radioactivity in blank 1 was typically ∼200 cpm. The radioactivity in the precipitated succinylated casein in the reaction mixture incubated with 1 mg of purified nonsynaptosomal mitochondria (see below) for 1 h at 37 °C was typically ≥2 times the blank value. Because of the inherently low activity of mitochondrial TGase, we also determined the radioactivity in blank 2. After incubation for 1 h at 37 °C,

the radioactivity in the precipitated protein was 238 ± 13 cpm ($n = 3$). This value is not different from blank 1, suggesting that non-Ca^{2+}-dependent mechanisms do not contribute to the incorporation of the label into succinylated casein in the presence of purified nonsynaptosomal mitochondria. The radioactivity in the positive control, i.e., complete reaction mixture, minus EDTA, plus mitochondria, was 399 ± 27 ($n = 3$); $p = 0.05$. Therefore, blank 1, except where noted, was used in the [^{14}C]putrescine-binding assays reported here.

Relative TGase activity was also determined using a new fluorescence procedure. The reaction mixture (final volume of 0.2 mL in a small, closed Eppendorf tube) consisted of 10 mM DTT, 10 mM $CaCl_2$, 100 μg of *N*,*N*-dimethylcasein, 25 μM (or in some cases 50 μM) fluorescein cadaverine, and 100 mM HEPES-NaOH at pH 8.0. The tube was heated to 37 °C, and the reaction was started by the addition of the enzyme source. After 2 h (or 16 h), the reaction was stopped by the addition of 0.4 mL of 0.4 M perchloric acid. The samples were then centrifuged at 10000*g* for 10 min at 4 °C to pellet coagulated protein. The supernatant was carefully discarded, and the pellet was washed with 0.4 mL of acetone/ethanol (1:1, v/v) brought to 0.1 M HCl with 12 M HCl. The pellet was again collected at 10000*g* and washed a further 2 times with acetone. The final pellet was air-dried and dissolved in 0.1 mL of 0.1 M NaOH followed by addition of 0.1 mL of 0.7 M boric acid (final pH ~7.2). The fluorescence of the samples (λ_{ex}, 488 nm; λ_{em}, 521 nm) was measured using Microfluor 2 Black "U" bottom microtiter 96-well plates (Thermolabsystems, Franklin, MA) and a Spectra MAX Gemini Plate Reader (Molecular Devices, Sunnyvale, CA). One set of controls consisted of the complete reaction mixture plus the enzyme source carried through the incubation and extraction procedures except that 20 mM EGTA or EDTA was included (to chelate Ca^{2+}). In another set of controls, boiled enzyme source was used in place of the active enzyme. The fluorescent method is sensitive, as shown by results obtained with commercial TGase 2 as a positive control. In this experiment, the relative fluorescence of the precipitated protein in the complete reaction mixture plus TGase 2 (2.5 μg incubated at 37 °C for 2 h) minus EDTA (100 000 RFU) was much greater than that of the control (complete reaction mixture plus TGase 2 plus EDTA) (~800 RFU). In comparison, a 10-min incubation using the [^{14}C]putrescine-binding assay (above) with this amount of enzyme generates a blank (complete reaction mixture plus enzyme plus EDTA) of about 200 cpm and a signal of about 40 000 cpm in the complete reaction mixture plus enzyme minus EDTA. Thus, the new fluorescence procedure with purified TGase 2 is of similar sensitivity to that of the radiochemical assay.

GDH, lactate dehydrogenase (LDH), and AspAT were measured spectrophometrically in 96-well plates as described by Park et al. (*41*). To distinguish between cytosolic (cyt) and mitochondrial (mit) isoforms of AspAT, a fraction of the biological sample was mixed with an equal volume of 200 mM Tris acetate buffer (pH 7.4) and 3.7 mM α-ketoglutarate. An aliquot was assayed for AspAT before and after heating at 65 °C for 15 min. This treatment completely destroys mitAspAT activity but has no effect on cytAspAT activity (*42*). To correct for NADH oxidase activity in the various brain fractions, the blanks for each enzyme assay contained the complete assay mixture plus the tissue fraction except that the α-keto acid substrate was omitted. γ-Cystathionase, which is present in high concentrations in liver (but not brain) cytosol, was measured by using L-homoserine in place of L-cystathionine as the substrate (*43*). The reaction mixture (50 μL) contained 20 mM L-homoserine, 100 mM potassium phosphate buffer (pH 7.2), and enzyme. After incubation at 37 °C, the reaction was terminated by the addition of 20 μL of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After incubation at 37 °C for 10 min, 130 μL of 1 M NaOH was added and the mixture was centrifuged for 2 min (at 10000*g*). The absorbance of α-ketobutyrate 2,4-dinitrophenylhydrazone at 430 nm of the supernatant was determined ($\epsilon = 16\,000\ M^{-1}\ cm^{-1}$) within 2 min against a blank carried through the same procedure. Blanks consisted of either 50 μL of buffer plus enzyme and no L-homoserine or 50 μL of buffer plus L-homoserine and no enzyme. To measure γ-cystathionase activity in the homogenate, 150 μg of homogenate protein was incubated for 5 min in the 50-μL reaction mixture before addition of the 2,4-dinitrophenylhydrazine. To measure the activity in the mitochondria (or mitoplasts), 150 μg of mitochondrial (or mitoplasts) protein was incubated for 30 min in the 50-μL reaction mixture before addition of the 2,4-dinitrophenylhydrazine reagent. KGDHC activity was determined fluorometrically in 96-well plates in the presence of saturating concentrations of thiamine pyrophosphate and was corrected for NADH oxidase activity essentially as described previously (*44*, *45*).

In pilot experiments, we found that purified nonsynaptosomal mitochondria stored on ice for up to 6 h exhibited a KGDHC-specific activity that was only about 15−20% that of mitochondria kept on ice for short periods (<1 h). Enzyme activity determinations, except for TGase (and γ-cystathionase in liver fractions), were carried out within 1 h after preparation of the individual subcellular fractions and storage on ice. Pilot experiments showed that the TGase and γ-cystathionase activities were not affected by freeze/thawing. Aliquots of brain and liver fractions were frozen at −80 °C within 1 h of preparation. Samples were thawed only once before TGase or γ-cystathionase assays. In all cases, except for the measurement of KGDHC activity, enzyme assays were carried out at 37 °C. In the case of KGDHC activity measurements, enzyme assays were conducted at 30 °C.

The continuous assay procedures for GDH, LDH, AspAT, and KGDHC provide initial rates. The γ-cystathionase and TGase assays involve end-point determinations. We have found that the γ-cystathionase assay with liver homogenates is linear for at least an hour (data not shown). Thus, the typical 10-min incubation used for this assay provides results that are indicative of initial rates. The specific activities of GDH, LDH, AspAT, KGDHC, and γ-cystathionase are expressed as milliunits/mg (nmol min^{-1} mg^{-1}) of protein, where these units represent initial rates. We have also found that the [^{14}C]putrescine-binding assay for measuring TGase activity with commercial guinea pig liver TGase 2 is linear for about an hour (data not shown). On the basis of our experience with this assay and the commercial TGase, we chose a 1-h incubation period for measuring TGase activity in the mouse brain and liver fractions. We report TGase activities in the various fractions as pmol h^{-1} (mg of protein)$^{-1}$. However, we have not yet investigated the time

course of the reaction with these fractions. Thus, in the case of TGase (unlike the other enzyme measurements), the activity units applied to mouse liver and brain fractions do not necessarily reflect initial rates but do indicate the presence of enzyme activities.

SDS−PAGE of N,N-Dimethylcasein Previously Incubated with Fluorescein Cadaverine and Mouse Liver Fractions. Samples of homogenate, mitochondria, and mitoplasts (each containing 0.5 mg of protein) prepared from fresh livers of C57BL/6N WT mice were incubated for 16 h at 37 °C in the 0.2 mL reaction mixture containing *N,N*-dimethylcasein and fluorescein cadaverine as described in the previous section. Blanks contained the complete reaction mixture plus the enzyme source plus 10 mM EDTA. The reaction was stopped by the addition of an equal volume of Laemmli buffer. The samples were then heated at 100 °C for 5 min and cooled, and aliquots containing 150 μg of protein were individually loaded into wells of 4−20% gradient Tris/glycine/SDS precast iGels (Life Gels, Clarkston, GA). The reservoir buffer was 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS at pH 8.3. For comparison with an "authentic" TGase, two lanes were reserved for a reaction mixture containing *N,N*-dimethylcasein and fluorescein cadaverine treated with TGase 2. In that case, 2.5 μg of commercial TGase 2 was used in place of the homogenate/mitochondrial sample and the boiled mixture containing 0.5 μg of TGase 2 was added to each well. Electrophoresis was carried out using a mini-gel unit (Bio-Rad, Hercules, CA). After electrophoresis was complete, the gel was removed from the electrophoresis chamber and washed twice with 20 mL of distilled water. Fluorescent bands were detected under UV light using a "Gel Logic 100" imaging system (Kodak, Rochester, NY). A photographic record of the images was obtained using the same system.

Western Blotting. Subcellular fractions were prepared as described above. Samples (50 μL) containing 100 mg/mL of protein were mixed with 50 μL of Laemmli buffer, incubated for 10 min at 100 °C, and loaded at ∼20−25 μg of protein (except where noted) per well onto precast 10−20% linear-gradient Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA) for SDS−PAGE. Electrophoresis was carried out using a mini-gel unit (Bio-Rad, Hercules, CA). The reservoir buffer was 25 mM Tris, 192 mM glycince, and 0.1% (w/v) SDS at pH 8.3. Proteins within the gels were transferred to 0.45 μm PVDF membranes (Pierce, Rockford, IL) using a mini-blot unit (Bio-Rad, Hercules, CA). Membranes were blocked with 5% (v/v) skimmed milk in TBST (Tris at pH 7.4, 150 mM NaCl, and 0.05% Tween 20) either for 3 h at room temperature or overnight at 4 °C. Blots were probed with primary antibodies either for 2 h at room temperature or overnight at 4 °C. This was followed by incubation with secondary antibodies for 1 h at room temperature. Signals were detected by using an alkaline phosphatase conjugate substrate kit or with an ECL Western blotting kit. Dilutions of the antibodies were performed according to protocols described by the manufacturers.

GTP-Agarose Pull Down of TGase 2. Nanda et al. (*37*) and Bailey et al. (*36*) have cautioned that commercially available antibodies to TGase 2 recognize proteins other than TGase 2. To overcome this problem, the GTP pull-down procedure of Bailey et al. (*36*) was used with minor modifications. GTP pull down was performed on brain and liver homogenates, mitochondria, and commercial TGase 2 samples. The brain and liver samples were homogenized at a ratio of 1 g of wet weight to 10 mL of the buffer used by Bailey et al. (*36*). Each sample (2 mg of homogenate or mitochondrial protein; 0.1 mg of commercial TGase protein) was incubated with 150 μL of GTP-agarose beads in the buffer used by Bailey et al. (*36*). In the last step of the procedure, bound proteins were eluted with 75 μL of immunoblotting loading buffer according to the procedure of Bailey et al. (*36*). This procedure has been used successfully for removing extraneous proteins in mouse brain homogenates (*36*). It should be noted, however, that the procedure may pull down other TGases. GTP has recently been shown to bind also to TGase 3 (*46*), TGase 4 (*47*), and TGase 5 (*48*), so that the pull-down procedure may not be specific for TGase 2. Denatured TGase 2 was removed from the GTP-agarose beads by boiling in the presence of blotting/loading buffer (*36*).

Protein Measurement. Protein concentrations were determined using a micro-Biuret assay kit from Sigma (St. Louis, MO) and BSA as a standard.

Statistical Analysis. Except where indicated, for determinations where $n \geq 3$, the mean ± the standard error of the mean (SEM) is reported. Statistical tests are noted in the text and tables. A p value of ≤ 0.05 was considered significant.

RESULTS

Confirmation of the High Purity of Nonsynaptosomal Mitochondria Isolated from Fresh Mouse Brain. Western blot analysis of various subcellular markers under two different conditions (parts A and B of Figure 1) showed that the untreated purified mitochondria (P6), mitoplasts (P7), and digitonin-treated mitochondria (P7*) were not detectably contaminated with ER or nuclear elements. The activity of LDH (cytosolic marker) in the P6 fraction was ≤0.05% of that in the original homogenate ($n = 10$). A strong signal was obtained with the mitochondrial matrix protein complex I in the P6, P7, and P7* fractions (Figure 1). Enrichment (ratio of specific activity in the mitochondria relative to that in the homogenate) of the mitochondrial marker enzyme KGDHC in the purified mitochondria was high (at least 7.6) (Table 2) but less so for the mitochondrial enzymes GDH and mitAspAT (∼3.5 and ∼4.4, respectively; Table 2). The lower enrichment of these enzymes may be due to their presence in extramitochondrial compartments (*49*, *50*) and/or to differing distribution between synaptosomal and nonsynaptosomal mitochondria (*51*).

Detection of Ca^{2+}-Dependent TGase Activity in Mouse Brain Nonsynaptosomal Mitochondria by the [^{14}C]Putrescine-Binding Assay. TGase activity, as measured by the [^{14}C]putrescine-binding assay, was detected in nine preparations of nonsynaptosomal mitochondria, three preparations from 6-month-old B6/CBA51/J mice listed in Table 2, two preparations from 14-month-old C57BL/6N mice [5 and 3.5 pmol h^{-1} (mg of protein)$^{-1}$, respectively], and four additional preparations from 6-month-old B6/CBA51/J mice (5.2 ± 0.4 pmol h^{-1} mg^{-1}). In only one case was TGase activity not detected in mouse brain mitochondria (mitochondria prepared from one batch of B6/CBA51/J mice). The average value, including the one zero value, was 4.4 ± 0.8, $n = 10$,

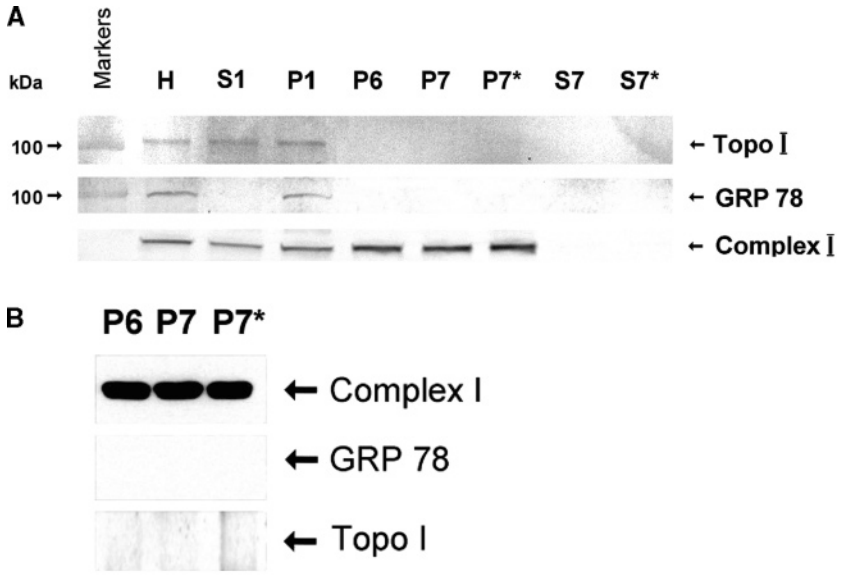

FIGURE 1: Western blots of various brain fractions obtained from WT mouse for specific subcellular markers. Topo I, GRP78, and complex I were used as nuclear, ER, and mitochondrial markers, respectively. The mitochondrial (P6) fraction was split into three portions: one portion (50 μL) was left unmodified; one portion (50 μL) was converted by osmotic shock to mitoplasts (P7); and another portion (50 μL) was treated with digitonin (P7*). S7 and S7* are the supernatants obtained after the preparation of P7 and P7*, respectively. Each lane contained 25 μg of protein. Western blots for each subcellular marker were carried out on separate gels, but with identical specimens, order of loading, and electrophoresis/transfer conditions. The blots shown were obtained with fractions isolated from fresh tissue. In A, the antibodies were from Topogen (Topo I), ABR (GRP78), and Molecular Probes (complex I). Detection was by the alkaline phosphatase procedure. In B, all three antibodies were from Santa Cruz Biochemicals, and detection was by the ECL procedure. A description of the various subcellular fractions is given in Table 1.

Table 2: Comparison of Ca^{2+}-Dependent TGase Activity with KGDHC, GDH, mitAspAT, and LDH Activities in Homogenate (H) and Highly Purified Nonsynaptosomal Mitochondria (P6) Obtained from Mouse Brain[a]

	total activity[b]		specific activities[c]		ratio of specific activities	
enzyme	H	P6	H	P6	P6/H	recovery in P6 relative to H (%)
KGDHC	8.8 ± 1.4	0.79 ± 0.24	10.4 ± 0.6	80 ± 26[d]	7.6 ± 2.2	8.8 ± 2.3
GDH	9.6 ± 2.4	0.39 ± 0.05	11.1 ± 1.8	38 ± 3[d]	3.5 ± 0.3	4.2 ± 0.8
mitAspAT[e]	70 ± 20	3.69 ± 1.10	79 ± 15	356 ± 91[d]	4.4 ± 0.5	4.5 ± 0.7
LDH	303 ± 74	≤0.15	353 ± 74	≤0.17[d]	≤0.05	≤0.05
TGase	25 ± 5	0.047 ± 0.007	33 ± 13	4.7 ± 0.6[d]	0.18 ± 0.05	0.21 ± 0.06

[a] The H and nonsynaptosomal mitochondria (P6) were prepared from the brains of 6-month-old B6/CBA51/J mice. The data are averages from three separate preparations. In each preparation, activities were measured in triplicate (except TGase, where activities were determined in duplicate). Each preparation was from 15 forebrains (~5 g of wet weight). [b] Units are μmol/min, except TGase, where activity is expressed as nmol/h. [c] Units are nmol min^{-1} (mg of protein)$^{-1}$, except TGase, where specific activity is expressed as pmol h^{-1} (mg of protein)$^{-1}$. [d] Significantly different from the H values with $p = 0.05$ by the Mann−Whitney U test. [e] Brain homogenates contain both cytAspAT and mitAspAT. The two activities were distinguished by their relative susceptibility to heat treatment (see the Experimental Procedures). Analysis for cytosolic and mitochondrial isoforms (see the Experimental Procedures) showed that 50 ± 2% of the total AspAT activity in the homogenate was due to mitAspAT.

significantly different from zero with $p < 0.001$ by the paired t test as well as by the χ^2 test; the 95% confidence interval was 2.67−6.15. The LDH (cytosolic marker) recovery in the mitochondrial (P6) fraction was 0.05%, a value less than the ~0.21% recovery of TGase activity (Table 2). Thus, the TGase activity in the mitochondria cannot be attributed to contamination with cytosolic TGase(s).

The relatively low recovery of TGase activity in the P6 fraction is due in part to the loss of nonsynaptosomal mitochondria during the isolation procedure. To estimate the fraction of nonsynaptosomal mitochondrial TGase activity in the brain homogenate, we used the KGDHC-specific activity data. Because KGDHC activity is strictly mitochondrial, the recovery of this enzyme activity in the nonsynaptosomal mitochondria (8.8%; Table 2) may be used as an approximate benchmark for calculating the amount of nonsynaptosomal mitochondrial TGase in the brain homogenates. Because the total recovery of brain TGase activity in the nonsynaptosomal preparation was 0.21%, about 2.4% [i.e., (0.21/8.8) × 100] of the total TGase activity (as assessed by the [^{14}C]putrescine-binding assay) in mouse brain homogenate was likely to be due to nonsynaptosomal mitochondria.

Ca^{2+}-Dependent TGase Activity in Mitoplasts Prepared from Mouse Brain Nonsynaptosomal Mitochondria. To determine whether the TGase activity is associated with the outer mitochondrial membrane or is present in the matrix/inner mitochondrial membrane, mitoplasts were prepared. Table 3 shows that TGase activity is present in the mitoplast fraction (P7). Recoveries of the mitochondrial matrix enzymes (GDH, KGDHC, and mitAspAT) in the mitoplast (P7) fraction were not different from those obtained with the intact mitochondria (P6), but the specific activities were higher (compare Tables 2 and 3).

The good recoveries and high specific activities (5−12-fold higher than in the homogenate) of marker matrix

Table 3: Comparison of Ca^{2+}-Dependent TGase Activity with KGDHC, GDH, and mitAspAT Activities in Mitoplasts (P7) Derived from Nonsynaptosomal Mouse Brain Mitochondria[a]

enzyme	specific activity[b]	recovery in P7 relative to homogenate (H) %
KGDHC	108, 129	4.0, 8.8
GDH	97, 52	3.5, 4.9
mitAspAT	657, 453	5.0, 4.3
TGase	4.3 ± 1.5[c] (6.4, 1.5, 5.0)	0.14 ± 0.04

[a] The data were obtained from two of the mouse brain preparations described in Table 2. In the case of TGase, activity was measured in an additional preparation so that the values shown for this enzyme are for $n = 3$. The actual values are shown in parentheses. The protein concentrations in the nonsynaptosomal mitochondrial fraction (P6; Table 2) and the mitoplast fraction (P7) were 48 and 58.4, and 20 and 37.8 mg/mL, respectively. [b] Units are nmol min^{-1} (mg of protein)$^{-1}$, except TGase, where specific activity is expressed as pmol h^{-1} (mg of protein)$^{-1}$. [c] Different from zero with $p < 0.05$ by the one-tailed t test; $p < 0.001$ by the χ^2 test; 95% confidence interval from -2.0 to 10.6.

enzymes showed that the preparation of mitoplasts was of high quality. Thus, the presence of TGase activity in the mitoplasts (Table 3) is unlikely to be an artifact. The similar recovery of TGase activity in the mitoplasts relative to that in the mitochondria (compare Tables 2 and 3) suggests that TGase in the nonsynaptosomal mitochondria is primarily in the matrix/inner mitochondrial membrane. In agreement with this conclusion, no TGase activity could be detected in the S7 fraction.

Detection of Cross-Reactivity to TGase 2 Antibodies in Mouse Brain Nonsynaptosomal Mitochondria. Full-length TGase 2 has a M_r of ~80 000. Western blotting with TGase 2 antibodies showed immunoreactivity toward a band corresponding to a M_r of ~80 000 in highly purified mouse brain nonsynaptosomal mitochondria (P6). This band was present when (a) CovalAb antibodies to TGase 2 were used and detection was with alkaline phosphatase or (b) antibodies to TGase 2 prepared previously (*3*) were used and detection was with ECL. The immunoreactive band at M_r of ~80 000 was also present in the P7 and P7* fractions but not in the S7 and S7* fractions.

These findings are consistent with the presence of TGase 2 in the various mouse brain fractions. However, Bailey et al. (*36*) recently showed that a band with a M_r of ~80 000 detected in mouse brain preparations with antibodies to TGase 2 is due to a nonspecific interaction. These authors recommended using NeoMarkers antibodies and pull down of TGase 2 with GTP-agarose beads to detect authentic TGase 2 in mouse brain homogenates. Accordingly, we used this procedure with minor modification (see the Experimental Procedures) to determine whether the purified mouse brain nonsynaptosomal mitochondria contain TGase 2. Figure 2 shows that an immunopositive band at M_r of ~80 000 was detected with NeoMarkers TGase 2 antibodies in the mitochondrial fraction. However, after the GTP-agarose pull-down procedure, this band was removed and two faint immunopositive bands were revealed at lower M_r values. These findings show that full-length TGase 2 is not detectable in the nonsynaptosomal mitochondria purified from normal mouse brain.

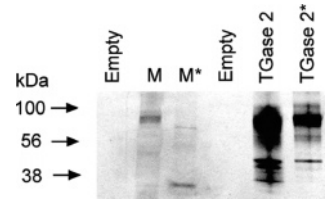

FIGURE 2: Western blot of nonsynaptosomal mitochondria purified from WT mouse brain. An untreated mitochondrial preparation and a mitochondrial preparation subjected to the GTP-agarose pull-down procedure were added to the lanes marked M and M*, respectively. Positive controls were commercial guinea pig liver TGase 2 and TGase 2 subjected to GTP-agarose pull down (lanes TGase 2 and TGase 2*, respectively). The amount of protein loaded was 20 μg in the lanes marked M and M* and 8 μg for the lanes marked TGase 2 and TGase 2*. NeoMarkers antibodies and the alkaline phosphatase procedure were used to detect immunopositive bands.

Ca^{2+}-Dependent TGase Activities in Mitochondrial Fractions Obtained from TGase $2^{-/-}$ (KO) and TGase $2^{+/+}$ (WT) Frozen Brain Samples. Analysis of brain homogenates obtained from TGase 2 KO mice and their WT controls (both on a SVJ129-C57BL/6 background) showed Ca^{2+}-dependent TGase-specific activities of ~5 and ~130 pmol mg^{-1} h^{-1}, respectively ([^{14}C]putrescine-binding assay, $n = 2$). In these experiments, the frozen brain was homogenized directly in isolation buffer at 0 °C. The baseline TGase activity in the WT SVJ129-C57BL/6 mouse brain [~130 pmol h^{-1} (mg of protein)$^{-1}$] was higher than that in brains from B6/CBA51/J mice [~33 pmol h^{-1} (mg of protein)$^{-1}$; Table 2]. This may be due to the use of different strains of mice. Nevertheless, the relative values obtained here for the TGase activity in TGase $2^{-/-}$ (KO) and TGase $2^{+/+}$ (WT) mouse brain are in accordance with those of Mastroberardino et al. (*21*). These authors, using the labeled putrescine-binding assay, showed that the TGase-specific activity in R6/1 (HD transgenic) TGase 2 KO mouse brain is ≤10% that of R6/1 TGase WT mouse brain.

TGase activity was also detected in the mitochondrial and mitoplast fractions prepared from the brains of both the TGase 2 KO and their WT controls. TGase-specific activities in nonsynaptosomal mitochondria and mitoplasts prepared from WT mice were 30.7 and 17.0 pmol h^{-1} (mg of protein)$^{-1}$, respectively. The corresponding values for the KO mice were 31.9 and 6.0 pmol h^{-1} (mg of protein)$^{-1}$, respectively ($n = 2$ separate preparations). The results are in accordance with the findings shown in Figure 2 that purified nonsynaptosomal mouse brain mitochondria contain TGase activity that cannot be assigned to TGase 2.

To ensure that the TGase activity in the nonsynaptosomal mitochondria detected in these experiments is not due to an artifact arising from the previous freezing of the brain, LDH was measured in the cytosolic and mitochondrial fractions. The LDH-specific activity in the nonsynaptosomal mitochondria obtained from frozen brain tissue was ~3% of that of the homogenate for both KO and their WT controls. This finding suggests that mitochondria prepared from previously frozen mouse brain are resistant to rupture and resealing induced by freeze/thawing of the intact tissue and that only a very small amount of cytosol is sequestered in these isolated brain mitochondria.

Confirmation of the High Purity of Liver Mitochondria Prepared from Fresh Mouse Liver. A small fraction of the total cellular LDH activity (~1%) is naturally present in rat

Table 4: Relative Activities of Ca^{2+}-Dependent TGase and Selected Enzymes in Homogenates, Mitochondria, and Mitoplasts Prepared from Mouse Liver[a]

enzyme/fraction	total units	recovery %	specific activity	specific activity relative to homogenate
GDH				
homogenate	110 ± 7	(100)	33.0 ± 2.5	(1)
mitochondria	7.5 ± 2.5	6.8	172 ± 32	5.4
mitoplasts	3.0 ± 0.5	2.7	695 ± 188	21.0
γ-cystathionase				
homogenate	36.4 ± 3.1	(100)	10.6 ± 0.9	(1)
mitochondria	<0.03	<0.10	<0.01	<0.001
mitoplasts	<0.03	<0.10	<0.01	<0.001
LDH				
homogenate	333 ± 98	(100)	97 ± 25	(1)
mitochondria	~10	~3	~3	~0.03
mitoplasts	<10	<3	<3	<0.03
mitAspAT				
homogenate	226 ± 28	(100)	68.0 ± 9.8	(1)
mitochondria	7.1 ± 1.5	3.1	160 ± 27	2.4
mitoplasts	4.7 ± 1.0	2.1	1060 ± 240	15.6
KGDHC				
homogenate	11.1 ± 0.4	(100)	3.33 ± 0.32	(1)
mitochondria	0.57 ± 0.05	5.1	7.98 ± 0.23	2.4
mitoplasts	0.060 ± 0.015	0.56	3.36 ± 1.36	0.99[b]
TGase				
homogenate	1336 ± 200	(100)	405 ± 74	(1)
mitochondria	0.070 ± 0.017	0.0052	9.7 ± 0.7	0.024
mitoplasts	0.047 ± 0.012	0.0035	10.0 ± 2.7	0.025

[a] Except in the case of Ca^{2+}-dependent TGase, total activity and specific activity are expressed as μmol/min and milliunits/mg of protein, respectively. In the case of TGase, total activity and specific activity are expressed as nmol/h and pmol h^{-1} (mg of protein)$^{-1}$, respectively. The Ca^{2+}-dependent activity was calculated using blank 2 (see the Experimental Procedures). A total of 18 6-month-old B6/CBA51/J mice were sacrificed in two lots of two on the same day. Thus, $n = 4$ separate preparations from the same group of mice. Each batch of mouse livers weighed ~5 g. All enzyme activities, except TGase, were measured in triplicate. TGase activity was measured in duplicate. However, $n = 4$ because only the average value for each enzyme activity in each preparation was used in calculating the mean. [b] The large loss of KGDHC activity during the preparation of mitoplasts from liver mitochondria does not appear to be related to protease activity. Protease inhibitor cocktail was present during the preparation of the mitoplasts. Moreover, KGDHC activity in brain mitoplasts was not destroyed during preparation from mitochondria (Table 3). The unusual susceptibility of liver KGDHC to damage during mitoplast preparation requires further study.

liver mitochondria (*52*). On the other hand, γ-cystathionase is exclusively cytosolic in rat liver cells (*53*). Thus, we used this enzyme as a cytosolic marker for liver subcellular fractions. The specific activity of γ-cystathionase in the mouse liver mitochondria (and mitoplasts) was <0.001 relative to that of the homogenate (Table 4). This finding shows that contamination of the purified mouse liver mitochondria by cytosolic elements is negligible. Further confirmation of the purity of the mouse liver mitochondria was obtained by analysis of mitochondrial marker enzymes. The specific activities of GDH and mitAspAT were greater in the mitochondrial fraction and much greater in the mitoplast fraction than in the homogenate (Table 4). The marked enrichment of mitAspAT and GDH attests to the high quality of the mouse liver mitochondria.

Detection of Ca^{2+}-Dependent TGase Activity in Subcellular Fractions Prepared from Fresh Mouse Liver. The TGase-specific activity in the mouse liver homogenate (~405 pmol h^{-1} mg^{-1}, Table 4) was much greater than that in the mouse brain homogenate (~33 pmol h^{-1} mg^{-1}, Table 2), in confirmation of previous findings of others (e.g., ref *2*). TGase activity was also detected in mouse liver mitochondria and mitoplasts (Table 4). The values (~9.7 and ~10.0 pmol h^{-1} mg^{-1}, respectively) are about twice those found for the mouse brain mitochondria and mitoplasts (Tables 2 and 3). As noted above, the specific activity of γ-cystathionase in the mouse liver mitochondria (and mitoplasts) is <0.001 of that of the homogenate (Table 4). This is significantly less than the specific activity of TGase in the mitochondria and mitoplasts relative to that in the homogenate (Table 4). The low yield of γ-cystathionase in the mitochondrial fraction was not due to an endogenous inhibitor. When 150 μg of mitochondrial protein was added to the 150 μg of homogenate protein, no inhibition of γ-cystathionase in the homogenate was observed (data not shown).

Recovery of Ca^{2+}-Dependent TGase Activity in Purified Mitochondria and Mitoplasts Prepared from Fresh Mouse Liver. From the absolute recovery of TGase activity in the purified mitochondria relative to that in the homogenate (0.52%; Table 4) and assuming that the recovery of mitochondria based on yields of GDH, mitAspAT, and KGDHC is about 3%, one can calculate the amount of TGase activity ([^{14}C]putrescine-binding assay) in rat liver accounted for by mitochondria to be about 0.17% of the total in the whole tissue [i.e., 0.0052 × (100/3)]. Similar calculations show that the amount of TGase in the liver homogenate accounted for by mitoplasts is about 0.14% [i.e., 0.0035 × (100/2.5)]. Thus, the TGase activity associated with purified liver mitochondria is largely in the inner membrane/matrix fraction, a finding similar to that obtained for brain mitochondria. The specific activity of TGase in the mouse liver mitoplasts (10.0 ± 2.7 pmol h^{-1} mg^{-1}; Table 4) is greater than that in the nonsynaptosomal brain mitoplasts (4.3 ± 1.5 pmol h^{-1} mg^{-1}; Table 2; $p = 0.025$; Mann−Whitney U test). However, the relative amount of TGase activity in the liver homogenates accounted for by mitoplasts (~0.14%) is considerably less than that accounted for by nonsynaptosomal mitoplasts relative to brain homogenates (~2.0%).

Ca^{2+}-Dependent TGase Activity in Mitochondria Prepared from Previously Frozen Liver Samples. Because TGase 2

activity cannot be present in the mitochondria prepared from the livers of TGase 2 KO mice, any TGase activity in these organelles must be due to a TGase isoform other than TGase 2. Because only frozen tissue was available for the preparation of mitochondria from the livers of TGase 2 KO mice, it was necessary to determine the extent to which the isolated mitochondria were contaminated with cytosolic elements. The specific activity of γ-cystathionase in the liver mitochondrial fraction prepared from previously frozen WT mouse liver was about 3 μmol min^{-1} mg^{-1} or about 30% of that of the homogenate. This value was obtained regardless of whether the mitochondria were prepared by method 1 or 2 (see the the Experimental Procedures) and contrasts sharply with that obtained with mitochondria prepared from fresh (i.e., not frozen) mouse liver ($<$0.01 μmol min^{-1} mg^{-1}; Table 4). Evidently, in contrast to mitochondria prepared from previously frozen mouse brain, the freeze−thawing process resulted in substantial damage and resealing of the liver mitochondrial membranes, entrapping some cytosolic components in the isolated mitochondria (cf. ref *54*). Despite this caveat, it was possible to show that TGase activity is naturally present in the liver mitochondria of both TGase 2 KO and wild-type mice. Thus, the average TGase activities in two separate liver homogenates prepared from WT and TGase 2 KO mice were ~673 (n = 2; 672, 674) and ~110 pmol h^{-1} (mg of protein)$^{-1}$ (n = 2; 94, 126), respectively. These data show that, as with the mouse brain, the predominant TGase activity (with the [^{14}C]putrescine-binding assay) in the whole mouse liver is due to TGase 2. The TGase activities in two preparations of mitochondria prepared from TGase 2 KO and two from their WT controls were ~30 (n = 2; 21.6, 39.7) and ~32 (n = 2; 25.7, 38.1) pmol h^{-1} (mg of protein)$^{-1}$, respectively. The TGase-specific activity in the liver mitochondrial fraction prepared from the TGase 2 KO mice is similar to that in the liver mitochondrial fraction prepared from WT mice, despite the overall 6-fold lower TGase-specific activity in the liver homogenate prepared from the KO mice. This finding makes it unlikely that the TGase activity in the liver mitochondria prepared from TGase 2 KO mice is due solely to "capture" of cytosolic elements during the purification of the liver mitochondria. These data show that a significant portion of the liver mitochondrial TGase activity is not due to a component encoded by the TGase 2 gene.

Western Blots of TGase 2 in Homogenates and Purified Mitochondria Obtained from Previously Frozen Mouse Liver. Western blotting (with NeoMarkers antibodies and ECL detection) of homogenates prepared from frozen livers obtained from both the TGase 2 KO mice and their WT controls revealed an immunopositive band at M_r of ~80 000 (Figure 3A). After GTP-agarose pull down of the WT liver homogenate, the band at M_r of ~80 000 (lane WT H*) was enriched relative to the untreated sample (lane WT H). In contrast, after GTP-agarose pull down, the band at M_r of ~80 000 was no longer present in the KO liver homogenate (compare lanes KO H and KO H* in Figure 3A). Essentially, similar profiles were noted when a separate liver homogenate was probed by Western blotting with NeoMarkers antibodies and alkaline phosphatase detection (compare lanes WT H and WT H* in Figure 3B with those in Figure 3A).

A M_r ~80 000 band was also prominent in the mitochondrial fraction prepared from frozen livers of WT mice (Figure 3B) before (lane WT M) and after GTP-agarose pull down (lane WT M*). Enrichment of this band in the WT M* lane relative to that in WT M lanes was observed. In KO mice, a very weak band with a M_r of ~80 000 was noted in the homogenate (lane KO H) and mitochondrial fraction (lane KO M). The intensity of this band was even weaker in the GTP-agarose pull-down material (lanes KO H* and KO M*) (Figure 3B). The results are consistent with some contamination of mitochondria with cytosolic elements as a result of freeze/thawing of the livers used to prepare the organelles. Presumably, the band in lane WT M (parts A and B of Figure 3) contained both cytosolic TGase 2 (as a contaminant) and a protein that reacted nonspecifically with TGase 2 antibodies. The increased intensity of the M_r of ~80 000 band in lane WT M* (Figure 3A) relative to that in lane WT M was due to the presence of contaminating cytosolic TGase 2. A M_r ~80 000 band was also present in the mitochondrial fraction prepared from livers obtained from KO mice (lane KO M), but the intensity of this band was considerably diminished in the mitochondria prepared from the frozen livers of TGase 2 KO mice (lane KO M*). The considerably diminished intensity of the M_r ~80 000 band in lane KO M* relative to that in lane WT M* is consistent with the lack of TGase 2 in the mitochondria prepared from the livers of KO mice. Overall, the data are consistent with the complete absence of TGase 2 in the livers of KO mice as expected. The intensity of the nonspecific M_r ~80 000 band in lane KO M* relative to that in lane KO M is consistent with some contamination of the mitochondria with cytosolic elements as determined by analysis of γ-cystathionase activity.

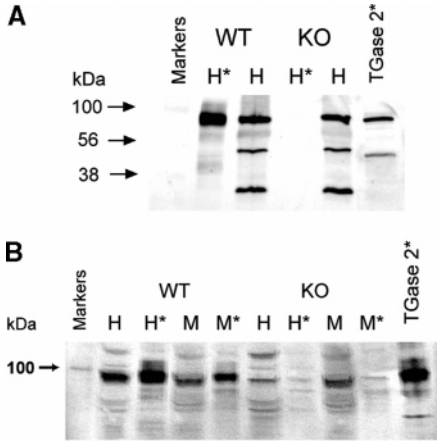

FIGURE 3: Western blots with NeoMarkers TGase 2 antibodies of homogenate (H) and mitochondrial (M) fractions prepared from frozen liver obtained from KO and WT mice. H* and M* represent GTP-agarose pull downs from H and M fractions, respectively. The lane labeled TGase 2* contained TGase 2 that had been subjected to the GTP pull-down procedure. (A) Detection by the ECL procedure. Each lane, except that marked TGase 2* (which was loaded with 4 μg), contained 25 μg of protein. In this experiment, frozen liver was ground in dry ice and the powder was suspended in buffer and homogenized at 0 °C. The mitochondria were purified from this suspension. (B) Detection by the alkaline phosphatase procedure. Each lane except that marked TGase 2* (which was loaded with 8 μg), contained 80 μg of protein. In this experiment, the frozen liver was thawed and then homogenized in buffer at 0 °C and the mitochondria were prepared directly from this suspension. In this figure and in Figure 2, no immunopositive bands were detected in the low M_r range.

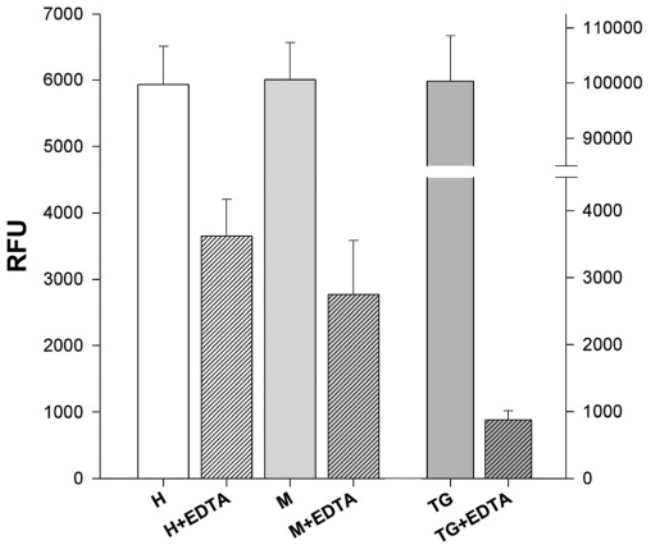

FIGURE 4: End-point fluorescein cadaverine assay for Ca^{2+}-dependent TGase activity in a liver homogenate and in liver mitochondria prepared from WT mice. The reaction mixtures, which contained Ca^{2+}, fluorescein cadaverine, *N*,*N*-dimethylcasein, and DTT, were incubated for 2 h at 37 °C. The increase in fluorescence resulting from covalent attachment of fluorescein cadaverine to *N*,*N*-dimethylcasein was then determined (see the Experimental Procedures). Reaction mixtures labeled H, M, and TG contained 150 μg of homogenate protein, 150 μg of mitochondrial protein, or 2.5 μg of commercial guinea pig TGase 2 protein, respectively. Controls (blanks, H + EDTA, M + EDTA, TG + EDTA) contained the complete reaction mixture plus liver homogenate, mitochondria, or purified TGase 2, plus EDTA. The homogenate and mitochondria were prepared from fresh liver. The data are from three separate experiments. TGase 2 was used as a positive control (see the Experimental Procedures). RFU, relative fluorescence in arbitrary units; TG, commercial guinea pig TGase 2. Note the break on the *y* axis for RFU of TG in the absence of EDTA. The RFU values for H versus H + EDTA, for M versus M + EDTA, and for TG versus TG plus EDTA are significantly different for each pair with *p* values of 0.03, 0.01, and 0.001, respectively, by the paired *t* test.

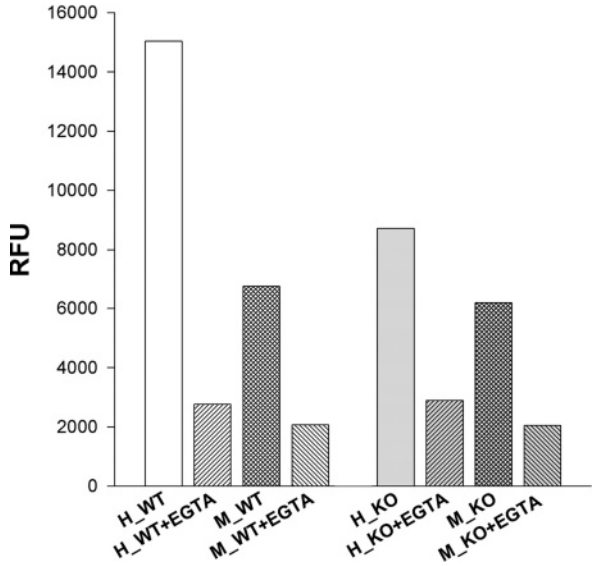

FIGURE 5: Comparison of end-point fluorescein cadaverine assay for Ca^{2+}-dependent TGase activity in liver fractions obtained from TGase 2 KO mice and their WT controls. Conditions were similar to those used in the caption of Figure 4, except that EGTA was used in place of EDTA. Six sets of experiments utilizing homogenates, and mitochondria prepared from previously frozen liver were carried out. In these experiments, protein amount and time of incubation were varied between 150 and 300 μg and between 2 and 16 h, respectively. When the data for all six experiments were combined and analyzed by the paired *t* test, significant increases in fluorescence of the incubation mixtures lacking EGTA versus those of the controls containing EGTA were noted for each pair. The *p* value for liver homogenates prepared from TGase 2 KO mice (H_KO) and for liver mitochondria prepared from WT mice (M_WT) versus their controls (H_KO+EGTA and M_WT+EGTA, respectively) was 0.01. The *p* value for liver homogenates prepared from WT mice (H_WT) and for liver mitochondria prepared from TGase 2 KO mice (M_KO) versus their controls (H_WT+EGTA and M_KO+EGTA, respectively) was 0.005. The example shown is from one of two experiments in which the reaction mixture contained 150 μg of protein and incubation was for 16 h.

Binding of Fluorescein Cadaverine to N,N-Dimethylcasein in the Presence of Mouse Liver Fractions. When the fluorescence assay (2-h incubation; 37 °C) was applied to mouse liver homogenates (150 μg of protein), the relative fluorescence values in the complete assay mixture minus EDTA (Figure 4, H) and in the complete assay mixture plus mitochondria minus EDTA (Figure 4, M) were significantly greater than in the corresponding controls, the complete reaction mixture plus homogenate/mitochondria plus EDTA (Figure 4, H + EDTA and M+ EDTA). When boiled samples of homogenate (150 μg of protein), mitochondrial preparation (150 μg of protein), or TGase 2 (2.5 μg) were added to the complete reaction mixture and incubated at 37 °C for 2 h, the relative fluorescence values were lower than those obtained with the complete reaction mixture plus active TGase source incubated under the same conditions (data not shown).

The fluorescence assay was also used to compare relative TGase activities of liver homogenates and mitochondria obtained from TGase 2 KO mice to those obtained with WT controls. A total of six experiments with varying protein and times of incubation were carried out. Data from one set of these experiments is depicted in Figure 5. In this experiment, the reaction mixture contained 150 μg of protein and incubation was for 16 h at 37 °C. For all six experiments combined, the RFU was significantly greater in the incubation mixtures lacking EGTA relative to those containing EGTA (controls) (see the caption of Figure 5). An important finding in the experiment depicted in Figure 5 is the presence of TGase activity in the mitochondria prepared from TGase 2 KO mice. The relative fluorescence of the complete reaction mixture containing WT mitochondria after correction for the blank is very similar to that obtained with the complete reaction mixture containing TGase 2 KO mitochondria. This finding is comparable to that noted above with the [^{14}C]putrescine-binding assay.

SDS−PAGE of N,N-Dimethylcasein Previously Incubated with Fluorescein Cadaverine and Mouse Liver Fractions. Protein precipitated from a reaction mixture containing *N*,*N*-dimethylcasein, fluorescein cadaverine, Ca^{2+}, and homogenate (or mitochondria) was subjected to SDS−PAGE (Figure 6). A band corresponding to *N*,*N*-dimethylcasein was tagged with a fluorescent label when this protein was incubated with fluorescein cadaverine, Ca^{2+}, and an aliquot of a liver homogenate obtained from WT mice (lane A). The intensity of this fluorescent band was greatly diminished when EDTA was present in the reaction mixture (lane B). Similar findings were obtained with highly purified mitochondria obtained from WT mouse liver (lanes C and D). Formation of fluorescent *N*,*N*-dimethylcasein was also evident when the

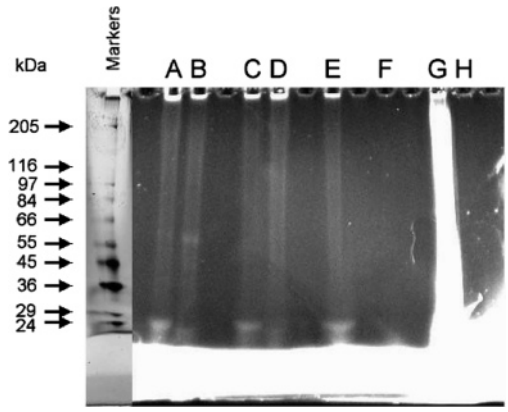

FIGURE 6: Fluorescent bands revealed when *N*,*N*-dimethylcasein is incubated with fluorescein cadaverine and liver fractions obtained from WT mice in the presence of Ca^{2+} and DTT. Blanks contained the complete reaction mixture plus liver fraction plus EDTA. The reaction mixtures were incubated for 16 h, and then aliquots were subjected to SDS−PAGE. The gel was then photographed under UV light. The positions of M_r markers are shown in a lane cut from a gel run in parallel, silver stained, and lined up with the unstained gel. The fluorescent band, which migrated in the M_r region of 24 000 to 29 000, corresponded to *N*,*N*-dimethylcasein as shown by silver staining of the parallel gel (not shown). The complete reaction mixture contained A, homogenate; B, homogenate plus EDTA; C, mitochondria; D, mitochondria plus EDTA; E, mitoplasts; F, fluorescein cadaverine only (amount corresponding to that in the other lanes); G, commercial TGase 2; and H, commercial TGase 2 plus EDTA. Proteins in the TGase 2 preparation, in addition to *N*,*N*-dimethylcasein, apparently acted as TGase substrates.

reaction mixture was incubated with highly purified mouse liver mitoplasts (lane E). A fluorescent band at M_r of ~60 000 was noted when the homogenate was incubated with *N*,*N*-dimethylcasein, fluorescein cadaverine, and liver homogenate (lane B). This band is not present in the lanes containing mitochondria (C and D).

DISCUSSION

Ca^{2+}-Dependent TGase Activity in Highly Purified Mouse Brain Nonsynaptosomal and Liver Mitochondria. One mechanism that may possibly contribute to the mitochondria-catalyzed binding of [^{14}C]putrescine to succinylated casein is oxidation of the polyamine to the corresponding aldehyde, which then forms an imine with a lysyl residue. Enzymes capable of oxidizing polyamines to aldehydes (diamine/polyamine oxidases) are well-documented (*55*). Another possibility for incorporation of labeled putrescine into succinylated casein is that redox metal cofactors (Fe, Cu) in the mitochondrial preparation catalyze the formation of ROS. The ROS then oxidize putrescine to an aldehyde, which covalently attaches to the protein. However, this mechanism is ruled out because the TGase reaction mixture invariably contains 5−10 mM DTT. Furthermore, these mechanisms do not account for the Ca^{2+} dependence of the incorporation of [^{14}C]putrescine into succinylated casein or for the Ca^{2+}-dependent attachment of fluorescein cadaverine to *N*,*N*-dimethylcasein. Moreover, the fluorescent label attached to *N*,*N*-dimethylcasein survives SDS−PAGE (Figure 6). Preparation of the samples for analysis entails boiling in a solution containing SDS plus DTT, which will ensure cleavage of disulfide bonds or aldehyde adducts.

The possibility was considered that the Ca^{2+}-dependent TGase-like activity is due to protein disulfide isomerase (PDI) or a related protein. In this regard, it was recently shown that PDI (and a number of homologues) from different organisms have Ca^{2+}-dependent TGase-like activity (*56*−*59*). Although most of the PDI activity in the mammalian cell is associated with the ER, a significant portion in the liver is present in mitochondria, where it has been reported to be associated with the outer membrane (*60*). However, PDI has not been shown to be present in the mitochondrial matrix and thus is unlikely to account for our findings of Ca^{2+}-dependent TGase activity in the mitoplasts.

We believe that overall our data provide strong evidence that mitochondria contain one or more members of the traditional Ca^{2+}-dependent TGase family. Most of the TGase protein and activity ([^{14}C]putrescine-binding assay) in rodent brain (*21*) and liver (*2*; present results) is due to TGase 2. TGase 2 is known to attach to macromolecules/particulate elements during subcellular fractionation (e.g., refs *61* and *62*). TGase-2-like protein has also been detected immunohistochemically in rat brain as dense aggregates attached to the outer mitochondrial membrane (*63*). Moreover, Rodolfo et al. (*64*) showed that, in human SK-N-BE(2) cells overexpressing TGase 2, about 50% of the expressed protein is localized to the outer mitochondrial membrane, where, upon induction of cell death, it cross-links many proteins including Bax. These findings suggested that the mitochondrial TGase detected in our experiments might be TGase 2. However, the carefully controlled experiments described here showed that TGase 2 is not present in the highly purified mouse liver and nonsynaptosomal mitochondria nor is it present in the mitoplasts. Conceivably, other TGases might bind to the outer membrane but this cannot account for the TGase activity in the mitoplasts. To the best of our knowledge, our work is the first to document the occurrence of a Ca^{2+}-dependent TGase-like activity in the matrix of liver and nonsynaptosomal mitochondria.

Ca^{2+}-Independent Attachment of Fluorescein Cadaverine to Proteins in Liver Preparations. Blanks in reaction mixtures containing liver homogenate (or purified liver mitochondria), DTT, Ca^{2+}, dimethylcasein, and EDTA (Figures 4 and 5) exhibited relatively high background fluorescence compared to those obtained with semipurified guinea pig liver TGase 2. This finding suggests that liver fractions contain an activity that can catalyze the Ca^{2+}-*independent* covalent attachment of a suitable amine to a Q protein substrate. Evidence in support of this hypothesis was obtained by SDS−PAGE analysis (Figure 6). Fluorescent labeling of *N*,*N*-dimethylcasein was noted in reaction mixtures containing EDTA, fluorescein cadaverine, and *N*,*N*-dimethylcasein incubated with liver homogenate (lane B) or with mitochondrial extract (lane D). The intensity of the fluorescent label attached to *N*,*N*-dimethylcasein, however, was less than that observed in the presence of Ca^{2+} (minus EDTA) (Lanes A and C, respectively). The data show that both the liver homogenate and liver mitochondria possess Ca^{2+}-dependent (i.e., TGase-like) and Ca^{2+}-independent activities capable of catalyzing attachment of fluorescein cadaverine to *N*,*N*-dimethylcasein.

Intestinal mucosal cells have been reported to contain a Ca^{2+}-independent enzyme that catalyzes the covalent attachment of labeled putrescine to a suitable Q substrate (*65*−

67). The enzyme responsible, which can utilize a number of different amines as attacking nucleophile, is not diamine oxidase (*66*). Our data (Figures 4−6) suggest that liver preparations can catalyze a Ca^{2+}-independent attack of an amine (fluorescein cadaverine) on *N*,*N*-dimethylcasein. Curiously, Ca^{2+}-independent fluorescent labeling of an additional protein (M_r of ∼60 000) was noted with the liver homogenate but not with purified liver mitochondria (Figure 6). This finding suggests that there may be distinct mitochondrial and extramitochondrial enzymes that catalyze Ca^{2+}-independent addition of amine to proteins and/or different substrates. The identities of these enzymes and substrates need to be established.

Possible Role of TGase in Mitochondria. Similar to the postulated role of TGase 2 in the formation of the cytoskeletal scaffold (*1*), mitochondrial TGase(s) may play a role in the formation of the "mitoskeletal" scaffold. The enzyme may also act normally to convert nonscaffold matrix proteins to forms with enhanced or reduced biological activity. The available data raise the speculative possibility that TGase has a role in the regulation of normal mitochondrial function and may perhaps contribute to mitochondrial dysfunction in response to the influx of calcium under pathophysiological conditions.

Mitochondrial TGase and Neurodegeneration. Neurodegenerative diseases are characterized in part by (a) elevated levels of Ca^{2+} in mitochondria (*68*) and (b) decreased ATP synthesis (*68*). Because TGases require Ca^{2+} for their cross-linking activity, increased Ca^{2+} will result in increased TGase activity in the mitochondria. If the mitochondrial activity is inhibited by GTP/ATP, as seems likely for a number of the TGases, this activity might be stimulated even further if ATP/GTP levels are decreased in the mitochondria (*33*). Damaged or partially denatured proteins may provide more exposed K and Q residue substrates for TGases. Therefore, increased TGase activity may be beneficial at first by removing damaged proteins, but with time, the activity may become deleterious by (a) removing essential proteins and peptides and (b) contributing to cytochrome *c* release because of oligomerization of Bax and induction of apoptosis.

Two enzymes present in the mitochondria may be susceptible to increased TGase activity in neurodegenerative disease. We previously showed that semipurified KGDHC is inactivated in the presence of TGase 2 and a glutaminyl substrate (*31*). The activity of the neural KGDHC is markedly decreased in a number of neurodegenerative diseases (e.g., ref *69* and references therein). If the mitochondrial TGase has properties similar to TGase 2, then it is possible that KGDHC components are normally cross-linked in brain mitochondria and that this cross-linking may be increased under conditions of oxidative stress in neurodegenerative diseases. Another mitochondrial protein that is especially susceptible to oxidative damage in neurodegenerative diseases is mitochondrial aconitase (Aco-2) (e.g., ref *70*). We have found that Aco-2 in homogenates of rat brain mitochondria is a substrate of TGase 2 (*32*). Damaged Aco-2 is a good substrate of LON (a mitochondrial protease) (*71*). It is possible that damaged or partially unfolded Aco-2 may be a better TGase substrate than is native, undamaged Aco-2. Therefore, it will be important to determine whether brain mitochondria subjected to oxidative stress contain cross-linked KGDHC subunits and Aco-2 within the matrix and whether such cross-linked species, if present, are substrates of LON.

We recently showed that at least 10 proteins could be detected in mouse brain nonsynaptosomal mitochondria by Western blotting with GGEL antibodies (*32*). More definitive statements on the role of TGase in brain mitochondria must await identification of these proteins and confirmation that they are indeed cross-linked in the mitochondrial matrix. Because the number of GGEL cross-links is increased in AD brain (*3*, *18*) and HD brain (*11*), it is possible that the extent of protein cross-linking in mitochondrial matrix proteins in these and other neurodegenerative diseases is also increased.

ACKNOWLEDGMENT

We thank Drs. Craig D. C. Bailey and Gail V. W. Johnson for their helpful advice regarding Western blotting of TGase 2 in tissue fractions.

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BI0500877