Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12604–12609, November 1997
Medical Sciences

Transglutaminase-catalyzed inactivation of glyceraldehyde 3-phosphate dehydrogenase and α-ketoglutarate dehydrogenase complex by polyglutamine domains of pathological length

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Edited by Louis Sokoloff, National Institutes of Health, Bethesda, MD, and approved August 28, 1997 (received for review April 24, 1997)

ABSTRACT **Several adult-onset neurodegenerative diseases are caused by genes with expanded CAG triplet repeats within their coding regions and extended polyglutamine (Q_n) domains within the expressed proteins. Generally, in clinically affected individuals $n \geq 40$. Glyceraldehyde 3-phosphate dehydrogenase binds tightly to four Q_n disease proteins, but the significance of this interaction is unknown. We now report that purified glyceraldehyde 3-phosphate dehydrogenase is inactivated by tissue transglutaminase in the presence of glutathione *S*-transferase constructs containing a Q_n domain of pathological length ($n = 62$ or 81). The dehydrogenase is less strongly inhibited by tissue transglutaminase in the presence of constructs containing shorter Q_n domains ($n = 0$ or 10). Purified α-ketoglutarate dehydrogenase complex also is inactivated by tissue transglutaminase plus glutathione *S*-transferase constructs containing pathological-length Q_n domains ($n = 62$ or 81). The results suggest that tissue transglutaminase-catalyzed covalent linkages involving the larger poly-Q domains may disrupt cerebral energy metabolism in CAG/Q_n expansion diseases.**

CAG expansion diseases include Huntington disease (HD), dentatorubralpallidoluysian atrophy (Smith disease), spinobulbar muscular atrophy (Kennedy disease), and spinocerebellar ataxias (SCA) type 1, 2, 3 (Machado–Joseph disease), and 7 (SCA-1, SCA-2, SCA-3, and SCA-7, respectively) (1–12). These genetic abnormalities lead to progressively worsening disorders of coordination or motor neuron degeneration with variable degrees of mental symptoms. The expanded CAG repeat disorders lead to the expression of polyglutamine (Q_n) expansions within the affected proteins. Q_n length is inversely correlated with age of onset of symptoms (6–10). Different proteins are specific for each disease (e.g., huntingtin, androgen receptor, and ataxin-1 in HD, spinobulbar muscular atrophy, and SCA-1, respectively). CAG/Q_n expansions (where $n \geq 40$) are believed to lead to a "toxic gain of function" (13, 14), but the mechanism is unknown. Several authors (e.g., refs. 14 and 15) have hypothesized that tissue transglutaminase (tTGase), an enzyme that catalyzes formation of a crosslink (isopeptide) between the γ-carboxamide group of a protein Q residue and an ε amino group of a protein K residue, may be involved in the toxic response. Kahlem *et al.* (16) showed that Q_n domains are good substrates of tTGase and that generally the longer the Q_n domain the greater the activity (16). The authors also reported that $R_5Q_{18}R_5$ forms highly insoluble aggregates in the presence of brain proteins and tTGase, and that brain homogenates catalyze incorporation of [^{14}C]glycine ethyl ester (a nucleophile substrate of tTGase) into a Q_{12}-containing peptide (16). In the work of Kahlem *et al.* (16) the largest Q_n domain studied was Q_{18}. We found that both a nonpathological-length Q_n domain ($n = 10$) and a longer, pathological-length Q_n domain ($n = 62$) are excellent substrates of tTGase (17, 18).

Burke *et al.* (1) showed that huntingtin, huntingtin-derived fragments, and the dentatorubralpallidoluysian atrophy protein bind selectively to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in human brain homogenates and to immobilized rabbit muscle GAPDH. Burke *et al.* also showed that GAPDH in a brain homogenate is selectively bound to an immobilized Q_{60} polypeptide, but not to an immobilized Q_{20} polypeptide. Koshy *et al.* (2) showed that the androgen receptor and ataxin-1 bind to rabbit muscle GAPDH. When assayed in the yeast two-hybrid system, GAPDH interacts selectively with ataxin-1 and with the androgen receptor (2). Binding of both wild-type and mutant (i.e., pathological) ataxin-1 to GAPDH is very strong and cannot be disrupted by 1 M NaCl (2). The region of ataxin-1 that binds to GAPDH is the N terminus containing the Q_n repeat sequence (2). Moreover, ataxin-1 interacts with a GAPDH protein fragment that contains the NAD^+-binding domain and the first 21 residues of the catalytic domain (2). Burke *et al.* (1) and Koshy *et al.* (2) suggested that interactions with Q_n tracts may disrupt GAPDH activity and thereby interfere with energy metabolism (2). Here we show that longer Q_n domains by themselves do not inhibit GAPDH activity, but do so in the presence of tTGase.

MATERIALS AND METHODS

Reagents. α-Ketoglutarate (sodium salt), glyceraldehyde 3-phosphate (GAP), NAD^+, NADH, BSA, bovine milk α-casein, hydroxylamine-HCl, DTT, coenzyme A (CoASH), thiamin pyrophosphate, and *N*-α-carbobenzoxyglutaminylglycine (CGG) were obtained from Sigma.

Enzyme Activity Measurements. Rabbit muscle GAPDH (crystalline suspension in 2.6 M ammonium sulfate; 1,000 units/mg of protein; 31 mg/ml) was purchased from Sigma. A single protein species (M_r of monomer ≈36,000) was detected by SDS/PAGE with Coomassie blue staining. The GAPDH assay mixture (0.8 ml) consisted of 50 mM Tris·HCl (pH 7.6), 1 mM NAD^+, 1 mM GAP, 10 mM DTT, 12.5 mM sodium arsenate, 0.2% (vol/vol) Triton X-100, and enzyme at 30°C. The initial rate of increase of absorbance at 340 nm of NADH ($\varepsilon = 6.23 \times 10^3$) was determined in a Cary 200 spectrophotometer. To ensure that

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Abbreviations: CoASH, coenzyme A; CGG, *N*-α-carbobenzoxyglutaminylglycine; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HD, Huntington disease; KGDHC, α-ketoglutarate dehydrogenase complex; Q_n, polyglutamine; SCA, spinocerebellar ataxia; tTGase, tissue transglutaminase.

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the reaction was linear with time the amount of enzyme added to the assay mixture was adjusted so that the initial absorbance change was no greater than 0.02/min. GAP was obtained as a 200 mM frozen solution. Because GAP is unstable it was thawed just before assay of GAPDH, and an aliquot then was added to the reaction mixture.

α-Ketoglutarate Dehydrogenase Complex (KGDHC). Pig heart (KGDHC) [0.5 units/mg of protein (11.1 mg/ml) in a solution containing 50% glycerol, 25% sucrose, 5 mM EGTA, 5 mM DTT, 1% Triton X-100, 0.01% sodium azide and 50 mM potassium phosphate (pH 6.9)] was obtained from Sigma. The assay mixture (0.8 ml) contained 1.0 mM α-ketoglutarate, 1.0 mM NAD^+, 0.3 mM thiamin pyrophosphate, 0.13 mM CoASH, 0.18% (vol/vol) Triton X-100, 10 mM $CaCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and 50 mM Tris·HCl buffer (pH 7.6) (19). The initial linear rate of appearance of absorption was measured at 340 nm in a Cary 200 spectrophotometer at 30°C. Except as noted, the volume of the standard GAPDH and KGDHC assay mixtures was 0.8 ml. In some experiments, activities of GAPDH and KGDHC were determined in a SpectraMax 250 96-well plate analyzer. In this case the standard assay volume was 0.2 ml.

tTGase. Guinea pig liver tTGase was obtained from Sigma as a lyophilized powder [specific activity of 1.6–2.6 units (hydroxaminolysis assay, 37°C)/mg of protein]. The preparation was not pure and exhibited multiple protein bands on SDS/PAGE. The hydroxaminolysis assay (which measures nucleophilic attack of hydroxylamine on CGG) was adapted (18) from that of Folk and Cole (20). An alternative assay that measures the tTGase-catalyzed covalent attachment of [1,4-^{14}C]putrescine to casein also was adapted (18) from that of Folk and Cole (20).

Ammonia free base (NH_3) interferes with tTGase assays (20). In some experiments, 10 μl of the GAPDH suspension in 2.4 M ammonium sulfate was added directly to 620 μl of 10 mM Tris·HCl (pH 7.2), and an aliquot of this solution (2 μl) was diluted into 25 μl of the final assay mixture. Under these conditions, the concentration of NH_4^+ is diluted to 3 mM, and the concentration of NH_3 ($pK_a \approx 9.4$) is negligible (≈50 μM, pH 7.6; ≈1 μM, pH 6.0). In other experiments where the concentration of GAPDH in the final assay mixture was ≈2–10 mg/ml, most of the ammonium sulfate was removed by pelletting the enzyme by centrifugation and dissolving the pellet in 10 mM Tris·HCl (pH 7.2). In each case, it was shown that the NH_3 carried over did not significantly inhibit the tTGase activity.

Construction of Different Length Q_n Clones. Q_n domains (n = 0, 10, 62, or 81) were expressed in *Escherichia coli* as glutathione *S*-transferase (GST)-fusion proteins (molecular mass = 29.86, 31.14, 37.80, and 40.24 kDa, respectively) (21) and purified as described (18). After freeze-thawing or on prolonged storage at 4°C the Q_{62} and Q_{81} fusion proteins tend to precipitate from solution. The $GSTQ_{62}$ and $GSTQ_{81}$ proteins can be resuspended by sonication, but $GSTQ_{81}$ reaggregates rapidly. The results reported here are for solutions of $GSTQ_{81}$ that were freshly prepared or used soon after sonication.

Measurement of GAPDH Activity in the Presence of Q_n Domains. To test for slow, but tight-binding, irreversible inhibition, 1 μg of GAPDH was incubated with 100 mM sodium phosphate buffer (pH 7.2), 10 mM 2-mercaptoethanol, and 10 μl of a stock solution of the $GSTQ_n$ fusion protein in a final volume of 25 μl. The mixture was incubated at 20°C, and at intervals over a 2-hr period 2-μl aliquots were withdrawn and assayed for GAPDH activity. To test for rapid, but reversible, inhibition of GAPDH, the $GSTQ_n$ fusion protein (0.5–2 mg/ml) was included in the 0.8-ml standard GAPDH assay mixture just before the addition of 0.1 μg of GAPDH. The initial rate of the dehydrogenase reaction was compared with that of a control lacking the $GSTQ_n$ fusion protein.

Measurement of GAPDH Activity After Exposure to Q_n Domains and tTGase. The reaction mixture consisted of 1 μg of GAPDH, 100 mM Tris·HCl buffer (pH 7.6), 10 mM $CaCl_2$, 20 mM glutathione (or DTT), guinea pig liver tTGase (20 milliunits; 2.6 units/mg of protein), and ≈12.5 μg of $GSTQ_n$ fusion protein (where indicated) in a final volume of 25 μl at 20°C. At intervals, 2 μl-aliquots were withdrawn and assayed for GAPDH activity by the standard procedure described above. Glutathione was chosen as the reductant in the initial experiments because it is present in solutions of the $GSTQ_n$ fusion proteins purified by affinity batch chromatography, and glutathione was used as a reductant in the earlier work of Folk and Cole (20). However, DTT gave more consistent results.

Measurement of KGDHC After Exposure to Q_n Constructs and tTGase. To determine if KGDHC is inhibited by $GSTQ_n$ plus tTGase, KGDHC (75 milliunits) was incubated at 20°C in a reaction mixture (0.1 ml) containing 8.5 milliunits of guinea pig liver tTGase (specific activity of 2.6 units/mg), 80 mM Tris·HCl buffer (pH 7.4), 10 mM $CaCl_2$, 10 mM DTT and $GSTQ_n$ construct. At intervals, 15-μl aliquots were removed and assayed for remaining activity.

Protein Measurement and Western blotting. Protein was measured by the method of Lowry *et al.* (22) by using BSA as a standard. Immunoblotting was carried out by using a mouse mAb against rabbit GAPDH (clone 65C) (Advanced Immunochemicals, Long Beach, CA) at a dilution of 1:1,000 and a polyclonal antibody against GST (Pharmacia) at a dilution of 1:500. A standard procedure that used alkaline phosphatase was used (23).

Data Analyses. All data are reported as the mean ± SEM. Single comparisons to a control value were carried out by using the Mann–Whitney U test. A P value of <0.05 was considered significant.

RESULTS

Inhibition of GAPDH by $GSTQ_n$ Fusion Proteins in the Presence of tTGase. Under the conditions outlined in *Materials and Methods*, no evidence was obtained to indicate that purified rabbit muscle GAPDH is inhibited by the various $GSTQ_n$ constructs in the absence of tTGase. However, if GAPDH possesses a K residue substrate of tTGase then crosslinking of GAPDH to $GSTQ_n$ constructs may occur, resulting in inactivation of GAPDH. To test this idea it was first necessary to determine if GAPDH possesses K and/or Q residue substrates of tTGase.

In the first experiment, labeled putrescine (Amersham) was used as attacking nucleophile. The reaction mixture (25 μl) containing 2.5 milliunits tTGase, 0.45 nmol [1,4-^{14}C]putrescine dihydrochloride (≈100,000 dpm), 10 mM DTT, 10 mM $CaCl_2$ and 100 mM Tris·HCl buffer (pH 7.6) was incubated for 10 min at 20°C. In this experiment, the radioactivity incorporated into trichloroacetic acid (TCA)-precipitable material (≈1,000 dpm) was greater than that of a control lacking tTGase (60 dpm). [The tTGase preparation contains Q-containing substrates (18).] When GAPDH was included in the reaction mixture at a final concentration of 2 or 10 mg/ml, label in the TCA-precipitable protein was decreased by ≈52 and 72%, respectively. When casein (4 mg/ml) was included in the reaction mixture the radioactivity in the TCA-precipitable pellet was ≈6,000 dpm and 3,100 dpm in the absence and presence of GAPDH (10 mg/ml), respectively. In the second experiment, the effect of GAPDH on the hydroxaminolysis reaction was investigated. The reaction mixture (0.1 ml) contained 2.5 milliunits of tTGase, 50 mM CGG, 10 mM DTT, 200 mM sodium acetate buffer (pH 6.0), 10 mM $CaCl_2$, and 100 mM hydroxylamine-HCl (previously neutralized with NaOH). After incubation at 37°C for 1 hr, hydroxamate formation was determined as described (18). At a concentration of 1 mg/ml, GAPDH inhibited this hydroxaminolysis reaction by 62 ± 4% (n = 3). These findings suggest that GAPDH is not a Q-donor substrate of tTGase, but that GAPDH possesses a K residue that can interfere with attack of nucleophiles (putrescine, hydroxylamine) on Q-containing substrates (CGG, endogenous proteins in the tTGase preparation, and casein).

In the next experiment, the possibility was investigated that GAPDH activity is altered by tTGase in the presence of Q donors. Fig. 1 shows that incubation of GAPDH with tTGase (0.8

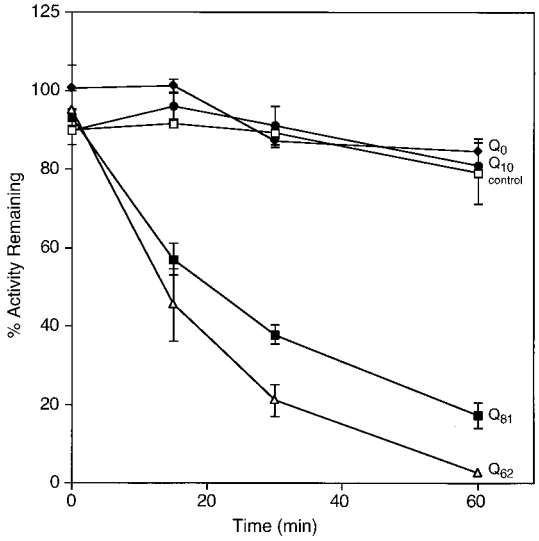

FIG. 1. Inactivation of GAPDH by $GSTQ_n$ fusion proteins plus tTGase. The control contained tTGase but no $GSTQ_n$ construct. The data are from three separate experiments. The decreases in activity at 15, 30, and 60 min in the presence of the $GSTQ_{62}$ and $GSTQ_{81}$ constructs are significantly different from the corresponding time zero values with $P = 0.05$. The decrease in activity at 60 min relative to the corresponding time zero value is significantly different for control, $GSTQ_0$ and $GSTQ_{10}$ values ($P = 0.05$). In a parallel experiment in which GAPDH was incubated in the absence of tTGase no inactivation occurred either in the presence or absence of the $GSTQ_n$ constructs over a 60-min time period (data not shown). Values are expressed as percentages relative to the Q_0 (zero time) value.

milliunits/μl) alone (i.e., in the absence of $GSTQ_n$) resulted in a small (≈12%), but significant, inhibition after 60 min. In this experiment, inhibition was not increased by addition of $GSTQ_0$ or $GSTQ_{10}$. As noted above, commercial tTGase contains proteins that are Q donors. Thus, when GAPDH was incubated with an even larger amount of commercial tTGase (2 milliunits/μl) the loss of activity after 30 min was appreciable (≈60%). The loss of GAPDH activity in the presence of Q-donor proteins within the tTGase preparation presumably is caused by formation of Q-K crosslinks.

Importantly, tTGase in the presence of either $GSTQ_{61}$ or $GSTQ_{81}$ inactivated GAPDH in a time-dependent manner, much more rapidly than did tTGase alone (Fig. 1). The inactivation with tTGase plus $GSTQ_{62}$ (or $GSTQ_{81}$) was also much more rapid than that observed with tTGase plus $GSTQ_0$ (or $GSTQ_{10}$) (Fig. 1). The results presented in Fig. 1 were obtained with glutathione as the reductant. Similar results were obtained with DTT as reductant and with different preparations of $GSTQ_n$ constructs. The reaction mixture (25 μl) in this case contained 1 mg GAPDH, 25 milliunits of tTGase, 10 mM DTT, 10 mM $CaCl_2$, and Q donors. After incubation for 60 min at 20°C, 2-μl aliquots were withdrawn and assayed for remaining GAPDH activity in a well plate analyzer. Percent activities relative to a contol lacking tTGase (100 ± 6) were 82 ± 6 (no added Q donor), 22 ± 2 (25 mM CGG), 14 ± 1 (casein, 10 mg/ml), 91 ± 8 ($GSTQ_0$, 0.5 mg/ml), 85 ± 6 ($GSTQ_{10}$, 0.5 mg/ml), 27 ± 2 ($GSTQ_{62}$, 0.5 mg/ml), and 40 ± 2 ($GSTQ_{81}$, 0.2 mg/ml); n = three separate determinations. CGG alone had no effect on the activity of GAPDH.

Effect of NAD^+, NADH, and GAP on tTGase-Catalyzed Inactivation of GAPDH. In the presence of a variety of Q donors, 1.0 mM NAD^+ strongly protected GAPDH against inactivation by tTGase (Table 1). GAP (1.5 mM) was also protective, but to a lesser extent (Table 1). Lower concentrations of NAD^+ (0.01 and 0.1 mM) and GAP (0.03 and 0.3 mM) were only marginally effective in protecting against inactivation of GAPDH by tTGase plus $GSTQ_{62}$ (Table 1). At a concentration of 1 mM, NADH strongly protected GAPDH against inactivation by tTGase plus $GSTQ_{62}$, but the effect was minimal at lower concentrations (0.01 and 0.1 mM) (Table 1).

Western Blot Analysis of GAPDH Incubated with tTGase and Casein or CGG. Western blot analysis (Fig. 2) showed that the intensity of the GAPDH band after incubation with both casein and tTGase (lane 9) was greatly diminished compared with a control lacking tTGase (lane 8). However, no additional bands

Table 1. Relative activities of GAPDH in the presence of tTGase, various Q donors and GAPDH substrates

	Minus GAPDH substrate	Plus GAPDH substrate	
		1.5 mM GAP	1.0 mM NAD^+
Experiment 1			
Q donor added			
Control (minus tTGase)	[100 ± 4]	–	–
None	83 ± 3	–	–
CGG (25 mM)	35 ± 5	65 ± 2*	101 ± 2*
Casein (4 mg/ml)	48 ± 1	80 ± 5*	90 ± 6*
$GSTQ_0$ (1.6 mg/ml)	43 ± 1	45 ± 1	72 ± 2*
$GSTQ_{10}$ (1.2 mg/ml)	53 ± 1	60 ± 2*	73 ± 2*
$GSTQ_{62}$ (1.7 mg/ml)	6 ± 1	35 ± 1*	78 ± 2*
Experiment 2			
Q donor added	8.2 ± 2	16 ± 3* (0.3 mM GAP)	
$GSTQ_{62}$ (1.7 mg/ml)		21 ± 10 (0.03 mM GAP)	
		19 ± 4* (0.1 mM NAD^+)	
		22 ± 4* (0.01 mM NAD^+)	
		64 ± 2* (1.0 mM NADH)	
		19 ± 4* (0.1 mM NADH)	
		22 ± 4* (0.01 mM NADH)	

The reaction mixture contained 100 mM Tris·HCl buffer (pH 7.6), 10 mM $CaCl_2$, 10 mM DTT, GAPDH (0.8 μg), tTGase (10 milliunits/units; specific activity of 2 units/mg of protein) and the indicated additions in a final volume of 20 μl. After 1 h at 20°C, 2-μl aliquots were withdrawn and assayed for GAPDH activity in a well plate analyzer. n = three or four separate determinations. All values in the "minus GAPDH substrate" column are significantly different from the control (i.e., minus tTGase) with a $P = 0.05$.
*Significant protection against the corresponding value obtained in the absence of added GAPDH substrate with $P = 0.05$.

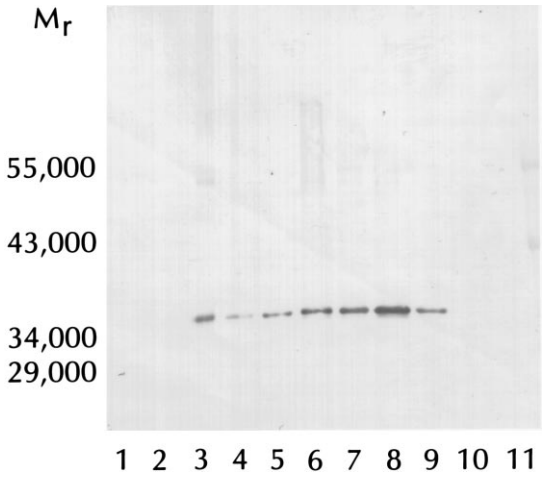

FIG. 2. Western blot analysis of GAPDH incubated with tTGase and casein or CGG. The reaction mixture (25 μl) contained 10 mM Tris·HCl buffer (pH 7.2), 10 mM DTT, 10 mM $CaCl_2$, and CGG or protein additions as indicated. After incubation for 0 or 90 min at 20°C, aliquots were withdrawn for determination of GAPDH activity and for SDS/PAGE immunoblotting analysis of GAPDH monomer. GAPDH activity was determined in duplicate 2-μl aliquots. To the remainder (21 μl) was added 5 μl of stop solution containing 60 mM Tris·HCl buffer (pH 6.8), 25% (vol/vol) glycerol, 14.4 mM 2-mercaptoethanol, and 0.14% (wt/vol) bromothymol blue. After boiling for 10 min, 20 μl was subjected to SDS/PAGE followed by Coomassie blue staining (not shown), and 5 μl was subjected to SDS/PAGE in a parallel gel followed by Western blotting. Lane 1, 6 milliunits guinea pig liver tTGase (1.6 units/mg), zero time; lane 2, 6 milliunits tTGase, 90 min; lane 3, GAPDH standard (0.6 μg), 90 min; lane 4, GAPDH standard (0.3 μg), 90 min; lane 5, GAPDH + 6 milliunits tTGase, 90 min; lane 6, GAPDH + 50 mM CGG, 90 min; lane 7, GAPDH + CGG + 6 milliunits tTGase, 90 min; lane 8, GAPDH + casein (100 μg), 90 min; lane 9, GAPDH + casein + 6 milliunits tTGase, 90 min; lane 10, casein, zero time; lane 11, casein, 90 min. M_r markers in lane 12 (not shown) were carbonic anhydrase (29,000), lactate dehydrogenase (34,000), ovalbumin (43,000), and glutamate dehydrogenase (55,000) (Diversified Biotech). Lanes 5–9 contained 0.6 μg of GAPDH. Relative activities (%) of GAPDH applied to lanes 3, 5–9 were [100 ± 0], 75 ± 1, 83 ± 3, 10 ± 2, 106 ± 4, and 8 ± 2, respectively (n = three separate experiments carried out in duplicate).

were detected in lane 9. Coomassie blue staining of a parallel-run gel showed that the casein preparation contained three heavily stained bands at M_r ≈26,000–30,000 that almost completely disappeared after incubation with tTGase. The disappearance was not associated with the appearance of new bands. The activity of GAPDH in the presence of casein and tTGase was decreased by 90%, and the intensity of the immunostained GAPDH band was similarly decreased compared with that of the control. A decrease in the intensity of the GAPDH band in the presence of casein and tTGase (Fig. 2, lane 9) compared with the control (Fig. 2, lane 8) also was noted in a parallel gel stained with Coomassie blue (data not shown). When GAPDH was incubated with CGG no difference between the intensity of the band in lane 6 (minus tTGase, control) and that in lane 7 (plus tTGase) was noted (Fig. 2), despite the fact that the activity of GAPDH was decreased by ≈90% in the presence of CGG plus tTGase.

Inhibition of KGDHC by $GSTQ_{62}$ and $GSTQ_{81}$ Fusion Proteins in the Presence of tTGase. Incubation of bovine heart KGDHC with the $GSTQ_n$ fusion proteins alone had no effect on the enzyme activity (Table 2). However, significant time-dependent loss of activity was noted in the presence of $GSTQ_{62}$ (or $GSTQ_{81}$) plus tTGase (Table 2). KGDHC also was strongly inhibited by tTGase plus the model Q donor CGG (Table 3). No significant inactivation of pig heart pyruvate dehydrogenase complex, pig heart mitochondrial malate dehydrogenase, and bovine liver glutamate dehydrogenase was noted when these enzymes were incubated with any of the $GSTQ_n$ constructs in the presence or absence of tTGase under conditions similar to those used in the KGDHC studies.

Table 2. Relative activities of pig heart KGDHC in the presence of various $GSTQ_n$ constructs with or without added tTGase

Addition	Concentration (mg/ml)	Relative Activities (%)*	
		−tTGase	+tTGase
None (control)	–	103 ± 2	[100 ± 3]
$GSTQ_0$	1	99 ± 3	107 ± 7
$GSTQ_{10}$	1	100 ± 2	95 ± 9
$GSTQ_{62}$	0.3	98 ± 5	59 ± 9†§
$GSTQ_{81}$‡	0.3	102 ± 3	70 ± 2†§
$GSTA_{61}$ (control)	1.2	95 ± 6	101 ± 3

*Relative activity remaining after 3 hr at 20°C.
†Significantly different from the control (i.e., a reaction mixture lacking GST construct but containing tTGase) with $P = 0.05$.
‡A sonicated preparation of the GST construct was used in this experiment.
§Relative activities at 1 and 2 hr were 85 ± 5 and 70 ± 6%, respectively, in the presence of $GSTQ_{62}$ plus tTGase, and 88 ± 3 and 82 ± 4%, respectively in the presence of $GSTQ_{81}$ plus tTGase.

In a separate experiment it was found that KGDHC (20 μl of the commercial preparation) stimulated the hydroxaminolysis reaction by 75 ± 10% (n = 3). The conditions were similar to those outlined above for measurement of the effect of GAPDH on the hydroxaminolysis reaction except that GAPDH was replaced by KGDHC. The stimulation was not caused by the nonprotein components in the commercial preparation because inclusion of these components in the hydroxaminolysis reaction mixture led to a 37 ± 3% decrease in the formation of γ-glutamylhydroxamate product. Possibly, the high protein content upon addition of KGDHC stabilizes tTGase and promotes the hydroxaminolysis reaction.

The possibility was considered that substrates and cofactors might protect KGDHC against inactivation by tTGase plus Q_n donor. The reaction mixture (20 μl) contained 10 milliunits of KGDHC, 20 milliunits of tTGase (specific activity of 1.6 units/mg of protein), 100 mM Tris·HCl (pH 7.6), 20 mM $CaCl_2$, 10 mM DTT, $GSTQ_{62}$ (1.7 mg/ml), and various substrates or cofactors as indicated. After incubation for 1 hr at 20°C, 5-μl aliquots were withdrawn and analyzed for KGDHC activity in a well plate analyzer. Percent activities of KGDHC relative to a control lacking tissue transglutaminase (100 ± 5) were 70 ± 3 (−$GSTQ_{62}$), 52 ± 6 (+$GSTQ_{62}$), 50 ± 4 (+$GTSQ_{62}$ + 0.1 mM NAD^+), 47 ± 3 (+$GSTQ_{62}$ + 0.01 mM NAD^+), 55 ± 6 (+$GSTQ_{62}$ + 1 mM α-ketoglutarate), 50 ± 4 (+$GSTQ_{62}$ + 0.1 mM α-ketoglutarate), 98 ± 5 (+$GSTQ_{62}$ + 1.2 mM CoASH), and 82 ± 4 (+$GSTQ_{62}$ + 0.12 mM CoASH); n = 3 separate determinations.

DISCUSSION

Crosslinking of Proteins to Q_n Domains. The present work shows that, despite tight association, Q_n constructs do not inhibit

Table 3. Relative activities of pig heart KGDHC in the presence of tTGase and CGG

Addition	% Activity	
	0 min	30 min
None	[100 ± 3]	98 ± 2
CGG (40 mM)	103 ± 5	104 ± 2
tTGase (10 milliunits)	98 ± 4	71 ± 4*
CGG (40 mM) + tTGase (10 milliunits)	103 ± 6	5 ± 1*

The reaction mixture contained 100 mM Tris·HCl buffer (pH 7.2), 20 mM $CaCl_2$, 10 mM DTT, KGDHC (10 milliunits) and the indicated addition(s) in a final volume of 20 μl at 20°C. At the indicated times, 2-μl aliquots were withdrawn and assayed for KGDHC activity. The specific activity of the guinea pig liver tTGase used in this experiment was 1.6 units/mg. n = four separate determinations.
*Different from control (no addition, 30 min) with $P = 0.05$.

GAPDH. However, once a Q residue is covalently linked to GAPDH, inactivation ensues. The Q residue can be supplied by the peptide CGG, protein Q donors within the tTGase preparation, and $GSTQ_n$ constructs. The extent of inactivation is greater with pathological-length Q_n inserts ($n = 61$ or 81) than with nonpathological length inserts ($n = 0$ or 10). The chances of a Q residue becoming favorably juxtaposed to a K residue of GAPDH for efficient tTGase-catalyzed crosslinking presumably is greater the longer the Q_n repeat. Moreover, because GAPDH is usually in the form of a tetramer, the chances of tTGase catalyzing multiple crosslinks between Q residues of the Q_n domain and K residues of separate monomers also presumably is greater the longer the Q_n domain. Q_n domains have a strong tendency to form β-pleated sheets (24), and this property is greater in the longer Q_n constructs. Nevertheless, this tendency appears to be offset by more favorable binding of GAPDH to the longer Q_n domains.

Although strong evidence is presented that GAPDH is a K substrate for tTGase-catalyzed covalent attachment to $GSTQ_n$, no bands corresponding to heteropolymers were detected on Western blotting by using either a mAb to GAPDH or a polyclonal antibody to GST (data not shown). It is unlikely that the epitope of GAPDH is blocked by covalent linkage to a Q residue of $GSTQ_n$ because the epitope was not blocked when GAPDH was inactivated by covalent linkage to CGG (Fig. 2). Most probably, covalent linkage between GAPDH and a protein containing a Q_n domain results in a highly insoluble product that does not enter the SDS gel during electrophoresis. Loss of GAPDH activity in the presence of tTGase and casein is associated with a corresponding decrease in the immunoreactivity of the 36-kDa GAPDH band on Western blots, but not with the appearance of higher M_r bands (Fig. 2). tTGase-catalyzed crosslinking of casein results in loss of protein on Coomassie blue-stained gels without the appearance of polymeric bands. Because casein possesses a free amino group that can act as a nucleophile (in addition to a Q electrophile) in crosslinking reactions, it is probable that GAPDH becomes interwoven in a large, highly insoluble polymeric complex in the presence of casein and tTGase.

GAP at high concentration (1.5 mM), but not at lower concentrations (0.03 and 0.3 mM), moderately protects GAPDH against inactivation by tTGase plus $GSTQ_{62}$. NADH at high concentration (1.0 mM), strongly protects, but not at lower concentrations (0.01 and 0.1 mM). At a concentration of 1 mM, NAD^+ is protective, but less so at lower concentrations (0.01 and 0.1 mM) (Table 1). The concentrations of GAP, NADH, and NAD^+ in tissues are $\approx$1–12 μM, 50–200 μM, and 30–900 μM, respectively (25). Therefore, it is unlikely that GAP or NADH will protect GAPDH against tTGase-catalyzed inactivation *in vivo* and that protection by NAD^+ will be small.

Inhibition of KGDHC activity occurs in the presence of tTGase and a suitable Q donor. The extent of inactivation, however, is a complicated factor of concentration of tTGase and exogenous Q donor in the reaction mixture. Although inactivation can occur with Q donors within the tTGase preparation (Table 3) or with $GSTQ_{10}$ (see the text), inactivation generally is more pronounced in the presence of longer Q_n domains (Table 2, text). The enzyme complex is not protected by its substrate α-ketoglutarate or by the cofactor NAD^+. KGDHC is, however, protected by 1.2 mM CoASH and, to a lesser extent, by 0.12 mM CoASH (see the text). The CoASH concentration *in vivo* is 10–80 μM (25). Thus, this cofactor is unlikely to provide much protection *in vivo*.

Several other enzymes tested are not inactivated by $GSTQ_n$ plus tTGase under the conditions used. It will be important to determine how commonly Q-K linkages involving extended Q_n domains occur *in vivo*. It also will be important to determine which are the susceptible K residues in GAPDH and KGDHC and the nature of the linkages.

Possible Metabolic Consequences Resulting from Inhibition of GAPDH and KGDHC. Huntingtin, GAPDH, and tTGase are widely distributed in tissues. Moreover, evidence suggests that the Q_n stretches are present *in vivo* in both normal and mutated huntingtin (26). In brain, huntingtin is present in cortical neurons in association with the matrix of the cytosol and the surroundings of vesicle membranes (27). Huntingtin also is associated with microtubules, and, to a lesser extent, with synaptic vesicles (28). The protein also has a nuclear targeting motif (29). GAPDH is predominantly in the cytosol associated with the cytoskeleton and membranes (1). A small amount is in the nucleus. Interestingly, human brain contains an enzyme that catalyzes ubiquination of huntingtin (30). Possibly, once covalently attached to another protein such as GAPDH, the resulting complex would be tagged for rapid degradation. tTGase is largely cytosolic with some present in the extracellular fluid (31). Inasmuch as huntingtin and GAPDH are present in the same cellular compartment they potentially can interact. However, GAPDH is abundantly expressed whereas huntingtin is expressed at a relatively low level. Therefore, it is not clear whether tTGase-catalyzed crosslinking reactions *per se* will lead to a marked deficit in total GAPDH activity in HD cells *in vivo*. In this regard, Browne *et al.* (32) recently reported that GAPDH activity is not significantly decreased in HD brain (frozen $\approx$14–16 hr postmortem) compared with control brain. In this report, GAPDH activity was measured in the reverse direction to that measured here. Moreover, the specific activity of GAPDH reported by Browne *et al.* is low, and the postmortem stability of the enzyme in the human brain was not determined. Thus, the possibility that a small amount of GAPDH is inhibited in HD brain cannot be excluded. Possibly, even a slight decrease in activity of GAPDH is deleterious, especially if a small, but crucial, compartment of the enzyme is affected. In this regard, a single mutation in 15% of GAPDH monomers can alter endocytosis in Chinese hamster ovary cells (33).

In its tetrameric form, GAPDH catalyzes an important reaction in glycolysis. In its monomeric form, GAPDH acts as a uracil DNA glycosylase (34). In addition, GAPDH binds to RNA, ATP, calcyclin, actin, tubulin, and amyloid precursor protein (see ref. 1 for original references). GAPDH has been implicated as a major protein involved in thiol/disulfide redox reactions in human lung carcinoma cells (35). Finally, GAPDH (36) and tTGase (37, 38) may play a role in apoptosis. GAPDH increases in cerebellar granule cells undergoing apoptosis; antisense oligonucleotides that block the expression of GAPDH delay the death of the granule cells (36). Thus, aberrant crosslinking reactions involving both GAPDH and tTGase may disrupt normal cell functions and the timing of cell death. Some authors have speculated that HD might be a disorder of inappropriate apoptosis (39) or inability to counterbalance apoptotic pathways (40).

KGDHC is a mitochondrial enzyme and therefore it is not known whether the assembled complex is a substrate of tTGase *in vivo*. However, crosslinking to one or more of the KGDHC subunits may occur before import into the mitochondrion. KGDHC activity is limiting for tricarboxylic acid cycle activity in brain (19). Thus, loss of this activity could result in metabolic impairment in expanded CAG/Q_n neurodegenerative diseases.

Possible Mechanisms for tTGase-Induced Neurotoxicity in Expanded CAG/Q_n Diseases. A small amount of tTGase is present in brain relative to liver (41). The activity is present in synaptosomes (42) and astrocytes in culture (42). Brain contains two tTGases, one of which may be unique (43–45). Ohashi *et al.* (45) purified a tTGase from rat brain that is distinct from that of the liver. As with other tTGases, this brain enzyme is a member of the GTP-binding family. Thus, expanded Q_n diseases may be caused in part by aberrant cell signaling. Alternatively, expanded CAG/Q_n repeats may promote tTGase-catalyzed reactions resulting in significant deficits in GAPDH and KGDHC activities. If this is the case, the expanded Q_n diseases would have similarities to other neurodegenerative diseases that are associated with impairment of energy metabolism (cf. refs. 46–49). Indeed, HD brain exhibits marked metabolic deficits (32). More-

over, irreversible inactivation of brain succinate dehydrogenase by 3-nitropropionate induces many of the motor and histological features of HD in experimental animals (50). The excitotoxin quinolinate (51) and neuronal NO synthase (in association with huntingtin binding protein) (52) also may play roles in the disease process. GAPDH possesses a sulfhydryl that is extremely sensitive to NO (53) and on prolonged exposure is irreversibly inactivated (53). NO levels also may affect GAPDH mRNA levels (54). If huntingtin, huntingtin binding protein 1, and neuronal NO synthase are associated *in vivo*, then mutated huntingtin, either through a tTGase-dependent or tTGase-independent processes, may lead to altered NO production and altered GAPDH activity. However, a unifying theory that ties together the metabolic deficits and excitotoxicity in HD brain is not yet available.

Finally, tTGase has been suggested to play a role in neurotransmitter release from synaptosomes (43, 44, 55), crosslinking of neurofilaments, glial filaments and myelin basic protein (56), crosslinking of filaments into insoluble polymers during aging (57), and the formation of paired helical filaments in Alzheimer disease (57). [See Lorand (58) for a recent commentary.] Thus, in addition to possible inappropriate apoptosis and metabolic derangements resulting from crosslinking to GAPDH and KGDHC, many other possible insults resulting from aberrant tTGase activity may exist in HD.

This work was supported by grants from the Will Rogers Foundation, the Overbrook Foundation, the Winifred Masterson Burke Relief Foundation, and National Institute of Aging (AG 09014).

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