INHIBITION OF α -KETOGLUTARATE- AND PYRUVATE DEHYDROGENASE COMPLEXES IN *E. coli* BY A GLUTATHIONE S-TRANSFERASE CONTAINING A PATHOLOGICAL LENGTH POLY-Q DOMAIN: A POSSIBLE ROLE OF ENERGY DEFICIT IN NEUROLOGICAL DISEASES ASSOCIATED WITH POLY-Q EXPANSIONS?

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ABSTRACT

At least seven adult-onset neurodegenerative diseases, including Huntington's disease (HD), are caused by genes containing expanded CAG triplets within their coding regions. The expanded CAG repeats give rise to extended stretches of polyglutamines (Q_n) in the proteins expressed by the affected genes. Generally, $n \geq 40$ in affected individuals and ≤ 36 in clinically unaffected individuals. The expansion has been proposed to confer a "toxic gain of function" to the mutated protein. Poly-Q domains have recently been shown to be excellent substrates of tissue transglutaminase. We investigated the effects of expression of glutathione S-transferase constructs containing poly-Q inserts of various lengths ($GSTQ_n$ where $n = 0, 10, 62$ or 81) on the activity of some key metabolic enzymes in the host *Escherichia coli* – an organism not known to have transglutaminase activity. *E. coli* carrying the $GSTQ_{62}$ construct exhibited statistically significant decreases in the specific activities of α -ketoglutarate dehydrogenase complex (KGDHC) and pyruvate dehydrogenase complex (PDHC). Previous work has shown that KGDHC and PDHC activities are reduced in the brains of Alzheimer's disease (AD) patients. Our results suggest that KGDHC and PDHC may be particularly

susceptible to the effects of a number of disparate insults, including those associated with AD and HD.

INTRODUCTION

Neurodegenerative diseases known to be caused by an expansion of CAG/poly-Q repeats include Huntington's disease (HD), dentatorubral pallidoluysian atrophy (DRPLA), spinobulbar muscular atrophy (SBMA), spinocerebellar ataxia types-1, 2, 3 (Machado-Joseph disease, MJD) and 7 (SCA-1, SCA-2, SCA-3, SCA-7) (1-5 and references quoted therein). In only one of the seven diseases has the normal gene product been assigned a recognizable biological function. The wild-type SBMA gene product is known to be an androgen receptor. Expansion of poly-Q repeats has been suggested to convey a "toxic gain of function" to the affected proteins. Huntingtin (the normal gene product of HD), DRPLA protein, androgen receptor, ataxin-1 (the normal gene product of SCA-1), and poly-Q constructs bind strongly to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1,2). It was suggested that interactions of extended poly-Q domains may disrupt the activity of GAPDH and interfere with energy metabolism (1,2). However, recent work from our laboratory showed that *E. coli* glutathione S-transferase constructs containing poly-Q inserts of various lengths ($GSTQ_n$ where $n = 0, 10, 62$ or 81) have no effect on the activity of GAPDH (6).

Several authors have suggested that individual glutamyl residues within poly-Q domains might be good substrates of tissue transglutaminase – an enzyme that catalyzes formation of a cross link (an isopeptide) between a protein glutamyl residue and a protein lysyl residue (7,8). Indeed, Kahlem et al. re-

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cently showed that small peptides containing inserts of up to 18 residues are excellent substrates of tissue transglutaminase (9). We independently showed that a protein-length construct containing a small number of Q repeats (i.e., GSTQ₁₀) is an excellent substrate of guinea pig liver tissue transglutaminase (10). Additionally, we showed that a construct containing a large number of Q repeats (i.e., GSTQ₆₂) is a good substrate of tissue transglutaminase (10). This finding may be relevant to the neurodegenerative diseases associated with poly-Q expansions because clinically affected individuals usually have ≥ 40 Q repeats in the mutated protein.

Extended poly-Q repeats have a strong tendency to form β -pleated sheets (11). Therefore, the formation of tight *non-covalent* interactions between extended poly-Q domains and key proteins may lead to disruption of energy metabolism and cerebral function by processes that do not involve transglutaminase. It is difficult to test this idea directly because tissue transglutaminase is widespread in mammalian tissues, including brain (12). Therefore, we chose to investigate the effects of extended poly-Q domains in a model system that does not contain a mammalian-type transglutaminase. Burke et al. previously showed that extended poly-Q domains are toxic to *E. coli* (13). In the present work *E. coli* colonies containing GSTQ_n constructs (where n = 0, 10, 62, 81) were grown under defined conditions, harvested at intervals and the specific activities of key metabolic enzymes were determined as an index of possible metabolic deficits. The results of these experiments are reported here.

METHODS

Transfection of E. coli Containing Different Length Poly-Q Constructs

E. coli colonies expressing various GSTQ_n proteins (where n = 0, 10, 62 or 81) were constructed according to the method of Onodera et al. (13). *E. coli* expressing GSTA₆₁ (13) was used as a control.

Preparation of E. coli Lysates

The cells were grown as described by Onodera et al. (13). After induction with isopropylthio- β -D-galactoside (IPTG), 1 ml aliquots of the cell culture were withdrawn at intervals and the suspensions were centrifuged at 10,000 g for 1 min at room temperature. The resulting pellet was then suspended in 1 ml of solubilization buffer [20 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, aprotinin (1.8 μ g/ml), leupeptin (1 μ g/ml), and pepstatin (1 μ g/ml)]. The suspension was cooled on ice and sonicated twice, each time for a period of 10 seconds, followed by addition of 2.5 μ l of 20% (v/v) Triton X-100. Aliquots (20-100 μ l) were withdrawn and assayed for activities of KGDHC, PDHC, GAPDH, and malate dehydrogenase (MDH).

Reagents

Protease inhibitors, sodium α -ketoglutarate, sodium

pyruvate, oxaloacetic acid, Coenzyme A (CoASH), thiamin pyrophosphate (TPP), glyceraldehyde 3-phosphate (200 mM frozen solution), sodium arsenate, bovine milk α casein, and *N*- α -carbobenzoxyl-glutaminyglycine (CGG) were obtained from Sigma Chemical Company (St. Louis, MO). All reagents were of the highest purity available.

Enzyme Assays

The assay buffer (AB) consisted of 50 mM Tris-HCl (pH 7.6), 10 μ M CaCl₂, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM MgCl₂, and 0.2% (v/v) Triton X-100. The procedures for enzyme assays are as follows. *KGDHC*: 0.66 ml of AB, 10 μ l of 80 mM sodium α -ketoglutarate, 10 μ l of 80 mM NAD⁺, 10 μ l of CoASH (10 mg/ml) and 10 μ l of 30 mM TPP. Lysate (100 μ l) was added and the rate of increase of absorbance at 340 nm was determined at 30°C in a Cary 200 Spectrophotometer. *PDHC*: As for KGDHC except that sodium α -ketoglutarate was replaced with sodium pyruvate. Preliminary experiments showed that lactate dehydrogenase activity (which would interfere with the assay if present in excess) is low in the lysate. *GAPDH*: 0.67 ml of AB, 10 ml of 80 mM NAD⁺, 10 μ l of 80 mM glyceraldehyde 3-phosphate, and 10 μ l of 1 M sodium arsenate. Lysate (100 μ l) was added and the rate of increase of absorbance at 340 nm was recorded at 30°C. Glyceraldehyde 3-phosphate is unstable and was therefore made up from the 200 mM frozen stock just before use. *MDH*: 0.75 ml of AB, 10 μ l of 10 mM NADH, and 20 μ l of freshly prepared 100 mM oxaloacetic acid neutralized with NaOH. Lysate (20 μ l) was added and the rate of decrease of absorbance at 340 nm was recorded at 30°C. The initial linear portion of the reaction was used in each of the above described assays to determine the enzyme activities; $\epsilon_{340\text{nm}}$ of NADH = 6.22×10^3 . Enzyme activities are expressed as units per mg of protein, where a unit is defined as the amount of enzyme that catalyzes the conversion of 1 μ mol of substrate to product per minute at 30°C. In preliminary experiments it was found that the rate of background oxidation of NADH in the *E. coli* extracts was low so that it was not necessary to add rotenone to the dehydrogenase assay mixtures. *Tissue transglutaminase*: Guinea pig liver tissue transglutaminase was obtained from Sigma as a lyophilized powder [specific activity of 2 units/mg of protein (hydroxaminolysis assay)]. The specific activity of the commercial preparation refers to the ability of the enzyme to catalyze the hydroxaminolysis of CGG at pH 6.0. In this assay the glutaminy residue of CGG mimics that of a protein and hydroxylamine mimics the attacking nitrogen nucleophile of a protein lysine group. The hydroxaminolysis assay procedure used in the current work was that of Cooper et al. (10) as adapted from that of Folk and Cole (14). An alternative assay makes use of the fact that casein is an excellent glutaminy-containing protein substrate and that the terminal amine(s) of polyamines (e.g. putrescine) can replace the ϵ nitrogen of lysine as the attacking nucleophile. The assay

procedure used in the current work was that of Cooper et al. (10) adapted from that of Folk and Cole (14). The procedure measures the tissue transglutaminase-catalyzed covalent attachment of [1,4-¹⁴C]putrescine (Amersham Life Science) to casein. In some cases casein was replaced by GSTQ_n at a concentration of 0.3–1 mg/ml. The commercial transglutaminase was used as a positive control in experiments in which transglutaminase activity was sought in *E. coli* lysates. Protein was determined according to the method of Lowry et al. (15) using bovine serum albumin as standard.

Statistical analyses

All values are reported as the mean ± SEM. Pairwise multiple comparisons were carried out by one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls procedure. The absolute values for the specific activity of a given enzyme in the *E. coli* extracts varied considerably among the different cultures and the distribution was non-normal. Therefore, ANOVA was also carried out on data that were log-transformed to satisfy the implicit statistical assumption of normal distribution with constant variance.

RESULTS

Lack of Tissue-Type Transglutaminase Activity in *E. coli*

In order for *E. coli* to be used as a model system for the study of possible deleterious effects of larger poly-Q inserts that are not related to the action of transglutaminase, it is important to ascertain whether the micro-organism possesses such an enzyme. Accordingly, we searched for this activity by using the hydroxaminolysis assay on aliquots of lysates obtained from four clones of *E. coli* for each of the five constructs (i.e. *E. coli* expressing GSTQ₀, GSTQ₁₀, GSTQ₆₂, GSTQ₈₁ or GSTA₆₁). In no case could activity be detected (<0.05 µmol/h/100 µg of protein, or <8 mU/mg of protein) in bacterial samples lysed at zero time and at 2 hours after induction with IPTG (data not shown). We also used a sensitive radio-chemical assay to measure covalent attachment of [1,4-¹⁴C]putrescine to casein in the presence of *E. coli* lysates. As with the previous assay, no activity (<5 pmol of [1,4-¹⁴C]putrescine attached to casein/h/100 µg of protein) could be detected by this procedure. Finally, we attempted to determine whether [1,4-¹⁴C]putrescine could be covalently attached to any of the GSTQ_n constructs in the presence of *E. coli* lysates. Again, no such activity could be detected in lysates of *E. coli*. Positive controls with commercial tissue transglutaminase gave the expected amount of product with the hydroxaminolysis and [1,4-¹⁴C]putrescine binding assays (data not shown).

Activities of Some Enzymes of Energy Metabolism in *E. coli* Expressing Various GST_n Constructs

The specific activities of KGDHC, PDHC, GAPDH, and MDH in *E. coli* extracts at 0, 1, 2, 3 and 6 hours after induction with IPTG are shown in Table 1. Four clones

were analyzed for each of the 5 constructs. Some of the enzyme activity values were quite variable and may have been related to clonal differences in the make-up of the enzyme constituents, levels of the expressed poly-Q domains, and to differences in the growth phase at time of sampling. The low activities of KGDHC and PDHC at time zero after induction in *E. coli* expressing GSTQ₆₂ relative to the corresponding activities in *E. coli* expressing other GSTQ_n constructs (Table 1) is consistent with leaky expression of GSTQ₆₂. This phenomenon has been noted previously (13). In order to obtain insights into trends in the degree of enzyme activity versus presence of construct the data for the five time points were combined and analyzed by one way ANOVA (Table 2). *E. coli* expressing the GSTQ₆₂ construct clearly demonstrated a reduction in KGDHC and PDHC activities compared with all other cultures (i.e., those expressing GSTQ₀, GSTQ₁₀, GSTQ₈₁ or GSTA₆₁). It was also noted that the activity of GAPDH is considerably lower in the *E. coli* GSTQ₆₂ construct compared with all others, but the difference was not statistically significant ($p = 0.06$). No differences in the activity of MDH among the *E. coli* colonies expressing the various constructs were noted.

Table 1: Specific activities (mU/mg of protein) of selected enzymes of energy metabolism in extracts of *E. coli* expressing GSTQ_n (where n = 0, 10, 62, or 81) or GSTA₆₂

Time after induction (Hours)	Enzyme	GST Construct				
		Q ₀	Q ₁₀	Q ₆₂	Q ₈₁	A ₆₂
KGDHC						
0		11±4	20±3	6 ±5	21±6	18±5
1		23±2	20±3	15±5	25±4	20±1
2		27±4	16±2	9±5	24±4	20±3
3		22±7	23±8	5±4	22±6	18±5
6		11±2	11±4	6±4	23±6	21±5
PDHC						
0		22±4	25±4	13±7	22±5	21±5
1		30±4	26±4	29±6	23±4	25±2
2		29±6	17±4	13±5	30±4	19±2
3		23±2	18±4	13±3	23±5	18±3
6		15±3	13±3	12±5	21±6	16±2
GAPDH						
0		114±44	55±22	50±22	99±42	79±29
1		64±31	64±10	42±9	86±15	89±16
2		75±30	46±14	30±4	79±12	69±26
3		91±34	65±15	39±11	54±27	70±22
6		56±21	43±7	43±7	75±33	58±15
MDH						
0		237±33	233±46	306±146	235±35	207±19
1		224±37	279±81	236±86	286±89	236±67
2		263±56	263±64	250±92	347±104	258±75
3		229±18	242±41	150±45	213±77	253±16
6		248±68	259±48	227±102	229±76	226±62

The data are the means±SEM from four different clones of each construct.

Table 2: Combined activities (mU/mg of protein) from 0 hour to 6 hours of selected enzymes of energy metabolism in extracts of *E. coli* expressing GSTQ_n (where n = 0, 10, 62, or 81) or GSTA₆₂

Enzyme	GST Construct				
	GSTQ ₀	GSTQ ₁₀	GSTQ ₆₂	GSTQ ₈₁	GSTA ₆₁
KGDHC	19±2	17±2	8±2*	23±2	19±2
PDHC	24±2	20±2	16±3**	24±2	20±1
GAPDH	80±14	54±6	39±5	79±12	73±9
MDH	236±20	240±19	260±24	234±41	262±33

Activities of the four enzymes were determined at 0, 1, 2, 3 and 6 hours after induction with IPTG in cultures separately expressing GSTQ₀, GSTQ₁₀, GSTQ₆₂, GSTQ₈₁ or GSTA₆₁. Thus a total of 20 activity measurements were made for each enzyme (5 combined time points for four separate clones). *P < 0.05 for pairwise comparisons against all other KGDHC values. **P < 0.05 for pairwise comparisons against all other PDHC values. †P = 0.006 compared to control (GSTQ₀) by ANOVA of the logarithmic function.

DISCUSSION

Decrease of KGDHC- and PDHC specific activities in E. coli expressing GSTQ₆₂

Reports of transglutaminase activity in microorganisms are few. *Streptovercillium sp.* (16) and *Candida albicans* (17) have recently been shown to possess transglutaminase activity. In these organisms, transglutaminase is possibly a catalyst for the strengthening of cell walls. We are unaware of reports that *E. coli* possesses an enzyme activity comparable to that of mammalian tissue transglutaminase. The present results indicate that *E. coli* does not possess a transglutaminase activity comparable to that present in mammalian tissues and does not catalyze cross-linking reactions with poly-Q domains. Thus, *E. coli* is a convenient model for investigating the effects of expanded poly-Q inserts unrelated to the action of transglutaminase.

Previous studies showed that GSTQ_n-fusion proteins containing short poly-Q_n stretches (n = 10-35) do not inhibit the growth of *E. coli* whereas GST-fusion proteins containing longer poly-Q stretches (n = 59-81) inhibit the growth of the bacterium (13). Interestingly, GST containing a long polyalanine construct (i.e., GSTA₆₁) has no effect on the growth of *E. coli* (13). In the present work we also noted that expression of the GSTQ₆₂- and GSTQ₈₁-fusion proteins causes a retardation of growth in the host *E. coli* whereas GSTA₆₁ does not (data not shown). These findings suggest that toxicity is associated with a property of the side chains of the extended poly-Q domain rather than with the possession of the plasmid. Since *E. coli* does not possess transglutaminase activity the inhibition of growth when n = 59-81 must be due to interactions of the extended Q residues other than to those resulting in covalent cross bridging to other proteins.

The specific activities of KGDHC and PDHC in *E. coli* expressing GSTQ₆₂ are significantly reduced compared

with the specific activities of these multi-enzyme complexes in bacteria transfected with GSTQ₀, GSTQ₁₀, GSTQ₈₁ or GSTA₆₁ (Table 2). Possibly, the extended Q domain of GSTQ₆₂ insinuates into *E. coli* KGDHC and PDHC disrupting the tertiary and quaternary structures of these large multi-enzyme complexes. Alternatively, the activities may be reduced secondarily to some other insult. Although *E. coli* KGDHC and PDHC share certain structural characteristics with the corresponding multi-enzyme complexes in mammals the activities are regulated very differently. Moreover, PDHC and KGDHC are mitochondrial enzymes whereas mammalian poly-Q domains (such as that in huntingtin) are not known to occur in these organelles. Therefore, it is unlikely that the present results can be extrapolated directly to the mammalian mitochondrial multi-enzyme complexes. The conclusion from the present work is that primary or secondary interactions involving extended poly-Q domains may lead to disruption of energy metabolism by processes that lead to inhibition of key enzymes of intermediary metabolism. In the case of *E. coli*, energy failure may be due to disruption of KGDHC and PDHC activities, but other enzyme activities may be compromised in mammalian tissues.

Unexpectedly, the presence of the GSTQ₈₁ fusion protein did not lead to a decrease in the specific activities of KGDHC or PDHC (Tables 1 and 2) despite the fact that the *E. coli* carrying this construct grew poorly. A possible explanation is that GSTQ₈₁ tends to form highly insoluble aggregates (6,10). Such aggregates will minimize the amount of free Q residues but may disrupt the physical properties of the cell.

Relevance to Neurodegenerative Diseases

Defects of energy metabolism are well documented to occur in the brains of patients with Alzheimer disease (AD) (reviewed in 18-21), Huntington's disease (HD) (21 and references therein) and Parkinson's disease (22). KGDHC and PDHC activities are reduced in AD brain (23-25). KGDHC activity has been reported to be reduced in AD fibroblasts (26,27). PDHC activity is reduced in HD brain (21), and KGDHC activity is reduced in cerebella of patients with spinocerebellar ataxia type I (28). KGDHC activity is decreased in affected regions of Parkinson's disease brain, possibly due to loss of neurons (22,29). Finally, the activities of KGDHC, PDHC and several other mitochondrial enzymes involved in energy metabolism are reduced in the brains of patients with several hereditary ataxias (30). Evidently, the multi-enzyme dehydrogenase complexes are especially vulnerable in neurodegenerative diseases.

As noted above, *E. coli* KGDHC and PDHC are distinct from their mammalian counterparts. Nevertheless, the present results suggest that – no matter what the cell type – the presence of extended poly-Q repeats may be enough to alter the activity profiles of key enzymes and to disrupt energy metabolism. This finding may have important ramifications in extended CAG/

poly-Q repeat diseases. Indeed, we have recently shown that alterations in enzymes of energy metabolism can be detected in mildly stressed cultured fibroblasts obtained from HD patients (unpublished data). Moreover, glyceraldehyde 3-phosphate dehydrogenase, a key glycolytic enzyme, is readily inhibited *in vitro* by extended poly-Q domains in the presence of tissue transglutaminase (6). In future work it will be important to determine the molecular basis (both transglutaminase mediated and transglutaminase independent) whereby extended poly-Q domains give rise to alterations in energy metabolism. Such work may provide an explanation for the subtle differences in the neuropathology of the various extended poly-Q diseases and may also suggest possible therapeutic interventions.

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