Structural characterization of transglutaminase-catalyzed cross-linking between glyceraldehyde 3-phosphate dehydrogenase and polyglutamine repeats

MARGHERITA RUOPPOLO,^{1,3} STEFANIA ORRÙ,¹ SIMONA FRANCESE,¹
IVANA CAPUTO,² AND CARLA ESPOSITO¹

¹Dipartimento di Chimica, Università di Salerno, Salerno, Italy

²Dipartimento di Pediatria, Università di Napoli "Federico II," Naples, Italy

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Abstract

The accumulation of abnormal polyglutamine-containing protein aggregates within the cytosol and nuclei of affected neurons is a hallmark of the progressive neurodegenerative disorders caused by an elongated (CAG)_n repeat in the genome. The polyglutamine domains are excellent substrates for the enzyme transglutaminase type 2 (tissue), resulting in the formation of cross-links with polypeptides containing lysyl groups. Enzymatic activity toward the Q_n domains increases greatly upon lengthening of such Q_n stretches (n > 40). Among the possible amine donors, the glycolytic enzyme glyceraldehyde-3-phosphate-dehydrogenase was shown to tightly bind several proteins involved in polyglutamine expansion diseases. Recently, the authors have shown that K191, K268, and K331, out of the 26 lysines present in glyceraldehyde-3-phosphate-dehydrogenase, are the reactive amine-donor sites forming cross-links with substance P, which bears the simplest Q_n domain (n = 2). The present study reports that synthetic peptides of both pathological and nonpathological length (n = 43 and 17, respectively) form cross-links with the same K residues located in the C-terminal region of glyceraldehyde-3-phosphate-dehydrogenase. In addition, it is shown that extra K residues present in the C termini of glyceraldehyde-3-phosphate-dehydrogenase are susceptible to cross-linking in the presence of transglutaminase. The present results indicate a possible modulating effect of Q_n stretches on tissue transglutaminase substrate specificity and mechanism of recognition.

Keywords: Glyceraldehyde 3-phosphate dehydrogenase; lysine residues; mass spectrometry; Q_n disease; transglutaminase

Reprint requests to: Carla Esposito, Dipartimento di Chimica, Università di Salerno, Via S. Allende, 84081 Baronissi, Salerno, Italy; e-mail: cesposito@unisa.it; fax: 0039-089-965296.

³Present address: Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli "Federico II," Naples, Italy.

Abbreviations: Asp-N, endoproteinase Asp-N; CD, circular dichroism; ESIMS, electrospray mass spectrometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GEE, glycine ethylester; HD, Huntington's disease; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Q, glutamine residue; K, lysine residue; LC/MS, liquid chromatography mass spectrometry; MALDI/MS, matrix assisted laser desorption ionization mass spectrometry; MDC, monodansylcadaverine; Q_n, polyglutamine repeat containing n Q residues; Q₁₇, peptide with sequence RPRPRQ₁₇RPRPR; Q₁₇*, peptide with sequence Q₁₇RPRPR; Q₄₃, peptide with sequence RPRPRQ₄₃RPRPR; Q₃₈*, peptide with sequence Q₃₈RPRPR; RP-HPLC, reverse phase high pressure liquid chromatography; TFA, trifluoroacetic acid; TIC, Total Ion Current; TG, transglutaminase; tTG, guinea pig liver tissue transglutaminase; Tris, Tris(hydroxymethyl)aminomethane.

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Transglutaminases (TGs) form a family of calcium-dependent enzymes, which catalyze an acyl transfer reaction between the γ -carboxamide T-group of a peptide-bound glutamine residue and the ϵ -amino group of a peptide-bound lysine, leading to an isopeptide bond (Folk and Finlayson 1977; Lorand and Conrad 1984). Nine different TG genes have been characterized in mammalian species so far (Grenard et al. 2001); however, the biological significance of some gene products is completely unknown. Although the specialized physiological function of the well-known TGs is to stabilize biological structures via cross-linking of proteins (Aeschlimann and Paulsson 1994; Aeschlimann and Thomazy 2000), recent data indicate that TGs are involved in neurodegenerative disorders such as Q_n/CAG repeat disorders, as well as Alzheimer's and Parkinson's diseases, and progressive supranuclear palsy (Kim et al. 2002).

Eight hereditary neuropathologies, such as Huntington's disease (HD), dentatorubral pallidoluysian atrophy, spinobulbar muscular atrophy, and spinocerebellar ataxias type 1, 2, 3, 6, and 7, are caused by the expansion of polyglutamine (Q_n) repeats in the encoded proteins (Paulson 1999; Perutz 1999; Zoghbi and Orr 2000). These proteins form insoluble intranuclear and cytoplasmatic aggregates in brains and in animal models of the disease, resulting in neuronal death and progressive neurodegeneration (Davies et al. 1997; DiFiglia et al. 1997; Paulson et al. 1997; Gutekunst et al. 1999; Li et al. 1999). In HD, such aggregates are formed when the expansion of Q residues exceeds a critical number ($n > 35\text{--}40$), leading to a "toxic gain of function" (Perutz 1999). It has been demonstrated that the expanded Q_n repeats are potent substrates for tissue TG (tTG) *in vitro*, resulting in the formation of covalently bonded aggregates with polypeptides that contain lysyl groups (Green 1993; Kahlem et al. 1996; Cooper et al. 1997a) with the rate of cross-linking increasing with the length of the glutamine repeats. It has been suggested that longer Q_n stretches not only increase the number of residues potentially available for tTG-mediated cross-linking, but also increase the apparent affinity of glutamines as tTG substrates, thus stimulating tTG activity (Cooper et al. 1999, 2002). Moreover, it was reported that the levels of expression and activity of tTG were increased in the cortex and cerebellum of HD patients (Lesort et al. 1999) simultaneously with significant increased levels of γ -glutaminyllysyl cross-links in nuclear inclusions of HD brain (Karpuj et al. 2002a), as well as in the cerebrospinal fluid of HD patients (Jeitner et al. 2001). Finally, overexpression of the tTG gene in human neuroblastoma cells strongly increases the number and the size of cellular aggregates (de Cristofaro et al. 1999), whereas the inhibition of tTG activity interferes with the accumulation of such cellular inclusions (Karpuj et al. 2002b).

To date, several key cellular proteins interacting with both normal and mutant polyglutamine-stretch-containing disease products have been described (Cha 2000). Such interacting proteins are candidate players in the pathogenesis of Q_n disease. In particular, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was shown to tightly bind several proteins involved in Q_n-expansion disease: huntingtin, dentatorubral pallidoluysian atrophy protein, ataxin-1, and the androgen receptor in human brain homogenates (Burke et al. 1996; Kosby et al. 1996). In addition, GAPDH was found covalently linked to the Q₆₀ sequence in a Balb-c 3T3 cell line overexpressing human tTG when the cell content was treated with the Q₆₀ polymer (Gentile et al. 1998).

Recently, we have reported the identification of GAPDH reactive lysines in a tTG-catalyzed reaction using Substance P peptide, a known tTG substrate (Esposito et al. 1995), which bears the simplest Q_n domain $n = 2$, (Orrù et al.

2002). K191, K268, and K331 were identified out of the 26 lysines present in GAPDH as the reactive NH₂-donor sites, thus indicating a high specificity of the tTG-catalyzed reaction. In the present paper, we describe further studies using synthetic peptides of pathological and nonpathological length ($n = 43$ and 17 , respectively). The aim of the present study is to verify whether tTG-mediated polymerization is a function of the glutamine-repeat length. In addition, we wanted to elucidate whether the different Q_n domains form cross-links with the same K residues involved in the presence of Substance P or determine an involvement of different lysines in tTG-catalyzed reactions.

Results

Characterization of Q₁₇ and Q₄₃ peptides

The chemical synthesis of Q_n peptides has proven to be a challenge, leading to polydisperse species as the number of glutamines in the sequence increases. Moreover, long Q_n stretches are prone to be insoluble and to form aggregates easily.

Two synthetic peptides of pathological and nonpathological length ($n = 43$ and 17 , respectively) were designed. The RPRPR sequence was chosen as the flanking region for both peptides in order to increase their solubility (Kahlem et al. 1996). To characterize the two synthetic peptides used in this work, a reverse phase HPLC and a MALDI/MS analysis of the two products were performed separately. The Q₁₇ species was dissolved as reported in a recent study on the solubilization of polyglutamine peptides (Chen and Wetzel 2000; Chen et al. 2001) and analyzed by HPLC. Its chromatographic profile revealed the presence of a very intense peak together with a very minor component. The two peaks were analyzed by MALDI/MS analysis. The mass spectrometric characterization of the major peak showed a unique component at m/z 3522.5, corresponding to the intact Q₁₇ peptide (molecular mass = 3521.8 D). On the other hand, the MALDI/MS characterization of the minor HPLC peak showed a heterogeneous mixture of synthesis fragments with masses <2000 D. Taken together, these results showed that the synthesis of Q₁₇ peptide yielded a $>90\%$ pure species.

As previously observed (Altschuler et al. 2000), the Q₄₃ peptide shows a very poor solubility. It was dissolved in a 1:1 HFIP/TFA solution as reported (Chen and Wetzel 2000; Chen et al. 2001). The solubilized peptide was then analyzed by HPLC. The chromatographic profile reveals two main peaks that were characterized by MALDI/MS. Mass data indicated that the first eluting peak corresponded to a broad mixture of synthesis fragments with masses <4000 D, whereas the last eluting HPLC peak contained a polydisperse species having an average molecular mass of 6444 ± 500 D (expected molecular mass of Q₄₃ peptide = 6853.2 D). As might be expected for peptides con-

taining a high number of Q in sequence, the Q₄₃ peptide did not appear to be a pure species, but its Q_n sequence ranged mainly from 36 to 43 residues, none of them being predominant.

The Q_n peptides were then submitted to an ultraviolet circular dichroism (CD). Q₁₇ was dissolved both in water and in 6 M guanidine. The CD spectrum of Q₁₇ run in water showed that the peptide consists of 25% α -helix, 32% β -sheets, and 43% random coil, whereas in the presence of 6 M guanidine, a 100% random-coil conformation was observed (data not shown). A high percentage of random-coil conformation was still monitored in an aqueous solution; however, it should be noted that polyglutamine peptides are able to adopt different conformations when flanked by different sequences (Perutz et al. 1994; Stott et al. 1995; Altschuler et al. 2000; Chen et al. 2001; Masino et al. 2002).

CD analysis of the Q₄₃ peptide indicates that it is in a random-coil conformation; however, the polydisperse nature of Q₄₃ makes CD data difficult to analyze. In any case, a detailed structural characterization of Q₁₇ and Q₄₃ using NMR or other techniques was out of the scope of this paper.

Reactivity of Q₁₇ peptide

Q₁₇ was incubated with the amino-donor glycine ethylester (GEE) in the presence of purified tTG. The reaction was monitored in a time-course experiment. The products of the tTG-catalyzed reaction were analyzed by MALDI/MS.

Figure 1 shows MALDI/MS spectra of the aliquots withdrawn at 1, 20, and 120 min. After 1 min, the MALDI spectrum shows the presence of the unmodified peptide together with four other signals corresponding to the Q₁₇ peptide covalently linked to one, two, three, and four molecules

Figure 1. MALDI/MS spectra of tTG catalyzed-reaction of Q₁₇ and GEE. Aliquots were withdrawn at 1, 20, and 120 min. In the insert of the spectrum registered after 120 min, an overview of the 4000–5000 *m/z* range is reported.

of GEE (molecular mass of GEE = 103.6 D). All the assignments are shown in Table 1. After 20 min, the MALDI spectrum shows that up to 12 GEE molecules were bound to Q₁₇ with a distribution centered on the species carrying 7 GEE molecules. After 120 min, up to 16 GEE molecules were found covalently linked to Q₁₇. The reaction profile did not change with prolonged incubation time. It has to be considered that every measured species could consist of a population of isomeric compounds, wherein different Q residues might be involved in the cross-links with the amino-donor substrate. These data hence indicate that all Q₁₇ glutamine residues are sterically accessible and reactive versus a small amino acceptor like GEE.

Q₁₇ was then incubated with a more sterically hindered amino-donor substrate such as monodansylcadaverine (MDC). The reaction was carried out by performing a time-course-MALDI/MS experiment as described above. The recorded MALDI mass spectra show that a growing number of MDC molecules are linked to the Q₁₇ peptide at increasing incubation times. After 120 min, up to 11 MDC molecules were added to Q₁₇. The reaction profile did not change with prolonged incubation times as observed in the previous experiment (data not shown). These results indicate that more than half of the glutamine residues in Q₁₇ were covalently linked to the bulky amino donor. However, a lower number of Q residues were involved in the cross-links formation, in comparison with the reaction in the presence of GEE. These results could be caused by the steric hindrance of MDC.

Similar experiments were carried out with Q₄₃ peptide to study its reactivity versus GEE or MDC. In both cases, the MALDI spectra showed a very complex mixture in which

each component of the polydisperse species had reacted with the amine-donor molecule. Experimental data indicated that GEE had an easy access to the Q₄₃ species, labeling from 30 to 35 Q residues after 120 min of incubation. A lower number of Q residues (20–25) reacted with the sterically hindered amino-donor substrate, thus confirming the trend observed with the Q₁₇ peptide.

Identification of reactive lysine residues of GAPDH in the presence of peptides containing nonpathological Q repeats

GAPDH was incubated with amino-acceptor Q₁₇ peptide in the presence of purified tTG. The mixture formed during the reaction, containing soluble and insoluble products, was hydrolyzed by endoproteinase Asp-N. The proteolytic enzyme produced a suitable digestion of GAPDH, as previously reported (Orrù et al. 2002). The resulting peptide mixture was separated by HPLC coupled on-line with an electrospray mass spectrometer (LCMS).

The Total Ion Current (TIC) profile of the peptide mixture is shown in Figure 2A. Every TIC fraction is related to a mass spectrum. TIC fractions containing the GAPDH peptides modified by Q₁₇ are numbered in Figure 2A. As an example, Figure 2B shows the mass spectrum related to fraction 1. The spectrum shows the presence of a species with mass 5663.4 ± 0.3 D. This signal cannot be assigned to any GAPDH peptide along the protein sequence on the basis of the molecular weight and the specificity of the enzyme. It was interpreted as GAPDH fragments D186–R194 and D323–E332 involved in the formation of a cross-link with a single Q₁₇ molecule via two different Q residues (expected molecular mass = 5664.3 D). Both GAPDH fragments contain a single K residue, K191 and K331, respectively, which were therefore identified unequivocally as tTG amino-donor sites. Table 2 shows the mass signals revealed by LCMS analysis and originated by the formation of cross-links between GAPDH peptides and Q₁₇.

Fraction 2 contains a molecular species with measured mass = 7210.5 ± 0.2 D. It was assigned to the two GAPDH fragments, D241–Y252 and D312–K331, covalently linked to the Q₁₇ peptide via two different Q residues (expected molecular mass = 7211.0 D). Fragment D241–Y252 contains two putative tTG amino-donor sites, K248 and K251, respectively, which could be involved in the formation of cross-links with Q₁₇ peptide. On the other hand, fragment D312–K331 contains a single K residue, K331, which is therefore unequivocally involved in the formation of the cross-link.

Fraction 3 contains a species with molecular mass = 5940.5 ± 0.4 D, corresponding to peptide D253–E275 covalently linked to the Q₁₇ peptide; the molecular mass shows the formation of four cross-links involving four different Q residues of the Q₁₇ peptide (expected molecular mass = 5942.6 D). K256, K257, K260, and K268, present

Table 1. MALDI/MS data of tTG-catalysed reaction of Q₁₇ and GEE

Signal	Mass (D)	Assignment
A	3522.5	Q ₁₇
B	3609.1	Q ₁₇ + 1 GEE
C	3695.7	Q ₁₇ + 2 GEE
D	3782.3	Q ₁₇ + 3 GEE
E	3868.9	Q ₁₇ + 4 GEE
F	3955.5	Q ₁₇ + 5 GEE
G	4042.1	Q ₁₇ + 6 GEE
H	4128.7	Q ₁₇ + 7 GEE
I	4215.3	Q ₁₇ + 8 GEE
L	4301.9	Q ₁₇ + 9 GEE
M	4388.5	Q ₁₇ + 10 GEE
N	4475.1	Q ₁₇ + 11 GEE
O	4561.7	Q ₁₇ + 12 GEE
P	4648.3	Q ₁₇ + 13 GEE
Q	4734.9	Q ₁₇ + 14 GEE
R	4821.5	Q ₁₇ + 15 GEE
S	4908.1	Q ₁₇ + 16 GEE

Letters refer to peaks shown in Figure 1.

Figure 2. (A) The LCMS chromatographic profile of Asp-N mixture generated by the in vitro tTG catalyzed reaction of GAPDH and Q₁₇. (B) Transformed ESMS spectrum of the fraction 1. Assignments of the mass signals to the corresponding peptides are shown.

in peptide D253–E275, were therefore unequivocally involved in the formation of cross-links.

Fraction 4 contains a species with molecular mass = $10,890.0 \pm 0.5$ D, which was assigned to GAPDH fragment D241–E275 linked to two Q₁₇ molecules (expected mass = $10,891.1$ D). Fragment D241–E275 contains six putative tTG amino-donor sites, K248, K251, K256, K257, K260, and K268, respectively, which could be involved in the formation of cross-links with the two molecules of Q₁₇ peptide.

Finally, fraction 5 contains a species with molecular mass

= 7757.0 ± 0.6 D, corresponding to GAPDH fragments D253–C281 and E264–T274 covalently linked with one Q₁₇ peptide (expected molecular mass = 7755.7 D). Peptide D253–C281 contains four putative tTG substrates, K256, K257, K260, and K268; whereas peptide E264–T274 contains a single K residue at position 268, which is therefore involved without ambiguity in the formation of cross-links. This mass signal shows the formation of a dimeric species involving fragments of two different GAPDH molecules covalently linked by the Q₁₇ peptide via two different Q residues.

Table 2. LC/MS data of Asp-N peptide mixture generated by tTG-catalyzed incubation of Q₁₇ and GAPDH

Fraction	Mass (D)	Assignment	Reactive K residues
1	5663.4 ± 0.3	D186–R194+Q ₁₇ +D323–E332	191, 331
2	7210.5 ± 0.2	D241–Y252+Q ₁₇ +D312–K331	248, 251, 331
3	5940.5 ± 0.4	D253–E275+Q ₁₇	256, 257, 260, 268
4	10890.0 ± 0.5	D241–E275+2Q ₁₇	248, 251, 256, 257, 260, 268
5	7757.0 ± 0.6	D253–C281+Q ₁₇ +E264–T274	256, 257, 260, 268

LCMS analysis was used to unambiguously identify K191, K256, K257, K260, K268, and K331 as tTG substrates and indicated the potential involvement of K248 and K251.

To confirm previous data, the reaction products were also hydrolyzed with trypsin, and the resulting peptide mixture was analyzed by LCMS. In this case, the proteolytic enzyme cleaved both GAPDH and the Q₁₇ peptide. Q₁₇ was hydrolyzed at the level of the first Q in sequence, producing a fragment Q₁₇RPRPR exhibiting a molecular mass of 2859.0 D. The fragment Q₁₇RPRPR was indicated with the abbreviation Q₁₇* in the following text. As a matter of fact, GAPDH fragments linked to Q₁₇* peptide were identified. Modified peptides of GAPDH identified by LCMS analysis are shown in Table 3.

Fraction 1 contains a species with molecular mass = 6841.9 ± 0.3 D, which was assigned to peptide K105–K114 linked to peptide L307–E332 via a covalent bond involving a molecule of Q₁₇* (expected molecular mass = 6840.5 D). The two peptides contain K110 and K331, respectively, involved in the formation of cross-links. The occurrence of a tryptic cleavage at the level of K114 ruled out the involvement of this residue in the formation of tTG-catalyzed cross-links. The same considerations can be extended to all the K residues reported in the following text whose peptide bond was cleaved by trypsin.

Fraction 2 contains two species with molecular masses of 5683.4 ± 0.4 D and 5021.2 ± 0.7 D, respectively. The first was assigned to GAPDH fragments L246–K260 and Y252–K260 linked via a cross-link with the Q₁₇* peptide (expected molecular mass = 5681.4 D). Peptide L246–K260 contains four putative tTG substrates, K248, K251, K256,

and K257; whereas peptide Y252–K260 contains two putative tTG substrates, K256 and K257. The presence of the cross-links between GAPDH peptides L246–K260 and Y252–K260 shows the formation of a dimeric species involving the fragments of two different GAPDH molecules covalently linked by the Q₁₇* peptide via two different Q residues. The species with molecular mass 5021.2 D contained in fraction 2 was interpreted as GAPDH fragments K105–K114 and K257–K268 linked via a cross-link with the Q₁₇* peptide (expected molecular mass = 5019.4 D). K110, contained in the peptide K105–K114, was unequivocally involved in the formation of the cross-link, whereas K257 and K260, contained in peptide K257–K268, could both act as tTG substrates.

Fraction 3 contains a species with mass = 4107.2 ± 0.4 D, which was assigned to GAPDH fragment K257–K268 covalently linked to Q₁₇* by means of two cross-links involving K257 and K260 (expected mass = 4108.5 D).

Finally, fraction 4 contains a species with molecular mass = 4713.5 ± 0.7 D, which was assigned to GAPDH fragments Y252–K257 and Y252–K260 linked via formation of a cross-link with the Q₁₇* peptide (expected molecular mass = 4715.2 D) involving K256 and K257. The occurrence of this cross-link confirms the presence of a dimeric species involving the fragments of two different GAPDH molecules.

Identification of reactive lysine residues of GAPDH in the presence of peptides containing pathological Q repeats

GAPDH was incubated in the presence of tTG with the Q₄₃ species with the aim of elucidating how the tTG-reactive

Table 3. LC/MS data of the tryptic peptide mixture generated by tTG-catalyzed incubation of Q₁₇ and GAPDH

Fraction	Mass (D)	Assignment	Reactive K residues
1	6841.9 ± 0.3	K105–K114+Q ₁₇ *+L307–E332	110, 331
2	5683.4 ± 0.4	L246–K260+Q ₁₇ *+Y252–K260	248, 251, 256, 257
	5021.2 ± 0.7	K105–K114+Q ₁₇ *+K257–K268	110, 257, 260
3	4107.2 ± 0.4	K257–K268+Q ₁₇ *	257, 260
4	4713.5 ± 0.7	Y252–K257+Q ₁₇ *+Y252–K260	256, 257

lysines are affected by a longer Q_n stretch. At the end of the reaction, the incubation tube contained a very large amount of insoluble products. The mixture was then submitted to treatment with a 1:1 HFIP/TFA solution as described previously (Chen et al. 2001), in order to improve solubility and to allow the subsequent enzymatic digestion to be more efficient. The treated mixture was submitted to two different extensive hydrolytic steps using endoproteinase Asp-N or trypsin as performed for the sample containing the Q_{17} peptide, described above. In contrast to what was observed with Substance P (Orrù et al. 2002) and Q_{17} (see above), the peptide mixtures obtained after the proteolytic digestions were characterized by the occurrence of a conspicuous amount of insoluble pellet. The peptide mixtures were then centrifuged, and the supernatant phases were then analyzed by LCMS. These analyses showed the presence of unmodified GAPDH fragments in the soluble fractions, whereas no signals related to a cross-link formation between the protein and Q_{43} species were found. The insoluble pellets were again submitted to a further treatment with a 1:1 HFIP/TFA solution in order to improve their solubility. The subsequent LCMS analyses showed the presence of GAPDH peptides modified by Q_{43} . However, they were characterized by a low TIC and very weak mass signals. It was therefore very difficult to obtain reliable data for the identification of GAPDH peptides involved in the formation of cross-links with Q_{43} peptide. It is also important to note that the Q_{43} peptide is not a single species but ranges from 36 to 43 residues, as reported before. Only two significant mass signals were observed, shown in Table 4. The peak at molecular mass = 9033.8 ± 0.7 D was assigned to GAPDH fragment D253–T274 (2359.7 D), covalently linked to the species RPRPR Q_{42} RPRPR (6725.1 D). The observed molecular mass showed the occurrence of three cross-links via three different Q residues (expected molecular mass = 9034.8 D). Fragment D253–T274 contains four putative tTG reactive lysine residues, K256, K257, K260, K268. On the basis of the molecular mass, it is possible to state that only three residues out of four are involved in the formation of the cross-links with the polyglutamine peptide. The species at molecular mass = $11,030.0 \pm 0.5$ D was assigned to GAPDH fragment V160–K212, covalently linked to the species Q_{38}^* (expected molecular mass = $11,029.0$ D). Q_{38}^* resulted from the tryptic hydrolysis of Q_{38} peptide at the level of the first Q in the sequence producing a fragment

Q_{38} RPRPR. Fragment V160–K212 contains two K residues, K183 and K191, one of them being involved in the cross-link. These data show that Q_{43} reacts with some of the same K residues that are involved in the formation of cross-links with its shorter homologous Q_{17} . However, the extreme insolubility of the samples under investigation only allowed a partial characterization of the cross-links formed in the presence of Q_{43} .

Three control incubations were carried out in order to understand the nature of the insoluble products formed in the tTG-catalyzed reaction between Q_{43} and GAPDH.

First, GAPDH was incubated alone under the experimental conditions described above. At the end of the incubation, the solution was centrifuged, and no pellet was observed.

In a second experiment, the Q_{43} peptide was incubated without tTG and GAPDH under the same conditions. At the end of the incubation, the solution was centrifuged, and the presence of a pellet was observed. The pellet was separated from the supernatant and submitted to treatment with a 1:1 solution of HFIP/TFA. This treatment allowed complete solubilization of the pellet.

Finally, the Q_{43} peptide was incubated with GAPDH without tTG under the conditions already described. At the end of the incubation, the mixture showed a pellet that was separated from the supernatant. The supernatant was hydrolyzed and characterized by LC/MS analysis as described above. This analysis showed the presence of GAPDH fragments together with signals of Q_{43} peptide. The pellet was submitted to treatment with a 1:1 solution of HFIP/TFA. This treatment led to a partial solubilization of the pellet, which was still visible in the test tube after a centrifugation step. These data indicate that GAPDH and Q_{43} form insoluble noncovalent aggregates, which are then cross-linked by the action of tTG (Perutz et al. 1994).

Discussion

The indications for the involvement of tTG in the etiology of $(CAG)_n/Q_n$ diseases, such as Huntington's disease (HD), depend on several pieces of evidence, such as the demonstration of elevated tTG activity in the affected regions of diseased brain and the colocalization of tTG with proteinaceous complexes in cells expressing truncated huntingtin (Lesort et al. 1999; Karpuj et al. 2002a; Kim et al. 2002). Furthermore, it has been demonstrated in vitro that the polyglutamine domains are excellent substrates for tTG, and the increased length of the Q_n stretches resulted in an increased ability of the peptide to be modified by tTG (Cooper et al. 2002). Several proteins have been identified that interact with huntingtin, including proteins involved in the cytoskeleton, membrane trafficking, and intercellular communication, as well as nuclear functions such as transcription and splicing (Cha 2000). Moreover, the glycolytic enzyme GAPDH was shown to tightly bind huntingtin and several

Table 4. LC/MS data of the peptide mixture generated by enzymatic hydrolysis of the tTG-catalyzed incubation of Q_{43} and GAPDH

Mass (D)	Assignment	Reactive K residues
9033.8 ± 0.7	D253–T274+RPRPR Q_{42} RPRPR	256, 257, 260, 268
11030.0 ± 0.5	V160–K212+ Q_{38}^*	183, 191

other proteins involved in polyglutamine-expansion disease (Burke et al. 1996; Kosby et al. 1996).

Recently, K191, K268, and K331 were identified out of the 26 lysines present in GAPDH as the reactive NH₂-donor sites in the tTG-catalyzed reaction (Orrù et al. 2002). The present paper reports the characterization of transglutaminase-catalyzed cross-linking between GAPDH and Q_n repeats of nonpathological ($n = 17$) and pathological ($n = 43$) length. The study confirmed that K191, K268, and K331 are, indeed, reactive NH₂-donor sites and led to the identification of K248, K251, K256, K257, and K260 as new electron-donor functionalities in GAPDH. Interestingly, both Q_n stretches form cross-links with the same K residues located in the C-terminal region of GAPDH. These results indicate that both Q_n stretches interact with the C-terminal domain of GAPDH. The structural characterization reported in this paper showed the occurrence of dimeric species involving different GAPDH molecules. The results indicate that Q_n domains can act as a bridge, via different Q residues, between two or more GAPDH molecules, giving rise to diverse multimers.

It is well known that a consensus sequence does not exist around the specific lysine residues acting as amino-donor sites in the tTG-catalyzed reaction; however, it has been suggested that the nature of the amino acid residues directly preceding the lysine may influence its reactivity. Indeed, uncharged, basic, polar, and small aliphatic residues enhance reactivity, which is decreased by residues such as D, G, P, H, and W (Groenen et al. 1994; Grootjans et al. 1995). In this context, the observed reactivity of GAPDH K248, K251, K256, K257, and K260 is in line with this trend. However, Substance P peptide, bearing the simplest Q_n domain ($n = 2$), was not able to recognize the above lysine residues as NH₂ donors, despite the fact that they are located in regions with sequences that should have enhanced their reactivity (Orrù et al. 2002). The observation that a higher number of GAPDH K residues are susceptible to cross-linking in the presence of tTG, when using Q_n stretches longer than Substance P, indicates that the interaction Q_n-GAPDH may cause conformational changes so that GAPDH becomes a better tTG substrate. Because insoluble proteinaceous deposits were formed with Q₄₃ and not with Q₁₇, a preferential interaction of GAPDH and Q₄₃ is indicated. The large formation of aggregates in the presence of pathological-length Q_n domains can be explained as a consequence of the peculiar kind of interaction between Q_n stretches and proteins. As proposed by Perutz (1999), the expanded Q_n repeats may interact with each other through a polar zipper, thus forming a nucleation site for noncovalent interactions in fibrillous aggregates containing Q_n stretches and interacting proteins. Such noncovalent aggregates would then become substrates for tTG.

Our findings (Orrù et al. 2002; data presented here) show that all the reactive lysines are present in the C-terminal

portion of GAPDH. Therefore, the hypothesis can be put forward that the selective reactivity of the GAPDH lysine residues could be caused by steric hindrance, which is the main factor in the recognition of lysine residues by tTG.

It is important to underline that the catalytic site of GAPDH is localized in the C-terminal domain of the enzyme (Sirover 1999). The fact that all the reactive lysines are located in this domain supports the previous observation that purified GAPDH was inactivated by tTG (Cooper et al. 1997b). This observation indicated that a slow decline in the energy metabolism of neuronal cells may trigger the degenerative process leading to cell death (Burke et al. 1996). Although a decrease in glycolysis seems to be ruled out in HD (Browne et al. 1997), there may be other aspects of GAPDH that are altered in HD brain. In fact, GAPDH has many other functions (apparently as a monomer), and a portion is found in the nucleus, where it appears to have some role in apoptosis (Sirover 1999).

Recently, it has been reported that the inactivation of GAPDH by tTG may contribute other than in HD, also in Alzheimer's disease (AD; Mazzola and Sirover 2001). In fact, tTG has been implicated not only in the formation of plaques and tangles in AD brain, but also may still play a significant role in AD by participating in apoptosis to cross-link proteins (Citron et al. 2001). Proteins not necessarily belonging to plaques, such as GAPDH and α -ketoglutarate dehydrogenase complex, two potential tTG substrates, might contribute to the progression of neurodegenerative disease, being inactivated by tTG in the presence of Q donors (Cooper 1997b). On the basis of the existing overlapping of aggregate composition in different diseases such as HD and AD involving both tTG and GAPDH, it is possible to suggest a key role played by tTG in the specific recognition of GAPDH.

Materials and methods

Materials

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (GAPDH), guinea pig liver tissue transglutaminase (tTG), glycine ethylester (GEE), monodansylcadaverine (MDC), dithiothreitol (DTT), α -cyano- α -hydroxycinnamic acid, and 1,1,1,3,3,3-hexafluor-2-propanol (HFIP) were purchased from Sigma. Q₁₇ peptide was purchased from Primm, whereas Q₄₃ peptide was purchased from Genemed Biotechnologies, Inc. Endoproteinase Asp-N sequencing grade and trypsin were purchased from Roche Diagnostic GmbH. RP-HPLC columns C₁₈ (25 $\times$ 0.21 cm) were purchased from Vydac (The Separation Group). All the other reagents and solvents were of the highest purity available from Carlo Erba. tTG was purified as described previously (Lee et al. 1989).

Chromatographic procedures

Chemically synthesized peptides were purified by performing an RP-HPLC analysis using a C₄ Vydac column (25 $\times$ 0.46 cm). The

eluting system consisted of 0.1% TFA (eluent A) and 0.1% TFA in acetonitrile (eluent B). Both Q₁₇ and Q₄₃ peptides were eluted by means of a linear gradient of eluent B in eluent A, from 5% to 95% in 45 min at a flow rate of 1 mL/min. Elution was monitored at 220 nm.

CD analysis of Q₁₇ and Q₄₃ peptides

Far-UV CD spectra were collected at 25°C on a Jasco J715 spectropolarimeter equipped with a Peltier thermostatic cell holder (Jasco model PTC-348), in a quartz cell of 0.1-cm light path. The temperature was measured directly in the quartz cell. Peptide solutions were filtered just before analysis on a 0.22-μm pore size PVDF membrane (Millipore) and baseline corrected. Spectra were recorded from 250 to 200 nm at 0.2-nm resolution, 16-sec response, at a scan rate of 20 nm/min. All data are the averages of three measurements, and the results are expressed as mean residue ellipticity [θ]. Sample concentration was estimated by quantifying the absorbance peak in reverse phase HPLC as already described (Chen et al. 2001)

Reactivity of Q₁₇ peptide

The reactivity of glutamine residues present in the Q₁₇ peptide was evaluated by incubating Q₁₇ peptide with glycine-ethylester (GEE) or with monodansylcadaverine (MDC). Both assays were carried out using 14.1 μg of Q₁₇ and 1.2 μg of GEE (molar ratio Q₁₇/GEE = 1/3) or 4.2 μg of MDC (molar ratio Q₁₇/MDC = 1/3) in the presence of 5 μg of tTG (molar ratio Q₁₇/tTG = 60/1). Assays were carried out in 125 mM Tris-HCl (pH 8.1), containing 10 mM DTT and 2.5 mM CaCl₂. Each assay mixture was incubated at 37°C in a total volume of 150 μL. Aliquots were withdrawn at 5, 10, 20, 30, 60, and 120 min. The reactions were stopped by freezing and then lyophilizing the samples. The mixtures were resuspended in 0.2% TFA and analyzed by MALDI/MS.

Identification of reactive lysine residues of GAPDH in the presence of peptides containing nonpathological and pathological Q repeats

GAPDH was incubated with Q₁₇ and Q₄₃ peptides using 50 μg of the protein and 6.4 μg of Q₁₇ (molar ratio GAPDH/Q₁₇ = 1/10) or 4.4 μg of Q₄₃ (molar ratio GAPDH/Q₄₃ = 1/10) in the presence of 5 μg of tTG (molar ratio GAPDH/tTG = 20/1). The reactions were carried out in 125 mM Tris-HCl (pH 8.1), containing 10 mM DTT and 2.5 mM CaCl₂. Each assay mixture was incubated at 37°C for 30 min. The reactions were stopped by freezing and then lyophilizing the samples.

Enzymatic and nonenzymatic hydrolysis

Samples were resuspended in 50 mM ammonium bicarbonate (pH 8.0). Asp-N hydrolysis was carried out using a 1/100 enzyme-to-substrate ratio (w/w) and 10% acetonitrile. Trypsin hydrolysis was carried out using a 1/50 enzyme-to-substrate ratio (w/w). Reactions were performed at 37°C for 18 h.

Samples deriving from the incubation of GAPDH and Q₄₃ were resuspended in 8 M guanidine and diluted to a 1 M guanidine final concentration with 50 mM ammonium bicarbonate (pH 8.0). The enzymatic hydrolyses were carried out as described above. The resulting peptide mixtures were lyophilized and then resuspended

with a 1:1 HFIP/TFA solution as previously described (Chen and Wetzel 2000; Chen et al. 2001). The peptide mixtures were then immediately analyzed by LCMS.

Mass spectrometry

MALDI mass spectra were recorded using a Voyager DE mass spectrometer (Applied Biosystem). A mixture of 1 μL of sample solution and 1 μL of α-cyano-4-hydroxycinnamic acid (10 mg/mL in acetonitrile/0.2%TFA = 7/3 [v/v]) was applied to the sample plate and allowed to dry. Mass calibration was performed using insulin (average molecular mass = 5734.6 D) and a matrix peak (379.1 D) as internal standard. Raw data were analyzed using computer software provided by the manufacturers and reported as average masses.

Liquid chromatography/mass spectrometry (LCMS) analyses were performed using an LCQ ion trap mass spectrometer (Finnigan Corp.), equipped with an electrospray ion source. The eluting system consisted of 5% formic acid, 0.05% TFA in water (eluent A), and 5% formic acid, 0.05% TFA in acetonitrile (eluent B). The 100-μL aliquots of each peptide mixture were injected onto an RP-HPLC C₁₈ column Vydac (25 × 0.21 cm; The Separation Group) and fractionated by performing a linear gradient of eluent B in eluent A from 5% to 65% B for 60 min, at a flow rate of 200 μL/min. Spectra were acquired from 500 to 2000 D. The resulting mass data were elaborated using the Excalibur software provided by the manufacturer. The mass range was calibrated using an ultramark solution provided by the manufacturer.

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