TISSUE TRANSGLUTAMINASE MEDIATED GLUTAMINE DEAMIDATION OF BETA-AMYLOID PEPTIDE INCREASES PEPTIDE SOLUBILITY, WHEREAS ENZYMATIC CROSS-LINKING AND PEPTIDE FRAGMENTATION MAY SERVE AS MOLECULAR TRIGGERS FOR RAPID PEPTIDE AGGREGATION.

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Tissue transglutaminase (TGase) has been implicated in a number of cellular processes and disease states, where the enzymatic actions of TGase may serve in both, cell survival and apoptosis. To date, the precise functional properties of TGase in cell survival or cell death mechanisms still remain elusive. TGase mediated cross-linking has been reported to account for the formation of insoluble lesions in conformational diseases. We report here that, TGase induces intramolecular cross-linking of beta-amyloid peptide (A$\beta$), resulting in structural changes of monomeric A$\beta$. Using high resolution mass spectrometry (MS) of cross-linked A$\beta$ peptides, we observed a shift in mass, which is, presumably associated with the loss of NH$_3$ due to enzymatic transamidation activity and hence intramolecular peptide cross-linking. We have observed that, a large population of A$\beta$ monomers contained an increase in mass of 0.984Da at a glutamine residue, indicating that glutamine Q$^{15}$ serves as an indispensable substrate in TGase mediated deamidation to glutamate E$^{15}$. We provide strong analytical evidence on TGase mediated A$\beta$ peptide dimerization, through covalent intermolecular cross-linking and hence the formation of A$\beta$$_{1-40}$ dimers. Our in depth analyses indicate that, TGase induced post-translational modifications of A$\beta$ peptide may serve as an important seed for aggregation.

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that is manifested as a gradual decline in cognitive function. The disease associated decrease in learning and memory accounts for the accumulation of the neuropeptide $\beta$-amyloid (A$\beta$). Several in vitro and in vivo studies have provided strong evidence that A$\beta$ peptide-induced loss in synaptic plasticity can be associated with A$\beta$ peptide oligomerization (1-4). Therefore, understanding peptide misfolding mechanisms plays a central role in trying to develop new therapies not only for AD, but also for other conformational diseases such as Parkinson’s (PD) and Huntington’s disease (HD). Several reports have suggested that tissue transglutaminase (TGase) may play a key role in the pathogenesis of AD (1,5-11). Moreover, post-mortem analyses of AD (12) and PD (13,14) brain tissue samples indicated the presence of intramolecular cross-links of TGase origin.

Aggregation of A$\beta$ peptide in AD and $\alpha$-synuclein ($\alpha$-syn) in PD is considered as a spontaneous property of these proteins, however, recent reports have raised the speculation that aggregation of these proteins may be strongly...
accelerated in the presence of TGase (1,15,16). Increased TGase activity appears to coincide with AD pathology (12) and, cerebrospinal fluid analysis for TGase levels (9), as well as changes in Aβ concentration and particular Aβ peptide fragment patterns have been suggested as useful biomarkers in AD (17).

TGase is an enzyme that catalyzes a calcium dependent transamidating reaction involving glutamine and lysine residues, which results in the formation of a covalent cross-link via ε-(γ-glutamyl) lysine bonds. TGase is localized in the cytoplasmic compartment of neurones (18) but it can also be found in nuclei and extracellular spaces (19). Its presence has been associated with cell survival and apoptosis (20), in which the recently identified serine/threonine kinase activity of TGase2 could be associated with the regulation of cell apoptosis (21).

Little is known about the functional role of TGase mediated cross-linking of Aβ peptide and the resulting changes in peptide structure and function in the pathogenesis of AD. Earlier studies have identified key regulatory residues as TGase substrates, using single and double site directed mutagenesis of Aβ peptide and α-syn protein (1,15,22). To date, no report provides strong analytical evidence on covalent Aβ dimer cross-linking. Moreover, it is known that, in the presence of suitable amine donors, TGase is able to form intramolecular cross-links in α-syn protein (15). To our knowledge, no report has focused on TGase induced glutamine deamidation and intramolecular cross-linking of Aβ peptide and its impact on peptide aggregation. In this study we have investigated TGase induced modifications of Aβ peptide and its associated effects on peptide aggregation, using high resolution mass spectrometry (MS). We provide strong analytical evidence that, TGase binding to Aβ peptide induces rapid and competitive deamidation of glutamine (Q15). Our findings suggest that, glutamine deamidation strongly increases the peptide’s solubility and hence reduces its affinity for aggregation. Using high resolution MS analysis of guinea pig and Chinese hamster ovary cell (CHO) TGase activity, we were able to provide analytical evidence on intramolecular cross-linking. We show that, intermolecular cross-linking of Aβ dimers is an activity predominantly found with guinea pig TGase. Collectively, our findings present an important repertoire of putative molecular triggers in the events of Aβ peptide aggregation. Our analytical approach by MS may serve as an indispensable tool and marker for future in vivo analyses of biological tissues. It is crucial to identify all possible biochemical and enzymatic reactions responsible in the formation of stable Aβ species, since Aβ dimers are known to form the building blocks of toxic protofibrils (23).

Experimental Procedures

β-amyloid peptide preparation. Aβ1-40 peptide (Bachem AG, Switzerland) and deamidated variant Aβ1-40 (E15) (Protein and Peptide Synthesis Facility, Biochemistry Department, University of Lausanne, Switzerland) were dissolved in hexafluoro-2-propanol (HFIP) at a concentration of 1 mg ml⁻¹, followed by 10 min sonication to break any preformed aggregates. HFIP was evaporated under a ventilated fume hood by applying a light stream of N₂ gas. The HFIP film containing the Aβ peptide was either directly re-suspended in a new buffer or stored at -20°C until use. Aβ peptide concentration was determined by UV absorbance at 280 nm, using the peptide’s theoretical molar extinction coefficient and by Micro BCA protein assay (Pierce, Thermo Scientific, Switzerland).

Solid-phase peptide synthesis. Aβ1-25 peptide was prepared by stepwise solid-phase synthesis (SPPS), using 9-fluorenylmethoxycarbonyl (Fmoc)-based and Wang resin techniques as described previously (24).

Guinea pig transglutaminase (TGase) cross-linking reactions. Synthetic human Aβ peptide wt or E15 variant (0.1 mg ml⁻¹) were cross-linked with 0.001 U of guinea pig tissue transglutaminase (TGase2, Sigma-Aldrich, Buchs, Switzerland) for 1 min to 1 h at 37°C unless otherwise stated in the results section. Reaction solutions contained 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM DTT, 25 mM HEPES, and 5 mM CaCl₂. For control samples, either CaCl₂ or enzyme was omitted from the reaction buffer, because preliminary experiments showed that the omission of CaCl₂ or TGase from the reaction produced similar results. Reactions were quenched
by the addition of excess EDTA (25 mM) and rapidly frozen at -80°C prior to analysis. The degree of transamidation (polymerization) was assessed by 15% and 18% Tris-Tricine SDS-PAGE, and liquid chromatography mass spectrometry (LCMS). Prior to gel loading, samples were mixed with standard Lämmli denaturing loading buffer and boiled at 100°C for 5 min.

**Far-UV circular dichroism spectroscopy.** Far-UV CD spectra were recorded at 25°C in a Jasco J-715 spectropolarimeter at peptide concentrations of 0.1 mg ml⁻¹ using a cuvette of 0.1 cm path length. The average of two spectra was collected for each sample in order to improve the signal-to-noise ratio. All spectra were corrected for the buffer baseline.

**Mass spectrometry.** Samples were infused at a flow rate of ~250 nl/min using a TriVersa NanoMate robotic ESI source (Advion BioSystems) (gas pressure 0.40 psi; ionisation potential 1.6-2.0 kV) equipped with a standard ESI chip (Advion, Ithaca, NY, USA). Ion detection was performed with a linear ion trap MS (LTQ XL MS, Thermo Scientific, Bremen, Germany). Samples (1.5-2 μg) were desalted and concentrated using C₁₈ Stage Tips (Proxeon, Odense, Denmark) and subsequently analyzed. Specific peptide sequence information was generated by collision induced dissociation (CID) of the ions of interest. The Ultra-Zoom SIM (single-ion monitoring) LTQ MS mode was used when higher resolution acquisitions were required. The GPMAW tool (version 7.1, Lighthouse data, Odense, Denmark) was used to generate a list of expected peptides from the different enzymatic digestions, as well as to compute their theoretical fragmentation patterns.

High resolution tandem mass spectrometry was performed on the hybrid LTQ Orbitrap FTMS (Thermo Scientific, Bremen, Germany) coupled with liquid chromatography or by direct infusion using TriVersa robot (as described above). Fragmentation of selected multiply charged ions was achieved by CID in the linear ion trap. Subsequently, product ions were transferred to the Orbitrap FTMS for high resolution detection. All samples were analyzed in a positive ion mode.

**In gel digestion of TGase and liquid chromatography LC-MS analysis of tryptic digests.** Gel pieces were first reduced and alkylated (DTE-Iodoacetamide). Proteolytic digestion was performed over night at 37°C using modified trypsin in 50mM ammonium bicarbonate (pH 8.3). Peptides were then extracted from gels and concentrated by speed vacuum prior to LC-MS measurements. Dried samples were resuspended in LCMS loading solvent (2% Acetonitrile, 0.1% formic acid) and separated by HPLC (nanoAcquity-Waters). Samples were first captured on a home-made capillary Pre-column (Magic C18; 3μm-200Å; 2cm x 100μm) prior to analytical separation. An 80 min biphasic gradient was run starting from 100% A solvent (2% Acetonitrile, 0.1% formic acid) to 90% B solvent (100% acetonitrile, 0.1% formic acid) on a homemade capillary column (Magic C18; 3μm-100Å; 12cm x 75μm ID at 250nl/min). Online mass spectrometric detection was performed on the hybrid LTQ Orbitrap FTMS.

**Data base searching for TGase sequences.** Tandem mass spectra were extracted and all MS/MS samples were analyzed using Mascot (Matrix Science, London, UK) database. Mascot was setup using the TGase fragments database assuming the digestion enzyme trypsin. Mascot and X! Tandem were searched with a mass tolerance of 0.50Da and a parent ion tolerance of 10.0ppm. Carbamidocysteine was specified in Mascot and X! Tandem as a fixed modification. Oxidation of methionine, acetylation of the N-terminus and phosphorylation of serine, threonine and tyrosine were specified in Mascot and X! Tandem as variable modifications.

**Criteria for protein identification.** Scaffold (v. 3.00.04) (Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at a greater than 95.0% probability. Protein identifications were accepted if they could be established a greater than 99.0% probability and contained at least 3 identified peptides.
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS). Aliquots (2µl) of intact or desalted samples were used for MALDI TOF/TOF MS (ABI 4800 model, Applied Biosystems, Rotkreuz, Switzerland) measurements. Matrix solution of a sinapinic acid (14 mg/ml in 0.1% TFA/ACN (1:1 v/v)) or alpha-cyano-4-hydroxycinnamic acid (7 mg/ml in ACN/0.1%TFA (1:1 v/v)) was used for sample deposition. The sample (1 µl) was mixed with 1 µl of matrix solution and then 1 µl of this mixture was deposited in duplicates on the target plate and allowed to air dry. Samples were analyzed in linear and reflectron modes. Theoretical isotopic envelopes of control, deamidated and transamidated Aβ peptides were drawn using the isotope calculator tool in Data Explorer Software (v.4.9).

SDS-Tris-Tricine polyacrylamide gel electrophoresis (PAGE) and gel staining. 15% or 18% gels were prepared using a standard Lämmli protocol. Voltage was set at 150 V at constant current for 70-90 min. Gels were stained with coomassie blue or silver stain as described previously (25).

SDS-PAGE and immunoblotting of Chinese hamster ovary (CHO) TGase. CHO lysate of 20µl volumes were mixed with standard Lämmli buffer and heated at 100°C for 5min prior to loading onto 12% SDS gels. The separated proteins were electroblotted onto a nitrocellulose membrane. The membrane was blocked for 1h at room temperature under constant rocking using Odyssey blocking buffer (Li-COR Biosciences, Bad Homburg, Germany) and diluted 1:3 in PBS. Membranes were incubated with the primary rabbit monoclonal antibody TGM2 (D11A6) (dilution: 1:1000) (Cell Signaling Technology, Switzerland) at 4 °C with constant rocking overnight. Membranes were washed four times with PBS-Tween (0.01% PBS containing Tween 20), followed by incubation with goat anti-rabbit IgM (dilution: 1:2000) conjugated to Alexa Fluor 680 and scanned in a Li-COR scanner at a wavelength of 700 nm.

Transmission electron microscopy (TEM). Samples of Aβ peptide (5 µl) were deposited on Formvar coated 200 mesh copper grids (Electron Microscopy Sciences, Hatfield, USA). Grids were washed with two drops of double-distilled H2O and stained with two drops of freshly prepared 1% (w/v) uranyl acetate (Electron Microscopy Sciences, Hatfield, USA). Specimens were viewed on a Philips CM10 electron microscope, operated at 80 kV. Digitized photographs were recorded with a slow-scan CCD camera.

Thioflavin T (ThT) fluorescence assay. A sample of Aβ peptide (50 µM, 300 µl) in 50 mM Tris-HCl 150 mM NaCl was incubated at 37°C. Samples for ThT readings and TEM were taken after 1-7 days of incubation, unless otherwise stated. Fibril formation was monitored by ThT fluorescence assay. Readings were carried out with both the peptide and ThT concentration of 5 µM each in 100 µl of 50 mM glycine-NaOH (pH 8.5). ThT fluorescence spectra were recorded on a Spex Fluorolog (Jobin-Yvon, Paris, France) at an excitation wavelength of 450 nm and an emission wavelength comprised between 460 - 600 nm. The relative fluorescence intensity at 485 nm was used as a measure of the amount of fibrillar aggregates formed in solution. Values are expressed as the percentage increase in ThT fluorescence of three independent measurements (means ± SD). Data were plotted using Prism Graph Pad (version 4.0).

Isoelectric Focusing (IEF) using the OFFGEL system: IEF of cross-linked Aβ1-40 was carried out as described before (26,27). Briefly, peptide samples were desalted and concentrated by protein phase separation and precipitation as described previously (28). Precipitated samples (pellets) were re-suspended in 50 µl of 0.2 M NaOH, followed by sonication for 15 min. The dissolved sample was then mixed with 3.6 ml of IEF sample buffer containing: 7 M urea, 2 M thiourea, 10% glycerol, 1% DTT and 1% carrier ampholytes. 24 x 150 µl of sample was loaded into a 24 well frame placed on a IPG strip (pH 3-10). Focusing was carried out at 4°C and at constant current [50 µA], with a linear voltage gradient up to 8000 V over 20 hrs. Following IEF, fractions at different IP’s (3-10) were collected into 1.5 ml eppendorf tubes and stored at -20°C prior to SDS-PAGE and MS analysis.
Assessing endogenous TGase activity in CHO cells. Chinese hamster ovary (CHO) cells were cultivated in DMEM/F-12 medium + 10% foetal calf serum (FCS) (Invitrogen). The cells were washed with PBS (Sigma Aldrich) and then collected by trypsination (0.05% trypsin-EDTA, 2min, Invitrogen) at a concentration of 1.2*10⁶-1.7*10⁶ cells/ml. Cells were centrifuged at 1200 rpm at 4°C during 4min and the supernatant was discarded. For TGase activity measurements CHO cells were re-suspended and lysed by 15min sonication in a total volume of 100µl buffer containing: 1% Triton, 150mM NaCl, 40mM Tris-HCl and protease inhibitors (Roche, Switzerland), pH 7.4. The reaction buffer (50µl) consisted of 40mM Tris-HCl, 150mM NaCl, 50mM Hepes pH 7.4 with Aβ1-40 peptide [12µM] (Bachem, Switzerland). The reaction was initiated by the addition of 2µl of CHO lysate and Ca²⁺ (5mM). Control conditions contained in addition 50mM EDTA.

Results

Tissue transglutaminase induces rapid polymerization of Aβ peptide. The appearance of stable Aβ dimers and trimers on the SDS-PAGE occurs within the first minute and increases over the time course of incubation (Fig. 1A). Low levels of stable tetramers can also be observed gradually, however, the trimeric and dimeric forms of this peptide seemed to dominate the presence of these soluble oligomers. Peptide morphology imaging by transmission electron microscopy (TEM) revealed an advanced peptide aggregation state, of a predominantly protofibrillar morphology following 60 min of incubation with TGase (Fig. 1B), whereas control incubations, in the absence of CaCl₂ or TGase did not result in peptide polymerization during the same time course of incubation (data not shown), which is an observation reported previously (1). Typical amyloid fibrils formation can be observed as early as 60 min, however, protofibrils appeared to be of the predominant peptide morphology, even after prolonged incubation times of 5 hrs and 24 hrs respectively (Fig. 1B). To determine if TGase mediated formation of dimers and trimers is dependent on peptide concentration, we carried out a series of cross-linking experiments at different peptide concentrations, ranging from 12 µM to 50 µM, but at constant TGase concentrations. We found that the formation of SDS-PAGE stable Aβ dimers is strongly dependent on initial peptide concentration, hence favoring a TGase mediated transamidation reaction, probably due to the close proximity of neighboring lysine residues serving as amide donors. Significantly increased amounts of SDS-PAGE stable dimer and trimer bands were observed at 50 µM Aβ peptide (Fig. 1C; lane 2), when compared with peptide concentrations at lower concentrations (lanes 3 and 4). No oligomerization was observed when CaCl₂ was omitted from the reaction buffer (Fig. 1C; lane 1).

The importance of lysine residues serving as amide donors in a TGase mediated Aβ peptide polymerization has been reported previously by Hartley et al., using a double mutant Aβ peptide with its two lysine residues (K¹⁶ & K²⁸) replaced with alanine residues (1). Lysine K¹⁶ appears to be indispensable for Aβ peptide polymerization, because mutation at lysine K²⁸ does not inhibit peptide aggregation (22). However, earlier studies did not provide sufficient analytical evidence of a transamidation reaction induced polymerization. Since a TGase mediated transamidation reaction is associated with the loss of ammonia (NH₃ → Δ mass = 17.026 Da), we were interested in analyzing the changes in mass of low molecular weight (LMW) oligomers using MS. This transamidation-associated loss of 17 mass units can be an ideal molecular tag in MS. MALDI TOF/TOF MS analysis in linear positive ion mode provides a quick screen of heterogeneous samples preparations (Fig. 2), prior to using more advanced analytical approaches by high resolution mass spectrometry. Therefore, transamidation may indeed have taken place, as suggested by the data shown in figures 1 and 2. However, the use of a large mass acquisition window in linear positive ion mode (4000 Da-15000 Da) may not meet the necessary mass accuracy for sound analytical proves. The measured average masses of the ions, potentially corresponding to the non-covalently formed dimers (av. m/z = 8662.53, [M+H⁺]) and trimers (av. m/z = 12997.07, [M+H⁺]) were found to be slightly increased when compared to the theoretical average masses of full-length Aβ dimers (av. m/z = 8660.72, [M+H⁺]) and trimers (av. m/z = 12990.58, [M+H⁺]), presumably due to
the large mass acquisition window selected (Fig. 2). A second population of dimer and trimer peaks was down-shifted by 17 mass units, which could account for a small population of intermolecular cross-linked peptides that formed dimers and trimers respectively. Acquisition in reflectron positive mode revealed the isotopic cluster of the Aβ monomer (monoisotopic peak at m/z = 4328.04 gives monomer monoisotopic mass ~4327.03 Da versus theoretical value of 4327.15 Da; 28 ppm mass accuracy) as well as a clearer analytical picture of its associated shift in mass (-17 Da) (Fig. 2; inset at top). This loss in NH₃ can be associated with intramolecular cross-linking of the monomer, similar to earlier reported findings with α-synuclein protein (15). It is important to note here that, sample preparation in acidic matrices for MALDI TOF MS analysis may result in breakdown of non-covalent oligomeric species, whereas increased peptide concentration, combined with increased laser intensity, may induce dimer and trimer artifacts.

TGase mediated deamidation of glutamine Q¹⁵ is highly competitive. To further probe for TGase mediated inter- and intramolecular cross-linking of Aβ peptide and to elucidate the peptide’s particular residues involved in these modifications we have carried out complementary analyses by direct infusion electrospray ionization (ESI) Orbitrap FTMS in conjunction with collision induced dissociation (CID). The outcome of these analyses provided additional important data on the TGase mediated intramolecular modifications found in Aβ peptide. In Fig. 3A we show a zoom of the measured isotopic envelope of unmodified, control Aβ (top; monoisotopic mass at m/z = 1082.79, [M+4H]⁴⁺ and most abundant isotopomer at m/z = 1083.30, [M+4H]⁴⁺) and TGase modified Aβ peptide (bottom: most abundant isotopomer at m/z = 1083.54, [M+4H]⁴⁺), indicating a shift in mass of 0.984 Da of the most intense peak (white arrow) following treatment with TGase. This shift in mass of 0.984 Da is highly associated with a TGase induced deamidation of glutamate at position Q¹⁵ resulting in the conversion to glutamate E¹⁵. We then applied CID in order to reveal the site of TGase mediated glutamine modification. For this purpose the peptide fragment of Aβ₁₋₄₀ following TGase treatment, and bearing the same mass shift of 0.984 Da, was selected as parent ion for CID (Fig. 3B). We selected this fragment to generate the smallest possible ions for identification and therefore reveal the residue involved in the mass shift of 0.984 Da. CID of this parent ion resulted in a host of b-type and y-type product ions of which y₉ ions (left zoom: m/z = 560.29, [M+2H]²⁺) and b₁₅ ions (right zoom: m/z = 905.38, [M+2H]²⁺) were of particular interest, since both fragments are associated with a shift in mass of 0.984 Da when compared to their theoretical, native fragments. As shown by the figure inset, the sequences of b₁₅ and y₉ ions overlap at residues H¹⁴ and E¹⁵ and therefore provide strong evidence for site specific deamidation at glutamine Q¹⁵. More importantly, we observed that conversion from glutamine (Q¹⁵) to glutamate (E¹⁵) now provides an additional cleavage site for Glu-C endoprotease proteolysis at residue E¹⁵, resulting in an additional proteolytic fragment of residues Aβ₁₆₋₂₂ as compared to control Aβ₁₂₋₂₂ (Q¹⁵) digests (data not shown). Similarly, we found that in substance P, glutamine Q³ is readily deamidated by TGase, whereas Q⁶ appeared to be a less favorable substrate, and glutamine deamidation to glutamate also introduces a new cleavage site for Glu-C proteolysis(29).

Lysine K²⁸ serves as a substrate in guinea pig TGase mediated intramolecular cross-linking and causes structural constraints in Aβ monomers. Since a transamidation reaction involves the loss of NH₃ from the peptide’s middle region, namely Aβ₁₋₄₀ following TGase treatment, and bearing the same mass shift of 0.984 Da, was selected as parent ion for CID (Fig. 3B). We selected this fragment to generate the smallest possible ions for identification and therefore reveal the residue involved in the mass shift of 0.984 Da. CID of this parent ion resulted in a host of b-type and y-type product ions of which y₉ ions (left zoom: m/z = 560.29, [M+2H]²⁺) and b₁₅ ions (right zoom: m/z = 905.38, [M+2H]²⁺) were of particular interest, since both fragments are associated with a shift in mass of 0.984 Da when compared to their theoretical, native fragments. As shown by the figure inset, the sequences of b₁₅ and y₉ ions overlap at residues H¹⁴ and E¹⁵ and therefore provide strong evidence for site specific deamidation at glutamine Q¹⁵. More importantly, we observed that conversion from glutamine (Q¹⁵) to glutamate (E¹⁵) now provides an additional cleavage site for Glu-C endoprotease proteolysis at residue E¹⁵, resulting in an additional proteolytic fragment of residues Aβ₁₆₋₂₂ as compared to control Aβ₁₂₋₂₂ (Q¹⁵) digests (data not shown). Similarly, we found that in substance P, glutamine Q³ is readily deamidated by TGase, whereas Q⁶ appeared to be a less favorable substrate, and glutamine deamidation to glutamate also introduces a new cleavage site for Glu-C proteolysis(29).

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involved in Aβ insight into the possible molecular mechanisms analytical data has been generated to provide TGase inhibitors (1,22). However, to date, no site directed mutagenesis of lysine residues or oligomers and by inhibiting oligomerization, using showing the presence of SDS-PAGE stable LMW species during SDS-PAGE analysis. We sought to overcome this phenomenon by enriching and protofibrils may eventually break down to smaller formation of protofibrils (Fig. 1), and that these of large aggregates, such as the above reported use of the OFF-GEL system provides an ideal tool for such purposes, since sample focusing is carried out under denaturing conditions, and thus is indispensable for dissociating larger, non-covalent aggregates from smaller, covalently cross-linked species. For this purpose we up-scaled our cross-linking experiments with 3 x 1 ml of Aβ1-40 [0.01 mg ml⁻¹] and TGase, in order to recover sufficient cross-linked material after IEF. Following cross-linking, samples were mixed and re-concentrated using 3 kDa MWCO filters, prior to re-suspension in IEF buffer. As expected, IEF resulted in a focusing of Aβ dimers in a fraction of pl 4.5 (Fig. 4A). FTMS analysis of this fraction also revealed the high abundance of deamidated Aβ monomers (m/z = 1083.54, [M+4H]⁴⁺ and 867.03, [M+5H]⁵⁺) (Fig. 4B). The two peptide fragments of Aβ1-34 and Aβ1-22 with deamidation at E15 (pl 4.84 and pl 4.8 respectively) (spectra not shown), as well as some trace amounts of intramolecular cl Aβ monomers (Fig. 4B, zoom: m/z = 1078.54 [M+4H]⁴⁺) were also found in this fraction. The pI value of this fraction is in close agreement with the calculated pI value of deamidated Aβ1-40 (E15) of 4.84. More importantly, this fraction contained a substantial amount of cross-linked Aβ dimers as can be seen by the presence of its corresponding gel band (Fig. 4B inset). Using FTMS, these dimers were detected at very low abundance at m/z = 1440.69 [M+6H]⁶⁺ (Fig. 4B). Nevertheless, by plotting the theoretical isotopic envelope of this dimer, we can confirm, for the first time, that these stable dimers are the result of TGase mediated intermolecular cross-linking. The measured isotopic distribution (Fig. 4C; bottom) matches the theoretical distribution (Fig. 4C; top), of which the calculated mass includes the loss of NH3 (-17.02 Da) as a byproduct of the transamidation reaction. Our MS findings on cross-linked Aβ1-40 dimers therefore confirm above reported observations on Aβ dimers found by SDS-PAGE analysis and are in agreement with earlier reports (1,22). Stable dimerization was also observed with a synthetic Aβ1-25 fragment (Fig. 5), where only one lysine (K16) residue serves as an amide donor, which further proves that this residue is indispensable for TGase mediated inter-molecular cross-linking of Aβ. Cross-linked Aβ1-40 dimers proved to be extremely stable, since CID MS/MS generated product ions only from a far C-terminal part of the peptide, e.g., b38 and b39, in addition to the frequently observed b₁ ion (supplemental Fig. I). CID of the Aβ1-25 dimer was more efficient probably due to higher peptide concentrations (Fig. 5; grey inset). It is a general observation in CID MS/MS of peptides and proteins that fragmentation efficiency, in terms of sequence coverage, drastically reduces with increased protein molecular weight. The use of the Aβ1-25 fragment peptide for studying intermolecular cross-linking proved to be indispensable, since intramolecular cross-linking was no longer observed with this fragment peptide. Therefore,
lysine K¹⁶ is not a suitable substrate for cross-linking to its adjacent glutamine Q¹⁵ residue probably due to sterical hindrance. Similar findings were reported with α-syn protein, where lysine K³⁰ is not cross-linked to its adjacent glutamine Q⁷⁹ residue, whereas Q⁷⁹ appeared to be a favorable substrate for TGase (15).

TGase induced Aβ peptide aggregation is associated with increased peptide fragmentation. Incubation times of up to 24 hrs or longer resulted in increased Aβ peptide fragmentation. This observation is based on the fact that, following IEF, a large pool of Aβ peptide fragments can be found in a fraction corresponding to their isoelectric point. A fragment of particular interest involves Aβ₁₋₂₂, which was found enriched in fraction pl 4.8, corresponding to the fragment’s calculated pl with deamidated glutamine (E¹⁵) (spectrum not shown). The “native” form of this fragment Aβ₁₋₂₂ (Q¹⁵) has a theoretical pl of 5.21 and was found in the same fraction as full-length wt Aβ₁₋₄₀ (pl = 5.25) (supplemental Fig. II), along with shorter Aβ fragments: Aβ₁₋₁₇, Aβ₁₋₁₈, Aβ₁₋₁₉, Aβ₁₋₂₀ and Aβ₁₋₂₁, with similar isoelectric points (pl = 5.21). In order to elucidate the impact of incubation time on Aβ peptide fragmentation, we carried out long time incubations of up to 8 days using a synthetic, deamidated variant Aβ₁₋₄₀ (E¹⁵), in the presence and absence of TGase. The advantage of using this variant is that, E¹⁵ Aβ peptide no longer serves as a substrate for TGase cross-linking reactions and therefore allows to study the sole events of peptide fragmentation in the presence of TGase. Incubated samples were analysed each day by MALDI TOF MS and a summary of the most relevant fragments observed is presented in supplemental Fig. III.

Glutamine deamidation decreases Aβ peptide affinity for fibril formation. The rapid aggregation propensity of Aβ peptide in the presence of TGase is an observation widely reported (1,30). It is believed that peptide aggregation may be triggered by the presence of “molecular seeds”, which could form a building block of larger aggregates. To determine whether glutamine (Q¹⁵) deamidation in Aβ monomers serves as a seed in peptide aggregation we investigated the aggregation ability of a synthesized variant Aβ₁₋₄₀ (E¹⁵) peptide. For this purpose, control (wt) Aβ₁₋₄₀ (Q¹⁵) and variant Aβ₁₋₄₀ (E¹⁵) [0.1mg ml⁻¹] were incubated separately at 37°C during 7 days. Under these conditions control Aβ₁₋₄₀ (Q¹⁵) formed typical amyloid fibrils within a few days, which was consistent with the gradual increase in Th T fluorescence measured (% Δ AU 254 ± 133, n=3) (Fig. 6). TEM imaging of control Aβ₁₋₄₀ (Q¹⁵) peptide revealed predominantly short fibrillar assemblies (200-300 nm length). Fibril formation was significantly reduced in the deamidated variant (E¹⁵) peptide over the same time course of incubation and this was in agreement with its lower levels of Th T fluorescence found (% Δ AU 9 ± 8, n=3). TEM imaging of the deamidated Aβ peptide did not show any major difference in fibril length or morphology, when compared with control Aβ peptide. Moreover, the decreased aggregation propensity observed with Aβ₁₋₄₀ (E¹⁵) peptide seemed to correlate with the remaining level of monomers found in the SDS-gel. Incubations of mixed Aβ peptide (Aβ Q¹⁵: Aβ E¹⁵) preparations at ratios of 1:1, 1:5 and 1:10 did not increase the speed of Aβ Q¹⁵ aggregation (data not shown). This suggests that glutamine deamidation per se does not account for the increased peptide aggregation in the presence of TGase.

Glutamine deamidation induces structural limitations in secondary structure transition. To further investigate the ability to induce changes in the peptide’s secondary structure following glutamine deamidation, Aβ₁₋₄₀ (E¹⁵) peptide was subjected to dissolution in trifluoro-ethanol (40% TFE / H₂O) and acetonitril (30% ACN / H₂O), in order to monitor the peptide’s properties to change its secondary structure. Changes in secondary structure of control and variant Aβ peptides were monitored by circular dichroism spectroscopy (CD), whereas solution induced changes in peptide morphology were monitored by TEM imaging. Both, control and deamidated variant peptides retained a random coil structure in a physiological solution (Tris-NaCl 25 mM/150 mM pH 7.4) (Fig 7A). Dissolution of control and deamidated Aβ peptide in TFE solution induced immediate and stable formation of typical α-helical structure in both peptides (Fig. 7A). The strength of α-helical structure was found to be increased in control Aβ peptide and seemed to retain over 4 days of incubation in TFE solution, whereas the CD signal
of deamidated variant Aβ peptide shifted to a typical β-sheet structure over the same time of incubation. Distinct morphological differences were observed with deamidated variant peptide, consisting of small aggregates with little protofibrillar structure, whereas control peptide formed typical amyloid fibrils, of straight and long morphology (2-4 µm) with few helical twists. Dissolution of control Aβ in ACN induced a rapid change from random coiled to typical β-sheet structure and remained stable over 2 days (Fig. 7B). However, β-sheet formation in deamidated Aβ was clearly suppressed even after 2 days of incubation. Following 4 days of incubation in ACN, both Aβ peptide samples showed strongly reduced ellipticity at a wavelength of 218 nm, indicating that advanced peptide aggregation or precipitation had taken place. Fibrillogenesis was more evident in ACN solution with the deamidated variant peptide, as compared to TFE solution; however, these fibrils appeared generally shorter and lacked lateral fibril association, as was generally found with control Aβ.

Changes in Aβ peptide morphology inhibits TGase substrate accessibility. Our findings above clearly indicate that glutamine deamidation is associated with reduced Aβ peptide aggregation. Based on these findings, we were interested in investigating the possible role of enzymatic dis-aggregation of Aβ fibrils through TGase mediated glutamine deamidation. For this purpose, control Aβ1-40 (Q15) and variant Aβ1-40 (E15) peptides were subjected to 7 days incubation at 37°C without TGase. Following 7 days of incubation, both samples were subjected to TGase cross-linking during 24 hrs and changes in peptide morphology were monitored using TEM imaging and SDS-PAGE, prior and after the addition of TGase. As expected, incubation of control and variant Aβ1-40 (E15) peptide resulted in the formation of abundant fibrils after seven days (Fig. 8; above). TGase cross-linking, however, did not result in a change of the predominantly fibrillar peptide morphology. It seemed that, pre-aggregated wt Aβ peptide was no longer available as a TGase substrate (Fig. 8; below: lane 5), whereas freshly prepared, soluble Aβ peptide readily formed typical low molecular weight aggregates, which migrated on the gel at the level of dimers, trimers or even tetramers (positive control, lane 6). TGase cross-linking activity in control Aβ (lane 6), was accompanied by the typical accumulation of protofibrillar and little fibrillar peptide morphology, whereas no changes in peptide morphology or fibril dissociation was observed in pre-aggregated control and deamidated Aβ peptides following TGase incubation (TEM images not shown). These findings indicate that changes in peptide morphology, as well as its associated changes in secondary structure strongly impede TGase substrate accessibility.

TGase induced Aβ peptide aggregation is not inhibited by the polyphenol myricetin. Several phenolic compounds, namely wine-related polyphenols, such as tannic acid (TA), curcumin (Cur) or myricetin (Myr) have been demonstrated to affect Aβ aggregation (31,32). It is well accepted fact, that such natural compounds may have the potential to reduce peptide induced cell toxicity. In order to investigate the inhibitory capacity of myricetin in the presence of TGase, control and deamidated variant Aβ peptide [0.1mg ml⁻¹] were subjected to TGase cross-linking in presence of myricetin [100 µM]. Following incubation at 37°C for 1 and 12 hours, samples were analyzed for levels of remaining monomers and typical low molecular weight oligomers using SDS-PAGE. Interestingly, TGase mediated aggregation of control Aβ1-40 (Q15) peptide remained highly increased in spite of the presence of Myr, and was manifested by a decrease in level of monomer bands (Fig. 9; lanes 3 & 7). It appeared that peptide aggregation was even strongly favored in the presence of Myr, since no monomers could be detected following 12 hours of incubation (lane 7) as compared to control Aβ1-40 (Q15) samples, were Myr was omitted from the reaction solution (lanes 4 and 8). More importantly, increased aggregation was also observed when calcium was omitted from the reaction solution (lanes 2 and 6), although, aggregation of this sample was slightly slower. TEM imaging of Aβ1-40 (Q15) peptide in the presence or absence of Myr, formed highly ordered fibrils of amyloid nature. Though, it seemed that in the presence of Myr, fibrils appeared as a dense cluster, which may account for the increased stability observed during SDS-PAGE analysis. The residual levels of Aβ
monomers found in the absence of Myr, could account for the usual level TGase induced deamidation (lanes 4 and 8). This observation is corroborated by the fact that, in deamidated Aβ peptide (E15) and the level of monomers remained unchanged following 12 hours of incubation (lanes 1 and 5). This strongly indicates that Myr induced aggregation is highly glutamine (Q15) dependent, whereas glutamate (E15) clearly impedes aggregation, probably due to the insertion of a site specific negative charge.

**Investigation of endogenous TGase cross-linking activity in CHO cells.** To investigate the functional role of endogenous TGase cross-linking activity, we selected a Chinese Hamster Ovary (CHO) cell line. CHO cells and neuroblastoma SH-SY5Y cells have been reported to express substantial endogenous TGase activity and TGase expression can be up-regulated in response to retinoic acid (RA) stimulation (33,34). MS analysis of Aβ1-40 peptide in the presence of CHO cells indicated that, endogenous levels of TGases are sufficiently active to induce stable Aβ peptide modifications. This was manifested by a rapid peptide bond fragmentation after residue G25 giving rise to the new fragment of Aβ1-25 (Fig. 10 A), which is an observation also made with guinea pig TGase. Following 10 min of incubation, this Aβ1-25 fragment was irreversibly modified by CHO TGase and the reaction appeared to be highly Ca2+ dependent, since it could be inhibited in the presence of 50mM EDTA (supplemental fig IV). Using nano-liquid chromatography coupled with high resolution MS (LC-MS) analysis we were able to reveal that, in contrast to guinea pig TGase, CHO TGase has a high affinity for intramolecular peptide cross-linking. This resulted in a shift in mass of both, Aβ1-40 m/z = 863.0732 [M+5H]+ (Fig 10B: bottom) and its fragment Aβ1-25 m/z = 972.5413 [M+3H]+ (Fig. 10C middle). However, these measured masses do not correspond exactly to the calculated mass of a typical transamidation reaction with an expected mass shift of 17.02Da (-NH3) (Fig. 10B & C top spectra). The monoisotopic masses for Aβ1-40 and Aβ1-25 are shifted by 0.2065Da (Fig 10B top spectrum) and 0.2709Da (Fig. 10C top spectrum) respectively. Deamidation was only observed in the Aβ1-25 fragment m/z = 978.5272 [M+3H]+ (Fig 10C middle spectrum, and supplemental fig. IV) but not in full-length Aβ1-40. Interestingly, this mass was also shifted (Δ mass = 0.2181Da) when compared to the calculated deamidation induced shift in mass m/z = 978.4545 [M+3H]+ (Fig 10C top spectrum). A large population of Aβ1-40 peptide remained unmodified after only 10 min of incubation (Fig. 10B middle spectrum) m/z = 866.4431 [M+5H]+, which corresponds to calculated Aβ1-40 mass of m/z = 866.4367 [M+5H]+ (Fig 10B top spectrum) (Δ mass = 0.032Da).

Fragmentation by CID no longer produces the typical “b & y” ion fingerprint found with control Aβ peptide or guinea pig TGase treated Aβ (data not shown), probably due to some unknown intramolecular peptide rearrangement. This may account for the difference in mass of ~0.2Da observed and may therefore explain the poor fragmentation behavior found by CID. This data strongly indicates that CHO TGase activity is to some extent distinct from guinea pig TGase, by showing strong preference for intramolecular cross-linking hence resulting in a structural rearrangement, similar to earlier reported peptide cyclization found in polypeptide antibiotics (35). This putative structural difference will be subject of our future studies, using electron transfer dissociation combined with CID fragmentation, in order to elucidate any intramolecular rearrangement following cross-linking with different TGase isoforms. Collectively one can state that, following in vitro and in situ cross-linking, the recovery and preparation of aggregated Aβ peptide for MS analyses is a difficult task, mainly due to poor peptide solubility and affinity binding to cell membranes and surfaces. This may explain why we could not detect any cross-linked dimers within the same sample preparations. Furthermore, it is reasonable to assume that Aβ may have been cross-linked to other cellular proteins, hence increasing sample complexity. Nevertheless, we were able to induce stable monodansylcadaverine (MDC) incorporation in Aβ peptide, through a transamidation reaction, using intact CHO cells (data not shown). MDC cross-linking with glutamine residues in Substance P in vitro has been recently been reported using the electron capture dissociation (ECD) analytical approach (29).
In order to further associate Aβ peptide modification with endogenous CHO TGase activity, we carried out immunoblotting on CHO cell lysates using a TGase monoclonal antibody. The presence of TGase was confirmed in two different CHO cell cultures and the results are presented in Figure 10D. In addition, guinea pig and CHO TGase were subjected to SDS-PAGE analysis. Gel bands (A to D) were cut at the migration height of guinea pig TGase (control) followed by trypsin proteolysis and LCMS/MS analysis to further elucidate any TGase sequence homology between CHO TGase or any other known TGase isoforms. MS/MS data analysis of tryptic digests resulted in a 72% sequence coverage for guinea pig TGase (control, band A) (Fig 10D; lane 5 and supplemental table I). In addition to band A, four more bands (B to D) at the migration level below A were cut, in order to identify any contamination, such as other proteolytic enzymes present in guinea pig (lane 5) or CHO TGase (lanes 6 & 7), that could account for the above reported Aβ peptide cleavage. MS/MS analysis of the trypsin digested bands only revealed the presence of TGase fragments in these additional gel bands (sequence coverage in bands, B: 53%, C: 28%, D: 14%, E: 26%) probably due to degradation occurring during sample boiling prior to SDS-PAGE analysis. No other known proteases were identified within these bands using the MS identification and selection criteria outlined in the methods.

Since the sequence of CHO TGase has not been mapped to date we were only able to assign identical fragments and hence compare them to sequence homology with respect to guinea pig TGase or any other known TGase isoforms. A total of 10 tryptic fragments were identified in CHO cells, bearing identical sequence homology to guinea pig TGase. No sequence homology could be associated with other known TGase isoforms also from different species. A complete list of all assigned fragments is provided in supplemental table I.

TGase mediated post translational modifications (PTM) and peptide fragment heterogeneity may collectively account for increased peptide aggregation. Fig. 11A summarizes our findings on TGase mediated cross-linking of Aβ peptide. The major TGase activities are classified by two main pathways, in which we distinguish between deamidation (left pathway; “a” & “b”) or transamidation (right pathway; “c” & “d”) reactions. Pathways “a” and “b” both result in a change in mass of 0.984 Da. The less favorable modification (“b”) involves the formation of “iso-glutamate (E15)” with an identical shift in mass, which may arise via a transient “glutarimide intermediate”, consisting of a labile six-membered ring structure (36). Aβ peptide isomerization at aspartyl residues D7 and D23 (37) as well as razemerization at serine (S26) (38,39) are well known PTM found in AD post-mortem brain tissue and these PTM’s have been shown to independently influence peptide aggregation behavior. Distinguishing the isoforms of amino acids in peptides, namely D versus iso-D as well as E versus iso-E, is now possible using electron capture dissociation (ECD) or electron transfer dissociation (ETD) tandem mass spectrometry by detecting signature product ions (40,41). Here, using the CID-based tandem mass spectrometry only, we cannot rule out the possibility of having residual amounts of iso-glutamate (E15) (pathway; “b”) or racemized glutamate (D-E15), which may display a completely different aggregation behavior as observed with the E15 Aβ variant peptide. Residue racemization is generally believed to induce changes to the peptide’s backbone structure which in turn may account for the increased peptide aggregation observed in the presence of TGase.

The right pathway sub-categorizes a transamidation reaction of either intramolecular (“c”) or intermolecular (“d”) nature. The structural constraints associated with intramolecular cross-linking is believed to decrease the propensity for aggregation (15). However, intermolecular cross-linking of Aβ dimers (“d”) may act as a molecular trigger in the early events of peptide aggregation and neurotoxicity.

Discussion

In order to clarify the significance of Aβ oligomerization in AD pathogenesis, it is important to identify key regulatory enzymes or molecular triggers that drive Aβ peptide oligomerization and its associated neurotoxicity. Numerous efforts have been undertaken to
determine the functional role of TGase activity in the pathogenesis of conformational diseases and the majority of these studies have identified key regulatory residues, using single and double site directed mutagenesis (1,15,22). In this work we sought to identify the regulatory mechanisms of TGase associated cross-linking of Aβ peptide using MS analysis. We provide insight into the highly competitive TGase activity found in the presence of Aβ peptide. Our findings clearly show that, Aβ peptide dimerization may derive from a TGase mediated transamidation reaction of glutamine Q 15 with a lysine K 16 residue of an adjacent monomer, hence forming covalently cross-linked dimers. Understanding the generation and properties of Aβ dimers is of fundamental scientific interest, since these species are known to form the building blocks of synaptotoxic protofibrils (23).

Previous work by Palmblad et al (42) reported that Aβ trimers and tetramers could only be detected at high concentrations, whereas detection of dimers and monomers was still possible at 4 nM, using ESI Fourier Transform Ion Cyclotron Resonance MS (FT-ICR MS). In this work we did not detect any stable trimers or tetramers by high resolution MS, although SDS-PAGE and MALDI TOF MS analysis indicated the low abundance of these soluble oligomeric species. Although dimerization of fragment Aβ1-25 was readily detected by MS, we were not able to favor cross-linking beyond dimers. It is possible that LMW oligomers rapidly form part of larger aggregates and are therefore mainly detected as a result of dissociation during SDS-PAGE analysis. We cannot rule out the possibility that, due to structural complexity in trimers and tetramers, lysine K 28 compensates as a substrate for lysine K 16, which would explain that cross-linking of fragment Aβ1-25 was limited to dimers only.

The neuroprotective role of the polyphenol myricetin (Myr) is known to act through inhibition of conformational changes in Aβ peptide monomers (43). Interestingly, we found that TGase induced formation of dimers and trimers was inhibited in the presence of Myr, whereas fibrillogenesis of wt Aβ1-40 was significantly increased. A recent study has associated the inhibitory effects of Cur and Myr at the monomer level by preventing the formation of A11-positive oligomers (31). These authors have suggested that, in the presence of oligomers, Myr has a strong seeding effect, by accelerating the formation of Aβ fibrils. These reports are in absolute agreement with our findings, since we did not detect any LMW oligomers, but a striking increase in fibril formation in the presence of TGase and Myr. We conclude that, glutamine Q 15 plays a crucial role in the events of Aβ peptide misfolding, since the Aβ E 15 variant peptide displays completely different properties under identical reaction conditions.

TGase mediated intramolecular cross-linking appears to be a highly favorable modification found in α-syn protein (15). More importantly, these earlier findings showed that, intramolecular cross-linking caused structural constraints in α-syn, hence preventing fibrillogenesis and therefore increasing protein solubility. To our knowledge no report has identified the presence of intramolecular cross-links in Aβ peptide before. Our MS data provide strong analytical evidence of intramolecular cross-linking in Aβ1-40 peptide, whereby lysine K 28 and K 16 serve as indispensable substrates for this transamidation reaction. Lysine K 16 appears to be a preferential amine donor for intramolecular cross-linking of Aβ1-25 by CHO TGase and this modification was associated with some putative structural rearrangement of Aβ peptide. In the presence of guinea pig TGase however, no intramolecular cross-linking could be observed in fragment Aβ1-25. Instead, glutamine deamidation appears to be a preferential PTM found with the guinea pig TGase isoform, which can be easily confirmed by CID fragmentation.

TGase mediated glutamine deamidation is a well known PTM found in dietary gluten protein, which in turn results in an increased T-cell response (44). The high glutamine substrate affinity for TGase cross-linking found in tau protein is of particular interest here (45), since glutamine deamidation may trigger misfolding of tau (46), α-synuclein and huntingtin protein. To our knowledge, we report here for the first time that, in addition to cross-linking, glutamine Q 15 deamidation is a highly competitive reaction in Aβ peptide. Residue deamidation and racemization is of particular importance in amyloidogenic peptides, since low levels of deamidation impurities seed the aggregation of pure peptides.
Spontaneous asparagine (N) deamidation in the prion protein PrP is thought to induce structural changes to its infectious PrP\textsuperscript{sc} form (48), and aspartic acid racemization accelerates Aβ peptide aggregation (49). Deamidation strongly decreases the peptide’s affinity for aggregation as shown by our synthetic variant of deamidated (E\textsuperscript{15}). This finding is corroborated by the observation that, in the synthetic E\textsuperscript{15} Aβ variant, secondary structure transition to β-sheet is impeded in acetonitrile solution, when compared to wt Aβ peptide. Although variant E\textsuperscript{15} Aβ peptide has the ability to form fibrils of amylodogenic nature, it seemed that fibrillogenesis was significantly reduced and that these fibrils are more prone to dissociation during SDS-PAGE analysis. Glutamine Q\textsubscript{15} has been reported to play a crucial role in the structural conversion from Aβ\textsubscript{1-42} oligomers to fibrils, where Q\textsubscript{15} forms an intermolecular contact with glycine G\textsubscript{37} within a β-turn-β unit in fibrils (50). This may indicate that, insertion of a negative charge (E\textsuperscript{15}) at glutamine Q\textsubscript{15}, disfavors intermolecular contacts during initial stages of fibrillogenesis. Glutamine deamidation, as a result of protein aging, is also known to account for the destabilization of aged-cryallins (51), which causes unfolding of lens proteins and eventually leading to cataract (52). It is tempting to suggest that TGase mediated glutamine deamidation could serve as a protective role by impeding charge dependent intermolecular contacts in monomers. This would be in line with the fact that in AD, TGase levels found in cerebrospinal fluid (CSF) are significantly increased (9,53). However, our findings rule out the possibility that TGase may induce unfolding of large aggregates, since incubation of TGase with pre-aggregated Aβ peptide did not result in structure dissociation or change in morphology. It is therefore reasonable to assume that TGase substrate accessibility is strongly impeded in aggregated Aβ, due to changes in the peptide’s secondary and consequently globular structure, which is in line with the knowledge that Aβ oligomers are resistant to proteolysis (1,54).

The striking increase in Aβ peptide fragmentation found in the presence of TGase has not been reported before and is of particular interest to us. Ikura and colleagues reported that cross-linking of Aβ peptide resulted in a new species of faster gel migration mobility, however, the identity of this fragment remains unknown (22). To date, we do not know if peptide fragmentation is due to some unknown TGase proteolytic activity or simply a result of the highly increased peptide aggregation phenomenon observed in the presence of TGase. Our IEF and MS analyses of cross-linked Aβ peptide indicate that significant peptide fragmentation can occur at residues glycine G\textsubscript{25} and glutamate E\textsubscript{22} respectively, since both complementary fragments, Aβ\textsubscript{23-40} and Aβ\textsubscript{26-40}, were also detected. The presence of smaller N-terminal fragments, such as Aβ\textsubscript{1-20} through Aβ\textsubscript{1-16}, may derive from the sequential breakdown of the larger parent fragments Aβ\textsubscript{1-25} (Fig. 11B). Cleavage after residue G\textsubscript{25} appears to be specific for both, guinea pig and CHO TGase, since this cleavage could be attenuated in the presence of EDTA and cleavage was not observed following prolonged incubation times without TGase (Supplemental fig. III and IV). However, in spite of the use of protease inhibitors during these reactions, we cannot rule out the possibility of contamination of other proteases. Calpain, for example, is known to induce cleavage of α-syn protein (55) and p35 (56). Nevertheless, cleavage of the Aβ\textsubscript{1-25} fragment is an interesting observation, since this fragment was earlier identified as a sphingolipid binding domain motif (57). This fragment has been shown to be rapidly internalized by neuronal cells in a cholesterol/sphingolipid-dependent manner (58) and, Aβ fragments have similar amylodogenic properties as full-length Aβ peptide (59).

The complex profile of 40 different soluble Aβ fragments found in APP transfected cells has been reported earlier (60), using immunoprecipitation combined with MS identification. Fragment Aβ\textsubscript{1-16} appears to be of particular interest when trying to associate Aβ peptide fragmentation with the early cognitive decline found in AD patients (61,62), because MS analysis of CSF shows a specific Aβ peptide fragment signature in sporadic AD patients. Interestingly, C-terminal fragments have recently been reported to affect the assembly of full-length peptide (63). Truncated Aβ is known to represent more than 60% of all Aβ species found in nondemented as well as in AD individuals (62). Our IEF data and MS analysis confirm the significant abundance of Aβ peptide fragments found in the presence of TGase \textit{in vitro}. This may suggest that
large oligomeric species could consist of a heterogeneous assembly of full-length as well as fragmented Aβ peptide, of which the latter may serve as a seed for aggregation. There are a number of experimental indications that, soluble oligomers might account for the AD associated decline in synaptic plasticity and memory (3,64) and that inhibition of natural Aβ oligomerization rescues long-term potentiation (LTP) (65). Moreover, TGase induced peptide oligomerization was shown to inhibit LTP in vitro (1), though attenuation of LTP observed with freshly prepared Aβ peptide may account for the rapid changes in peptide morphology in vivo (2). LTP is not affected by the Aβ1-16 fragment per se (61), however, collectively these findings do not rule out the possibility that other Aβ fragments may exert cellular toxicity independently or through an orchestral function with the full-length peptide.

Since little is known about antecedent biomarkers in AD, it is vital to identify relevant markers before the development of pathological symptoms. CSF analysis for TGase levels (9), as well as changes in Aβ concentration or particular Aβ peptide fragment signatures, have been suggested as useful biomarkers in AD (17). Truncated Aβ species can be considered as early, potential pathological markers in AD (61) and therefore represent additional antigen targets for vaccination (62). In this work we highlight the important observation on TGase induced PTM as well as show new Aβ peptide fragment signatures and therefore provide crucial information for understanding and targeting the inhibition of Aβ peptide aggregation.

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The abbreviations used throughout this article are: TGase, tissue transglutaminase; CHO, Chinese hamster ovary; Aβ, beta-amyloid; AD, Alzheimer disease; MS, mass spectrometry; MALDI TOF/TOF, matrix assisted laser desorption ionisation time of flight/time of flight; FTMS, Fourier transform mass spectrometry; ESI, electrospray ionisation; CID, collision induced dissociation; ThT, thioflavin T; TEM, transmission electron microscopy; CD, circular dichroism; cl, cross-link.

**FIGURE LEGENDS**

**Fig. 1.** TGase induces rapid polymerization of Aβ peptide. A) SDS-PAGE, silver stained gel: Rapid polymerization of Aβ monomers to putative dimers (2), trimers (3) and tetramers (4) is enhanced in the presence of TGase (lanes 2-8) but are not present at time zero (lane 1). Low molecular weight oligomers are formed in the first few minutes of incubation and the level was stabilized after 60 min (lane 8). B) Transmission electron microscopy (TEM): image top left; In the presence of TGase, Aβ peptide forms predominantly protofibrillar structures after only 60 min of incubation. Right; TGase alone at a 10 fold increased concentration forms amorphous aggregates, which are morphologically different to Aβ protofibrils. Prolonged incubation times of Aβ and TGase for 5 and 24 hrs results in large clusters of oligomeric and protofibrillar aggregates (below; images left & right). C) Formation of stable Aβ dimers is strongly dependent on peptide concentrations and hence favoring a TGase mediated transamidation reaction, probably due to the close proximity of adjacent lysine residues. Significant amounts of putative dimers and trimers are observed with 50 µM Aβ peptide, whereas peptide polymerization seems to decrease at lower peptide concentrations (lanes 2-4). No polymerization was observed where Ca²⁺ was omitted from the reaction solution (lane 1). Equal amounts of total protein was loaded onto each well.

**Fig. 2.** Detection of stable cross-linked low molecular weight oligomers by MALDI-TOF-TOF. Broad acquisition in linear positive mode of TGase cross-linked Aβ peptide, showing the presence of abundant monomers (M) as well as low levels of dimers (D) and trimers (T). Inset above; zoomed acquisition of Aβ monomer (M) in reflectron positive mode showing the isotopic distribution of monomers and an isotopic cluster associated with a shift in mass of -17 Da, which may account for intramolecular cross-linked monomers. Gel inset (left); denaturing SDS-PAGE analysis of the same sample showing the level of dimers and trimers with respect to monomers present in the sample.

**Fig. 3.** TGase induced glutamine (Q¹⁵) deamidation is a highly competitive reaction. A) High resolution MS (Orbitrap) analysis of cross-linked Aβ peptide. Top; Isotopic envelope of control Aβ¹⁻⁴⁰ peptide m/z = 1083.3021 [M+4H]⁴⁺ (black). Below; The shift in mass (Δ m/z = 0.24 [M+4H]⁴⁺ → 0.984 Da) of the most intense peak within the isotopic cluster (white arrow) m/z = 1083.5421 [M+4H]⁴⁺, accounts for a TGase mediated deamidation at residue Q¹⁵. The appearance of a new isotopic cluster (left) m/z = 1078.5406 [M+4H]⁴⁺, with a mass shift of -17 Da, indicates the presence of an intramolecular cross-link (red spectrum). The isotopic envelope of TGase treated Aβ also revealed an appearance of a new, monoisotopic peak (m/z = 1082.5394 [M+H]⁴⁺) (black arrow) and is associated with a loss of 1 Da in Aβ monomer. This may account for some putative molecular reorganization at the site of residue modification. (inset at top: depiction of Q to E conversion involving the loss of NH₂ followed by an OH adduct). B) Collision induced dissociation (CID) of fragment Aβ¹⁻²² generated two new ions of y₉ (m/z = 560.29 [M+2H]²⁺) and b₁₅ (m/z = 905.38 [M+2H]²⁺) of which both ions bear a mass shift of 0.984 Da, due to deamidation at residue Q¹⁵ → E¹⁵. C) Comparison of fragmentation behavior of control Aβ (black spectrum) and intra-molecular cross-linked (red spectrum) Aβ peptide. CID of control Aβ generated abundant b and y ions throughout the whole peptide sequence. However, fragmentation of intramolecular
cross-linked peptide was strongly inhibited probably due to C- with N-terminal overlapping and hence protecting the peptide’s mid region.

**Fig. 4.** Aβ dimers are formed through a TGase mediated intermolecular transamidation process. A) SDS-PAGE analysis of IEF fractions of pl from 3.0 to 7.8. Aβ peptide fragments were found in fractions of pl 3.9 to 6.0, whereas dimers and deamidated monomers were predominantly focused in fraction pl 4.5. B) High resolution MS analysis of Aβ dimers following TGase cross-linking and iselectrofocusing. Aβ dimers were found in the same fraction as deamidated monomers (inset; gel after IEF), which corresponded to a pl of 4.5. Deamidated monomers were generally observed at three different charge states of: \( m/z = 1444.38[M+3H]^{3+} \), \( m/z = 1083.54[M+4H]^{4+} \), \( m/z = 867.03[M+5H]^{5+} \), whereas Aβ dimers were only found at one charge state of \( m/z = 1440.69[M+6H]^{6+} \). C) Zoom of cross-linked dimer. The measured mass corresponds exactly to the theoretical dimer (top isotopic envelope) with a loss of -17.02Da (NH₃) for the (Q¹⁵ & K¹⁶) transamidation reaction, as well as a shift in mass of 0.984Da for deamidation taking place on the second glutamine residue.

**Fig. 5.** ESI MS analysis of cross-linked Aβ₁₋₂₅ dimer. TGase cross-linking of a fragment Aβ₁₋₂₅ peptide (monoisotopic mass = 2931.36Da) increases the formation of stable dimers, since this fragment peptide lacks lysine K₂₈ for competitive, intramolecular cross-linking. Above; calculated theoretical isotopic cluster of wt Aβ₁₋₂₅ dimer (black cluster: \( m/z = 1173.6[M+5H]^{5+} \)), calculated cluster of cross-linked (cl) Aβ₁₋₂₅ dimer with deamidation at the second glutamine Q¹⁵ (gray cluster: \( m/z = 1170.3[M+5H]^{5+} \)) and cl Aβ₁₋₂₅ dimer without deamidation (white cluster: 1170.1[M+5H]⁵⁺). Below; high resolution ESI-MS analysis of the isotopic cluster from in vitro TGase cross-linked Aβ₁₋₂₅ dimers, of which isotopic mass \( m/z = 1170.32[M+5H]^{5+} \) corresponds to the mass of 2(2931.36 Da) – (17.026Da (NH₃)), of which the second glutamine Q¹⁵ is deamidated to glutamate (E¹⁵) + (0.984Da). Inset; MSMS fragmentation of cross-linked Aβ₁₋₂₅ dimer. The presence of abundant “b” ions confirmed the identity of this cross-linked fragment peptide, of which ion b₇ is always readily observed by CID fragmentation. Larger fragments involved the loss of C-terminal residues A₂₁ to G₂₅ within a whole Aβ₁₋₂₅ dimer D-(21-25). A summary of all identified ions is depicted in the diagram adjacent: For the sake of simplicity fragment ions are only depicted on one side of the dimer, but could take place on either monomer or a combination of both. The scheme of the cl dimer shows a deamidation at position E¹⁵ (-NH₂ +OH), whereas glutamine Q¹⁵ on the lower monomer, serves for cross-linking (transamidation: –NH₃) with K₁₆ and hence accounts for stabilisation of the dimer.

**Fig. 6.** Glutamine deamidation accounts for decreased peptide aggregation. A) Thioflavin T (Th T) fluorescence spectroscopy of wt (Q¹⁵) and variant (E¹⁵) Aβ peptide following 7 days incubation. Values represent the mean ± SD of three individual experiments. B) ThT fluorescence of variant E¹⁵ from day 1 to 7. Incubation of E¹⁵ (50µM) at 37°C resulted in a steady increase in ThT fluorescence signal over 7 days. However, peak levels of Th T binding were significantly lower (<50%) when compared with wt Aβ peptide after 7 days. C) TEM imaging and SDS-PAGE; The decreased levels in Th T binding levels found with E¹⁵ peptide, seemed to be in line with the lower amounts of typical amyloid fibrils (right image), as well as the decreased stability of these fibrils found by SDS-PAGE analysis (gel inset; 2nd lane). Incubation of wt Aβ peptide resulted in the formation of typical amyloid fibrils (left image).

**Fig. 7.** Glutamine deamidation has an impact on secondary structure transitions of Aβ peptide. Far-UV circular dichroism (CD) analysis and transmission electron microscopy (TEM) imaging of wt and E¹⁵ variant Aβ peptide. Left; Solubilization of wt and E¹⁵ Aβ peptides in 40% trifluoroethanol (TFE) induced strong α-helical structure in both peptides. However, the α-helical structure in E¹⁵ peptide appeared to be less stable over the time course of incubation, showing a more significant transition to β-sheet structure, accompanied by a decrease in signal ellipticity after 4 days. Right; Peptide solubilization in 30% acetonitrile (ACN) solution induces rapid β-sheet conformation in wt Aβ peptide, showing a typical ellipticity minimum at a wavelength of 220nm. β-sheet conformation appeared to be stable over two days.
prior to decreasing in signal strength after 4 days of incubation at 25°C. However, the ability to induce β-sheet structure in variant (E15) Aβ peptide was strongly reduced. Solubilization of E15 peptide in ACN shifted the minimum to 203nm. CD signals at this wavelength have been associated with possible β-turn structure in peptides. The strong decrease in secondary structure signal after 4 days of incubation is associated with peptide precipitation and hence an increase in solution turbidity. Insets left & right; TEM imaging of E15 peptide in either solution shows increased peptide precipitation, as seen by the presence of amorphous aggregates and the lack of typical amyloid fibrils as compared to wt peptide. (arrow: site of two Aβ wt fibrils associating with each other forming a typical helical twist).

Fig. 8. Peptide aggregation reduces TGase accessibility to glutamine substrates. Advanced peptide aggregation and changes in peptide morphology reduced TGase substrate recognition. Above; TEM imaging confirmed the presence of abundant fibrils following incubation of Aβ wt and E15 variant for 7 days. In the case of E15 peptide, fibrils appeared long and straight in morphology (left image) as compared to wt fibrils of short and dense morphology (middle image). Below; SDS-PAGE analysis of these fibrils resulted in breakdown to monomers (lanes 2 & 3), whereas no SDS-stable low molecular weight oligomers were found. Addition of TGase to wt and E15 fibrils did not result in the formation of SDS-stable oligomeric species (lanes 4 & 5), as is typically found with freshly prepared wt Aβ peptide, which readily formed dimers, trimers and tetramers in the presence of TGase (lane 6). No change in peptide morphology was observed in aggregated wt or E15 variant following the addition of TGase (images not shown). Abundant protofibrils and few fibrils were observed in freshly prepared wt Aβ peptide following incubation with TGase for 1 day (above: right image).

Fig. 9. TGase induced fibrillogenesis is strongly increased in the presence of myricetin. Left; SDS-PAGE analysis (silver stained); TGase induced cross-linking of LMW oligomers is no longer observed in the presence of myricetin (100µM) after 1hr or 12hrs (lanes 2 & 3, 6 & 7). However, fibrillogenesis is strongly increased in the presence of myricetin, as seen by the appearance of dense fibril clusters (TEM image top) as well as by the striking decrease in monomers found with these samples (gel; lane 7). Fibrillogenesis is also highly accelerated in the presence of TGase (TEM image bottom), however, a substantial amount of monomeric Aβ remained soluble (gel; lane 8), which may account for the usual amount of deamidated monomers found after cross-linking. In variant E15 peptide, the presence of Myr does not induce aggregation after 1hr & 12hrs of incubation, as seen by the abundant levels of monomers present in the gel (lanes 1 & 5).

Fig. 10. Analysis of endogenous TGase activity in CHO cells. A) MALDI-TOF-TOF acquisition (reflectron mode) of Aβ1-40 peptide following incubation with CHO cells. Significant peptide fragmentation at residue G25 was observed after only 10min of incubation, giving rise two N-terminal fragments of Aβ1-25 bearing deamidation and transamidation like modifications of a m/z = 2932.93 [M+H]+ and m/z = 2914.98 [M+H]+ respectively. B) Liquid chromatography coupled with FT-MS analysis of the same sample as shown in (A) with zoom on Aβ1-40. The measured isotopic envelope of Aβ1-40 m/z = 866.4431 [M+5H]5+ (middle spectrum) corresponds to calculated Aβ1-40 mass of m/z = 866.4367 [M+5H]5+ (top spectrum) (Δ mass = 0.032Da, 7ppm). The residual isotopic envelope with a m/z = 866.4431 [M+5H]5+ accounts for a typical loss of H2O (18.01Da) found in Aβ1-40. A new isotopic envelope m/z = 863.0732 [M+5H]5+ (bottom spectrum) with a retention time (RT) of 64.4min was revealed. This monoisotopic mass does not match (Δ mass = 0.2065Da) the typically associated transamidation reaction found with TGase (-NH3), as can be seen by the calculated monoisotopic mass of m/z = 863.0319 [M+5H]5+ (top spectrum). C) Liquid chromatography coupled with FT-MS analysis of the same sample as shown in (A) with zoom on fragment Aβ1-25. The measured isotopic envelope of Aβ1-25 m/z = 978.5272 [M+3H]3+ (middle spectrum: right) does not match the calculated deamidation induced shift in mass m/z = 978.4545 [M+3H]3+ (top spectrum) (Δ mass = 0.2181Da), whereas the measured monoisotopic mass of control Aβ1-25 m/z = 978.1287 [M+3H]3+ (bottom spectrum) corresponds to its calculated mass of wt Aβ1-25 m/z = 978.1265 [M+3H]3+ (Δ mass = 0.006Da, 2.25ppm). A similar shift in mass was observed for
the second population of Aβ₁₋₂₅ (middle spectrum: left), where the measured monoisotopic mass \( m/z = 972.5413 \text{[M+3H]}^3+ \) does not account for the typical transamidation reaction found with TGase as seen by the calculated mass with a -NH₃ induced shift \( m/z = 972.4510 \text{[M+3H]}^3+ \) (Δ mass = 0.2709Da). Retention times are significantly shifted in both Aβ₁₋₂₅ fragments (RT = ~64min), when compared with control Aβ₁₋₂₅ fragment (bottom spectrum, RT =44.25min). D) Immunoblotting and SDS-PAGE analysis of guinea pig and CHO transglutaminase. Following cell lysis, significant amounts of TGase could be detected in two different batches of CHO cells (lanes 3 & 4). As a positive control for immunoblots, guinea pig TGase was loaded at two different concentrations (lanes 1 & 2). SDS-PAGE: Bands at the migration height of guinea pig TGase (A = ~78kDa) were cut and subjected to trypsin proteolysis followed by LCMS analysis and de novo sequencing to further confirm the presence of TGase in CHO cells (lanes 5-7). In addition to band A, four more bands (B to D) at the migration level below A were cut, in order to identify any contamination present in guinea pig (lane 5) or CHO TGase (lanes 6 & 7). (TGase sequence coverage of bands in lane 5: B= 53%, C= 28%, D= 14%, E= 26%) (TGase sequence homology found in bands A of lane 6 & 7: A= 23% & 17%).

Fig. 11. Summary of TGase mediated modifications observed in Aβ peptide. A) Left pathway; TGase selectively binds to glutamine at position \( Q^{15} \). In the absence of suitable amide donor/substrate, such as peptide bound lysine residues, the enzyme induces a highly favorable deamidation reaction, involving hydrolysis of the amide group on glutamine (NH₂) followed by an insertion of OH to form glutamate \( E^{15} \). The isomerized form “iso-glutamate (\( \beta E^{15} \))” with an identical shift in mass of 0.984Da, may arise from the formation of a transient “glutarimide intermediate”, consisting of a labile six-membered ring structure. Following glutamine deamidation, conversion of glutamate to iso-glutamate, or vice versa, is possible through its glutarimide intermediate. right pathway; If a lysine residue is within the proximity of the enzyme’s active site, such as at high peptide/substrate concentrations, then a transamidation reaction may be favored, where lysine (K) serves as an amide donor. The β-turn at residues 22-23 in Aβ monomer may favor K₂₈ as a substrate for intra-molecular cross linking, by bringing this residue to the close proximity of Q₁⁵. Lysine K₁₆ is indispensible for inter-molecular cross-linking and hence the formation of stable dimers or LWM oligomers. Both transamidation reactions are associated with a loss of -17.04Da (NH₃), which serves as a “molecular tag” in mass spectrometry. Aβ₁₋₄₀ peptide is depicted with a broken surface structure at residues 15-25. The proximity of glutamine Q₁⁵ and lysine K₁₆ residues are highlighted in yellow. B) Aβ peptide post-translational modifications and peptide fragmentation may play a causal role during increased aggregation. Aβ peptide aggregation is accompanied with abundant peptide fragmentation. The increase in concentration of N-terminal fragments such as Aβ₁₋₂₅, Aβ₁₋₂₂ and Aβ₁₋₁₆ (counter clock wise) or its complementary C-terminal fragments of Aβ₂₂₋₄₀ and Aβ₂₃₋₄₀ respectively (clock wise) may play a causal role during accelerated peptide aggregation. The presence of shorter Aβ fragments such as Aβ₁₋₁₆ may directly influence the overall speed of peptide aggregation (left pathway), by serving as a “molecular seed” for full-length Aβ₁₋₄₀ peptide. The polyphenol Myricetin (Myr) may specifically influence peptide aggregation by serving as a seed at an oligomeric level. N-terminal fragments may aggregate to form amyloidogenic aggregates on their own, whereas the complementary C-terminal fragments (right pathway) may affect aggregation of full-length peptide. Post-translational modification such as: serine (S²⁶) phosphorylation or racemization, methionine oxidation (M³⁵), aspartic acid isomerization (D⁷ & D²³), TGase cross-linking of dimers and pyro-glutamate formation (\( \beta E^{5} \)) are known to influence the affinity of peptide aggregation. On the other side, TGase mediated glutamine deamidation (E¹⁵) increases the threshold of peptide aggregation. (Aβ peptide surface structures were drawn using YASARA v. 6.8.13 software).
Figure 7

A)

B)

Figure 8
Figure 9

| myricetin | + | + | + | + | + |
| tTGase   | + | + | + | + | + |
| CaCl     | + | + | + | + | + |
| time (h) | 1 | 1 | 1 | 12 | 12 |
| lane     | 1 | 2 | 3 | 4 | 5 |

Figure 10

A)

B)

C)

D)
Figure 11
Tissue transglutaminase mediated glutamine deamidation of beta-amyloid peptide increases peptide solubility, whereas enzymatic cross-linking and peptide fragmentation may serve as molecular triggers for rapid peptide aggregation.

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