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Deamidation and Transamidation of Substance P by Tissue Transglutaminase Revealed by Electron-Capture Dissociation Fourier Transform Mass Spectrometry

Luca Fornelli,[a] Adrien W. Schmid,[a, c] Luigino Grasso,[b] Horst Vogel,[b] and Yury O. Tsybin*[a]

Abstract: Tissue transglutaminase (tTGase) catalyzes both deamidation and transamidation of peptides and proteins by using a peptidyl glutamine as primary substrate. A precise consensus sequence for the enzyme is unknown and the ratio between deamidated and transamidated (or cross-linked) reaction products is highly substrate-dependent. Due to its overlapping body distribution with tTGase and ease of manipulation with tandem mass spectrometry, we used the neuropeptide substance P as a model to investigate the associated enzymatic kinetics and reaction products. Online liquid-chromatography Fourier-transform ion-cyclotron-resonance mass spectrometry (FT-ICR MS) combined with electron-capture dissociation (ECD) was employed to study the tTGase-induced modifications of substance P. A particular strength of ECD for peptide-enzyme reaction product monitoring is its ability to distinguish isomeric amino acids, for example, Glu and iso-Glu, by signature product ions. Our studies show that the primary reaction observed is deamidation, with the two consecutive glutamine residues converted sequentially into glutamate: first Gln_5, and subsequently Gln_6. We then applied ECD FT-ICR MS to identify the transamidation site on an enzymatically cross-linked peptide, which turned out to correspond to Gln_5. Three populations of substance-P dimers were detected that differed by the number of deamidated Gln residues. The higher reactivity of Gln_5 over Gln_6 was further confirmed by cross-linking SP with monodansylcadaverine (MDC). Overall, our approach described herein is of a general importance for mapping both enzymatically induced post-translational protein modifications and cross-linking. Finally, in vitro Ca-signaling assays revealed that the main tTGase reaction product, the singly deamidated SP ($RPKPEQFFGLM\text{-}NH_2$), has increased agonist potency towards its natural receptor, thus confirming the biologically relevant role of deamidation.

Keywords: enzymes · fragmentation · peptides · radical ions · reaction dynamics

Introduction

Tissue transglutaminase (type 2 transglutaminase; tTGase) is a ubiquitously expressed enzyme that is found extra- and intracellularly.[1] tTGase is the only member of its protein family to be expressed in human brain.[2] It is involved in several biological processes[1] and acts as a GTPase[3] and a kinase.[4] The most studied biological function of tTGase enzyme is the formation of peptidase-resistant isopeptidic bonds in a Ca^{2+}-dependent acyl-transfer reaction.[1,5–7] Under physiological conditions, the acyl-donor group can be the γ-carboxamide of a peptidyl-glutamine (Gln) residue, whereas the acyl-acceptor group can be either a low-molecular-weight primary amine or the ε-amino group of a lysine (Lys). In the latter case, the transamidation reaction (also known as a cross-linking) yields an ε-(γ-glutamyl)−lysine

[a] L. Fornelli,[+] Dr. A. W. Schmid,[+] Dr. Y. O. Tsybin
Biomolecular Mass Spectrometry Laboratory
Ecole Polytechnique Fédérale de Lausanne
1015 Lausanne (Switzerland)
Fax: (+41) 121-693-9700
E-mail: yury.tsybin@epfl.ch

[b] L. Grasso, Dr. H. Vogel
Laboratory of Physical Chemistry of Polymers and Membranes
Ecole Polytechnique Fédérale de Lausanne
1015 Lausanne (Switzerland)

[c] Dr. A. W. Schmid[+]
Current address: Proteomics Core Facility
Ecole Polytechnique Fédérale de Lausanne (EPFL)
1015 Lausanne (Switzerland)

[+] Contributed equally to this work.

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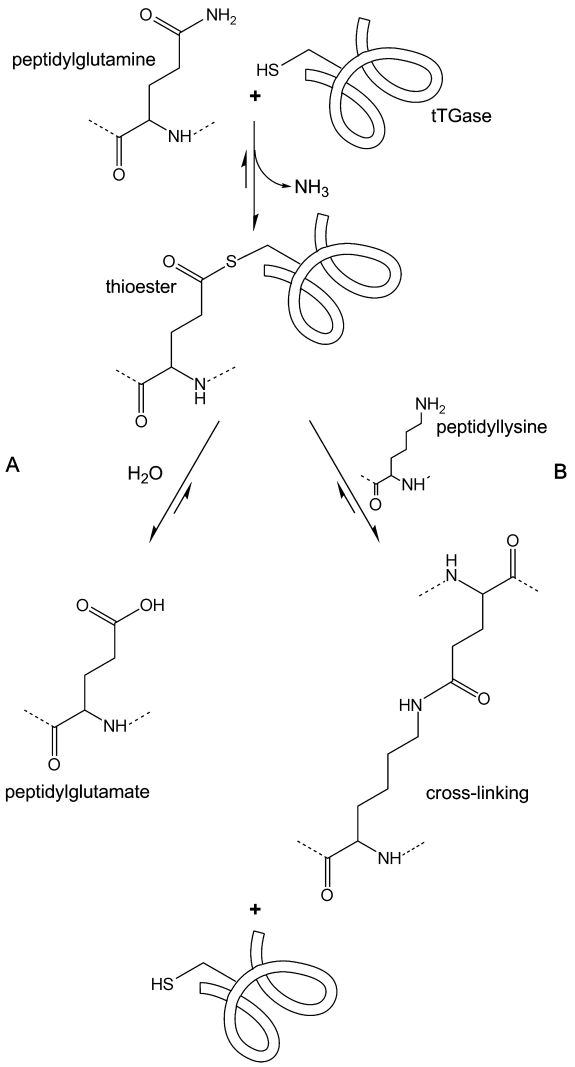
bond. In the absence of a good acyl acceptor, tTGase can also deamidate Gln to glutamic acid (Glu)[8] by using a water molecule to react in place of the primary amine with the Gln–enzyme intermediate (Scheme 1). The tTGase acyltransfer activity, which can result in both inter- and intramolecular protein cross-linking, has been widely investigated in relation to the onset of several neurodegenerative pathologies, characterized by the presence of protein aggregates.[9] Specifically, tTGase has been proven to induce the formation of protease-resistant aggregates, which are thought to play a crucial role in the initiation and development of Alzheimer disease, using both Aβ-40 and Aβ-42 at physiologically relevant concentrations in vitro.[10] Moreover, tTGase-mediated intramolecular cross-linking of α-synuclein has been shown to prevent the formation of typical amyloid aggregates.[11]



Scheme 1. Representation of two calcium-ion-dependent peptide-modifying reactions catalyzed by tissue transglutaminase (tTGase): A) deamidation and B) transamidation using the γ-acyl group of Gln as a first substrate, and water or ε-amino group of Lys as a second substrate.

On the other hand, glutamine deamidation is a post-translational modification (PTM) that introduces the charged amino acid Glu in place of the uncharged Gln within a polypeptide chain. Gln-to-Glu substitution can strongly influence protein–protein or protein–receptor interactions by altering signaling pathways and inducing pathological insurgence. An example of the latter case is tTGase-mediated deamidation of gliadin, a protein found in wheat gluten.[12,13] Deamidation of gliadin protein was reported to be responsible for the inflammatory responses associated with celiac disease.[14] Deamidation-induced formation of new disease-associated protein complexes might be recognized as neoepitopes by T-cell receptors and lead to autoimmune response.

The substrate specificity of tTGase has been widely investigated recently.[15–17] Although a number of generic requirements have been reported, no conclusive amino acid sequence pattern for substrate–enzyme recognition has been found yet, neither for the acyl-donor nor for the acyl-acceptor group.[18,19] The lack of a defined consensus amino acid sequence that indicates the glutamines and lysines that can serve as substrates for tTGase, together with the fact that not all the actual substrates show the same affinity for the enzyme, requires the development and use of specific tools for determining the residues that are deamidated or transamidated by transglutaminase on peptides and proteins of interest, including pathology-related neuropeptides.[20,21] Traditional strategies for the identification utilize cross-validation approaches, as they comprise both the use of mutant peptides or proteins and bottom-up experiments, which consist primarily of mass-spectrometry-based analysis of protein digests sometimes coupled with collision-induced dissociation (CID) for the biomolecular ion fragmentation.[11]

Taking into account the current understanding of the central biological and pathological role of the tTGase-induced modification of peptides and proteins, we selected tTGase-catalyzed transamidation and deamidation of the neuropeptide substance P (SP)[22] to demonstrate the applicability of the tandem mass spectrometry for the characterization of tTGase reaction products. The undecapeptide SP belongs to the tachykinin neurotransmitter family.[23,24] It is distributed both in the peripheral nervous system,[25] where it mediates different physiological responses, including smooth muscle contraction,[26] vasodilation,[27] and increase in vascular permeability,[28] as well as in the central nervous system,[29] where it is involved mainly in inflammatory response[30] and pain perception.[31] SP action is regulated through its binding to the specific neurokinin-1 receptor (NK-1R), a G-protein-coupled transmembrane protein.[32–34] Binding to NK-1R results in phospholipase C activation and to the subsequent increase in the intracellular Ca^{2+} concentration.[35] Moreover, stimulated neurokinin-1 receptor can affect different neuronal pathways.[23] For example, analgesic response of the opiates can be influenced by NK-1R activation, mediated by its primary agonist SP, to modulate the reward (addition) mechanism of the opiates.[36,37] The use of SP as a substrate for tTGase is widely described in the literature. Particularly,

the presence of two consecutive Gln residues in the SP sequence (positions 5 and 6) has been exploited to induce the formation of cross-linked species with low-molecular-weight fluorescent amines, for example, spermidine, putrescine, and monodansylcadaverine.[38–40] First, this method allows to detect SP–amine complexes in different assays using the fluorescent properties of the amines.[38] Secondly, peptides modified with low molecular weight compounds can be efficiently analyzed by low-resolution mass spectrometry.[39] Although the overlapping body distribution with tTGase suggests that SP could be a natural substrate for this enzyme, no conclusive analysis of tTGase-induced SP deamidation and transamidation have been conducted. Importantly, as the C-terminal part of SP (residues 5–11) is involved in the NK-1R binding process,[41,42] SP deamidation could change the properties of the peptide sufficiently to affect its biological function.

The current study is thus aimed to provide in-depth analysis of tTGase-mediated deamidation of SP by using liquid chromatography (LC) coupled with high-resolution tandem Fourier-transform ion-cyclotron-resonance mass spectrometry (FT-ICR MS).[43] Identification of residue-specific modifications was carried out by electron-capture dissociation (ECD).[44–46] Electron addition to the multiply charged ions in the gas phase during the ECD process leads to formation of intermediate charge-reduced radical species. These metastable species readily dissociate by the cleavage of the N–C_α backbone bonds of the peptides, whereas the lateral chains of the amino acids and also labile post-translational modifications (PTMs) are typically preserved. Importantly, ECD FT-ICR MS has been recently shown to be able to differentiate aspartic acid from iso-aspartic acid[47] and glutamic from γ-glutamic acid.[48] The differentiation is based on the specific side-chain loss that is characteristic for the structure of the residues.

This study first confirms the previously reported higher reactivity of Gln_5 compared to Gln_6.[38] Second, we report the detection and characterization of SP dimers.[39] Both results are accompanied by the kinetics studies of tTGase deamidation and also transamidation. The latter point is of particular importance, considering that the mechanism of the highly substrate-dependent tTGase acyl-donor recognition is not completely understood.[15,49] Finally, to determine a putative biological role of tTGase-mediated deamidation of SP, a singly deamidated variant of SP was used to reveal changes in agonist potency upon binding to neurokinin-1 receptors.

Results

Kinetics of tTGase-induced deamidation of substance P: Figure 1 shows the kinetics of the reaction between substance P and tTGase monitored by LC–MS. More specifically, the time dependence of the population of singly deamidated substance P versus the unmodified peptide is shown. According to the literature, tTGase exhibits high specificity toward acyl-donor group recognition.[15] Theoretically, both

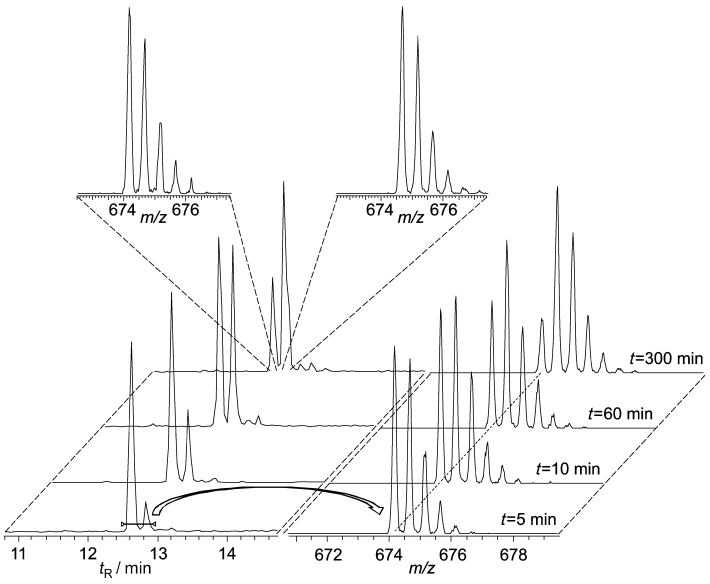


Figure 1. Time-course LC–MS analysis of substance-P deamidation catalyzed by tissue transglutaminase (tTGase). Four timepoints are considered, namely, 5, 10, 30, and 60 min. Left: Total ion current chromatograms exhibit a first peak that corresponds to unmodified (or control) substance P, and a second one that corresponds to the singly deamidated substance P. Right: Combined isotopic distribution of both peaks (the two isotopic distributions are partially overlapping) demonstrates the relative abundance of the unmodified and deamidated species. The inset shows separate isotopic distributions for the two species that correspond to the LC–MS of the 300 min sample. The m/z scale of the LC ESI LTQ MS data shown was only externally calibrated (for doubly charged SP, m/z: calcd: 674.37; found: 674.18).

glutamine residues in SP, namely, Gln_5 and Gln_6, could be deamidated. To monitor the deamidation order and rate, a reaction was performed with relatively low concentrations for both substrate (74 μM) and enzyme (0.002 U).

As depicted in Scheme 1, the deamidation reaction occurs when a water molecule enters the active site of tTGase after the formation of an intermediate between enzyme and Gln residue of the substrate. A low concentration of SP should favor the deamidation process over transamidation, whereas low enzyme concentration was chosen to allow the study of a fast process. Figure 1 shows that the singly deamidated substance P is the main reaction product under the employed reaction conditions (both SP and tTGase concentrations are low) at 300 min from the reaction onset. Figure 1, left, displays two baseline-resolved peaks from a total-ion chromatogram related to substance P control (substrate retention time ≈12.6 min) and singly deamidated substance P (product retention time ≈12.9 min). The combined isotopic distribution of the two peaks that differ in molecular weight by 0.98 Da demonstrates their relative abundance change as a function of time (Figure 1, right).

Competition between deamidation and transamidation of substance P: The results presented in Figure 1 demonstrate that substance P is a good substrate for tTGase, but do not clarify which one of the two Gln residues is deamidated.

The reaction pathway that leads to peptide transamidation with subsequent formation of dimers or polymers is also not considered in Figure 1. Previous findings by Ferrándiz et al. have shown that Gln_5 serves as an indispensable tTGase substrate and is more reactive as an acyl donor.[38] Thus, we used the LC–MS/MS to investigate if both Gln residues can be tTGase substrates and elucidate the possible reaction sequence as a function of time. Electron-capture dissociation (ECD) was chosen as a tandem mass spectrometry method mainly due to the efficient and easy-to-interpret fragmentation of substance P it induces. Furthermore, the specific character of ECD allows one to distinguish amino acid isomerization that may occur during the enzymatic reaction (vide supra). According to a study by Marino's group, substance P dimers should not be formed.[39] However, this finding contrasts with the general understanding of tTGase catalytic activity, which demonstrates a relatively good specificity for the peptidic sequence that contains the acyl donor (Gln), but not for the acyl acceptor (Lys).[18]

We assume that transamidation, which can be thought of as a competitive reaction with respect to deamidation, can be promoted by increasing the probability of two substance P monomers to be recognized in rapid succession by the enzyme. This result was achieved by using a high substrate concentration. To accelerate the reaction and increase the enzyme activity, we used higher substrate and tTGase concentrations and increased the temperature. Figure 2 shows

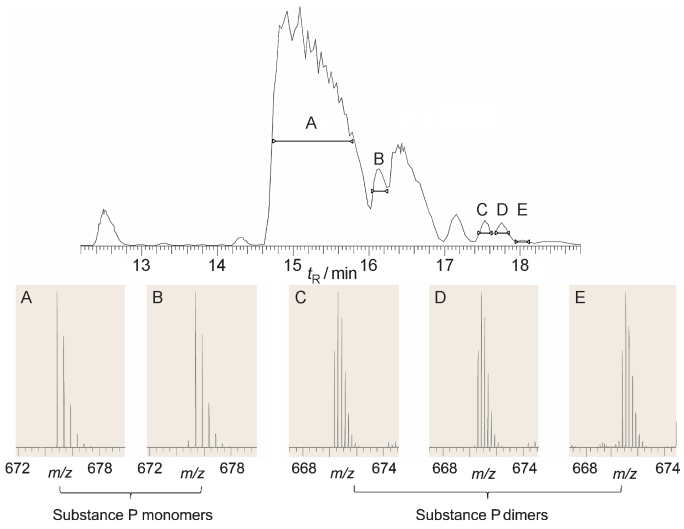


Figure 2. Reverse-phase HPLC separation of the tTGase-catalyzed substance-P deamidation and transamidation reaction products.

the total ion current (TIC) chromatogram of reaction products obtained after 5 h incubation of a solution of 222 μM SP with 0.02 U of tTGase at 37 °C. The species of interest were detected by LC FT-ICR MS (Figure 2, insets). The singly deamidated SP elutes at retention time, $t_R = 15$ min, followed by doubly deamidated SP at $t_R = 16.2$ min. The two peaks at $t_R = 16.4$ and 17.2 min correspond to the truncated forms, which lack the first two residues at the N terminus, of singly and doubly deamidated SP, respectively. Degradation of SP after the second residue is observed normally after incubation for several hours at 37 °C and represents a cleavage at the C-terminal side of proline residue. These species are followed by three distinct peaks of lower intensity that correspond to the three different populations of SP dimers ($t_R = 17.55$, 17.75, and 18.1 min). Under these experimental conditions, the reaction was completed without detection of unreacted SP.

The substantially higher intensity of the peaks assigned to deamidated SP compared to the cross-linked SP suggests that the Lys residue of SP is not a preferred substrate for the enzyme. A similar conclusion can be made by comparing the reaction described here and the one between SP and the primary amine monodansylcadaverine (MDC) (vide infra).

Selective deamidation of Glu_5 and Glu_6: ECD mass spectra collected for singly and doubly deamidated SP in the data-dependent LC FT-ICR MS/MS experiment are shown in Figure 3. The expected and almost complete sequence coverage is achieved in both cases. The absence of the c_1^+/z_{10}^+ or

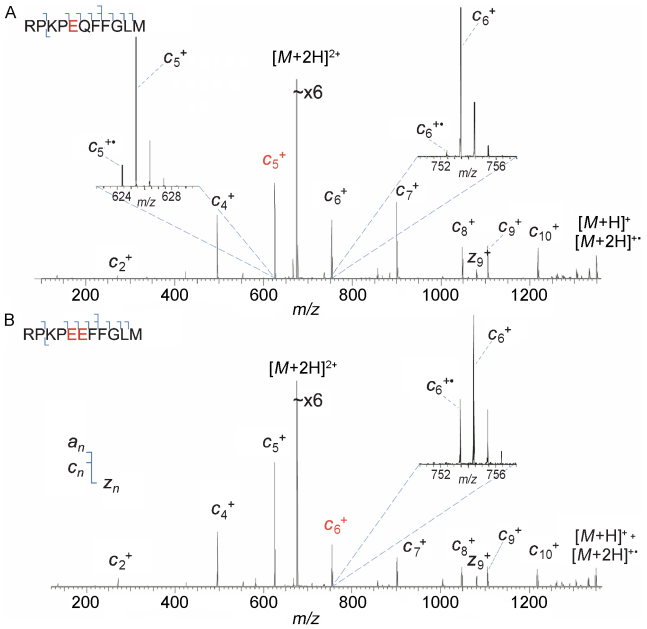


Figure 3. Tandem mass spectra of tTGase-catalyzed (top) singly and (bottom) doubly deamidated substance P, acquired with LC ECD FT-ICR MS.

c_3^+/z_8^+ ions is due to the ring structure of a proline residue, which prohibits the formation of the corresponding ECD product ions. The fragmentation patterns are similar to those of nondeamidated SP.[50] The predominance of *c*-type ions over *z*-type ions is, presumably, due to the position of the charged residues close to the N terminus of the peptide.

ECD reveals that the singly deamidated SP has a single Gln-to-Glu substitution that occurs exclusively at position 5 (Figure 3A). The inset in Figure 3A shows the presence of a

radical species, $c_5^{+\cdot}$, at the amount typically obtained in ECD of the unmodified SP for c_5^+ ions. Moreover, all the heavier *c*-type ions, from c_6^+ to c_{10}^+, show a 0.98 Da mass shift in comparison to the corresponding ions of unmodified SP, as demonstrated by c_6^+ isotopic distribution (Figure 3A, inset). The ECD mass spectrum of doubly deamidated SP shown in Figure 3B correlates with the ECD pattern for the singly deamidated SP species for c_2^+, c_4^+, and c_5^+ ions. Consistently, an additional 0.98 Da shift in mass is observed for the rest of the c_n^+ ion series, for $n>5$.

Importantly, no population of SP with a single deamidation at position 6 is observed. Therefore, ECD FT-ICR MS results, coupled with the intensity ratio of the TIC chromatographic peaks for the two modified SP species, suggest that either the deamidation of Gln_5 is necessary for the subsequent deamidation of Gln_6, or that Gln_6 is a much less probable substrate for tTGase than the Gln_5 residue.

Substance-P transamidation products: As displayed in Figure 4, the three populations of SP dimers revealed by LC–MS differ by a 0.98 Da mass shift, which corresponds to a single deamidation event.

Calculation of the expected molecular weight of cross-linked dimers from transamidation (twice molecular weight of SP minus ammonia; 17.024 Da) indicates the three dimers to be singly, doubly, and triply deamidated species. Proposed sequences for each population inferred by the measured *m*/*z* values are reported between the two isotopic distributions for the two charge states, 4+ and 5+, observed in the broadband mass spectra (Figure 4). Note that the intensities of the first two dimer populations are similar and higher than the one of the third population, Figure 2.

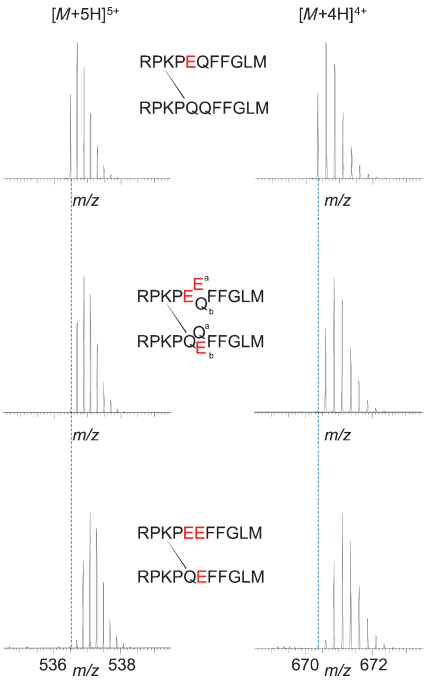


Figure 4. Isotopic distributions of the (left) quintuply and (right) quadruply charged populations of (top) singly, (middle) doubly, and (bottom) triply deamidated SP dimers formed by transamidation and observed by LC FT-ICR MS. Insets show the corresponding suggested structures of the dimers.

ECD FT-ICR MS was performed on every isolated SP dimer population by using the $[M+4H]^{4+}$ ions as precursors (Figure 5). The FT-MS was set to operate in a targeted

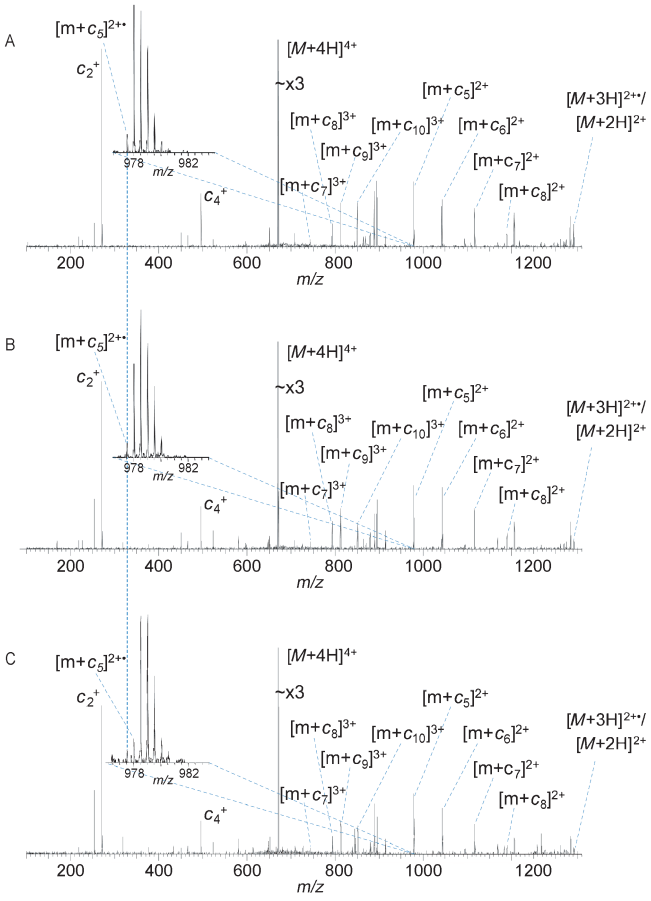


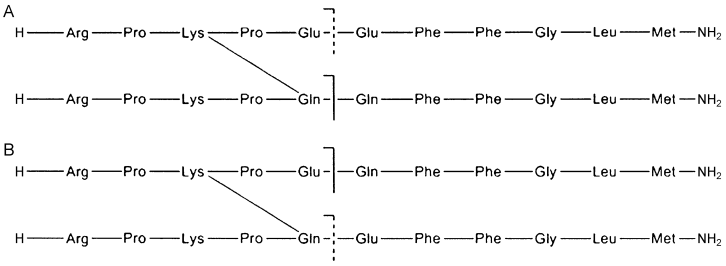
Figure 5. ECD FT-ICR MS of (top) singly, (middle) doubly, and (bottom) triply deamidated dimers of substance P formed by tTGase-induced transamidation ("m" indicates the monomeric form of SP). Tandem mass spectra were acquired under standard operating conditions in the LC–MS/MS experiment.

mode to isolate (isolation window of 5 Th) and fragment species at *m*/*z* 670.10. In all three ECD mass spectra reported in Figure 5, a total absence of c_5^+ ion, that could originate from an SP monomer if cross-linked at position 6, is observed. Therefore, only Gln_5 is involved in the transamidation reaction. The low reactivity of Gln_6 revealed by the study of ECD mass spectra of the two deamidated forms of monomeric SP (Figure 3) supports this conclusion. The remarkably high abundance of c_2^+ ions in comparison to other product ions, specifically c_4^+ ions, reflects the unique possibility for both monomeric units that form the dimers to contribute.

ECD product ions that contain the transamidation-reaction-produced covalent bonding between an intact mono-

meric SP and a *c*-type fragment of the second SP monomer are assigned in Figure 5 as $[m+c_n]^{k+}$. The analysis of the series of $[m+c_n]^{k+}$ ions gives an important indication about the position of deamidated residues. The ECD fragmentation pattern obtained from the singly deamidated SP dimer population contains a characteristic $[m+c_5]^{2+}$ ion that indicates the deamidation of Glu_5 (Figure 5A, inset). The first deamidation of the Gln_5 residue is further confirmed by the fact that one of the two Gln_5 residues in the dimer is involved in the formation of the cross-linking bond, and a population of $[m+c_5]^{2+}$ ions without a corresponding mass shift is not observed.

ECD mass spectra for the SP dimer population with two deamidated residues is shown in Figure 5B. Depending on both the position of the second Glu residue and the fragmentation site, two possible $[m+c_5]^{2+}$ fragment ions can be produced by ECD: one with the same *m*/*z* as observed for the singly deamidated dimers, and a heavier one (Scheme 2).



Scheme 2. Possible deamidation and fragmentation sites of doubly deamidated SP dimers for obtaining $[m+c_5]^{2+}$ ions ("m" indicates the monomeric form of SP); dashed lines indicate the lighter product ions, solid lines the heavier ones. A) Dimer with both the deamidation on the same monomer; B) dimer with deamidation on both SP monomers.

A partial overlap between the two product ion isotopic distributions can be observed (Figure 5B, inset). Finally, as a consequence of deamidation of all the three originally available Gln residues in the third population of SP dimers, a further mass shift in the $[m+c_5]^{2+}$ isotopic distribution is apparent (Figure 5C and inset).

Substance-P cross-linking with MDC: The above-illustrated results demonstrate that the tTGase-induced deamidation of Glu_6 can occur not only after the deamidation of Glu_5, but also after a cross-linking between Glu_5 and a peptydil lysine. To further investigate this process, a tTGase reaction with SP was performed in the presence of a primary amine, monodansylcadaverine (MDC).[38] The acyl-acceptor MDC was used in a 10-fold excess amount to promote the transamidation between SP and MDC in a competition with SP deamidation. Kinetics studies were performed by using the same algorithm as for the SP-tTGase reaction described above. We were able therefore to monitor both the formation of transamidated SP-MDC complexes and the results of the competition between deamidation and transamidation reactions. In contrast to the previous reports on the kinetics of SP interaction with tTGase, the reaction product analysis described here did not take advantage of MDC fluorescent properties but was obtained through LC–MS analysis.[38]

The advantage of the LC–MS method is in the detailed characterization of peptide modifications, specifically, Gln deamidation, on transamidated SP-MDC complexes. The time-course analysis clearly indicates that the main reaction product is the cross-linked MDC-SP ($t_R = 11$ min, *m*/*z* 833.439 for the doubly charged species, as displayed on the left inset of Figure 6), in presence of both higher (right) and lower (left) concentration of substrates. After approximately one hour from the reaction start, the abundant peak of MDC-SP complex is followed by a less abundant peak ($t_R =$ 11.2 min, *m*/*z* 833.931, doubly charged), which corresponds to an MDC-SP with deamidated Gln_6 (Figure 6, central inset). Although the intensity of the latter peak increases with time, it remains low. Nevertheless, the presence of this peak demonstrates that the second glutamine of SP can be deamidated also in presence of an isopeptidic bond on Gln_5 side chain.

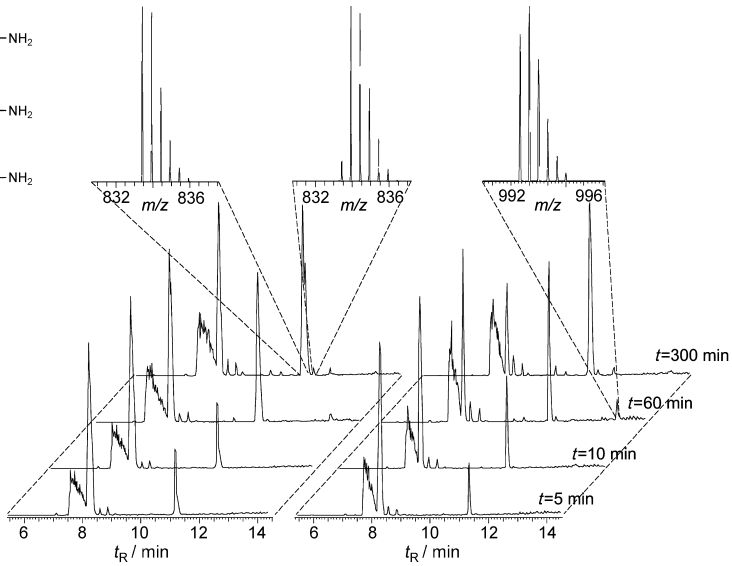


Figure 6. LC FT-ICR MS of a tTGase-catalyzed reaction between substance P and monodansylcadaverine (MDC) used in a 10-fold excess amount. Left: 74 μM SP; right: 148 μM SP.

Abad and co-workers investigated the reactivity of Gln_6 in SP during its tTGase-catalyzed reaction with MDC detecting the reaction products by reverse-phase HPLC combined with fluorescence spectroscopy.[38] They observed a high propensity of SP to be cross-linked to both Gln_5 and Gln_6 when the reaction is performed at 37 °C with an approximately 100-fold excess amount of MDC over SP. On the contrary, we show that under our reaction conditions, the formation of a doubly cross-linked SP ($(MDC)_2$-SP) is not favored and that this reaction product is probably unstable. After 60 min from the beginning of the reaction, a peak related to $(MDC)_2$-SP is detected for both reactions ($t_R =$

13.3 min, m/z 992.508 for the doubly charged species; spectrum on Figure 6, right inset).

The reasons for the low abundance of $(MDC)_2$-SP can be found in the competition between deamidation and transamidation and in a possible hydrolytic activity of the enzyme towards isopeptidic bonds, as reported by Sollid et al.[51] This tTGase reaction could explain the absence of $(MDC)_2$-SP peak at the later reaction times.

Biochemical confirmation of substance-P deamidation: To verify the data collected with LC–MS/MS, and to determine possible modifications that occurred at SP as an effect of side reactions of tTGase, we performed enzymatic digestion of SP with endoproteinase Glu-C from *Staphylococcus aureus* V8 (Figure 7).[52] Glu-C can selectively cleave the peptidic bond on the C-terminal side of a glutamic acid residue when used in ammonium bicarbonate 0.1 M, pH 8.0. A solid-phase-synthesized Glu_5-SP variant was used to compare its Glu-C digestion products with the ones obtained by using the tTGase-deamidated SP as a substrate for the endoproteinase. In summary, the following substrates were compared by the proteolitic digestion: SP after reaction with tTGase, Glu_5-SP after tTGase deamidation, and Glu_5-SP alone, without previous reaction with tTGase. All the samples were tested in the presence (positive control) and absence (negative control) of Ca^{2+}. Our enzymatic digestion described before was carried out using the products of upscaled tTGase reactions (performed in 1 h with 0.05 units of enzyme to prevent possible aggregation of the products that could occur after many hours from the start) without intermediate purification. The substrates for Glu-C were dissolved in a buffer that contained Tris-HCl to obtain the final concentration in the new reaction mixture of around 110 μM. The synthetic Glu_5-SP was used for the reaction in presence of the same concentration and composition of the buffer. The detection of the digested peptides was performed by LC–MS by using the linear trap quadrupole (LTQ) mass spectrometer.

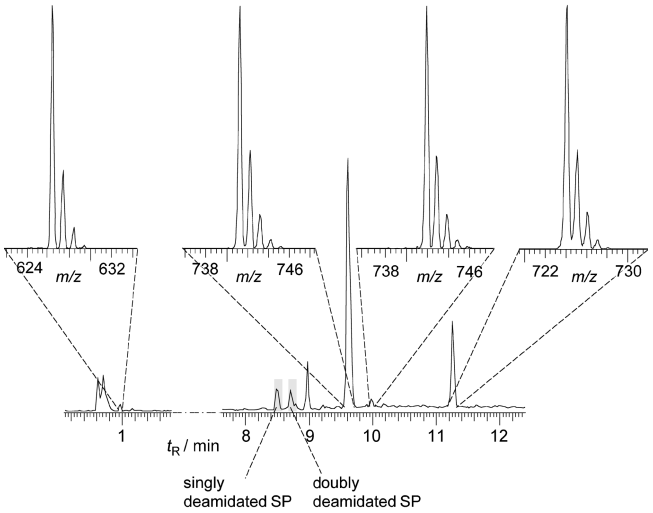


Figure 7. LC–MS of Glu-C digestion of reaction products of substance P with tTGase.

All the screened samples provided the expected fragments: no cleavage for SP negative control, 2 main fragments, SP [1–5] and SP [6–11], for the singly deamidated SP (both enzymatically obtained from unmodified SP and a synthetic modified peptide), and, more interestingly, the same two fragments for the doubly deamidated SP, with a shift in mass of 0.98 Da in the SP [6–11] fragment in comparison with the one obtained from singly deamidated SP. This result, combined with the lack of detection of the SP [1–6] or SP [7–11] fragment, reveals that Glu-C cleaves specifically only after the Glu_5 residue (see Figure S1 in the Supporting Information). Figure 7 illustrates the products detected for the upscaled tTGase reaction of SP after 24 h. The highly hydrophilic SP [1–5] peptide elutes very early, and most probably it is partially undetected, whereas the above-mentioned variants of the SP [6–11] fragment elute after more than 9 min. Note that the second most abundant species in the TIC is at m/z 724.12. We suggest that it corresponds to the SP [6–11] fragment in which the N-terminal Gln or Glu residue is converted into pyroglutamic acid (pGlu) through a nucleophilic attack reaction of the free N-terminal amine to the γ-carboxylic group. The mass difference between this peak and the most abundant SP [6–11] peptide is 17.1 Da. The formation of pGlu after proteolitic cleavage of peptides and proteins has been previously reported, but only for tryptic digestion.[53] The LC–MS data alone is not sufficient to determine if only the N-terminal Gln residue is converted into the pGlu or if the N-terminal Glu of the second and less abundant SP [6–11] fragment also contributes. Literature reports suggest that the pGlu conversion is more favorable in the presence of an N-terminal glutamine, which correlates here with the observation of the more abundant of the two species.[54,55]

Conformational studies on substance P and singly deamidated substance P: To determine potential differences in the conformation of unmodified SP and its singly deamidated Glu_5-SP variant in solution, circular dichroism (CD) spectra were recorded for both peptides (Figure 8).

Both peptides in aqueous solution show CD spectra typical for a random coil conformation (Figure 8A) as reported previously.[56–58] Apparently, a single amino acid substitution as in the Glu_5-SP variant does not induce the formation of a stable secondary structure. However, it is known that SP can acquire an ordered secondary structure when dissolved in organic solvents or bound to detergent micelles. Figure 8B and C represent SP (solid line) CD spectra in 50% 2,2,2-trifluoroethanol (TFE) and 30 mM sodium dodecyl sulfate (SDS), respectively. In the former case, the spectra collected in 50% TFE are characterized by a minimum in ellipticity at 205 nm and a maximum at 195 nm, thus indicating a β-turn or mixed β-turn/α-helix structure.[57] Upon binding to negatively charged SDS micelles, SP forms predominantly

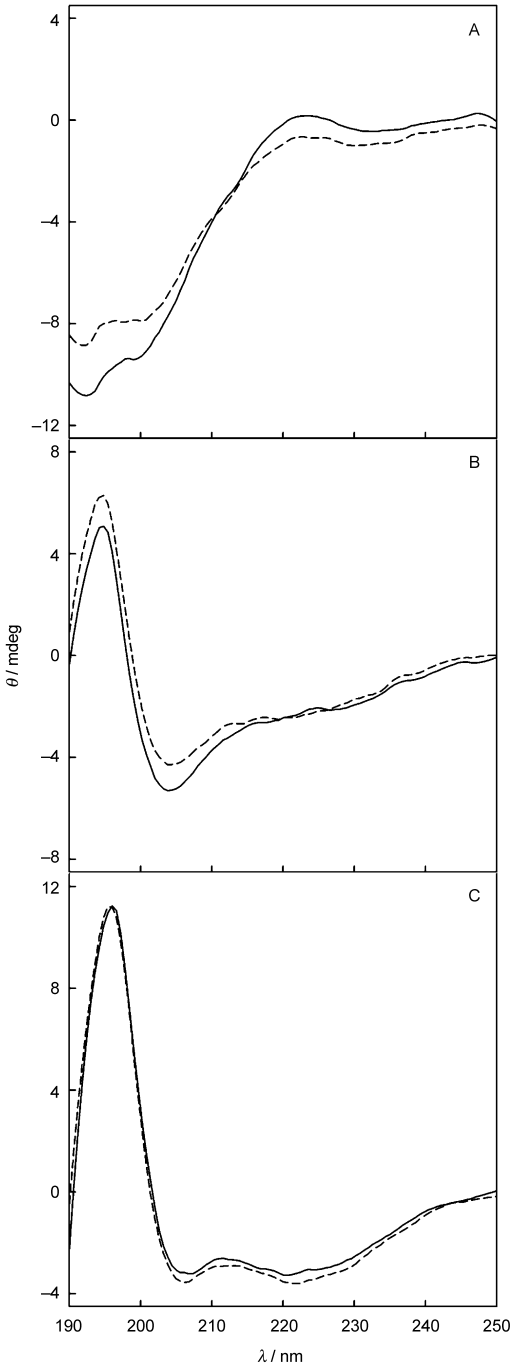


Figure 8. Circular dichroism spectra of SP (solid line) and Glu_5-SP (dashed line) in different solutions: A) water, B) 50% TFE–water, C) 30 mM SDS in water.

Although peptide structure assignment based on the CD spectra in SDS and TFE solution is not unambiguous, the high similarity of the spectra trends for SP and Glu_5-SP suggests that the substitution at the fifth residue does not alter the peptide structure in solution. Nevertheless, conclusive indications with regards to Glu_5-SP receptor binding characteristics could not be derived from our CD data.

Receptor activation studies: To demonstrate the biological relevance of our structural investigations further, we investigated the capability of Glu_5-SP to induce a cellular response through binding to the neurokinin-1 receptor (NK1-R). For these studies, HEK293 cells that stably expressed the NK1-R were used. We employed the fluorescence assay based on the signal transduction pathway, which is activated after the binding of an agonist to NK1-R. This process leads to an increase in the free cytosolic concentration of Ca^{2+} and subsequently to a fluorescent response mediated by Fluo-4 Ca^{2+} indicator dye.

First, a confocal microscopy-based analysis was carried out to compare the cellular response to the two peptides and the morphological changes induced by the potential stimulation (Figure 9).[60]

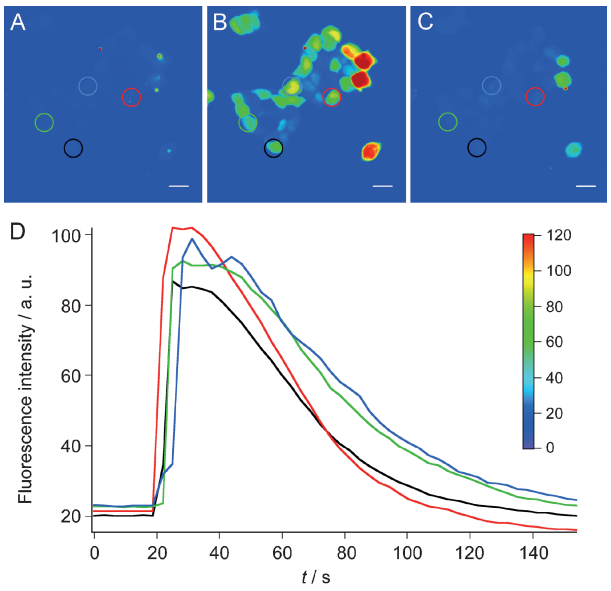


Figure 9. Ca-ion concentration mobilization in HEK293 after the addition of Glu_5-SP. The confocal images show the fluorescence of the Ca-ion indicator Fluo-4 inside of the HEK cells at A) 0 s, B) 30 s, and C) 110 s after the addition of Glu_5-SP to a final bulk concentration of 20 nM. D) Time dependence of fluorescence intensity of Fluo-4.

an α-helical conformation in agreement with published data.[57,59] The synthetic singly deamidated SP variant shows a similar behavior (Figure 8C). The CD spectra of the two peptides in the presence of 30 mM SDS are almost identical, whereas in 50% TFE, Glu_5-SP shows minor structure variations in comparison to the unmodified SP, in particular in the 215–230 nm region.

As shown in Figure 9, the addition of Glu_5-SP produces a quick increase of fluorescence with the cellular response duration, approximately 100 s, comparable with the reported values[61,62] and those observed for SP (data not shown). Moreover, the binding of SP to NK1-R eventually results in the formation of blebs on the cell membrane.[63] The same phenomenon is observed for Glu_5-SP.

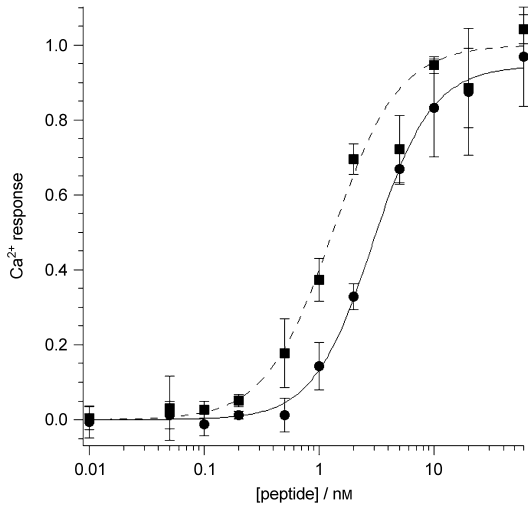


Figure 10. Change of the intracellular Ca-ion concentration at different bulk concentrations of SP (●) and Glu_5-SP (■) obtained as described in Figure 9. Lines are fits of the experimental data to the Hill equation. Experimental data are expressed as mean (± standard deviation) of four measurements.

We subsequently compared the cellular Ca-ion responses in a dose-dependent stimulation for the two agonists.[64] Figure 10 shows the concentration-response curves that indicate that Glu_5-SP induces the intracellular response at slightly lower concentrations than the unmodified counterpart. The calculated EC_{50} values for the wild-type and deamidated SP are (2.9 ± 0.5) and (1.3 ± 0.2) nM, respectively. The repetition of the experiment resulted in a substantial confirmation of the ratio between the EC_{50} values.

Discussion

The first biology-related question we addressed here by LC FT-ICR MS/MS concerns tTGase activity on SP. Although it is known that tTGase can catalyze two competitive reactions, one that involves both glutamine and lysine (transamidation), the other glutamine and a water molecule (deamidation), the reactivity of lysine residues in SP is a source of controversy in the literature. Whereas some reports claim that no dimers could be detected[39] and that lysines preceded by a proline are not good acyl acceptors, Pastor et al. mentioned the detection of intermolecular cross-linking of SP analogues, however, without delivering further information.[49] Here we have shown that the reactivity of Lys_3 is low. We confirmed it first by the kinetic study on SP only, which shows that the deamidation is much favored on this substrate over transamidation, and second by the analysis of SP–monodansylcadaverine reaction products, which on the contrary highlight the strong propensity of tTGase for cross-linking this primary amine to the neuropeptide.

However, we demonstrated that at the specific reaction conditions, for instance, at an increased substrate concentration, tTGase efficiently produces SP dimers, although deamidated SP remains the main reaction product. Moreover, tTGase can modify the structure of the monomers and create different populations of intermolecularly cross-linked SP. ECD FT-ICR MS analysis demonstrates the presence of three different subpopulations of dimers that are distinguished by the number of deamidated glutamines they contain, from 1 to 3 (three is the maximum number of Gln residues in SP dimer that may be modified after the formation of one isopeptidic bond).

As indicated by previous studies[38] and confirmed here by tandem mass spectrometry, in the case of SP, the two glutamine residues can both act as substrates for tTGase, but strictly sequentially. The Gln_6 residue can be either deamidated or transamidated, but only after the modification of the Gln_5 residue. This conclusion is based on the observation that, as displayed in Figure 2, in the attempt to force the tTGase-mediated production of dimers, the main reaction products are singly and doubly deamidated SP, and the former of these species is deamidated at position 5 only. This is revealed by the corresponding ECD mass spectra, which present a c_5^+ ion that corresponds to a Gln_5-to-Glu_5 substitution. The corresponding isotopic distribution is the one expected for a c^+ ion that terminates with Glu (RPKPE) and not the one terminated with Gln (RPKPQ), despite the presence of a radical species. The characterization of the SP dimers by ECD reaffirms this conclusion. ECD mass spectra show that the transamidation takes place only at position 5 of the first SP monomer, and the first of the three detected deamidations proceeds at Gln_5 of the second SP monomer. The Gln_6 residue is modified only afterwards. Consistently, in the MDC–SP reaction, the Gln_6 residue is deamidated only in doubly deamidated SP or in a small population of MDC–SP dimers in which the acyl donor for cross-linking remains Gln_5.

The fact that the modification that occurs at Gln_5 can be either a deamidation or a transamidation suggests that the probable reason for Gln_6 to start acting as a substrate is not the deamidation-induced local charge modulation (increase of a negative charge upon conversion from glutamine to glutamate). Although in the presence of two consecutive glutamines in the primary structure the one closer to the C-terminal end is normally less reactive than the other, this cannot be considered to be a general rule.[15] Presumably, Gln_6 starts to be reactive after a modification of Gln_5 as a consequence of a conformational change in the peptide that leads to improved steric accessibility to the active site of tTGase. Therefore, a modification at position 5 might also affect SP properties in other binding processes. Generally, it is believed that SP assumes a partial α-helical conformation when interacting with membranes and proteins such as receptors. The attainment of an α-helical secondary structure has been previously indicated by CD experiments.[56,57] Recent NMR spectroscopic studies suggested that in a membrane-bound form SP is organized with a central α-helix and a flexible N-terminal end.[65] We confirmed the fact SP rearranges itself in an α-helix by CD performed in 30 mM SDS, a membrane-mimicking environment (see Figure 8). Not

surprisingly, the same behavior is retained also by Glu_5-SP. The activity of Glu_5-SP was a subject of the studies reported here due to the high rate of its formation by tTGase from SP. The capability of SP analogues to reach an α-helical conformation despite single and even double sequence mutations has been demonstrated by NMR spectroscopy.[65,66]

To reveal the capability of Glu_5-SP to interact with a protein, we chose a calcium-ion signaling assay in live HEK293 cells that stably express the neurokinin-1 receptor (NK1-R), the natural agonist of which is SP. We demonstrated that Glu_5-SP acts as an agonist for NK1-R, with an activity that is even higher than that of unmodified SP. NMR spectroscopic studies conducted on polypeptides that represent the predicted second and third extracellular loops (named EC2 and EC3, respectively) of NK1-R showed that both peptides are involved in SP binding; specifically Gln_5 is among those SP residues that interact with EC3 and, presumably, that maintains a solvent-accessible position.[67,68] This is in agreement with the case of the NK2 receptor, which is very closely related to the NK1-R, in which the binding site for agonist and antagonist peptides has been localized with high precision in living cells also at the extracellular side of the receptor.[69,70] On the other hand, Perrine et al. demonstrated that in cells that express the rat NK1-R, the Gln_5-to-Ala_5 substitution only slightly reduces the capability of SP to induce intracellular calcium-ion responses, thus suggesting that the residue at the fifth position is involved in the binding to the receptor but is not essential for the activation of the downstream signal transduction.[66] Nevertheless, the latter study considers a nonconservative substitution, whereas the tTGase-induced deamidation results in a less dramatic modification. In the Gln-to-Glu substitution, the main variation is associated with the introduction of a negative charge into SP, whereas the steric hindrance of the two side chains is similar. The results obtained here suggest that the absence of the amide group in the lateral chain of glutamate slightly alters the interaction of the fifth residue with both the solvent and, more notably, the third extracellular loop of the receptor without compromising at the same time the general structure of the peptide. These findings offer the prospect for further analysis directed toward the elucidation in detail of the differences in the interaction of substance SP and Glu_5-SP with EC3.

Conclusion

By coupling reverse-phase liquid chromatography, for the separation of tissue transglutaminase (tTGase) reaction products, with online electron capture dissociation high-resolution mass spectrometry, we were able to unambiguously determine the deamidation and transamidation sites on the neuropeptide substance P (SP). The identification of these sites on our model peptide was unambiguous even without using traditionally employed strategies such as testing modified peptides that carry point mutations. In fact, ECD offered a complete fragmentation pattern of both deamidated and transamidated SP (without, in the latter case, cleavage of the newly formed isopeptidic bond). Therefore our proposed method has the potential of being used in the elucidation of structural features of larger peptides and proteins, for example, amyloid-beta peptide,[71] subjected to both enzymatic "zero-length" cross-linking and post-translational modifications.

Taken together, this study shows that under different conditions the major reaction products are always singly and doubly deamidated SP, tereby highlighting the low propensity of Lys_3 of SP to act as a substrate in a cross-linking reaction. Moreover, ECD spectra have demonstrated that both the two consecutive glutamine residues of the substrate (Gln_5 and Gln_6) can be deamidated, but strictly sequentially, and with Gln_5 as a preferred substrate for tTGase. In fact, when the reaction conditions allow the formation of SP dimers, ECD reveals that the cross-linking site is on Gln_5 only, whereas Gln_6 can react the same but, again, only after Gln_5 modification (deamidation or transamidation). This results in the formation of three different dimers populations with one, two, or three deamidated Gln residues, respectively. With regards to the identification of the isomeric residues, for example, glutamate to γ-glutamic acid isomerization, the results reported here did not demonstrate the presence of characteristic product ions in ECD FT-ICR MS specified by O'Connor and co-workers.[48] Therefore, the reaction catalyzed by tTGase indeed does not induce isomerization and deamidated SP species do not undergo spontaneous Glu isomerization after 5 h in solution at 37 °C. Finally, we discovered that the cellular response of SP upon binding to the natural receptor NK1-R is not decreased but, on the contrary, slightly increased by a single deamidation event that occurs at residue 5, as proven by an in vitro Ca^{2+} assay with HEK293 cells that express the receptor.

Experimental Section

Samples and reagents: Water, acetonitrile (HPLC MS grade), NaCl, and $CaCl_2$ were purchased from Fluka analytical (Buchs, Switzerland). Formic acid was obtained from Merck (Zug, Switzerland). Tris-HCl buffer was purchased from Calbiochem (Nottingham, United Kingdom). Guinea pig liver transglutaminase, endoprotease Glu-C from *Staphylococcus aureus* V8, and substance P were purchased from Sigma–Aldrich (Buchs, Switzerland). Dulbecco's modified Eagle medium (DMEM), GlutaMAX medium, fetal calf serum (FCS), and Dulbecco phosphate-buffered saline (D-PBS) were purchased from Invitrogen (Breda, Netherlands). Fluo-4 NW calcium assay kit was obtained from Invitrogen (Carlsbad, CA, USA). Substance P variant modified at position 5 (E_5-SP) with the substitution of the glutamine residue by glutamic acid, RPKPEQFFGLM-NH_2, was produced by solid-phase synthesis (Protein and Peptide Chemistry Facility, University of Lausanne, Switzerland). E_5-SP crude peptide was purified by reverse-phase HPLC chromatography, whereas other reagents and samples were used without further purification.

Transglutaminase-catalyzed reactions: In a typical reaction catalyzed by Guinea pig liver transglutaminase (tTGase), the reaction mixture contained 40 mM Tris-HCl (pH 7), 70 mM NaCl, and 2 mM $CaCl_2$. For the kinetics studies of substance-P deamidation following interaction with tTGase, tTGase (0.002 units) was mixed with a solution of substance P

(74 μM) at an incubation temperature of 30 °C. In total, 7 fractions were collected at the following time points: 5, 10, 15, 30, 60, 120, and 300 min. For substance-P deamidation and transamidation, the peptide concentration was increased to 222 μM. For this reaction and those described below, tTGase (0.02 units) was used at 37 °C unless otherwise stated. The reaction was stopped after 24 h with fraction collection performed after 1, 2, and 5 h. For the monodansylcadaverine (MDC)–substance P cross-linking reaction, substance P at two concentrations, 74 and 148 μM, was used. A 10-fold excess amount of MDC was employed to yield the corresponding final MDC concentrations of 740 and 1480 μM, respectively. Several fractions were collected following the scheme used for substance-P deamidation and transamidation. The enzymatic catalysis was always quenched by immediately storing the liquid samples at −80 °C.

Liquid chromatography mass spectrometry (LC–MS): Analysis of the reaction products was performed by liquid chromatography combined online with high-resolution tandem mass spectrometry (LC–MS/MS). For the reverse-phase liquid chromatography, a Surveyor LC pump (Thermo Fisher Scientific, Bremen, Germany) was used. After a 1:2 dilution in water, samples (10 μL) were loaded onto a silica-based Hypersil Gold (100×1 mm, 1.9 μm particle size) C_{18} resolving column (Thermo Fisher Scientific, Bremen, Germany). Eluents consisted of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B). The employed flow rate was 50 $\mu L\,min^{-1}$ with an initial flow of 5% B maintained for 2 min followed by a linear gradient up to 80% B in 20 min.

Tandem mass spectrometry analysis was performed using a linear-ion-trap Fourier-transform ion-cyclotron-resonance mass spectrometers (LTQ FT-ICR MS) equipped with a 7 and 12 T superconducting magnet (Thermo Fisher Scientific). Eluting analytes were electrosprayed by using a standard electrospray ion source (ESI). The LTQ FT-ICR MS were operated in the data-dependent mode to automatically switch between broadband MS and MS/MS acquisitions. Peptide cations of interest were isolated in the LTQ (isolation window of 5 Th, target charge counts 5×10^5) according to a predefined mass list and then transferred to the ICR ion trap for subsequent tandem mass spectrometry. Electron-capture dissociation (ECD) was performed under standard experimental parameters: selected precursor ions were irradiated with low-energy electrons for approximately 50 ms before product ion excitation and detection. Ion magnetron motion correction was taken into account by introducing an appropriate delay prior to electron injection.[72] The minimum signal-to-noise (S/N) ratio for selection of precursor ions was set to 500. Broadband MS spectra (m/z 500–800) were collected using transient recording over 768 ms (a resolution of 100000 at m/z 400 for 7 T LTQ FT-ICR MS configuration), whereas ECD was performed with transient recording over 192 ms (a resolution of 25000 at m/z 400 for 7 T LTQ FTMS configuration) across an m/z window of 100–2000 Th. Reported ECD mass spectra normally average 10 scans. Data analysis was performed using Xcalibur software, version 2.0.7 (Thermo Fisher Scientific).

Proteolytic digestion with Glu-C: Endoproteinase Glu-C digestion was carried out at 37 °C in 0.1 M ammonium bicarbonate at pH 8.0 by using an enzyme-to-substrate ratio of 3:100 (w/w). Reaction mixture aliquots were collected after 6, 10, and 24 h. Different substrates were tested at 110 μM concentration, including substance P with and without modifications: wild-type substance P; reaction products of tTGase catalytic activity (same conditions previously described except for the enzyme concentration, 0.05 U instead of 0.02) on substance P with and without Ca^{2+}; reaction products of tTGase on synthetic Glu_5-SP (same conditions mentioned above) with or without Ca^{2+}; synthetic Glu_5-SP in the same Tris-HCl final concentration of the previous sample, with and without Ca^{2+}. The GPMAW tool (version 8.0, Lighthouse data, Odense, Denmark) was used for generating a list of expected peptides from the enzymatic digestion. Reactions were always quenched by immediate sample storing at −80 °C.

CD measurements: Far-UV circular dichroic spectra were collected using a Jasco J-810 spectropolarimeter (Jasco Corporation, Japan) at 37 °C with a cuvette with a path length of 0.1 cm. CD spectra were recorded between 190 and 250 nm, with 0.2 nm wavelength steps and a bandwidth of 1 nm. Samples (SP and Glu_5-SP) were dissolved to a final concentration of 50 μM in water, 50% 2,2,2-trifluoroethanol (TFE) and 30 mM sodium dodecyl sulfate (SDS). For every reported spectrum, six scans were averaged to improve signal-to-noise ratio. All spectra were corrected for the buffer baseline.

Confocal imaging: Adherent HEK293 cells that stably expressed NK1 receptors[60] were grown in DMEM/F-12+GlutaMAX medium that contained 10% FCS and Hygromycin B (100 $\mu g\,mL^{-1}$) in a humidified atmosphere with 5% CO_2 at 37 °C. For confocal imaging, cells were seeded on glass coverslips in six-well dishes in DMEM/FCS (2 mL). After 24 h, cells were incubated at 37 °C for 30 min with 5 mM Fluo-4 AM (Invitrogen, Carlsbad, CA, USA) in serum-free medium, followed by 30 min incubation with fresh medium that contained 10% FCS. Agonist-containing solution was added to the selected well during the experiments. Cells were imaged using confocal fluorescence microscopy with a Zeiss LSM510 confocal microscope equipped with a C-apochromat ×63/1.2 water-immersion objective (Zeiss, Germany). Excitation was at 488 nm (Ar laser) and the Fluo-4 fluorescence was monitored using a 505 nm longpass emission filter at a rate of 1 image every 2 s.

Calcium-signaling assay: For monitoring the receptor activation, HEK293 cells that stably expressed NK1 receptors were seeded in DMEM/FCS into a clear-bottom 96-well plate for 24 h at 37 °C and 5% CO_2. Growth medium was removed and replaced with fluorescent Ca^{2+}-indicator solution (Invitrogen, Carlsbad, CA, USA). Cells were subsequently incubated for 30 min at 37 °C followed by another 30 min at room temperature before the measurements, without removing the dye-loading solution. The increase of fluorescence intensity upon addition of SP or Glu_5-SP in 11 different concentrations (60, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.01 nM) in assay buffer (1× Hank's balanced salt solution (HBS), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)) was measured on a fluorescence plate reader (FLEX Station, Molecular Devices) at room temperature. The fluorescence emission was recorded at (525±4.5) nm upon excitation at (485±4.5) nm, and final values of Ca^{2+} response were the result of averaging the measurements of 4 wells for each peptide concentration. The EC_{50} for SP and Glu_5-SP were obtained by fitting a sigmoidal dose–response equation to the Hill equation [Eq. (1)]:

$$E_L = E_0 + (E_{max} - E_0)/(1 + (EC_{50}/[L])^{n_H}) \quad (1)$$

in which E_L is the fluorescence intensity for a given ligand concentration [L]; EC_{50} is the concentration that produces 50% of the maximal response E_{max}; E_0 is the baseline intensity in the absence of ligand; n_H is the Hill coefficient. Experimental data were fitted to the Hill equation using Igor Pro software (WaveMetrics Inc., Lake Oswego, OR). Experiments were performed in duplicate. To assess a possible statistical difference among the two dose–response curves, an F-test was performed, whereas a t-test was used for the comparison of EC_{50} values.

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