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Addressing substrate glutamine requirements for tissue transglutaminase using substance P analogues

María Teresa Pastor, Antonio Diez, Enrique Pérez-Payá, Concepción Abad*

Departament de Bioquímica i Biologia Molecular, Universitat de València, Dr Moliner 50, E-46100 Burjassot, Spain

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Abstract We have investigated the effect on the substrate requirements for guinea pig liver (tissue) transglutaminase of a set of 11 synthetic glutamine substitution analogues making up the full sequence of the naturally occurring tissue transglutaminase substrate substance P. While a number of peptide sequences derived from proteins that are well-recognized as tissue transglutaminase substrates have been studied, the enzyme activity using substitution analogues of full-length natural substrates has not been investigated as thoroughly. Thus, our set of substance P analogues only differs from one to other by one amino acid mutation while the length (of the peptide) is maintained as in the natural parent peptide. Our results indicate that a glutamine residue is not recognized as substrate by the enzyme whether it is placed at the N- or C-terminal or between two positively charged residues or between two proline residues. To further address the effect on enzyme activity of charged amino acids in the vicinity of the reactive glutamine residue, a new set of synthetic charge replacement analogues of substance P has been also studied. Together, the results have identified new minimal requirements for modification of a particular glutamine residue in a polypeptide chain. It would be of interest to set up a full set of such requirements in order to highlight potential glutamine residues as enzyme targets in the growing list of proteins that are being described as transglutaminase substrates.

Key words: Transglutaminase; Substance P analogue; Multiple peptide synthesis

1. Introduction

Tissue transglutaminase (tTG, type II TG) catalyzes a Ca^{2+}-dependent acyl transfer reaction in which new γ-amide bonds are formed between γ-carboxamide groups of peptide-bound glutamine residues and a variety of primary amines [1]. Although the most obvious function of tTG is to stabilize biological structures, there is increasing evidence that this enzyme is involved in many additional, more subtle processes, like apoptotic cell death, regeneration events, receptor-mediated endocytosis, cell signal transduction [2] and in the pathology of neurodegenerative diseases [3]. In the reaction catalyzed by tTG, a glutamine residue serves as acyl donor and the ε-amino group of lysine residues as well as some polyamines are the physiological acyl acceptors, although non-physiological amines can also be used by the enzyme. Thus, when incorporated into peptides and proteins, fluorescent amines such as monodansylcadaverine (MDC) have proved to be useful tools for structural analysis [4–7] and peptide-membrane interaction studies [8,9].

The number of protein glutaminyl substrates for tTG is highly restricted, whereas the tolerance to structural differences in acyl acceptors is considerable. Although reactive glutamine residues are preferentially located in a flexible extension of the molecule, very often, at both the N- and C-terminal segment [2,10], so far, it has not been possible to derive a consensus sequence around the specific glutamine residues from the numerous TG substrates characterized. The impact of the primary structure surrounding a potential reactive glutamine on TG-catalyzed reactions has been investigated in early studies using peptide sequences derived from proteins that are well-recognized as TG substrates [11–13]. Based on these studies and some more recent contributions, Coussons et al. [14] have proposed a set of minimal requirements for the modification of a particular glutamine residue in a polypeptide chain. This glutamine must satisfy an accessibility criterion (being located on solvent-exposed or flexible areas of the protein) and in the amino acid sequence around the glutamine, there must be an absence of discouraging features. However, despite these extensive efforts, few studies have dealt with the role of individual amino acid residues on enzyme activity using substitution analogues of full-length natural substrates.

We have previously shown that both adjacent glutamines of the naturally occurring peptide substance P (RPKPQQ-FFGLM-NH_2) are substrates for tTG in a consecutive reaction [15]. Thus, in order to determine if the proposed requirements are of general applicability, we decided to test as TG substrate a set of substitution analogues of substance P. The analogues were designed with the following criteria, (i) we replaced Gln-6 of substance P by Asn in order to have only Gln-5 as reactive glutamine to simplify substrate recognition by the enzyme, we will refer to this peptide as the parent peptide or S2 (Table 1), (ii) the glutamine residue was then walked all along the parent peptide sequence, thus generating a set of 10 glutamine substitution analogues of substance P (analogues S4–S13, Table 1), (iii) the two positively charged residues of the parent peptide, namely Arg-1 and Lys-3, were systematically replaced by glycine or serine or omitted, thus generating a set of seven new substance P analogues (analogues S14–S20, Table 1). The full set of peptides would address how the relative position of the reactive glutamine influences the extent of modification in an invariant peptide sequence as well as the role of the positively charged residues at the N-terminal vicinity of the potential reactive residue. The overall analysis of the behavior of the full set of substance P analogues as substrates of tTG has revealed new substrate glutamine requirements for tTG-mediated protein modification.

*Corresponding autor. Fax: (34) (96) 3864635.
E-mail: cabad@uv.es

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2. Materials and methods

2.1. Materials

MDC (*N*-(5-aminopentyl)-5-dimethylamino-1-naphtalene sulfonamide) was obtained from Serva (Heidelberg, Germany). Guinea pig liver tTG (*R*-glutaminyl-peptide:amine-γ-glutamyltransferase, EC 2.3.2.13) was from Sigma (St. Louis, MO, USA). Salts, buffers and reagents were of the highest purity available.

2.2. Peptide synthesis

The peptides were synthesized by simultaneous multiple peptide synthesis [16] according to standard solid phase techniques by using 9-fluorenylmethoxycarbonyl chemistry [17]. The peptides were purified by preparative reverse phase HPLC. Analytical reverse phase HPLC and laser desorption time of flight mass spectroscopy were used to determine the purity and identity of the peptides.

2.3. TG-mediated chemical modification of peptides

The tTG-mediated chemical modification of the peptides was carried out using 0.4 mM peptide, 0.6 U/ml tTG, 20 mM dithiothreitol, 40 mM calcium chloride and 5 mM MDC in 100 mM Tris-HCl (pH 8.0) buffer. Reaction mixtures were incubated at 37°C and stopped at different reaction times by addition of a volume of 5% trifluoroacetic acid (TFA) containing water:acetonitrile (7:3, v/v). The extent of peptide modification was quantitatively assessed by HPLC as previously described [15].

3. Results

Fig. 1 shows the time course of the tTG-mediated modification with MDC of representative SP analogues (sequences for each peptide are given in Table 1). The parent peptide (S2) apparently behaves as the native substance P sequence and it is a good substrate for tTG. Thus, after 2 h of reaction, 94% of the peptide has incorporated the probe on its Gln-5. On the contrary, analogue S13 could be described as a poor glutaminyl substrate and no amine incorporation was detected for analogue S6 even after a long incubation time (24 h). Analogues S7, S9 and S11 are good substrates of tTG and the percentage of modified peptide gradually increased with time, except for analogue S7 where the degree of modified peptide decreased for long incubation times. Furthermore, for this analogue, we observed the appearance of high molecular weight aggregates paralleled with the decrease in MDC incorporation indicative of peptide cross-linking. In this respect, it should be mentioned that all five reactive analogues in Fig. 1 have a lysine residue in its sequence that can participate as an intrinsic acyl acceptor in competition with the external MDC amine. This lysine residue is preceded by proline that would be considered as an unfavorable residue for intrinsic cross-linking, based in the recently described requirements about acyl acceptor lysines in proteins [18]. In this sense, no cross-linking was detected for those analogues (S2, S9, S11 and S13) that have lysine flanked by prolines (-PKP-). In contrast, the analogue S7 that has a glutamine adjacent to lysine (-PKQ-) was able to participate in cross-linking reactions. It seems that in this case, the adverse effect of a proline residue preceding a lysine could be counteracted by a strong positive effect due to the adjacent glutamine residue. In fact, cross-linked lysine residues with an adjacent glutamine at the C-terminal side are frequently found in a variety of proteins in cornified cell envelopes [19,20].

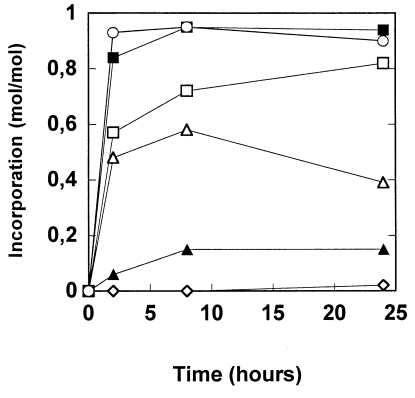

Fig. 1. Time course of tTG-mediated modification of synthetic analogues of substance P with MDC. (○) S2, (◇) S6, (△) S7, (■) S9, (□) S11, (▲) S13. Peptide codes are listed in Table 1.

Depending on the relative position of the glutamine residue, the different synthetic substance P analogues showed pronounced differences as tTG substrates. Table 1 summarizes the percentage of MDC-labelled peptide obtained from the different analogues after 2 h of tTG incubation. At this reaction time, the level of peptide cross-linking was negligible for all 20 peptide analogues. A close examination of the results presented in Table 1 pointed to factors that could be related with enzyme specificity at the amino acid level for a glutamine residue located in a defined peptide sequence. As expected, the analogue S3 that has the two potential reactive glutamines substituted by asparagines was not a substrate for the enzyme. In those analogues where glutamine was placed at the first, second or third position from the N-terminal end, we could not detect probe incorporation even after a long (24 h) incubation time (analogues S4, S5 and S6). Conversely, those analogues in which either Pro-4, Asn-6, Phe-7, Phe-8, Gly-9 or Leu-10 was replaced by a Gln (analogues S7–S12) were found to act as substrates in a different extent. Those analogues

Table 1
tTG-catalyzed incorporation of MDC into synthetic analogues of substance P[a]

Code	Peptide sequence	mol of MDC incorporated/ mol of peptide
S1	RPKP**QQ**FFGLM-NH_2	1.00
S2	RPKP**Q**NFFGLM-NH_2	0.94
S3	RPKPNNFFGLM-NH_2	0.00
S4	**Q**PKPNNFFGLM-NH_2	0.00
S5	R**Q**KPNNFFGLM-NH_2	0.00
S6	RP**Q**PNNFFGLM-NH_2	0.00
S7	RPK**Q**NNFFGLM-NH_2	0.49
S8	RPKPN**Q**FFGLM-NH_2	0.73
S9	RPKPNN**Q**FGLM-NH_2	0.84
S10	RPKPNNF**Q**GLM-NH_2	0.81
S11	RPKPNNFF**Q**LM-NH_2	0.58
S12	RPKPNNFFG**Q**M-NH_2	0.82
S13	RPKPNNFFGL**Q**-NH_2	0.06
S14	GPKP**Q**NFFGLM-NH_2	0.90
S15	SPKP**Q**NFFGLM-NH_2	0.70
S16	PKP**Q**NFFGLM-NH_2	0.90
S17	RPGP**Q**NFFGLM-NH_2	0.85
S18	RPSP**Q**NFFGLM-NH_2	0.88
S19	RPP**Q**NFFGLM-NH_2	0.80
S20	SPSP**Q**NFFGLM-NH_2	0.80

[a]We have followed the proposal of Coussons et al. [14] to number the amino acid residues that could influence the reactivity of a defined glutamine residue in the peptide sequence. As an example in analogue RPKPNQFFGLM-NH_2, glutamine is designated as position 0, thus asparagine will be at −1, proline at −2, lysine at −3, etc. and phenylalanine at +1, phenylalanine at +2, glycine at +3, etc.

designed to study the influence of the presence of positive charges in the surrounding of the potential reactive residue (i.e. analogues S14–S20) could be described as good substrates for the enzyme showing minor differences in reactivity (Table 1).

4. Discussion

We have stressed in the introduction the apparent absence of a consensus recognition sequence for those peptides and proteins that can act as substrate of tTG. Thus, studies addressing the sequence requirements that would define a glutamine residue as tTG substrate could be of interest in order to identify the reactive glutamine in the growing list of proteins that are currently being described as TG substrates. In the present paper, we have undertaken one of such studies by means of the design and assay, as tTG substrates, of a set of different analogues of substance P, a naturally occurring substrate of tTG. Variations in the tTG activity on the different analogues of substance P have provided new information on the specific role of a series of features that could influence the recognition of a defined glutamine residue by the enzyme. These features can be grouped, based on the following criteria: (i) the potential reactive glutamine residue is at the N- or C-terminus, (ii) the presence of proline residues surrounding the target glutamine residue, (iii) the presence of positive charges around the target glutamine residue and (iv) how the replacement or omission of a positive charge at the −4 or −2 position from the target glutamine residue might affect its quality as tTG substrate. The discussion below is structured following these criteria.

The two peptide analogues where the potential reactive glutamine residue is located as the N-terminal residue (S4) or as the C-terminal residue (S13) can be described as non-tTG substrates although, as discussed later, in S4, the presence of a proline residue at +1 could have some influence.

When the glutaminyl residue is located in the second or third position away from the N-terminus, it can be recognized by the enzyme (reviewed in [10]) even when a proline residue precedes the glutamine residue. As reported examples that also follow that feature, we could mention β-casein [21], analogues S14–S20 in the present work, gliadin peptides [22] or osteonectin where Gln-3 in the sequence APQQEAL- was identified as a major substrate in the TG-catalyzed cross-linking of differentiating cartilage [23]. In contrast, glutamine residues having a proline residue in the +1 position [20,24,25] are rarely major targets in the TG-catalyzed cross-linking of polypeptide chains. As a representative example, the sodium potassium ATPase inhibitor, a protein with a single glutamine residue in its sequence followed by a proline residue (-Q^5P-), was not cross-linked by TG [26] although the protein has four potential acyl acceptor lysines exhibiting favorable requirements to be used by the enzyme [18]. On the other hand, from the proteins that have been reported to have polypeptide sequences with proline flanking the glutamine residue, none of such proteins were a substrate for the enzyme [20,25]. Thus, the lack of enzyme recognition of glutamine-3 in our synthetic analogue S6 (Table 1) can be ascribed to the presence of the prolines around the glutamine residue that do not allow a correct orientation of the glutaminyl substrate.

Analogue S5, that was not recognized as a substrate by the enzyme, bears in its sequence also a proline residue at +2 that could be considered, initially, as a potential discouraging factor. However, it has been described that a number of glutamine residues in sequences -QXP- are efficient substrates of TG in both peptides [27] and proteins [6,10,21,26]. So, there should be other factors that could explain why this peptide is not a substrate for tTG. In fact, analogue S5 has positively charged residues around the glutaminyl residue (arginine at −1 and lysine at +1) previously described as a discouraging feature, particularly that at the C-terminal side of the glutamine side chain [14]. In contrast, it should be mentioned that glutaminyl residues with a positively charged side chain in the −1 position [6,25,28] or +1 position [10,20,21,29–31] serve as substrate for TG. Thus, in order to reconcile our results with the above mentioned references, we should consider that S5 is not a tTG substrate because the glutamine is sandwiched between two positively charged residues. To the best of our knowledge, only one example has been reported where a glutamine flanked by basic residues (-KQK-) acts as substrate for tTG. This is found in cross-linked peptides derived from cell envelopes [20], although the amine donor lysine involved in the cross-linking is generally placed in a sequence with a high content of negatively charged residues (Asp, Glu) that can act as charge neutralizing residues.

Replacement by glycine (or serine) of either the arginine or the lysine or both residues at positions −2 and −4 has a minor influence on peptide modification (analogue series S14–S20, Table 1). Furthermore, small changes in MDC incorporation were observed after elimination of the basic residue in each −4 (analogue S16) or −2 (analogue S19) position. Thus, the positively charged residues at the N-terminal side of the glutamine in substance P are not of importance for determining the specificity. These results are in agreement with those obtained earlier by Gorman and Folk [13] who reported that positively charged residues to the C-terminal side of a reactive glutamine in a synthetic model decapeptide have a low impact on the activity of TG enzymes. In particular, it was found that the substitution of a lysine residue in the +2 position by different amino acid residues as alanine, glycine or leucine did not modify the specificity of the human plasma factor XIII nor of the guinea pig liver enzyme.

Basically all the glutamine residues acted as amine acceptors in the reaction with MDC, as the target residue was 'walked' from the fourth N-terminal position towards the C-terminal one (analogues S7–S12), except for the analogue S13 where the reactivity strongly declined. Analogues S9, S10 and S12 improved markedly its quality as substrates relative to S8 and S11. It is tempting to speculate that glycines adjacent of the target glutamine could favor the substrate accessibility. In this sense, it has been described a number of tTG reactive glutamines in different proteins and synthetic peptides that have glycine either at the −1, +1 or +2 position (for discussion see [6,7,10,12,13,24,26]. On the other hand, peptides S7, S8 and S9 and the series S14–S20 provide an example about the role of asparagine around glutamines in determining the specificity. Thus, all peptides with a single Asn in either the −1 (S9) or +1 (S14–S20) position are good substrates of TG. Analogue S7 (two asparagines to the C-terminal side of the glutamine) was not as satisfactory as analogue S9 (two asparagines to the N-terminal side), which could reflect some influence of these residues adjacent to the target glutamine [32]. As a whole, the set of substance P analogues that have been used in the present study provides an opportunity to

assess the role of the protein substrate's primary structure on the specificity of tTG while the native secondary structure is appropriately retained. This approach is useful in order to minimize the effect of some other parameters on the enzyme activity, like the overall peptide conformation, that could occur when a peptide sequence is analyzed outside its natural protein environment.

Comparison of the results obtained in the present work with those from Coussons et al. [14] revealed that new minimal requirements for modification of a particular glutamine residue in a polypeptide chain could be proposed in addition to those previously reported: (i) a N- or C-terminal glutamine residue should be considered as adverse for modification by tTG, (ii) protein (peptide) sequences having the glutamine residue placed both between two positively charged residues or between two proline residues should be considered as discouraging features for correct interaction with the enzyme. In contrast, the presence of positively charged amino acids at two or four residues away from the glutamine towards the N-terminal side seems not to affect the specificity of tTG.

Finally, although the basis for glutamine specificity in the tTG-catalyzed reaction remains unclear, it seems that both the sequence around the potential target glutamine and the conformation of adjacent regions of the protein could determine whether a glutamine residue can be reactive. To further our understanding on the role that the relationship sequence-conformation plays in the requirements that would define a glutamine residue as tTG substrate, studies are in progress towards the design and synthesis, by multiple peptide synthesis techniques, of peptide sequences with a pre-determined secondary structure having glutamine residues at specific positions. Similar approaches have proved to be crucial in elucidating the structural determinants of a number of biological processes [33,34].

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