Localization of Potential Transglutaminase Cross-Linking Sites in Bovine Caseins

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Transglutaminase acyl donor sites in the four individual bovine caseins were determined. Purified caseins labeled with a radioactive site-specific probe in a guinea pig liver transglutaminase-catalyzed reaction were subjected to enzymatic digestion followed by reversed-phase HPLC separation of the resulting peptides. By a combination of sequence and mass spectrometric analysis of the major radioactive fractions, the following TG-reactive glutamine residues were found: α_{s1}-casein, Gln13, Gln108, Gln130, and Gln140; α_{s2}-casein, Gln79, Gln169, Gln185, and Gln187; β-casein, Gln54, Gln56, Gln72, Gln79, and Gln182; κ-casein, Gln29, Gln45, Gln114, and Gln163. In addition, an intra- or intermolecular cross-link between Gln45 and each of two adjacent lysine residues, Lys21 and Lys24 in monomeric κ-casein, was identified.

Keywords: *Caseins; transglutaminase*

INTRODUCTION

Transglutaminases (TG, EC 2.3.2.13) have been extensively studied and catalyze an acyl transfer reaction between protein-bound glutaminyl residues and a variety of primary amines [reviewed by Aeschlimann and Paulsson (1994)]. When protein-bound lysyl residues act as acyl acceptors, the reaction leads to the formation of intra- and/or intermolecular ϵ-(γ-glutamyl)lysine isopeptide bonds. The capacity of TG to mediate the attachment of specific compounds, e.g. amino acids or carbohydrates, and to introduce cross-links in or between proteins has been applied as a potential means of improving the nutritional value and the functional properties of food proteins.

Ikura et al. (1981, 1985) reported that TG can be used to introduce methionine into casein and soybean proteins and lysine into wheat gluten. Moreover, Bercovici et al. (1987) incorporated lysine oligomers into casein using guinea pig liver TG to prepare branched polypeptide chain proteins.

There have been a number of reports concerning the TG-mediated polymerization of food proteins, such as α-lactalbumin and β-lactoglobulin (Aboumahmoud and Savello, 1990), soybean proteins and casein components (Ikura et al., 1980a,b), and pea legumin (Larré et al., 1993). The solubility and hydration properties of polymerized proteins were greatly modified (Motoki et al., 1984). Likewise, the solubility of the vegetable proteins, pea legumin, and wheat gliadins has been improved by TG-mediated incorporation of glycosyl units to the glutamine residues of these proteins (Colas et al., 1993). In addition, TG has been shown to induce gelation of food proteins resulting in gels with different rheological properties (Chanyongvorakul et al., 1994; Sakamoto et al., 1994).

The bovine caseins are important food proteins and are widely used as additives in numerous food products (e.g., in baked products and cereals, coffee creamers, desserts, and pasta products) and casein and its derivatives have been employed in a variety of pharmaceutical applications as well (Southward, 1989). In milk, the caseins exist as stable calcium phosphate protein complexes termed micelles, which are composed of the four caseins: α_{s1}-, α_{s2}-, β-, and κ-casein [reviewed by Swaisgood (1992) and Mercier and Vilotte (1993)]. Although the exact quarternary structure of the casein micelle remains unknown, it is generally accepted that the micelles are composed of submicelles held together by calcium phosphate.

The caseins are well-known substrates for TG. Besides the above-mentioned studies, these proteins have been widely used in assays for TG activity. Furthermore, β-casein has been applied in the studies of the specificity of TG-catalyzed modification of glutamine residues in proteins (Gorman and Folk, 1980; Coussons et al., 1991). Finally, β-casein and TG have also been used to prepare neoglycoproteins for the study of different aspects of glycoprotein structure in lectin−sugar interactions (Yan and Wold, 1984). However, TG cross-linking sites in the other caseins have not yet been investigated.

In the present study, guinea pig liver TG was used to incorporate putrescine, a site-specific probe, to localize potential TG-reactive glutamines in the individual bovine caseins.

MATERIALS AND METHODS

Reagents. Pepsin, chymotrypsin, and *N*-tosyl-L-phenylalanine chloromethane-treated trypsin were obtained from Worthington Biochemical Corp., U.K. [1,4-^{14}C]Putrescine was from Amersham, U.K. Guinea pig liver transglutaminase, γ-glutamyl-ϵ-lysine, angiotensin II, and adrenocorticotropic hormone fragment were from Sigma Chemical Co., St. Louis, MO. Vydac C_{18} (10 μm) and PD-10 columns were from The Separations Group, Hesperia, CA, and Pharmacia, Uppsala, Sweden, respectively. Reagents used for sequencing were purchased from PE Applied Biosystems, Foster City, CA. Fresh bulk milk (Black and White Danish dairy cattle) was

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supplied by MD Foods Research and Development Centre, Brabrand, Denmark. All other chemicals were of the best grade commercially available.

Purification of Bovine Caseins. Multimeric κ-casein was purified from acid-precipitated whole casein on a Sepharose Cl-6B column as described (Rasmussen and Petersen, 1991; Rasmussen et al., 1992). α_{s1}- and β-casein as well as monomeric α_{s2}-casein were purified by ion-exchange chromatography on a Mono S column as described (Rasmussen et al., 1994).

Transglutaminase-Catalyzed Reactions. The individual caseins (approximately 1 mg) were labeled with [^{14}C]-putrescine (83 nmol) by guinea pig liver TG in a 1:100 (mol/mol) enzyme/substrate ratio. The reactions were performed in 0.1 M Tris-HCl, 2.5 mM $CaCl_2$, and 10 mM dithioerythritol, pH 8.5 (reaction volume 1 mL), at 37 °C for 3 h. In the case of κ-casein, the multimers were reduced to monomer form before they were labeled with the radioactive probe. TG activity was inhibited by addition of 20 mM ethylenediaminetetraacetate, and the mixtures were desalted on a PD-10 column in 50 mM NH_4HCO_3 (α_{s1}-, α_{s2}-, and β-casein) or in 5% HCOOH (κ-casein).

Generation and Characterization of [^{14}C]Putrescine-Labeled Peptides. The radiolabeled caseins were digested with the following enzymes: pepsin in 5% HCOOH for 18 h (κ-casein); trypsin in 0.1 M NH_4HCO_3 for 18 h (α_{s2}-casein) or 24 h (α_{s1}- and β-casein). β-Casein was further digested with chymotrypsin for 9 h. All digestions were performed at an enzyme/substrate ratio of 1:100 at 37 °C followed by freeze-drying. Some of the trypsin/chymotrypsin-derived peptides from β-casein containing radioactivity were pooled, freeze-dried, and subdigested with pepsin at a concentration of 2 μg/mL in 5% HCOOH at 37 °C for 2 h and lyophilized. The resulting peptides were separated by reversed-phase HPLC, and incorporation of radiolabeled putrescine was determined by liquid scintillation counting of aliquots from each fraction on a Beckman LS 1801 instrument.

Amino acid sequence analysis was carried out on an ABI 477/120 A protein sequencer (PE Applied Biosystems). Part of the phenylthiohydantoin (PTH) derivate sample left over after on-line injection was used for radioactivity detection as decribed above.

Plasma desorption mass spectrometry (PDMS) was carried out on a BioIon 20K plasma desorption time-of-flight instrument (BioIon AB, Uppsala, Sweden). The samples were dissolved in 0.1% trifluoroacetic acid (TFA) and applied to nitrocellulose-covered targets, spin-dried, and microrinsed as described (Nielsen et al., 1988). Spectra were recorded at 15 kV for 10^6 fission events. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out on a Bruker Biflex instrument (Bruker-Franzen, Bremen, Germany) equipped with a reflector and a nitrogen ultraviolet laser. Samples were dissolved in 0.1% TFA and mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA/acetonitrile in a matrix-to-sample ratio of 1:1 (v/v). The mixture (0.9 μL) was applied to a target and dried at ambient air. Human adrenocorticotropic hormone fragment (18−39) and angiotensin II were used as molecular mass calibrants. Theoretical peptide masses were calculated using the GPMAW program (Lighthouse Data, Odense, Denmark).

RESULTS AND DISCUSSION

Identification of TG-Reactive Glutamines in the Caseins. The strategy used for the localization of potential TG-reactive glutamine residues in the bovine caseins was as outlined: purified caseins were labeled with [^{14}C]putrescine, a lysine analogue, in a reaction catalyzed by guinea pig TG. The labeled proteins were digested with specific proteases, and the resulting peptides were separated by reversed-phase HPLC to unequivocally locate the radiolabeled glutamines. The radioactive peptides were identified by liquid scintillation counting, repurified, and subjected to sequence and mass spectrometric analysis. Figure 1 shows the reversed-phase HPLC separations of the enzymatic digests of the individual [^{14}C]putrescine-labeled monomeric caseins. The recovery of [^{14}C]putrescine after reversed-phase chromatography was calculated to be approximately 83%, 91%, 86%, and 87% for α_{s1}-, α_{s2}-, κ-, and β-casein, respectively. Several major radioactively labeled peaks were found in the digests (α_{s1}-casein, A1−A5; α_{s2}-casein, B1−B3; κ-casein, C1−C5; β-casein, D1−D3). In the case of β-casein, the peaks in D3 were pooled, subdigested with pepsin, and separated by reversed-phase HPLC and analyzed as decribed above. In the sequence analysis of the radioactive peptides, yields of some of the PTH-Gln were relatively low or absent, indicating putrescine-linked glutamines. Scintillation counting of the PTH amino acids as well as mass spectrometric analysis was used to confirm incorporation at these positions, and the combined results are shown in Table 1. The radioactivity in the first eluting peak (A1−D1) in each of the digests originated from unbound [^{14}C]putrescine not totally removed by the desalting method used. The following TG-reactive glutamine residues were found: α_{s1}-casein, Gln 13, Gln108, Gln130, and Gln140; α_{s2}-casein, Gln79, Gln169, Gln185, and Gln187; β-casein, Gln54, Gln56, Gln72, Gln79, and Gln182; κ-casein, Gln29, Gln45, Gln114, and Gln163. In addition, mass spectrometric analysis revealed that one of the radioactively labeled peaks (C5) in the digest of κ-casein also contained two cross-linked peptides: Glu18−Tyr30 and Tyr42−Leu50. The cross-link was between Gln45 and either Lys21 or Lys24 as indicated by the absence of Gln45 in the sequence analysis as well as the presence of a peak, which eluted at the same position as the dipeptide ϵ-(γ-glutamyl)lysine, in the cycles corresponding to the two lysine residues. Since the cross-linked peptides could have originated from the same κ-casein molecule or from different molecules, it is not possible to decide whether the cross-link is intra- or intermolecular.

Reactivity of Glutamines and Lysines in TG-Catalyzed Reactions. Only TG-reactive glutamines in β-casein have been identified in earlier studies. Coussons et al. (1991) identified Gln79 as the major TG-reactive residue in a guinea pig liver TG-catalyzed reaction. According to the authors, at least three other significant radioactive peptides were present in the reaction mixture, indicating the presence of other modified glutamines as well. Opposed to this, the primary incorporation site by factor $XIII_a$ in β-casein has been identified as Gln167 (Gorman and Folk, 1980). This variation could be related to differences in specificities between the two enzymes due to the fact that distinct transglutaminases may recognize the same protein as substrate, but often with different affinity and/or with specificity for different glutamine residues (Gorman and Folk, 1984). In amidinated β-casein, Gln54, Gln56, Gln79, Gln167, Gln175, Gln182, and Gln194 have been shown to be incorporation sites for carbohydrates in a reaction also catalyzed by guinea pig liver TG (Yan and Wold, 1984). The modification of Gln54, Gln56, Gln79, and Gln182 is in agreement with our results. In the present study, only the major labeled peaks in the various digests were investigated. Therefore, the other reactive glutamines found by Yan and Wold (1984) may be present in some of the minor labeled peaks in the β-casein digest.

The number of proteins acting as glutaminyl substrates for TGs is restricted, as both primary structure and conformation appear to determine whether a

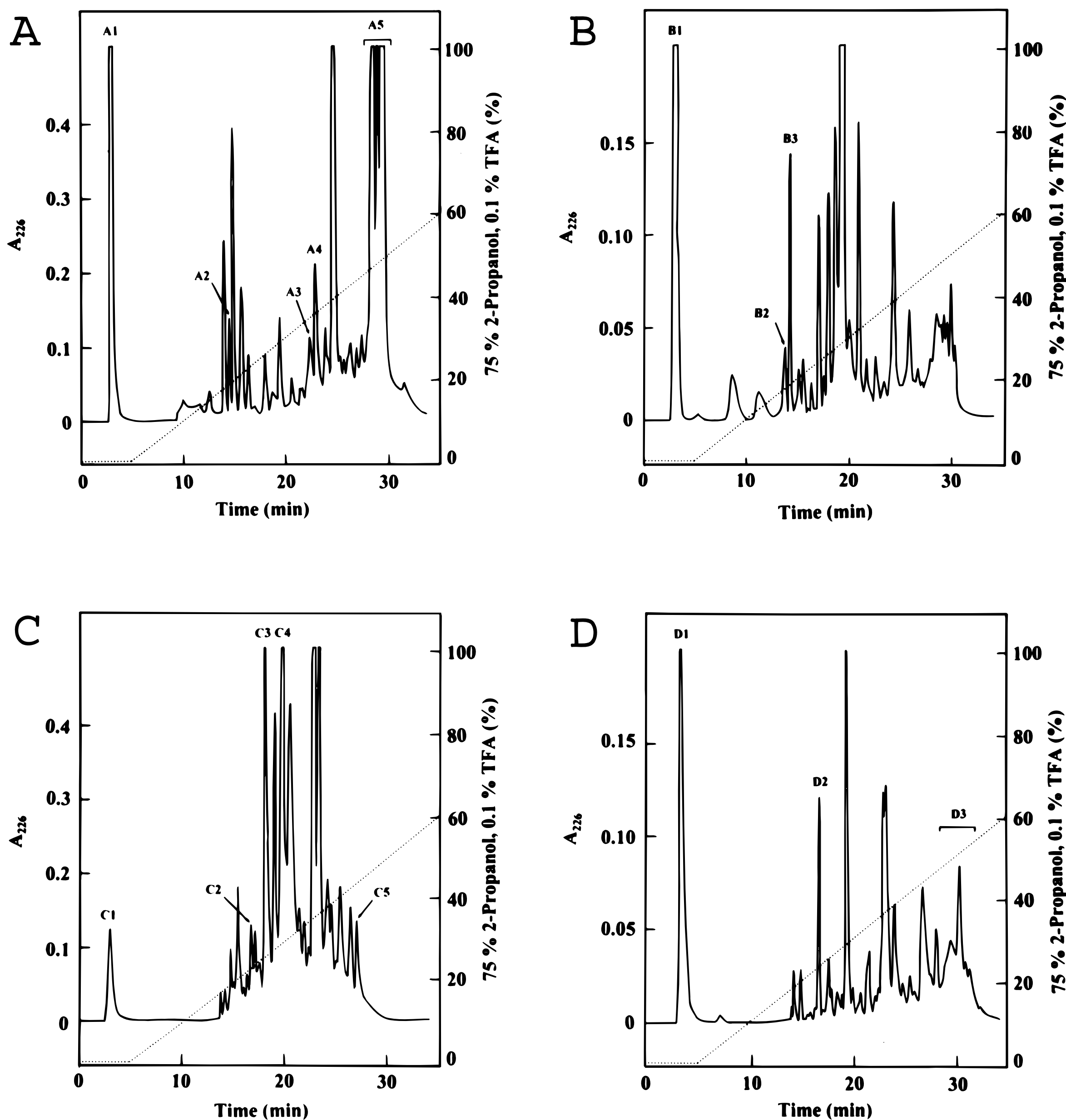

Figure 1. Reversed-phase HPLC separation of enzymatic digests of [^{14}C]putrescine-labeled (A) α_{s1}-casein, (B) monomeric α_{s2}-casein, (C) monomeric κ-casein, and (D) β-casein. The reaction conditions are given under Materials and Methods. Peptides were eluted with a linear gradient of 75% propanol in 0.1% TFA (dotted line) on a Vydac C_{18} (10 μm) column (4 × 250 mm). The flow rate was 0.85 mL/min, and the column temperature was 40 °C. Peptides were detected in the effluent by recording the absorbance at 226 nm (continuous line) and collected manually. Major radioactivity-containing peaks (marked by capitals) were identified by scintillation counting as described under Materials and Methods.

glutamine residue can be reactive (Aeschlimann and Paulsson, 1994). Although a considerable number of reactive glutamine residues have been identified in proteins functioning as TG substrates, a comparison of the amino acid sequences surrounding these sites has not revealed a clear sequence motif for this modification. As summarized by Aeschlimann et al. (1992), it seems to be a common feature that these glutamines are located in solvent-exposed surface regions or flexible extensions of the proteins, as patches of hydrophilic residues are often found in their vicinity. This also applies to the TG target site sequences found in the caseins as shown in Table 1.

On the basis of the pattern of neighboring charged amino acids in TG site sequences, it has been proposed that the presence of positively charged amino acids (Lys and Arg) in a window of five residues C-terminal to a glutamine residue discourages the ability of the glutamine to be modified by guinea pig liver TG (Coussons et al., 1992). However, as seen in Table 1, several of the TG-reactive glutamines in the caseins (i.e., Gln130 in α_{s1}-casein; Gln79, Gln169, Gln185, and Gln187 in α_{s2}-casein; Gln29, Gln45, and Gln114 in κ-casein; Gln182 in β-casein) do not follow these charge rules.

The reactivity of two adjacent glutamines (Gln-Gln or Gln-Xxx-Gln) has been reported for a number of proteins [reviewed by Aeschlimann et al. (1992) and Hohenadl et al. (1995)] and is well-illustrated in the caseins. In κ-casein, only the second of two consecutive glutamines, Gln45, is recognized as an acyl donor. The opposite is seen in α_{s1}-casein, where it is the first glutamine, Gln130, that acts as the acyl donor. The situation, Gln-Xxx-Gln, in which both residues act as acyl donors at the same time or only one at a time, is seen in β-casein (Gln185-Thr-Gln187) and α_{s2}-casein (Gln54-His-Gln56), respectively.

Table 1. Localization of TG-Reactive Glutamines in Bovine Caseins by Sequence and Mass Spectrometric Analyses

peak designation[a]	sequence[b]	obsd MW[c]	calcd MW[d]
α_{s1}-casein			
A2	^{125}EGIHA**Q**QK^{132}	984.9 (P)	985.0 (1 putrescine (putr))
A3	^{104}YKVP**Q**LEIVPNS*AEER^{119}	2026.9 (P)	2027.1 (1 putr + H_2PO_3*)
A4	^{8}HQGLP**Q**EVLNENLLR^{22}	1834.9 (P)	1835.0 (1 putr)
A5a[e]	^{136}IGVN**Q**ELAYFYPELFR^{151}	2035.4 (L)	2034.2 (1 putr)
A5b[e]	^{125}EGIHAQQKEPMIGVN**Q**ELAYFYPELFR^{151}	3285.3 (L)	3283.6 (1 putr)
	^{133}EPMIGVN**Q**ELAYFYPELFR^{151}	2393.1 (L)	2391.7 (1 putr)
α_{s2}-casein			
B2	^{166}KIS**Q**R^{170}	705.2 (P)	705.7 (1 putr)
	^{77}HY**Q**K^{80}	649.9 (P)	649.6 (1 putr)
B3	^{182}TVY**Q**H**Q**K^{188f}	978.4 (P)	978.0 (1 putr)
κ-casein			
C2	^{162}V**Q**VTSTAV^{169}	–[g]	–
C3	^{42}YYQ**Q**KPVAL^{50}	1184.3 (L)	1184.3 (1 putr)
C4	^{106}MAIPPKKN**Q**DKTEIPTINT^{124}	2138.9 (L)	2139.5 (1 putr)
C5	^{18}FSDKIAKYIPI**Q**YVL^{32}	1873.5 (L)	1873.2 (1 putr)
	^{18}FSDKIAKYIPIQY^{30h}	2678.7 (L)	2678.2 ($-NH_3$)[i]
	^{42}YYQ**Q**KPVAL^{50h}		
***β*-casein**			
D2	^{177}AVPYP**Q**R^{183}	905.3 (L)	905.0 (1 putr)
D3[j]	^{53}A**Q**T**Q**SLVYPFPGPIPN^{68}	1880.6 (L)	1879.0 (2 putr)
	^{69}SLP**Q**NIPPLT**Q**^{79f}	1281.6 (L)	1282.4 (1 putr)
	^{69}SLP**Q**NIPPLT**Q**T^{80}	1460.3 (L)	1458.5 (2 putr)
	^{73}NIPPLT**Q**TPVVVPPFLQPEVM^{93}	2391.6 (L)	2391.8 (1 putr)

[a] Peak designations correspond to those of Figure 1. [b] Only peptides containing glutamine residues labeled with [^{14}C]putrescine are included, and the identified TG-reactive glutamines are shown in bold. [c] Molecular mass determined by mass spectrometric analysis. (P) and (L) denote mass determined by PDMS and MALDI-TOF-MS, respectively. [d] Calculated average masses including the number of putrescine units and phosphate groups given in parentheses. [e] Repurification of the peaks in A5 resulted in two major radiolabeled peaks: A5a and A5b. [f] The two TG-reactive glutamines are only labeled one at a time. [g] Not resolved. [h] Identified as an intra- or intermolecular cross-link between Gln45 and either Lys21 or Lys24 (underlined). [i] The calculated mass of the two peptides minus an NH_3 group. [j] The peaks in D3 (Figure 1) were pooled, subdigested with pepsin followed by reversed-phase HPLC, and subjected to sequence and mass spectrometric analysis.

A common notion is that TGs are much less selective toward amine donor lysine residues in proteins than they are to the glutamine substrate (Greenberg et al., 1991; Aeschlimann and Paulsson, 1994). Recently, recombinant αA-crystallin mutants have been used to study the influence of the residue preceding the amine donor lysine in reactions catalyzed by tissue TG and factor XIII (Grootjans et al., 1995). It was found that Gly or Asp before the amine donor lysine has the strongest adverse effects on substrate reactivity, whereas Pro, His, and Trp were less favorable. In constrast, Val, Arg, and Phe and to a lesser extent Ser, Ala, Leu, Tyr, and Asn were shown to have an enhancing effect. This pattern of preference was shown to be largely in agreement with that observed for the limited number of characterized amine doner lysines in protein substrates for TGs. However, this does not totally apply for κ-casein, in which an aspartic acid is present directly N-terminal to the identified TG-reactive lysine residues, Lys21.

In conclusion, the present results show that there are a limited number of potential TG-reactive glutamine residues in the bovine caseins despite their relatively high content: in the case of α_{s1}- and α_{s2}-casein, κ-casein, and β-casein, only 4 of 15, 4 of 14, and 5 of 21 glutamines, respectively. In addition, to employ TG to improve or create food proteins with new functional and rheological properties, TG-mediated cross-linking and incorporation of site-specific labels might be a useful approach in the analysis of the structure of the casein micelle.

ABBREVIATIONS USED

MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MS, mass spectrometry; PDMS, plasma desorption mass spectrometry; PTH, phenylthiohydantoin; putr, putrescine; TG, transglutaminase; TFA, trifluoroacetic acid.

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