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Modification of Glutamine and Lysine Residues in Holo and Apo α-Lactalbumin with Microbial Transglutaminase

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The molecular structures determine the physical properties of milk proteins and are important for the texture of many dairy-based foods. Bovine α-lactalbumin (α-LA) is a globular 123 amino acid Ca^{2+} binding milk protein. Modification with microbial Ca^{2+} independent transglutaminase (MTGase) was used to modify lysines and glutamines in holo and apo α-LA. At 30 °C no lysines or glutamines are modified in holo α-LA, whereas in apo α-LA lysines 13, 16, 108, and 114, and glutamines 39 and 43, are modified. At 50 °C lysines 13, 16, 108, and 114, but no glutamines, are modified in holo α-LA, whereas in apo α-LA lysines 5, 13, 16, 108, and 114, and glutamines 39, 43, 54, 65, and 117, are modified. The methods presented here offer the possibility to manipulate the availabilities of residues in α-LA to the MTGase reaction and enable the preparation of α-LA species with different degrees of modification and hence with different physical properties.

KEYWORDS: Transglutaminase; α-lactalbumin; calcium; mass spectrometry; protein structure

INTRODUCTION

Globular proteins such as α-lactalbumin (α-LA) and *β*-lactoglobulin are major components in whey and are major protein sources in the western diet. The physical properties of these proteins are important for the texture of many dairy-based foods. The enzymatic modification of proteins results in changes of protein structure and may therefore result in changes in the functional properties such as solubility, gelation, emulsion formation, and emulsion stabilization. Transglutaminase (TGase; protein-glutamine *γ*-glutamyltransferase, E. C. 2.3.2.13) may be particularly useful to modify food proteins. Globular milk proteins in their native conformation are, however, resistant to enzymatic modification by TGase.

Bovine α-lactalbumin is a 123 amino acid Ca^{2+} binding regulatory protein of lactose synthase, that modulates the affinity of the catalytic component UDP-galactose *β*-*N*-acetylglucosaminidine *β*-1,4-galactosyltransferase I, for acceptor substrates through a reversible protein−protein interaction (*1*). The native structure of α-LA is stabilized by the binding of Ca^{2+}. Upon removal of the Ca^{2+} ion, the tertiary structure becomes less rigid, but the secondary structure of the protein remains intact (*2*).

TGase is a ubiquitous enzyme that is present in mammals, plants, and microorganisms. TGase catalyses acyl-transfer reactions in which the *γ*-carboxamide group of glutamyl residues in proteins or in peptides is the acyl donor (see **Figure 1**). Depending on the acceptor, TGase can catalyze three different reactions. First, the *ϵ*-amino group of lysine residues in proteins or peptides can serve as acceptor, which yields inter- and intramolecular *ϵ*-(*γ*-glutamyl)−lysine isopeptide bonds. With this reaction it is possible to cross-link proteins or link small glutamine-containing peptides to the lysines of proteins. Second, other primary amines, including the α-amino group of glycine, can serve as acceptor. This second reaction is known as the acyl transfer reaction and results in the attachment of primary amines to the glutamine residues of proteins. In the third reaction catalyzed by TGase, in the absence of amine donors, water serves as the acceptor resulting in the deamidation reaction in which glutamine is converted into glutamic acid (*3*). The enzymatic reactions of Ca^{2+}-dependent TGases have been well studied, especially the blood coagulation TGase FXIIIa and TGases from guinea pig liver (*4*). The microbial Ca^{2+}-

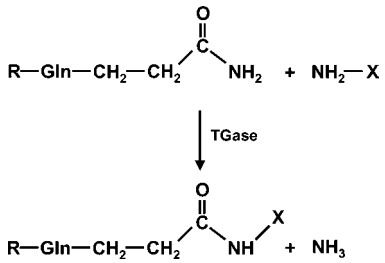

Figure 1. TGase reaction; X = alkyl, lysine, or glycine, and R = peptide or protein.

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independent TGase from *Streptoverticillium mobaraense* (MTGase) has a broader substrate specificity for amine acceptor glutamine substrates in proteins than the mammalian TGases (*5*−*8*). The enzyme displays its maximum activity between pH 6 and pH 7. MTGase can be used to improve food protein functionality (*9* and references therein, *10*) and to produce hypoallergenic flour (*11*).

The investigation of the TGase acyl transfer reaction is hampered by the protein cross-linking side reaction. By using a large molar excess of primary amines or glutamine-containing peptides compared to the protein concentration, the cross-linking reaction can be suppressed, the acyl transfer reaction can be studied, and the modified residues in the protein can be identified.

Until now a thorough study of the enzymatic modification of holo and apo α-LA with transglutaminase under different nonreducing reaction conditions has not been described. We investigated the modification of lysine and glutamine residues in holo and apo α-LA and assessed the accessibility of the residues to the MTGase reaction with small model peptides and amines under different modification conditions. The modification of lysine and glutamine residues depends on the reaction conditions that are used. This makes it possible to prepare α-LA species with a defined number of modifications and hence with different physical properties.

MATERIALS AND METHODS

Materials. Microbial transglutaminase (MTGase) from *Streptoverticillium mobaraense* was produced as described previously (*12*). α-Lactalbumin (α-LA; Type I from bovine milk), dithiothreitol (DTT), α-*N*-carbobenzyoxy-glutamine-glycine (Z-Gln-Gly), sinnapinic acid (4-hydroxy-3-methoxy cinnamic acid), α-hydroxy cinnaminic acid, *N*-ethylmaleimide, butylamine, hexylamine, octylamine, 4-aminobutyric acid, 6-aminohexanoic acid, and 8-amino-octanoic acid were from Sigma (Zwijndrecht, The Netherlands). Acetic acid, tris(hydroxymethyl)aminoethane (Tris), disodium ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, calcium chloride, 3-[*N*-morpholino]-ethanesulfonic acid (MES), boric acid, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from Merck (Amsterdam, The Netherlands). Guanidine·HCl (p.a.) was from Acros (Geel, Belgium). Glycine-glycine-O-ethyl ester (Gly-Gly-OEt), glycine-glycine-glycine (Gly-Gly-Gly), glycine-tyrosine-OH (Gly-Tyr-OH), and glycine-leucine-OH (Gly-Leu-OH) were from Bachem (Bubendorf, Switzerland). Sequencing grade Glu-C protease was purchased from Roche Biochemical (Almere, The Netherlands). All other chemicals were of analytical grade. C18 Zip-Tips were from MilliPore (Etten-Leur, The Netherlands).

Modification of α-Lactalbumin. *Influence of Ca^{2+} and pH on the Modification Reaction of α-Lactalbumin.* α-LA (1 mg/mL) was dissolved in 100 mM MES buffer (pH 6.0), 100 mM HEPES buffer (pH 7.0), 100 mM Tris buffer (pH 8.0), 100 mM sodium borate buffer (pH 9.0), or 100 mM sodium borate buffer (pH 10.0). To the solutions, Gly-Gly-OEt (6 mg/mL) or Z-Gln-Gly (6 mg/mL) were added, and the pH was readjusted when necessary. Reactions were carried out in the presence of $CaCl_2$ (10 mM) or EDTA (10 mM) at 50 °C with 10 μg/mL MTGase. Because MTGase is slowly deactivated at elevated temperatures (*4*), extra enzyme (10 μg/mL) was added after 5 h. Samples were taken at 0, 5, 10, 15, 30, 60, 120, and 240 min and after 24 h. The reactions were stopped by heating the samples to 95 °C for 10 min. Of all pH values tested in these modification reactions, the optimum was found to be pH 6.0 and all further experiments were therefore performed at that pH (see Results section).

Modification of α-Lactalbumin with Different Primary Amines, Amino Acids, and Peptides. α-LA (1 mg/mL) was dissolved in 100 mM sodium acetate buffer (pH 6.0) containing $CaCl_2$ (10 mM) or EDTA (10 mM), and butylamine, hexylamine, octylamine, 4-aminobutyric acid, 6-aminohexanoic acid, 8-amino-octanoic acid (50 mM each), Gly-Gly-Gly, Gly-Tyr-OH, or Gly-Leu-OH (6 mg/mL each) were added. The pH of the solutions was readjusted when necessary. The solutions were warmed to 50 °C, MTGase (10 μg/mL) was added, and samples were taken after 0, 5, 10, 15, 30, 60, 120, and 240 min and 24 h. After 6 h extra MTGase (10 μg/mL) was added. For analytical purposes the reactions were stopped by heating the samples to 95 °C for 10 min. All other reactions were stopped by the addition of the irreversible MTGase inhibitor *N*-ethylmaleimide.

Sequential Modifications of α-Lactalbumin. To investigate the possible influence of the first modifications with Gly-Gly-OEt (or Z-Gln-Gly) on the second modification with Z-Gln-Gly (or Gly-Gly-OEt), α-LA (1 mg/mL) was dissolved in 100 mM sodium acetate buffer (pH 6.0) with 10 mM EDTA or 10 mM $CaCl_2$. Z-Gln-Gly (6 mg/mL) or Gly-Gly-OEt (6 mg/mL) and MTGase (10 μg/mL) were subsequently added. Reactions were carried out at 50 °C. After 6 h extra MTGase (10 μg/mL) was added. After 24 h the reaction was terminated by adding the irreversible TGase inhibitor *N*-ethylmaleimide (1 mM). The reactions were stopped without heating in order to prevent changing the tertiary structure of the protein. Excess peptides, *N*-ethylmaleimide, $CaCl_2$, and EDTA were removed by dialysis against sodium acetate buffer (pH 6.0) at 4 °C. The Gly-Gly-OEt (or Z-Gln-Gly) modified α-LA was then modified in the second modification reaction with Z-Gln-Gly (or Gly-Gly-OEt) in the presence of $CaCl_2$ or EDTA under reaction conditions identical to those of the first modification reaction. Reactions were stopped after 24 h by heating the reaction mixtures to 95 °C for 10 min.

Digestion of α-Lactalbumine for Mass Spectrometric Analysis. To reduce and alkylate α-LA, the protein was dissolved in freshly made 6 M guanidine·HCl, 100 mM NH_4HCO_3 (pH 7.8) with 10 mM DTT, and was incubated at 56 °C for 1 h. After this was cooled to room temperature, iodoacetamide from a 1 M freshly made stock solution was added to yield a final concentration of 50 mM. The alkylation was allowed to proceed for 1.5 h in the dark at room temperature. After dialysis against demineralized water, the protein was lyophilized. The reduced and alkylated protein was dissolved in NH_4HCO_3 buffer (pH 7.8, ∼100 μg/mL) or in sodium phosphate buffer (pH 7.8, ∼100 μg/mL) containing 10% acetonitrile (v/v). Glu-C (2 μg/100 μg) was added and the proteolysis was allowed to proceed for 16 h at 37 °C. The digests were diluted with an equal volume of water, and peptides were isolated with Zip-Tip extraction (vide infra).

Protein Analysis. *Circular Dichroism (CD) Spectroscopy.* Far-UV spectra of ∼0.1 mg/mL nonmodified and modified α-LA species in 10 mM Tris/HCl buffer (pH 7.0, with 1 mM EDTA or 1 mM $CaCl_2$) were recorded on a Jasco J-715 spectropolarimeter at 20 °C in the range from 190 to 260 nm with a spectral resolution of 0.1 nm. The scan speed was 100 nm/min, the response time was 0.125 s, and the bandwidth was 1 nm. Quartz cells with an optical path of 0.1 cm were used. The spectra were corrected for the corresponding protein-free sample. The spectra were analyzed for the secondary structure content of the proteins using a standard nonlinear least-squares fitting procedure (195−260 nm).

Near UV CD spectra of ∼1.2 mg/mL α-LA in 10 mM Tris/HCl (pH 7.0, with 1 mM EDTA or 1 mM $CaCl_2$) were recorded at 20 °C from 250 to 350 nm with a spectral resolution of 0.5 nm. The scan speed was 100 nm/min and the response time was 0.25 s using a bandwith of 1 nm. Quartz cells with an optical path of 1 cm were used. The spectra were corrected for the corresponding protein-free sample. In the CD experiments Tris/HCl buffers were used, not the for-CD-spectroscopy preferred phosphate buffer, to allow for the determination of the effect of Ca^{2+} on protein structure.

Matrix-Assisted Laser Desorption and Ionization−Time-Of-Flight Mass Spectrometry (MALDI−TOF-MS) Analyses. MALDI mass spectra of nondigested α-LA were recorded on a PerSeptive Voyager DE-RP (PerSeptive Biosystems, Framingham, MA) mass spectrometer equipped with delayed extraction technology. The spectra were recorded in the linear positive ion mode. Spectra from summations of 256 laser shots were used. The following instrument settings were used: accelerating voltage 25 000 V, grid voltage 91.5%, guide wire 0.3%, and delayed extraction time 200 ns. Sinnapinic acid was used as matrix. The matrix solution was prepared by dissolving 10 mg of sinnapinic acid in 1 mL of 50% aqueous acetonitrile (v/v) containing 0.3% trifluoracetic acid (v/v). Samples were prepared by mixing 9 μL of matrix solution with

1 μL of sample solution (~1 mg protein/mL). Aliquots of 1 μL were transferred to a gold-coated welled sample plate and were allowed to crystallize under atmospheric pressure at room temperature. Spectra were calibrated externally using the m/z $[M + H]^+$ and m/z $[M + 2H]^{2+}$ peaks in the spectrum of a mixture of bovine insulin (5734.6 Da), thioredoxin (11674.5 Da), and apo myoglobin (16952.6 Da) (calibration mixture 3, Perseptive Biosystems) which were recorded under identical conditions. All protein samples were dialyzed against demineralized water before analysis.

The peptides formed by Glu-C digestion were analyzed using a TofSpec 2EC mass spectrometer (Micromass, Whyttenshawe, U.K.) equipped with a 2 GHz digitizer.

The spectra were recorded in the reflectron positive ion mode. Peptides were collected on ZipTip μC18 pipet tips, washed with 1.0% formic acid, and eluted with 60% acetonitrile/1.0% formic acid. For MALDI analyses of the digests 0.5-μL aliquots of the peptide-containing eluate were mixed with 0.5 μL of a 10 mg/mL α-hydroxy cinnaminic acid solution in a 1:1 (v:v) mixture of acetonitrile and ethanol. Samples (1 μL) were transferred to stainless steel welled sample plates and samples were allowed to crystallize under atmospheric pressure at room temperature.

Q-TOF MS/MS Mass Spectra. When needed, electrospray MS and low-energy collision-induced dissociation (MS/MS) analyses were performed on a Q-TOF (Micromass, Whyttenshawe, UK) mass spectrometer with a Z-Spray orthogonal ESI source. The eluates were directly infused, using gold-coated nano electrospray capillaries (New Objective, Woburn, MA).

Analysis of Mass Spectrometric Data of α-Lactalbumin Digests. After internal mass calibration (better than 40 ppm), the MALDI spectra were isotope deconvoluted using the MaxENT 3 algorithm (MaxENT Solutions, Cambridge, U.K.). The MS data were scanned for digest fragments that were modified with the used amine or peptide in the enzymatic modification reaction. MS data of nonmodified protein were available with each experiment. A custom-made software tool called FindLink supported these analyses. The FindLink program (*13, 14*) generates a mass fragment database based on the input of the residue sequence of the studied protein, the selectivity of the digest cleavages, and the presence of modifiable amino acid residues in the peptide. Database entries include all fragment candidates for surface label modifications. Each database entry was automatically matched within a mass tolerance better than 40 ppm with the experimentally obtained mass lists. The matches for surface labeling were systematically documented as output of the analyses. After this analysis some peptides containing the modifier were selected using the Q-TOF to generate additional MS/MS data for sequence confirmation and the existence of the modified amino acid residue.

Polyacrylamide Gel Electrophoreses (SDS−PAGE). Modified and nonmodified proteins were analyzed under denaturing conditions with 15% SDS−PAGE analysis as described previously (*15*). Reaction mixture samples were denatured by incubation for 5 min at 100 °C in 2% SDS and 1% DTT. Gels were stained for protein with Coomassie Brilliant Blue G250. A high-molecular-weight calibration kit (Pharmacia) was used to derive the molecular masses.

RESULTS

To investigate the accessibility of the lysine and glutamine residues of α-LA to the MTGase reaction, both the Ca^{2+} containing holo and the Ca^{2+} free apo form of α-LA were modified with the glutamine-containing peptide α-*N*-carbobenzyloxy-glutamine-glycine (Z-Gln-Gly) and various primary amines. These small model compounds allow for accurate biochemical and mass spectrometric analysis of the modified proteins. The modifications were performed at different pH values and different temperatures. Because the most effective modification reactions were found to occur at 50 °C, we performed the experiments at that temperature unless stated otherwise.

Influence of Ca^{2+} and pH on the Modification Reaction. Because the native structure of α-LA in its Ca^{2+}-ion containing holo form is different from that in its Ca^{2+}-free apo form (*2*), the modification of both the holo and the apo proteins were studied. Both the holo and apo proteins were modified with the primary amine glycine-glycine-O-ethyl ester (Gly-Gly-OEt) and the peptide Z-Gln-Gly at different pH values. Gly-Gly-OEt is covalently attached via its α-amino group to the glutamines and Z-Gln-Gly is covalently attached to the Nε of lysines in α-LA. At different time points samples were taken to follow the kinetics of the reaction. The modified proteins were analyzed both with SDS−PAGE and MALDI-TOF mass spectrometry.

Modification of Glutamine Residues with Gly-Gly-OEt. At 30 or 50 °C in the presence of Ca^{2+} at pH 6.0, 7.0, 8.0, 9.0, or 10.0, there was no detectable modification of α-LA with Gly-Gly-OEt, nor was the protein cross-linked. This indicates that in α-LA in its native conformation no glutamines are available to MTGase under the conditions that were used.

The MALDI-TOF-MS spectra (**Figure 2**, $t = 0$) show the molar mass of nonmodified α-LA at m/z 14179, which is in good agreement with the theoretical mass of 14178 Da. The matrix adducts at m/z 14402 of sinnapinic acid (Mw = 223) can be seen as small signals next to the main α-LA peaks. In the MTGase reaction in the presence of EDTA a mixture of α-LA species modified by 3, 4, and a trace of 5 Gly-Gly-OEt groups (mass increment of 143 Da per modification) was formed at 50 °C. The mass peak of α-LA containing 5 Gly-Gly-OEt groups is almost totally obscured by a sinnapinic acid adduct. Because of the high peptide concentration that was used in the MTGase reaction, protein cross-linking was completely blocked (SDS−PAGE analysis; data not shown). The highest reaction rate was observed at pH 6.0 and pH 7.0 where after 60 min all protein is modified with at least one Gly-Gly-OEt group, and after 24 h the MTGase reaction was almost complete. Approximately 80% of the modified protein contained 4 modifications and 20% contained 3 modifications. At pH 8.0 the reaction proceeded slightly slower, whereas at pH 9.0 and 10.0 no modifications were observed. MTGase is not optimally active at these alkaline pH values, and in addition the ethyl ester may have been hydrolyzed thus making the peptide a less effective acyl donor. At 30 °C the reactions proceed very slowly and only incomplete modification reactions were observed.

Modification of Lysine Residues with Z-Gln-Gly. In the presence of Ca^{2+} at 50 °C a slow attachment of a maximum of two Z-Gln-Gly groups (mass increase of 320 Da per modification) to α-LA takes place at pH 6.0, 7.0, 8.0, and 9.0, whereas at pH 10.0 there was no modification. At pH 6.0 and 7.0 the fastest modification reactions are observed, and after 4 h all protein carries at least 1 Z-Gln-Gly group. After 24 h a minor product (ca. 10%) with 2 modifications was visible in the MALDI-TOF-MS spectrum at m/z 14820 (**Figure 2B**) of the proteins modified at pH 6.0, 7.0, and 8.0. At 30 °C only at pH 6.0 a trace of a mono modified α-LA species was detected.

At 50 °C a maximum of 5 Z-Gln-Gly groups is attached to α-LA in the presence of EDTA at pH 6.0 (**Figure 2C**). At pH 7.0 and 8.0 the reaction proceeds slower, and after 24 h a maximum of 4 attached Z-Gln-Gly groups could be detected. At pH 9 hardly any modification occurs and at pH 10 no modified protein can be detected. At 30 °C the reactions proceed slowly and are incomplete with a maximum of 2 modifications at pH 6.0.

In **Figure 2** the modifications of α-LA with Z-Gln-Gly and Gly-Gly-OEt at pH 6.0 and 50 °C in the presence or absence of calcium are shown.

Modification with Different Primary Amines, Amino Acids, and Peptides. To test the acyl donor specificity of

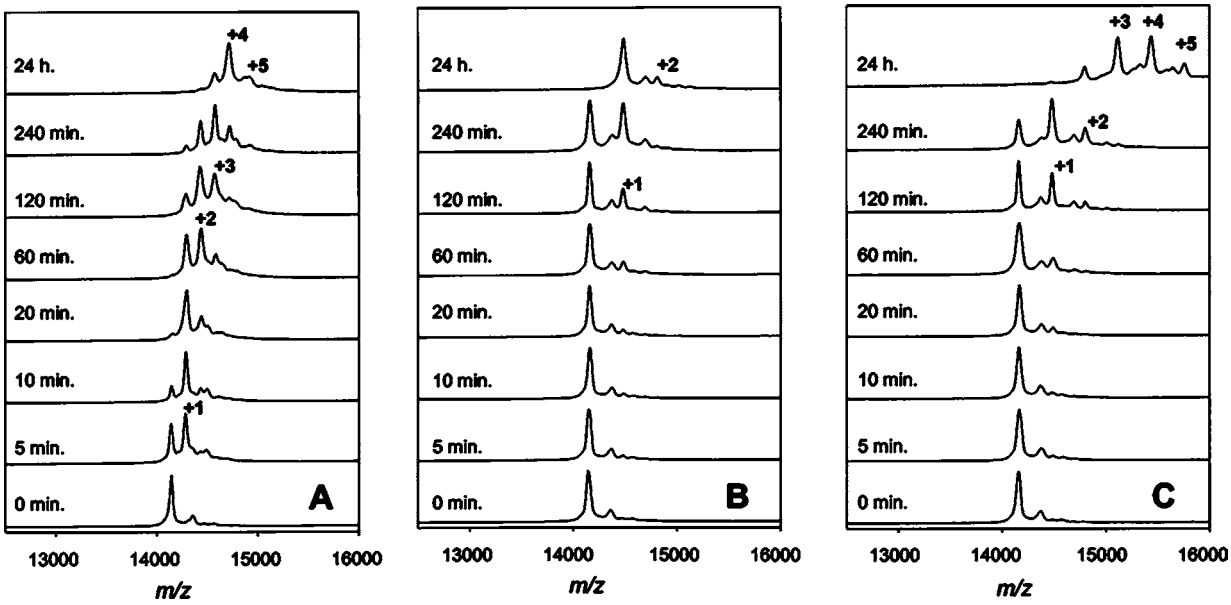

Figure 2. MALDI-TOF-MS spectra of α-LA modified by MTGase at pH 6.0 and 50 °C with (A) Gly-Gly-OEt in the presence of 10 mM EDTA, (B) Z-Gln-Gly in the presence of 10 mM $CaCl_2$, and (C) Z-Gln-Gly in the presence of 10 mM EDTA.

Table 1. Modification of α-LA with Different Primary Amines

acyl acceptor	structure	number of modifications per α-LA molecule
1-aminobutane	$CH_3–CH_2–CH_2–CH_2–NH_2$	5
1-aminohexane	$CH_3–CH_2–CH_2–CH_2–CH_2–CH_2–NH_2$	3
1-amino-octane	$CH_3–CH_2–CH_2–CH_2–CH_2–CH_2–$ $CH_2–CH_2–NH_2$	2
4-aminobutyric acid	$HO–C(O)-CH_2–CH_2–CH_2–NH_2$	3
6-aminohexanoic acid	$HO–C(O)-CH_2–CH_2–CH_2–CH_2–CH_2–NH_2$	5
8-amino-octanoic acid	$HO–C(O)-CH_2–CH_2–CH_2–$ $CH_2–CH_2–CH_2–CH_2–NH_2$	5
glycine-glycine-glycine	Gly-Gly-Gly	5
glycine-tyrosine-OH	Gly-Tyr-OH	5
glycine-leucine-OH	Gly-Leu-OH	5
glycine-glycine-O-ethyl ester	Gly-Gly-OEt	5

MTGase in the modification reaction of α-LA, 1-aminobutane, 1-aminohexane, 1-amino-octane, 4-aminobutyric acid, 6-aminohexanoic acid, 8-amino-octanoic acid, Gly-Gly-Gly, Gly-Tyr-OH, and Gly-Leu-OH were used as acyl donors. The reactions were performed at pH 6.0 and 50 °C in the presence of 10 mM EDTA or 10 mM $CaCl_2$. In the presence of Ca^{2+} there were no detectable modifications, even after 24 h, just as was observed for Gly-Gly-OEt. In the presence of EDTA, however, modification by MTGase took place with all primary amines tested. In **Table 1** the results are summarized. The reactions were comparable with that of the modification with Gly-Gly-OEt as is shown in **Figure 2A**. The modification reactions with 1-aminohexane, 4-aminobutyric acid, and 1-amino-octane proceed very slowly and a maximum of 3 modifications with 1-aminohexane and 4-aminobutyric acid and a maximum of 2 modifications with 1-amino-octane were observed after 24 h MTGase treatment. The reaction with the aliphatic amines is probably limited by their low solubility and the negatively charged carboxylic group close to the amine group in 4-aminobutyric acid may hinder the reaction.

Sequential modifications

To investigate whether the first modification of the lysines with Z-Gln-Gly or the glutamines with Gly-Gly-OEt in α-LA influences the subsequent modification of the glutamines or lysines in a second modification reaction, a modification scheme was followed as shown in **Figure 3**. In **Figure 3** the maximum number of attached groups per α-LA molecule found after each modification reaction is shown. To start the second modification reaction with completely modified α-LA species (i.e. no unmodified α-LA left) the first modifications were executed at 50 °C at pH 6.0. In the first reaction α-LA was modified with Z-Gln-Gly or Gly-Gly-OEt, both in the presence or absence of Ca^{2+}. The first reaction was terminated after 24 h by the addition of *N*-ethylmaleimide (NEM), a thiol reactive compound that blocks the active site Cys of MTGase. In this way the structure of α-LA is not altered by heat denaturation. After removal of excess peptides and NEM by dialysis, in a second reaction the Z-Gln-Gly or Gly-Gly-OEt modified proteins were further modified with Gly-Gly-OEt or Z-Gln-Gly, respectively. The second modifications were also executed in both in the presence or absence of Ca^{2+}. After the second modification reaction complex mixtures of α-LA species with different numbers of attached groups are formed. In **Figure 4** a typical example of

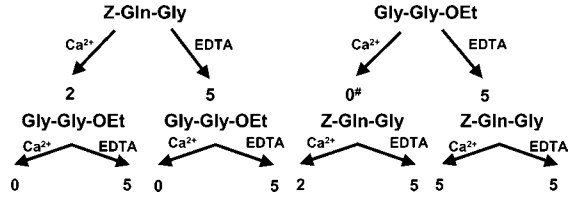

Figure 3. Reaction scheme of the sequential modifications of α-LA. The number of introduced modifications that were visible in the MALDI-TOF-MS spectra of the modified intact α-LA molecules after each 24 h modification reaction are indicated. (#) trace of mono modified protein.

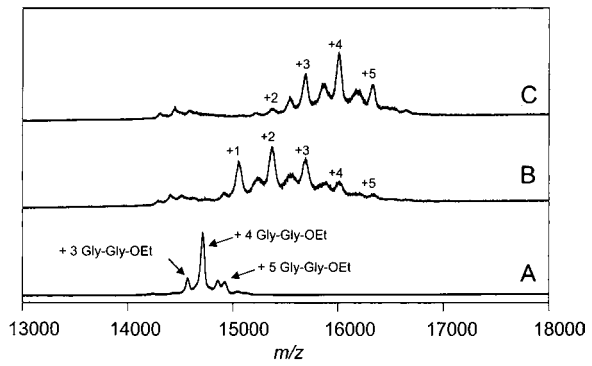

Figure 4. Sequential modification of α-LA: (A) α-LA modified with Gly-Gly-OEt in the presence of EDTA; (B) second modification of the modified protein with Z-Gln-Gly in the presence of Ca^{2+}, and (C) in the presence of EDTA. All spectra were of samples that had reacted with MTGase for 24 h in both the first and the second modification reactions.

a complex mixture obtained after two sequential 24 h MTGase reactions is shown.

Because α-LA cannot be modified with Gly-Gly-OEt in its native conformation, only the second modification of α-LA that had been modified with Gly-Gly-OEt in the absence of Ca^{2+} in the first reaction was studied. Such α-LA molecules (see also **Figure 2A**) are modified with a maximum of 5 Z-Gln-Gly groups both in the presence and absence of Ca^{2+} in the second reaction. The reaction in the absence of Ca^{2+} is, however, faster than the reaction in the presence of Ca^{2+} (see **Figure 4 B** and **C**). This may indicate that in the first modification reaction a glutamine residue is modified that weakens the Ca^{2+} binding in α-LA. α-LA that was modified with Z-Gln-Gly in the presence of Ca^{2+} in the first reaction (see also **Figure 2B**), was modified with Gly-Gly-OEt in the second reaction in the presence of Ca^{2+} and only a trace amount of α-LA containing one Gly-Gly-OEt group could be detected. The second reaction in the presence of EDTA attached five extra Gly-Gly-OEt groups to each of the modified protein species regardless of the number of groups introduced in the first modification reaction.

α-LA modified with Z-Gln-Gly in the presence of EDTA in the first reaction (see also **Figure 2C**) was not modified with Gly-Gly-OEt in the second reaction in the presence of Ca^{2+}. The second modification reaction in the presence of EDTA introduced five additional Gly-Gly-OEt groups.

Circular Dichroism (CD) Analysis. The Z-Gln-Gly, Gly-Gly-OEt, the sequentially modified, and the nonmodified α-LA species were analyzed in the presence of Ca^{2+} and EDTA with near UV CD and far UV CD to analyze the tertiary and secondary structures of the proteins, respectively.

The shapes of the near UV CD spectra in the presence of EDTA, with extremes around 272, 292, and 297 nm, were very similar for the nonmodified and modified α-LA species. In the presence of Ca^{2+} the shape of the spectra is not significantly affected, but the intensity of the spectra increases. The data indicate that the globular tertiary structure of α-LA is not affected to a large extent by the modifications.

The shape of the far UV CD spectra in the presence of EDTA or Ca^{2+} is similar for the nonmodified and modified α-LA species. All spectra have a broad extreme around 220 nm, with a shoulder around 208 nm and a zero crossing around 201 nm. These data indicate that the secondary structure of α-LA is not affected to a large extent by the modifications. Curve fitting showed that the random coil in the presence of Ca^{2+} is decreased by 5−10%, indicating a structuring effect of the Ca^{2+} binding.

Identification of the Modified Residues in α-Lactalbumin. Modified α-LA was treated with Glu-C protease in sodium phosphate buffer (pH 7.8) or in ammonium bicarbonate buffer (pH 7.8). In phosphate buffer Glu-C cleaves proteins C-terminally of Glu and Asp residues (**Table 2**, V series ions), whereas in ammonium bicarbonate the protease preferentially cleaves C-terminally of Glu residues (**Table 2**, E series ions). The α-LA peptide fragments were analyzed with MALDI-TOF-MS and electrospray ionization quadrupole-TOF tandem mass spectrometry (Q-TOF MS/MS spectrometry). By comparing MALDI-TOF spectra of modified and nonmodified α-LA with the theoretical spectra that were calculated with the software tool "Findlink" (*13, 14*), most of the modified residues were identified. All modified peptides and additional modifications were identified with Q-TOF MS/MS analysis. In **Table 2** the results of the MALDI-TOF-MS analyses are summarized.

Modified Lysine Residues. Analysis of the MALDI-TOF-MS data showed that lysine residues Lys13 or Lys16, Lys98 or Lys108, and Lys114 are modified with Z-Gln-Gly in the presence of Ca^{2+}. This indicates that the MALDI-TOF spectrum of nondigested modified α-LA (see **Figure 2B**) is a mixture of different modified protein species where on average one or two lysine residues are modified. Lysine residues Lys5, Lys13 or Lys16, Lys98 or Lys108, and Lys114 are modified with Z-Gln-Gly in the presence of EDTA. The modified residues in peptides that contain two or more modifiable residues cannot be identified unambiguously with MALDI-TOF-MS. Fragmentation of these peptides with Q-TOF MS/MS can be used for the identification.

In the Q-TOF spectra of digests of α-LA modified with Z-Gln-Gly in the presence of Ca^{2+} or EDTA an extra doubly charged ion at *m*/*z*2 1058.4 (i.e., *m*/*z* 2116.8) was detected that was not visible in the MALDI-TOF-MS spectrum. The peptide was further fragmented and could be identified as peptide Leu12−Glu25 modified with two Z-Gln-Gly groups attached to Lys13 and Lys16. Other fragments in which the location of the modified residue could not be established with MALDI-TOF-MS, because they contained two or more modifiable residues, were also fragmented further by tandem MS to locate the modified residues within the peptide. Modified peptide Leu12−Glu25 with modified Lys13 or Lys16 at *m*/*z* 1795.9 in the MALDI-TOF-MS spectrum was visible at *m*/*z*2 898.40 in the Q-TOF spectrum. After fragmentation of the doubly charged ion it was not possible to identify whether Lys13 or Lys16 was modified because no discriminating fragment ions were present in the spectrum. The ion at *m*/*z* 1795.9 is probably a mixture of two nearly identical peptides in which Lys13 or Lys16 is modified. Peptide Lys98−Glu113 at *m*/*z* 2209.2 with modified Lys98 or Lys108 was visible at *m*/*z*2 1104.90 in the Q-TOF spectrum. Fragmentation of the peptide clearly showed that Lys108 is modified. A small mass peak at *m*/*z* 2529.2, with less than 2% intensity, in the MALDI-TOF-MS spectrum pointed to a peptide K98−E113 with modified Lys98 and Lys108.

The MALDI-TOF-MS spectra of intact, undigested modified α-LA (see **Figure 2C**) showed that at least five different lysine residues of α-LA are modified with Z-Gln-Gly. We have identified five enzymatic modifiable lysines (i.e. 5, 13, 16, 108, and 114).

Modified Glutamine Residues. The glutamine residues can only be modified by MTGase in apo α-LA. Analysis of the MALDI-TOF-MS data of α-LA modified with Gly-Gly-OEt and 6-aminohexanoic acid showed that glutamine residues 39, 43, 54, 65, and 117 of α-LA are modified (see **Table 2**). Up to five different glutamine residues of α-LA can be modified with

Table 2. Deconvoluted Peptide Masses of the Modified α-LA Peptides and Identification of the Modified Residues

additive	modification	Glu-C digestion buffer	experimental peptide mass $[M + H]^+$(Da)	deviation of theoretical mass (ppm)	fragment[a]	peptide	modified residue
Ca^{2+}	Z-Gln-Gly	NH_4HCO_3	1652.8	7	E7–8	Lys114-Leu123	Lys114 or Lys122
		NH_4HCO_3	1795.9	17	E4	Leu12-Glu25	Lys13 or Lys16
		NH_4HCO_3	4551.1	2	E4–5	Leu12-Glu49	Lys13 or Lys16
		Na-phosphate	1411.6	28	V19–20	Lys114-Glu121	Lys114
		Na-phosphate	1795.9	12	E4 (V4–5)	Leu12-Glu25	Lys13 or Lys16
		Na-phosphate	2209.1	2	V18	Lys98-Glu113	Lys108
EDTA	Z-Gln-Gly	NH_4HCO_3	1227.5	49	E1–2	Glu1-Glu7	Lys5
		NH_4HCO_3	1652.8	7	E7–8	Lys114-Leu123	Lys114 or Lys122[b]
		NH_4HCO_3	1758.8	17	E1–3	Glu1-Glu11	Lys5
		NH_4HCO_3	1795.9	12	E4	Leu12-Glu25	Lys13 or Lys16
		NH_4HCO_3	2327.2	9	E3–4	Val8-Glu25	Lys13 or Lys16
		NH_4HCO_3	4551.3	48	E4–5	Leu12-Glu49	Lys13 or Lys16
		Na-phosphate	1227.5	8	E1–2	Glu1-Glu7	Lys5
		Na-phosphate	1411.7	21	V19–20	Lys114-Glu121	Lys114[c]
		Na-phosphate	1795.9	27	E4 (V4–5)	Leu12-Glu25	Lys13 or Lys16[c]
		Na-phosphate	2209.2	39	V18	Lys98-Glu113	Lys108[c]
EDTA	Gly-Gly-OEt	NH_4HCO_3	3060.4	11	E5	Trp26-Glu49	Gln39 and Gln43[c]
		NH_4HCO_3	4517.1	2	E4–5	Leu12-Glu49	Gln39 and Gln43
		Na-phosphate	1234.6	27	V19–20	Lys114-Glu121	Gln117
		Na-phosphate	3060.4	24	E5 (V6–8)	Trp26-Glu49	Gln39 and Gln43
EDTA	$NH_2(CH_2)_5COOH$	NH_4HCO_3	3002.3	21	E5	Trp26-Glu49	Gln39 and Gln43
		Na-phosphate	1205.6	10	V19–20	Lys114-Glu121	Gln117
		Na-phosphate	3002.3	21	E5 (V6–8)	Trp26-Glu49	Gln39 and Gln43[c]
		Na-phosphate	3768.6	37	V9–11	Tyr50-Asp78	Gln54 and Gln65

[a] De α-LA fragments generated by the Glu-C cleavage in NH_4HCO_3 buffer are indicated as the E series, and the fragments generated in sodium phosphate buffer are indicated as the V series. When a fragment is formed by the proteolysis in both buffers the second fragment is indicated between brackets. [b] Q-TOF MS/MS analysis showed that only Lys114 is modified. [c] These modifications are formed at 30 °C and 50 °C. All other modifications were formed at 50 °C.

Gly-Gly-OEt (see **Figure 2A**) of which we identified four, and one additional modified glutamine (Gln65) was identified in the MALDI-TOF-MS and Q-TOF MS/MS spectra with 6-aminohexanoic acid modified α-LA.

DISCUSSION

To assess the accessibility of the lysine and glutamine residues in α-LA to the MTGase reaction we modified the substrate protein with the glutamine-containing peptide Z-Gln-Gly and a number of different primary amines. A maximum of 5 lysines and 5 glutamines can be modified by MTGase depending on temperature, pH, and the presence or absence of Ca^{2+}.

To explain the differences in the number of modifiable residues in α-LA under the different reaction conditions, we describe briefly the structural elements of α-LA that may be relevant in this context. The 123 amino acid bovine α-LA has no free SH groups, but has 4 disulfide bridges and a Ca^{2+}-binding site. The protein consists of two structural domains: a large α-helical domain and a small β-sheet domain that are separated by a cleft (*17*). The Ca^{2+}-binding site is formed by the carboxylic group of Asp84 in the helical domain and the carbonyl oxygen of Lys79 in the β-sheet domain (1HFZ.pdb, *16*). The apo protein loses its native conformation above 320 K (47 °C), whereas the holo protein keeps its native conformation. In the apo form the β-sheet domain of the protein is significantly unfolded, whereas the α-helical domain retains its helices and tertiary fold (*18*). The holo and apo proteins have a similar globular shape (*17*). The effect of the temperature on the protein structure with respect to the modification reaction is discussed below.

As we have shown, of the 12 lysines and 6 glutamines of α-LA at least 5 lysines and 5 glutamines can serve as MTGase substrate.

At 30 °C no lysines can be modified in the holo protein, whereas at 50 °C lysines 13, 16, 108, and 114 can be modified. These lysines are all highly solvent exposed in the X-ray structure. According to the analysis of the α-LA structure (1HFZ.pdb) with swiss pdb viewer (accessible area tool; swiss pdb viewer V3.7; www.expasy.ch/spdbv/), their side chains have solvent-accessible areas of at least 42%. The side chain of Lys5, which can only be modified in the apo protein at 50 °C, has a solvent accessible area of 21%. All these lysines are located in the helical domain of the protein.

At 30 and 50 °C no glutamines are modified in the holo protein. In the apo protein at 30 °C glutamines 39 and 43, and at 50 °C also glutamines 54, 65, and 117, are modified. The modifiable glutamine residues are solvent exposed and their side chains have solvent-accessible areas of at least 29%. Only the side chain of Gln54 has an accessible area of less than 7%. The nonmodifiable Gln2 has an accessible area of 40%. Gln39, Gln43, and Gln65 are part of the β-sheet domain and Gln117 is part of the helical domain. Glutamine 54 is part of the β-sheet domain and is located in the cleft between the two domains of the protein.

Apparently the structural differences between the holo and apo protein, and not the accessible surfaces, determine whether the glutamines and lysines are modified. The progressive unfolding of the β-sheet domain of the apo protein at increasing temperatures may explain the modification of the glutamines. Furthermore, other secondary structural elements in the holo and apo proteins may become more flexible with increasing temperature, making modification possible.

Interestingly, the Ca^{2+} dependence of the sequential modification with Z-Gln-Gly greatly disappears after modification of the maximum number of glutamines with Gly-Gly-OEt. Gln 54 is located in the cleft between the α-helical and β-sheet domain. Because the Ca^{2+} binding site is formed by two residues from each domain, modification of Gln 54 with a bulky substituent may widen the cleft and weaken the Ca^{2+} binding, particularly at elevated temperatures. As a consequence the

structure of α-LA may be more flexible, thus allowing similar modification in the presence or absence of Ca^{2+}.

According to the CD analyses in the presence or absence of Ca^{2+}, there are no major conformational changes, and the tertiary and secondary structures of the modified α-LA species do not differ significantly from those of the nonmodified protein. Small local changes in the protein structure, that for instance influence Ca^{2+} binding, however, cannot, be detected with this technique.

The protein structure and solvent accessibility cannot fully explain the possible modification, as the lysines and glutamines that are not modified are also present in β-sheets or helices and also have large solvent-accessible areas (between 18 and 50%). Others observed that glutamines in proteins are generally modified by TGase if they are located at the end of β-sheets or in unstructured regions, and that negatively charged amino acids adjacent to the glutamine residues may block the TGase reaction (*19*). Indeed, glutamine residues Gln2 and Gln65 that are next to Glu1 and Asp64, respectively, are not modified. We, however, observed that Gln117, next to Asp116, is modified in the apo α-LA. For the modification of the lysine residues in α-LA it seems irrelevant whether they are next to charged amino acids or hydrophobic amino acids, as lysines next to Glu, Asp, His, Lys, Thr, Ile, Gly, Ala, and Leu are modified. It was observed by several authors (*20*, *21*) that N-terminal Asn, Phe, Tyr, and Arg residues restrict the MTGase modification of lysine. In this study the lysines that are not modified have N-terminal Asn, Cys, Val, or Asp residues.

The reduced and heat denatured α-LA is insoluble, and hence the accessibility of more lysine or glutamine residues could not be tested.

It is probably not only the primary and secondary structure, but also the tertiary structure of the protein that is important for the modification with MTGase. The latter is corroborated by the fact that other globular proteins such as β-lactoglobulin, ovalbumin, and bovine serum albumin are not substrates for TGase in their native conformations. These proteins are only TGase substrates after reduction of their disulfide bonds and loss of their tertiary structure (*4*, *22*, *23*).

Recently, the modification of Lys5 and Gln54 in reduced α-LA (i.e., in the presence of DTT) by MTGase has been reported. Under reducing conditions, however, large structural changes in α-LA occur when the disulfide bonds are broken (*24*). The enhanced susceptibility to the transglutaminase reaction of nonreduced apo α-LA has been described (*25*). In the latter study no thorough comparison of the modifications of the glutamines and lysines in the apo and holo α-LA was made and only the modification of Gln54 and three unidentified lysines was reported. The modifications were performed at pH 7.5 and 37 °C. At 37 °C both the holo and the apo α-LA are mainly in the native conformation (*18*), which may explain why fewer modifications were found. The deamidation of Gln39, Gln43, Gln54, and Gln65 in apo α-LA at pH 7.0 and 42 °C with protein-glutaminase from *Chryseobacterium proteolyticum* has been reported (*26*). This is in good agreement with the glutamine residues that were modified in the present study. We have shown that five glutamines (39, 43, 54, 65, and 117) and five lysines (5, 13, 16, 108, and 114) are available for modification by MTGase in apo α-LA, whereas no glutamines and four lysines (13, 16, 108, and 114) are available in the holo protein. Furthermore, we have shown that a wide variety of primary amines, including peptides, can be used in the MTGase modification of α-LA. The possibility to direct the modification sites in α-LA and the broad substrate specificity of MTGase opens the way to make well-defined α-LA derivatives with different physical properties. Preliminary data indicate that with Z-Gln-Gly, and especially with 6-aminohexanoic acid, modified α-LA can form foams that are much more stable than the foams of the native protein. Furthermore, the modified proteins have better emulsifying properties.

The flexibility of lysine- and glutamine-containing protein regions greatly determines the possibility to use MTGase in protein modifications. Ways to increase the flexibility of other globular proteins that are resistant to modification by MTGase are currently under investigation.

ABBREVIATIONS USED

α-LA, bovine α-lactalbumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Glu-C, endoproteinase Glu-C from *Staphylococcus aureus* strain V8; Gly-Gly-OEt, glycine-glycine-O-ethyl ester; MALDI-TOF-MS, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry; MTGase, Ca^{2+} independent microbial transglutaminase from *Streptoverticillium mobaraense*; Q-TOF MS/MS, quadrupole time-of-flight tandem mass spectrometry; TGase, transglutaminase; Z-Gln-Gly, α-*N*-carbobenzyloxy-glutamine-glycine.

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