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ARTICLE

Identification of the Peroxidase-Generated Intermolecular Dityrosine Cross-Link in Bovine α-Lactalbumin

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The peroxidase-mediated oxidation of calcium-depleted bovine α-lactalbumin generates a mixture of covalently bound protein oligomers with interesting foaming properties. Here, we isolated the initially formed covalent α-lactalbumin dimer and studied its mode of cross-linking. Liquid chromatography−Fourier transform mass spectrometry (LC−FTMS) of proteolytic digests revealed the unambiguous identification of a peroxidase-catalyzed covalent link between Tyr18 and Tyr50. This shows that, although the radical reaction is often regarded as a random reaction, the initial product formation is specific. Protein structural modeling indicates that the conjugation reaction between these tyrosines is sterically favored and involves initial noncovalent protein complex formation through charge compensation, facilitating intermolecular cross-linking. The identification of the Tyr18−Tyr50 cross-link supports the view that the peroxidase-mediated oxidation of apo α-lactalbumin is a sequential process, involving the formation of linear trimers and higher order oligomers.

KEYWORDS: α-Lactalbumin; molten globule; dityrosine; horseradish peroxidase; protein cross-linking

INTRODUCTION

Enzymatic cross-linking is an emerging tool for changing the functionality of food proteins (*1*−*4*). One such cross-linking procedure involves the peroxidase-mediated one-electron oxidation of tyrosine residues with the subsequent generation of (iso)dityrosine bonds (*5*−*7*).

Recently, it was demonstrated that the holo form of the bovine whey protein α-lactalbumin is insensitive to peroxidase-catalyzed cross-linking, whereas the apo form is cross-linked (*8*). The cross-linking of the apo form was quantified in-line with ultraviolet (UV) detection of the dityrosine bond. Furthermore, when the reaction was studied as a function of pH and ionic strength, it was established that the size distribution of the cross-linked α-lactalbumin products can be directed toward the protein oligomers desired (*9*).

Bovine α-lactalbumin contains four tyrosine residues with different accessibilities and reactivities (**Figure 1**). In holo α-lactalbumin, Tyr18 is sufficiently exposed to be photosensitized by chemically induced dynamic nuclear polarization (*10*). In the flexible acid molten globule state, also Tyr36 and Tyr50 were found to be available for photosensitizing (*11*). Nuclear magnetic resonance (NMR) measurements of gradually denaturing acid molten globule α-lactalbumin showed an order in accessibility for tyrosine residues: Tyr36 becomes accessible first, followed by Tyr50, Tyr18, and last Tyr103 (*12*). The crystal structure of apo α-lactalbumin reveals a more open structure at the location of Tyr103 in the α domain compared to the structure of holo α-lactalbumin (*13*). Thus, it is not obvious which tyrosine residues are exposed and involved in the peroxidase-mediated dityrosine cross-linking process of apo α-lactalbumin.

To understand of the mechanism of peroxidase-catalyzed protein cross-linking, we address in the present work the mode of cross-linking between α-lactalbumin monomers. Until now, the oxidative formation of protein dityrosine cross-links has mainly been demonstrated by amino acid analysis of complete protein hydrolysates (*14*, *15*) and separated peptides (*16*) or by indirect evidence from site-directed mutagenesis (*17*, *18*). For the reaction of sperm whale myoglobin with hydrogen peroxide, it was established that the radical transfer from the heme co-factor to the protein results in the formation of intra- and intermolecular isodityrosine and dityrosine bonds (*19*). In the case of α-lactalbumin, the oxidation is catalyzed by horseradish peroxidase. To determine the specificity of dityrosine formation in this system, we isolated a covalent α-lactalbumin dimer fraction and analyzed its proteolytic digest by liquid chromatography−Fourier transform mass spectrometry (LC−FTMS). The mass spectrometric data were subsequently screened for dipeptides, allowing for the unambiguous identification of the covalent link between Tyr18 and Tyr50. The results are discussed in relation to the structural properties of α-lactalbumin.

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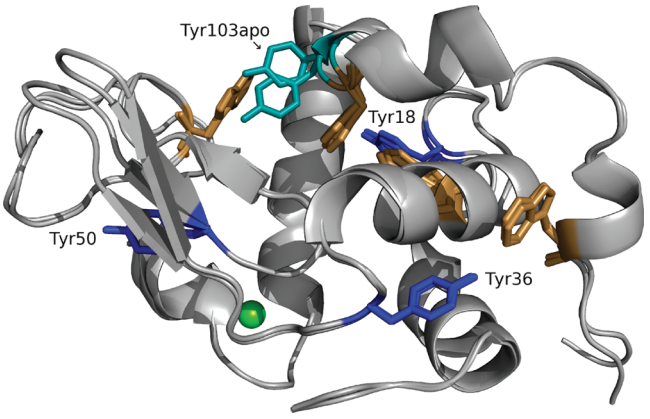

Figure 1. Superimposition of the crystal structures of bovine holo α-lactalbumin [Protein Data Bank (PDB) accession code 1f6s] and bovine apo α-lactalbumin (PDB accession code 1f6r) (*13*). Tyr18, Tyr36, and Tyr50 are indicated in blue, and Tyr103 is indicated in cyan. Tryptophan residues are shown in gold, and the calcium ion in holo α-lactalbumin is depicted as a green sphere.

MATERIALS AND METHODS

Materials. Apo bovine α-lactalbumin (BioPURE) was obtained from Davisco Foods International, Inc. (Le Sueur, MN). According to the manufacturer, the material contains 95% (w/w) protein [of which 90% (w/w) α-lactalbumin] and 0.55% (w/w) calcium. Besides α-lactalbumin, the commercial preparation was found to contain traces of bovine serum albumin (BSA) and β-lactoglobulin (β-LG) (*8*). Horseradish peroxidase (HRP)-type VI-a (P6782) was obtained from Sigma (Sigma Chemical Co., St. Louis, MO). Trypsin (Trypsin Gold, MS grade) was from Promega (Madison, WI), and endoproteinase Glu-C (Glu-C, Protease V8 sequencing grade) was from Roche Biochemical (Basel, Switzerland). The surfactant *Rapi*Gest was from Waters (Milford, MA). All other (bio)chemicals were of analytical grade and purchased from Sigma or Merck (Darmstadt, Germany).

Apo α-Lactalbumin Cross-Linking. Apo α-lactalbumin (10 mg dissolved in 1 mL of 10 mM ammonium acetate at pH 6.8) was incubated for 60 min at 37 °C in the presence of HRP (50 μL, 10 mg/mL) and hydrogen peroxide (2 μL, 0.5 M). For preparative purposes, the cross-linking reaction was repeated 20 times and the products obtained were combined in one stock. This stock was stored at −20 °C and thawed before fractionation.

Size-Exclusion Chromatography (SEC). Cross-linked protein samples (20 μL, 5 mg/mL) in 0.1 M ammonium acetate at pH 6.8 were applied to a Superdex 75 10/300 GL column connected to an Äkta Purifier system (GE Healthcare, Uppsala, Sweden) at room temperature. The column was equilibrated and eluted with 0.1 M ammonium acetate at pH 6.8 at a flow rate of 0.9 mL/min. The eluate was monitored at 280 nm. Calibration of the column was performed with a low-molecular-mass SEC calibration kit (GE Healthcare). The kit contained blue dextran (2000 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Preparative Anion-Exchange Chromatography (AEC). Cross-linked apo α-lactalbumin was fractionated on a preparative scale (4 × 50 mg) using a 6 mL Resource Q column running at 6 mL/min in an Äkta Purifier system (GE Healthcare). The column was equilibrated for 5 min with 20 mM Tris-HCl at pH 7.4. After sample injection (5 mL, 10 mg/mL), the column was washed for 3 min, followed by a 20 min linear gradient from 0 to 0.5 M NaCl in 20 mM Tris-HCl at pH 7.4. The column was regenerated with 1.5 M NaCl in 20 mM Tris-HCl at pH 7.4 for 5 min, followed by a 5 min re-equilibration of 20 mM Tris-HCl at pH 7.4. The eluate was monitored at 280 and 318 nm, and 0.5 mL fractions were collected. Fractions that had similar composition as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were pooled, and fractions containing dimers and oligomers were dialyzed against water and lyophilized. This resulted in a yield of 30 mg of the dimer pool, which was stored at −20 °C and thawed before digestion.

SDS−PAGE. The peroxidase-catalyzed formation of covalently linked apo α-lactalbumin oligomers was analyzed with SDS−PAGE using a Phast system (GE Healthcare) according to the instructions of the supplier. PhastGels Gradient 8-25 (GE Healthcare) was used. Before application, protein samples (5 mg/mL) were treated with β-mercapthoethanol (1.25%, v/v) and heated for 5 min at 100 °C in the presence of SDS (1.25%, w/v). Proteins were stained with Coomassie Brilliant Blue, and a protein molecular-weight marker (article 17-0446-01; GE Healthcare) was used for calibration.

Digestion of α-Lactalbumin Monomer and Dimer. Stock solutions (1−10 mg/mL in water) were made of the commercial apo α-lactalbumin material and the isolated apo α-lactalbumin monomer and dimer fractions after the cross-link reaction. A double digestion was performed by either starting with trypsin or with Glu-C in the presence of 0.1% (w/v) *Rapi*Gest (a surfactant that can promote digestion and is compatible with mass spectrometry analysis) or with a single digestion with Glu-C in the presence or absence of 0.1% (w/v) *Rapi*Gest.

The samples were diluted to a concentration between 20 and 100 μg of protein using 0.1 M NH_4HCO_3 in a volume of 50 μL. After the addition of 5 μL *Rapi*Gest from a 1% stock and 5 μL of 100 mM dithiothreitol, reduction was performed for 1 h at 55 °C. After cooling, alkylation was performed by adding 6 μL of 0.3 M iodoacetamide, allowing for 45 min of reaction at room temperature.

For double digestion, the samples were divided into two parts of equal volume. With one part, the digestion was started by trypsin with a 1:50 (protease/protein, w/w) ratio and incubated overnight at 37 °C. With the other part, the digestion was started by Glu-C at the same w/w ratio and incubated overnight at room temperature. The next day, both samples were again divided in a 50:50 ratio split. One part of the trypsin and Glu-C digestion was boiled for 10 min in a water bath, and the other half was acidified with trifluoroacetic acid (TFA) to a final concentration of 1% (v/v). The second digestion was performed with the cooled boiled samples with a w/w ratio of 1:25 trypsin (37 °C) or Glu-C (room temperature) for 6 h. The reaction was stopped by adding TFA to a final concentration of 1% (v/v). All samples were depleted from the *Rapi*Gest following the instructions of Waters and were collected after desalting with an 80 μg Omix C18 tip (Agilent Technologies, Palo Alto, CA) in 20 μL of 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA. Therefore, for each sample, we obtained four different digestions: trypsin, Glu-C, trypsin followed by Glu-C, and Glu-C followed by trypsin.

The single digestion with Glu-C with and without *Rapi*Gest was performed with the same digestion conditions and purification method as described above.

Mass Spectrometric Analyses. Mass data were acquired using an ApexQ Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a CombiSource coupled to an Ultimate 3000 (Dionex, Sunnyvale, CA) HPLC system with a 100 μm inner diameter, 150 mm monolithic reverse-phase column (Onyx C18, Phenomenex, Torrance, CA).

Samples with up to 1−2 pmol of digest were injected as 3 μL of 0.1% (v/v) TFA aqueous solutions and directly loaded onto the analytical column. After injection, a linear gradient was applied in 30 min from water containing 0.1% (v/v) TFA to water containing 0.1% (v/v) TFA and 40% (v/v) acetonitrile at a flow rate of 2 μL/min. During elution, a chromatogram based on about 600 high-resolution electrospray ionization (ESI) FTMS spectra was recorded using a MS duty cycle of about 3 s.

LC tandem mass data were recorded by MS data-dependent selection of peptide ions in the Q sector followed by collisional fragmentation in the collision hexapole at an argon pressure of about 5×10^{-6} mbar and then detection of peptide fragment ions in the FTMS cell. For this, alternating MS and MSMS experimental sequences were executed with duty cycles of 1 and 3 s, respectively.

MS Data Processing and Analysis. The MS data were processed using the Data Analysis 3.4 software program (Bruker Daltonics). In a batch procedure, the 600 mass spectra were extracted from the chromatogram and the monoisotopic masses of the peptides were determined using Brukers peak recognition technology SNAP II. Mass calibration was achieved by selectively extracting and then summing about four mass spectra from the chromatogram showing MSMS confirmed unmodified peptides from trypsin + GluC-digested α-lactalbumin. With the theoretical masses of these calibrant peptides, the summed spectrum was mass-calibrated,

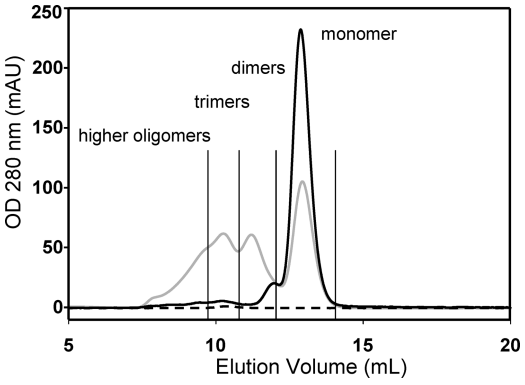

Figure 2. Size-exclusion chromatogram of untreated apo α-lactalbumin (black line) and HRP (dashed black line) in 10 mM ammonium acetate at pH 6.8 using a Superdex 75 10/300 GL column. Apo α-lactalbumin was incubated at 37 °C for 1 h in the presence of peroxidase and hydrogen peroxide (gray line).

and the resulting calibration parameters were applied to all spectra in the chromatogram. This resulted in a mass calibration of better than 1.5 ppm over the entire chromatogram for all analyses. For each FTMS analysis, the resulting array of up to 600 monoisotopic mass lists was exported as a MASCOT generic file (mgf). Ion abundances in the exported array of monoisotopic mass lists were the sum of abundances of all isotopes over all charge states for each peptide.

The exported mgf file was imported in the CoolToolBox software program, which is a major update of the in-house developed VIRTUALMSLAB program (*20*, *21*).

From the imported array of up to 600 monoisotopic mass spectra, the CoolToolBox program constructed up to 500 peptide ion chromatograms. For each peptide ion chromatogram, the mass and retention time were taken at the apex of the chromatogram profile and the abundance was summed over the ion chromatogram profile. The ultimate LC−MS data processing resulted in a peptide monoisotopic mass list with abundance and LC retention. Peptide assignments of the ion masses were made by matching the processed LC−FTMS data from the peptide trypsin + GluC-digested α-lactalbumin within a mass window of 1.5 ppm, with the peptide masses in a database obtained from a corresponding *in silico* digestion/chemical modification of bovine α-lactalbumin, bovine β-LG, and BSA (*20*). The latter two proteins were detected with tandem MS as minor contaminants in the α-lactalbumin protein used.

Modified and cross-linked peptides were assigned by matching the experimental ion masses with the masses of all *in silico* generated theoretically assumed peptides and cross-linked peptide pairs from chemical modification and dehydrogenative cross-linking between tyrosine residues (*20*).

The tandem MS data were processed using the Data Analysis 3.4 software program (Bruker Daltonics). The processed MSMS peptide fragmentation data were individually analyzed as peptide residue sequences with chemical modification.

Protein Modeling. Three-dimensional models of the crystal structures of holo bovine α-lactalbumin (PDB accession code 1f6s) and apo bovine α-lactalbumin (PDB accession code 1f6r) (*13*) were visualized using PyMol (The PyMOL Molecular Graphics System, version 1.3, Schrödinger, LLC). The charge distribution of the apo α-lactalbumin monomer was calculated using the program APBS (*22*).

RESULTS

Preparation of the Dimeric α-Lactalbumin Fraction. The peroxidase-mediated cross-linking of apo α-lactalbumin in 10 mM ammonium acetate at pH 6.8 results in the apparent molecular-mass distribution shown in **Figure 2**. The percentage of monomer, dimer, trimer, and higher oligomers, as determined by SDS−PAGE (no data shown), was about 35, 22, 22, and 22%, respectively, taking equal weight-based response factors into account (*9*). To determine the mode of cross-linking between α-lactalbumin monomers, peroxidase-treated α-lactalbumin monomers and

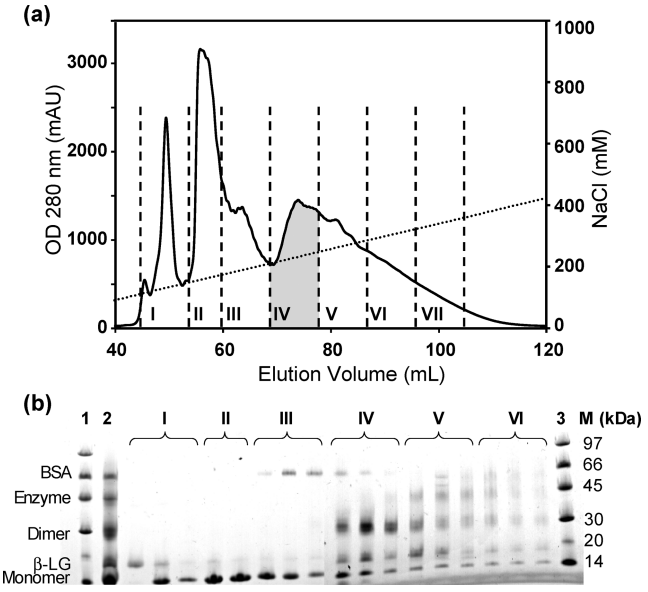

Figure 3. Preparative isolation of the apo α-lactalbumin dimer. (a) Separation of cross-linked apo α-lactalbumin by AEC. The Roman numbers indicate the different pools. Pool IV (gray) contains the α-lactalbumin dimer. The dotted line indicates the NaCl gradient. (b) SDS−PAGE of collected fractions. Lanes 1 and 3, marker proteins with their molecular mass denoted at the right; lane 2, cross-linked α-lactalbumin mixture; BSA, bovine serum albumin; enzyme, HRP; β-LG, β-lactoglobulin.

covalent α-lactalbumin dimers were fractionated using AEC (**Figure 3a**). As can be seen from the SDS−PAGE pattern in **Figure 3b**, monomeric α-lactalbumin elutes between 126 and 176 mM NaCl in two peaks (pools I and II). In pool II, a slight increase in optical density (OD) of 318 nm was observed (no data shown), and this might indicate the formation of an intramolecular dityrosine cross-link (*8*). Most of the α-lactalbumin dimers elute between 210 and 250 mM NaCl (pool IV). Despite the lack of base-peak separation, it was possible to obtain an enriched dimeric α-lactalbumin preparation [30 mg of 15% (w/v) starting material]. This preparation contains BSA (2%), β-LG (29%), and monomeric α-lactalbumin (23%), as determined via protein standards in SDS−PAGE, but no other α-lactalbumin oligomers. The protein contaminations present in the dimer pool were, therefore, used as internal standards in further MS experiments.

Identification of Dityrosine Cross-Link(s) in Dimeric α-Lactalbumin. To identify the mode of cross-linking between α-lactalbumin monomers, a peptide mapping strategy was developed. Prior to digestion with trypsin and Glu-C, disulfides were blocked with dithiothreitol and capped with iodoacetamide. After proteolytic cleavage, α-lactalbumin peptides were separated by reverse-phase high-performance liquid chromatography (RP-HPLC) and identified by FTMS.

The digestion profiles of both α-lactalbumin monomers and dimer after the first single digestion showed an increased contamination in the treated monomer fraction. The main difference observed in these digestion profiles is the amount of BSA- and β-LG-derived peptides (results not shown). The peptides obtained after a single tryptic or Glu-C digestion were too large to reveal the location of the dityrosine cross-link. Therefore, a second digestion was performed with the less contaminated, untreated monomer and dimer fractions. After analysis with RP-FTMS (**Figure 4a**) and application of the CoolToolBox program, the peptides were annotated to the different milk proteins. The theoretical and experimental masses of the peptides found for untreated and dimeric α-lactalbumin are listed in **Table 1**,

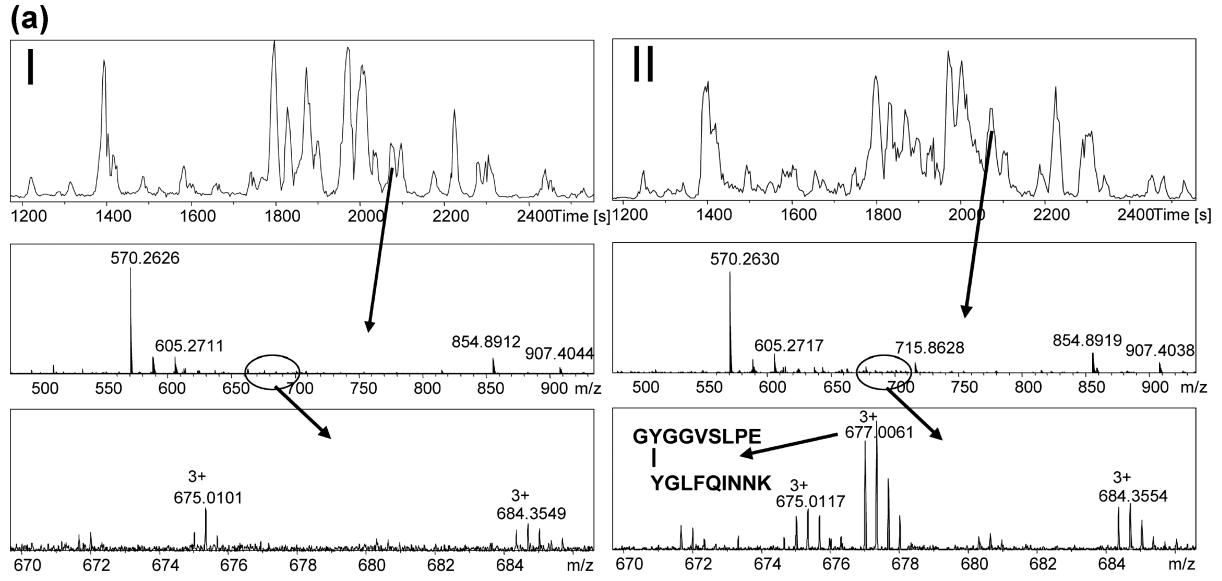

Figure 4. (a) Total ion current of untreated (I) and dimeric (II) α-lactalbumin separated and analyzed on a RP-FTMS system. The mass spectra and expansions thereof at the elution time of the cross-linked peptide are shown for both samples. (b) Sequence of the dityrosine peptide.

Table 1. α-Lactalbumin Peptides Found in Digested α-Lactalbumin Monomer and Dimer with Their Retention Time on RP-HPLC

peptide fragment	retention time (s)	theoretical mass (Da)	experimental peptide mass $[M + H]^+$ (Da)
1−5	926	618.346	618.345
6−10	1344	710.329	710.329
8−10	1000	421.256	421.256
14−16	1028	375.224	375.224
17−25	2003	878.425	878.425
26−49	2027	2774.200	2774.201
50−58	1974	1096.579	1096.578
59−62	1253	606.307	606.307
63−79	1404	2003.818	2003.817
80−93	2443	1699.755	1699.753
95−98	1025	488.308	488.307
99−108	1872	1200.652	1200.652
109−114	930	707.339	707.339
114−121/115−122	1804	1091.519	1091.519

showing a sequence coverage of 96%. Only the amino acids 11−13 and 94 could not be detected. The presence of all tyrosine-containing peptides can be caused by the presence of residual monomeric α-lactalbumin. The β-LG impurity was used as an internal standard and showed a 100% coverage with minor miscleavages (no data shown).

Monomeric bovine α-lactalbumin contains four tyrosines at positions 18, 36, 50, and 103, which in theory can form 10 different dityrosine bonds (**Table 2**). Of the possible dipeptides, one mass was found that unambiguously matched with a mass of a peptide containing a cross-link between Tyr18 and Tyr50 (**Table 2** and **Figure 4**). Because of the low signal, it was not possible to perform a MS^n analysis on this peptide. Extensive searches for the other dityrosine cross-links and Tyr−Trp, Tyr−Phe, Tyr−His, Trp−Trp, Trp−His, Trp−Phe, Phe−His, Phe−Phe, and His−His cross-links did not result in any match. This fact and the identification of even minor modifications in α-lactalbumin (described below) strengthen the results obtained. Thus, in the α-lactalbumin dimer, only a single dityrosine cross-link was found.

Table 2. Theoretical and Experimental Masses of α-Lactalbumin Dipeptides Containing the Dityrosine Cross-Link

possible dipeptides	sequence	theoretical mass (Da)	experimental peptide mass $[M + H]^+$ (Da)
Y18−Y18	17−25 + 17−25	1753.899	ND[a]
Y18−Y36	17−25 + 26−49	3593.816	ND
Y18−Y50	17−25 + 50−58	1972.201	1971.981
Y18−Y103	17−25 + 99−108	2076.356	ND
Y36−Y36	26−49 + 26−49	5433.733	ND
Y36−Y50	26−49 + 50−58	3812.118	ND
Y36−Y103	26−49 + 99−108	3916.273	ND
Y50−Y50	50−58 + 50−58	2190.503	ND
Y50−Y103	50−58 + 99−108	2294.658	ND
Y103−Y103	99−108 + 99−108	2398.812	ND

[a] ND = not detected.

Structural Model of the Dityrosine Cross-Linked α-Lactalbumin Dimer. The abundance of the Tyr18−Tyr50-containing dipeptide ions and the absence of other possible dityrosine peptide ions in the array of mass spectra indicate that only these two tyrosines are able to come in close vicinity of each other in an apo α-lactalbumin dimer and form an intermolecular cross-link. To determine if such a cross-link is plausible from a structural point of view, dimer models were made using the crystal structure of apo α-lactalbumin (PDB accession code 1f6r). All charges on the surfaces of both monomers were labeled, and subsequently, one monomer was rotated and translated in space to obtain an orientation in which both the distance between two tyrosines of a different monomer was minimal and the charge compensation for both monomers was maximal. Only the Tyr18−Tyr50 combination yields a short intertyrosine distance (**Figure 5a**) and a very good charge compensation, as shown in **Figure 5b**. It can be seen that the side chain of Tyr18 is located in a predominantly

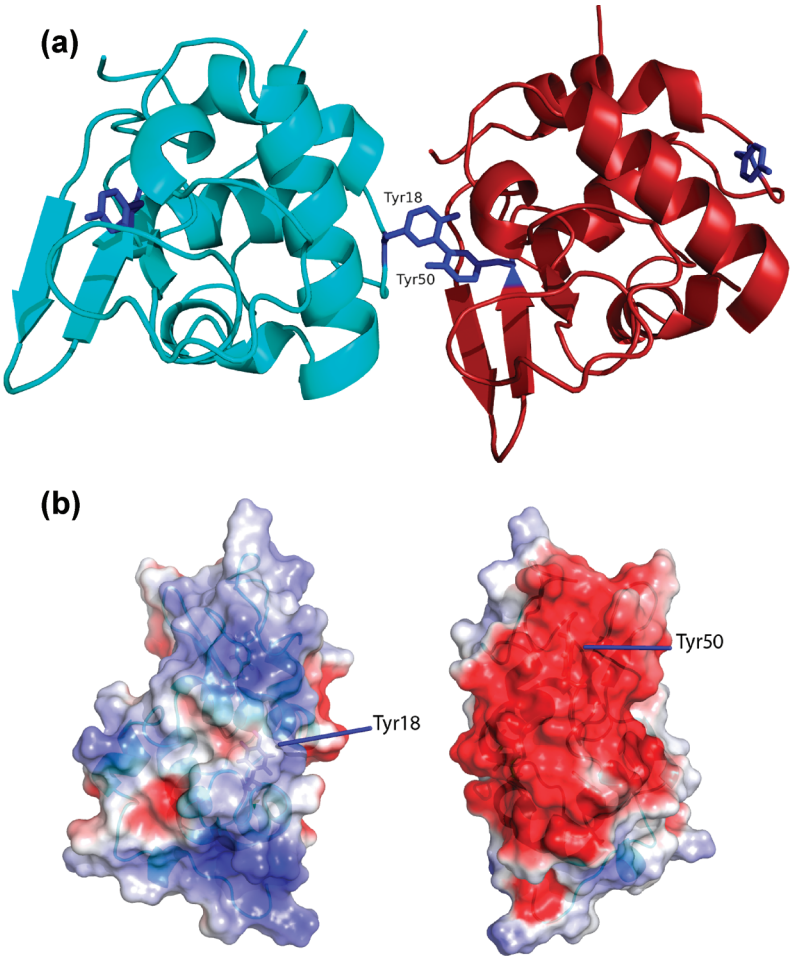

Figure 5. (a) Model of the apo α-lactalbumin dimer with coupled Tyr18 and Tyr50 residues. (b) Surface charge distribution of the apo α-lactalbumin monomer: (left) predominantly positive area around Tyr18 (blue) and (right) mainly negative region around Tyr50 (red).

positive area (blue), in contrast to that of Tyr50, which resides in a mainly negative region (red) of apo α-lactalbumin.

Other Modifications in the Cross-Linked α-Lactalbumin Dimer Fraction. Besides the one-electron oxidation of tyrosine side chains and the subsequent formation of dityrosine links, HRP might also catalyze the oxidation of other amino acid residues (*19*, *23*). Therefore, the digestion profiles of HRP-treated α-lactalbumin monomer and cross-linked α-lactalbumin dimer fractions were analyzed for other modifications, such as oxidation, deamidation, reduction, and acetylation. The single methionine (Met90) was found to be oxidized for 60%, and for tryptophan, 3.5% of all residues were dehydrogenated. Besides from the dityrosine link, these were the only modifications found in the cross-linked α-lactalbumin dimer fraction.

DISCUSSION

This research has identified the tyrosines involved in peroxidase-catalyzed cross-linking of apo α-lactalbumin. LC−FTMS of proteolytic digests revealed the unambiguous identification of a molecular covalent link between Tyr18 and Tyr50. Tyr18 is located in the α-helical domain, and Tyr50 is located in the *β*-sheet domain (**Figure 1**), making the intramolecular conjugation between these residues rather unlikely. Previously, we showed that holo α-lactalbumin is not sensitive to HRP-catalyzed cross-linking. This suggests that radical transfer from the peroxidase heme center to Tyr18 and/or Tyr50 is not efficient or that Tyr18 and Tyr50 of separate holo α-lactalbumin molecules cannot come in close contact. The crystal structure of apo α-lactalbumin reveals a more open structure at the location of Tyr103 in the α domain compared to the structure of holo α-lactalbumin (*13*) (**Figure 1**). However, Tyr103 is not involved in the formation of the molecular dityrosine link. The structure determination was performed on crystals grown in high concentrations of ammonium sulfate (*13*), conditions that are clearly different from our cross-linking experiments. Previous research has shown the importance of ionic strength on the tertiary conformation of α-lactalbumin (*9*, *24*). Our results show that the reaction of HRP with apo α-lactalbumin in low ionic strength is a rather specific reaction. Of the available amino acids, only tyrosine and methionine are extensively modified, with one other minor modification: tryptophan. Preliminary experiments show that the covalent α-lactalbumin dimer displays strong fluorescence at 420 nm, indicative for a dityrosine rather than an isodityrosine bond (*19*).

The assignment of the Tyr18−Tyr50 cross-link fits with the results obtained from our previous research (*8*). In that study, it was shown that the cross-linking of apo α-lactalbumin is a consecutive process ultimately leading to the formation of polymers. The initial formation of a Tyr18−Tyr50 cross-linked dimer is in agreement with such a sequential process. After formation of the covalent dityrosine bond, there is always a spare Tyr18 and Tyr50 left for continuation of the cross-linking reaction. This sequential process is schematically presented in **Figure 6**. The cross-link formation can be described by a clustering and reaction process. In the clustering process, two α-lactalbumin molecules form a complex through favorite charge interactions.

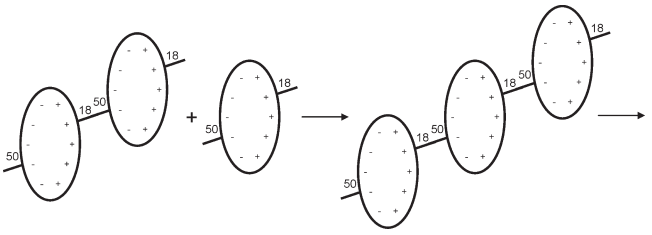

Figure 6. Model of the apo α-lactalbumin dimer reacting to higher oligomers.

In this complex, Tyr18 and Tyr50 are at about 4 Å distance (**Figure 5a**), which is considered to be close enough to form a cross-link in the reaction process. The HRP-mediated oxidation of both tyrosine residues most likely occurs before complex formation. Thus, we conclude that, at low ionic strength, the flexible structure of apo α-lactalbumin and charge−charge interactions are the driving force for the generation of the Tyr18−Tyr50 cross-link.

ABBREVIATIONS USED

BSA, bovine serum albumin; HRP, horseradish peroxidase; FTMS, Fourier transform mass spectrometry; Glu-C, endoproteinase Glu-C; β-LG, β-lactoglobulin; HPLC, high-performance liquid chromatography, PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid.

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