Selective Sulfenylation of Tryptophan Residues in α-Lactalbumin of Bovine Milk*

Yoram Shechter, Abraham Patchornik, and Yigal Burstein

From the Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel

SUMMARY

Tryptophan residues of bovine α-lactalbumin, one of the two protein components of lactose synthetase, were selectively sulfenylation with 2-nitrophenylsulfonyl chloride. When the reaction was performed in 0.1 M acetic acid for 1 hour, a modified protein containing 1.0 nitrophenylsulfonyl tryptophan residue per α-lactalbumin molecule was formed and isolated in 42% yield. Upon reduction, carboxymethylation, and trypsin digestion, this sulfenylated α-lactalbumin (NPS-αLA) yielded two yellow peptides. Amino acid composition of these peptides indicated that in NPS-αLA, tryptophan residues 60 and 118 were equally sulfenylation to the extent of 0.5 nitrophenylsulfonyl tryptophan per α-lactalbumin. NPS-αLA cross-reacts with anti-α-lactalbumin antibodies, but, unlike the native protein, it has no biological activity in the lactose synthesis reaction, and its electrophoretic mobility on polyacrylamide gels is different from that of native α-lactalbumin.

Lactose synthetase is an enzyme which catalyzes the final step in the biosynthesis of lactose (1, 2): UDP-galactose + glucose → lactose + UDP. This enzymatic reaction requires two proteins for significant activity, α-lactalbumin (3-5) and galactosyltransferase (6). Galactosyltransferase alone will synthesize lactose only at high glucose concentrations (7, 8) but in the presence of α-lactalbumin the apparent Km for glucose is much lower and biosynthesis of lactose is much more effective. Brew et al. (6) have shown that galactosyltransferase catalyzes the transfer of galactose to N-acetylglucosamine: UDP-galactose + GlcNAc → NAc-lactosamine + UDP. This reaction can be inhibited by α-lactalbumin, which will allow lactose synthesis in the presence of glucose as an acceptor. These and other recent kinetic studies suggest that α-lactalbumin modifies galactosyltransferase in a substrate-dependent interaction (9).

α-Lactalbumin of bovine milk is a single polypeptide chain composed of 123 amino acids with a molecular weight of 14,500 (10, 11). Studies on chemical modifications of α-lactalbumin might shed light on the role of certain amino acid residues in the activity of this protein as a “specifier” for galactosyltransferase. α-Lactalbumin has 4 tryptophan residues located at sequence positions 26, 60, 104, and 118 (10, 11). Solvent perturbation studies suggested that 2 of these 4 residues are buried and the other 2 are exposed to the solvent (12, 13). Denton and Ebner (14) have shown that upon iodination or nitration of α-lactalbumin, tryptophine, histidine, and tryptophan residues were modified and activity of the protein was lost. In that study, loss of activity was attributed to the modification of the tryptophine residues. Tyrosinase oxidized 1 tryptophan residue in α-lactalbumin with practically no loss of activity in the lactose synthesis reaction (14). Robbins and Holmes (15) have shown that whereas 1 tryptophan residue of α-lactalbumin forms a complex with N-methylacetamide at neutral pH, 2 tryptophan residues form such complexes at pH 2, due to partial acid denaturation. Barman (16) studied the reaction of α-lactalbumin with 2-hydroxy-5-nitrobenzyl bromide and found that at pH 2.7 and 6.0, tryptophan residues 26, 104, 118 were partially modified, while tryptophan residue 60 remained intact.

The purpose of this study was to examine the role of the tryptophan residues of α-lactalbumin in its biological function, by sulfenylation of those residues with NPS CI, a specific reagent (17). The results clearly indicate that sulfenylation of tryptophan residue 60 or 118 results in loss of the biological activity of α-lactalbumin in the biosynthesis of lactose, but its immunological properties remain unchanged.

MATERIALS AND METHODS

Proteins

Bovine α-lactalbumin was isolated from raw skimmed milk by a modification of the method of Aschaffenberg and Drewry (18) as described by Castellino and Hill (19). A pure protein was obtained as judged by disc gel electrophoresis (20) and amino acid composition (11). Partially purified galactosyltransferase was isolated from bovine milk according to Brodbeck and Ebner (3) and Brew et al. (6). It had a specific activity of 27 nmoles of galactose transferred per min per mg with glucose (and saturated with α-lactalbumin). l-1-Tosylamido-2-phenyl-ethyl chloromethyl ketone-treated trypsin was prepared by treating trypsin (lyophilized, twice crystallized, Lot TRL6295) with 2-thio-2-nitrophenyl)tryptophan; 8CM-αLA, α-lactalbumin derivative in which all 4 disulfide bonds were reduced and carboxymethylated; 4NPS-αLA, α-lactalbumin derivative in which all 4 tryptophan residues were sulfenylated.

1 The abbreviations used are: αLA, α-lactalbumin of bovine milk; NPS-Cl, 2-nitrophenylsulfonyl chloride (α-nitrobenzenene-sulfenyl chloride); NPS-Trp, 2-thio-2-nitrophenyl)tryptophan; 8CM-αLA, α-lactalbumin derivative in which all 4 disulfide bonds were reduced and carboxymethylated; 4NPS-αLA, α-lactalbumin derivative in which all 4 tryptophan residues were sulfenylated.
with L-1-tosylamido-2-phenylethyl chloromethyl ketone (both purchased from Worthington Biochemicals Corp.) according to Carpenter (21). Pyruvate kinase type 1 from rabbit skeletal muscle (obtained from Sigma Chemical Co.) contained nucleoside-diphosphokinase and lactate dehydrogenase necessary for the spectrophotometric assay of UDP. Hen egg white lysozyme (three times recrystallized, diazoylated, and lyophilized, Lot 10918010) was purchased from Sigma Chemical Co. Rabbit antiserum to α-lactalbumin was a gift of Dr. E. Maron. Iodinated 125I-α-lactalbumin (1.2 × 10⁵ cpm per μmole) and NPS-αLA (9 × 10⁵ cpm per μmole) were prepared according to Hunter and Greenwood (22).

Reagents

UDP-galactose, ATP, and Tris were products of Sigma Chemical Co. Dithioerythritol was a product of Cyclo Chemicals. NPS-Cl was obtained from Eastman Organic Chemicals. NADH was purchased from P-L Biochemicals, Inc. D-Glucose and analytical grade urea were products of BDH Ltd. Urea was crystallized from 95% ethanol; only freshly prepared solutions were used. Phosphoenolpyruvate was obtained from Boehringer GmbH.

Methods

Analytical Procedures

Spectrophotometric Measurements—These measurements were performed with a Cary 14 and a Beckman Acta V spectrophotometer. Quartz cells with 1-cm light path were used.

Amino Acid Analyses—Analyses were performed on a Beckman-Spinco model 120C automatic amino acid analyzer after hydrolysis in 6 N HCl for 22 hours (23, 24). Analyses for tryptophan were performed spectrophotometrically according to Spies and Chambers (25). The content of tryptophan in the presence of NPS-tryptophan was determined from its absorption at 280 nm after subtraction of the absorption of NPS-Trp at that particular wavelength (E₂₈₀ = 15,000, in acetic acid, Scoffone et al. (17, 26)).

Acrylamide Disc Electrophoresis—Disc electrophoresis was performed on a Shandon apparatus, using 7% gels in Tris-glycine buffer (pH 8.9). Protein separations were at 5 mA per gel at a running pH of 9.5 for 30 min, according to Davis (20).

High Voltage Paper Electrophoresis—Paper electrophoresis and descending paper chromatography were performed according to Katz et al. (27). The electrophoretic separations at pH 6.5 were performed on Whatman No. 3MM filter paper, for 60 min at 3000 volts.

Experimental Procedures

Sulfenylation of α-Lactalbumin and Lysozyme—To a solution of the protein in 0.1 M acetic acid (2 mg per ml), solid NPS-Cl was added (0.5 mg per ml) in the dark, at room temperature, with constant stirring. After varying reaction times, excess reagent was centrifuged down and the modified protein was purified by gel filtration on a Sephadex G-25 column (2 × 80 cm) using 0.1 M acetic acid as eluent. Fractions containing the protein were pooled and lyophilized. 4NPS-αLA was prepared according to the procedure of Scoffone et al. (11): to a solution of 1 μmole αLA in 2 ml of 30% acetic acid, 40 eq of NPS-Cl dissolved in 1 ml of glacial acetic acid were added at room temperature with constant stirring. After 1 hour the modified protein was precipitated with acetone-1 M HCl (39:1) at 0°. The precipitate was separated by centrifugation and was washed several times with acetone and ether. The final product was dried in vacuo over P₂O₅.

Determination of Extent of Sulfenylation of α-Lactalbumin—Concentrations of the covalently bound chromophore (NPS) were determined spectrophotometrically at 305 nm using E₃₀₅ = 4,000 mole⁻¹ cm⁻¹ in 80% acetic acid according to Scoffone et al. (17). Protein concentrations were determined by amino acid analysis assuming 12 residues of lysine and 1 residue of arginine (11). αLA concentrations were determined spectrophotometrically at 280 nm, using E₂₈₀ = 29,100 mole⁻¹ cm⁻¹.

Characterization of Sulfenylated Lysozyme Derivatives—Determination of extent of sulfenylated lysozyme, purification of the sulfenylated derivative, and identification of the sulfenylated residues were performed according to Shechter et al. (28).

Purification of Sulfenylated α-Lactalbumin—A 15-mg protein sample was separated on a DEAE-cellulose column (1 × 15 cm) equilibrated with 0.05 M Tris-hydrochloride buffer (pH 8.3). The elution was performed with a linear gradient of NaCl (0.05 to 0.5 M) in the same buffer, at a rate of 1 ml per min; fractions of 5 ml were collected. The protein fractions were pooled, diazoylated against 0.05 M ammonium bicarbonate, and rechromatographed on a Sephadex G-25 column using 0.05 M ammonium bicarbonate as eluent.

Reduction and Alkylation of Disulfide Bonds of α-Lactalbumin Derivatives—The proteins were reduced with dithioerythritol and alkylated with 2-iodoacetic acid as described by Shechter et al. (28). The reduced and alkylated protein derivatives were purified by gel filtration on Sephadex G-25 using 0.05 M ammonium bicarbonate as eluent. Fractions containing the protein were pooled and lyophilized.

Trypsin Digestion of 8CM-αLA Derivatives—Reduced and alkylated derivatives of αLA (5 mg per ml) were dissolved in 0.03 M Tris-hydrochloride buffer (pH 8.3). To this solution, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (1 mg per ml, in water) was added in three portions up to a final concentration of 2% (w/w of αLA). The digestion was performed at 37° with continuous shaking for 4 hours. The extent of digestion was determined by subjecting a portion of the trypsic digest to a further digestion with carboxypeptidase B. The released COOH-terminal lysine and arginine residues were determined by amino acid analysis.

Assay Procedure—Activity of purified α-lactalbumin and its derivatives in promoting lactose synthesis in the presence of galactosyltransferase (A-protein) and glucose was assayed according to the method of Brodbeck and Ebner (3). By this method, UDP formation in the lactose synthesis reaction was followed spectrophotometrically according to the method of Davidson (29). A standard assay mixture contained in a final volume of 2 ml: Tris-hydrochloride buffer (pH 7.5), 100 μmole; phosphoenolpyruvate, 2 μmole; ATP, 0.2 μmole; MnCl₂, 8 μmole; NADH, 0.4 μmole; UDP-α-D-glucose, 0.4 μmole; α-glucose, 150 μmole; pyruvate kinase (a crude preparation, containing lactate dehydrogenase), 1 mg; and various amounts of galactosyltransferase and α-lactalbumin as required. Assays were performed at 25° and were corrected for endogenous activity by placing in the reference cuvette all reagents except substrate. The rate of conversion of NADH to NAD⁺ was followed spectrophotometrically at 340 nm. Activities of native and modified α-lactalbumin were compared under assay conditions in which linear response could be obtained with respect to the concentration of the protein (20 to 80 μg per assay mixture).

Immunological Procedure—To a fixed amount of iodinated...
Fig. 1. Course of sulfenylation of α-lactalbumin by NPS-Cl in 0.1 M acetic acid (pH 2.9). Sulfenylation was performed at a protein concentration of 2 mg per ml with solid NPS-Cl (0.5 mg per ml). A second portion of NPS-Cl (0.5 mg per ml) was added after 4 hours as indicated by the arrow. NPS-Trp was determined spectrophotometrically according to Scoffone et al. (17).

Supplementary data are presented as a miniprint supplement to the JBC Document number, the form desired, microfiche or full size photocopies, these same data are available as JBC Document No. 73M-839. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC Document number, the form desired, microfiche or full size photocopy of 6 pages and the number of copies desired. Orders should be accompanied by remittance to the order of the Journal in the amount of $2.50 for microfiche or for photocopy.
17 to 58 in α-lactalbumin, was isolated; its amino acid composition is presented in Table II. The second yellow peptide of Peak I (Fig. 3B), which contained 23% of the optical density of this peak at 365 nm, was isolated by high voltage paper electrophoresis at pH 6.5. The amino acid composition of this peptide (Peptide 3BII) indicated that it corresponded to residues 99 to 114 in α-lactalbumin (Table II) and resulted from incomplete tryptic cleavage at lysine 108.

Characterization of Tryptic Peptides Containing NPS-Trp in NPS1-αLA

Fig. 3C illustrates the separation of 1.6 μmoles of tryptic peptides derived from reduced and carboxymethylated NPS1-αLA (Peak b in Fig. 2B). This modified protein contained 1.0 residue of NPS-Trp per α-lactalbumin molecule. Six discrete peaks were observed (Fig. 3C). The two NPS-Trp-containing peaks (Peaks II and III, Fig. 3C) were eluted at the same elution volumes as were Peptides 3BI and 3BIII (Fig. 3B); their NPS-Trp-content is presented in Table I. The other 4 tryptophan-containing peptides were eluted at the same elution volumes as were Peptides A1, AII, AIII, and AIV of Fig. 3A. Identification of the sulfenylated tryptophan residues of NPS1-αLA was performed in the following way. Fractions corresponding to Peaks II and III (Fig. 3C) were pooled, concentrated, and subjected to high voltage paper electrophoresis at pH 6.5 (Fig. 5). As expected, Peak III contained a single peptide, Peptide 3CIII, which was yellow (Fig. 5B). The amino acid composition of this peptide corresponded to residues 59 to 62 in α-lactalbumin. Peptide 3CI, the only yellow peptide of Peak II (Fig. 3C), was eluted from the electrophoretogram and its amino acid composition corresponded to residues 115 to 122 in α-lactalbumin. The amino acid composition of these peptides is presented in Table II. From these results it seemed that in NPS1-αLA 2 tryptophan residues, residues 60 and 118, were partially but equally sulfenylated to the extent of about 0.5 NPS-Trp. In order to confirm this finding, the other 4 unsulfenylated tryptophan-containing areas of Fig. 3C, Fractions 20 to 40 and 53 to 72, were combined, lyophilized, and resulfenylated with NPS-Cl in 80% acetic acid. The solvent was removed by rotary evaporation and lyophilization, and the resulfenylated peptide mixture was fractionated on a Sephadex G-25 column. The separation pattern of this mixture is illustrated in Fig. 3D; NPS-Trp content of each fraction is presented in Table I. As can be seen from this figure, 4 NPS-Trp-containing peaks again were formed. From the NPS-Trp content of each peak (Table I), one could conclude that the ratio of the resulfenylated tryptophan residues Trp60-Trp61-Trp118-Trp119 was about 1:0.5:1:0.5, respectively, thus proving that in NPS1-αLA, 0.5 residue of each of tryptophans 60 and 118 had been sulfenylated.

Sulfenylation of Hen Egg White Lysozyme

Lysozyme was sulfenylated with NPS-Cl in 0.1 M acetic for 3 hours. Only one sulfenylated derivative, a monosulfenylated one (referred to as 1NPS-lysozyme) was formed. 1NPS-lysozyme was purified by ion exchange chromatography on a Bio-Rex 70 column and was then reduced, carboxymethylated, and digested by trypsin. The tryptic peptides were separated by gel filtration on Sephadex G-25 and the NPS-Trp-containing peptide was isolated. Amino acid and sequence analyses of this peptide indicated that in 1NPS-lysozyme, tryptophan 62 was the only sulfenylated residue (for detailed procedures see Shechter et al. (28)).

Properties of NPS1-αLA

Activity—The activity of NPS1-αLA to promote lactose synthesis in the presence of galactosyltransferase (A-protein) and glucose was measured and compared with that of the native protein. NPS1-αLA had less than 5% of the activity of native α-lactalbumin.

Electrophoretic Mobility—NPS1-αLA migrated on polyacrylamide gels (at pH 8.9) as a single band with a mobility different from that of the native protein (Fig. 6).

Immunological Properties of NPS1-αLA—The precipitin curve of the reaction of iodinated 125I-NPS1-αLA with rabbit antiserum to α-lactalbumin is presented in Fig. 7. For comparison, the
DISCUSSION

_d-_-_lactalbumin was sulfenylated with NPS-Cl in 0.1 M acetic acid (pH 2.9) and a monosulfenylated derivative (NPS-rLA) was isolated in 42% yield. NPS-rlLA contained 1.0 NPS-tryptophan residue per a-lactalbumin molecule and had almost no biological activity. NPS-rlLA yielded a single protein band on disc gel electrophoresis and was eluted as a single monomeric peak from Sephadex G-25 and G-100 columns as well as from DEAE-cellulose columns. However, fragmentation of NPS-rlLA followed by isolation and identification of the sulfenylated NPS-tryptophan residues indicated that NPS-rlLA was composed of a 1:1 mixture of two monosulfenylated a-lactalbumin derivatives, namely NPS-Trp,-rlLA and NPS-Trp,-rlLA. Both derivatives were inactive in the lactose synthesis reaction, indicating that blocking either of these tryptophan residues prevented the proper interaction of -lactalbumin with galactosyl transferase in the presence of glucose.

Bradshaw and DemMLEAU (30), found that the charge transfer probe N-methyl nicotinamide interacts very weakly with a-lactalbumin to form one charge-transfer complex. These authors proposed that tryptophan 60 was responsible for this interaction. Robbins and Holmes (15) showed that, although at pH 6 about 1 tryptophan residue of a-lactalbumin forms such a charge-transfer complex, 2 tryptophan residues form a complex at pH 2. Circular dichroism measurements, as well as absorption spectra and fluorescent studies by Robbins and Holmes (32), all indicate that changes in the pH result in changes in the environment of certain tryptophan residues of a-lactalbumin. Solvent perturbation studies by Robbins and Holmes (15) indicated that an average of 2 tryptophan residues in a-lactalbumin are exposed and it was suggested that these residues are tryptophans 104 and 118. Although no definite information is available concerning the actual three-dimensional structure of a-lactalbumin, the molecular model proposed by Browne et al. (33), which is based on the striking similarity between the amino acid sequences of bovine a-lactalbumin and hen egg white lysozyme, could be useful in considering the environment of the 4 tryptophan residues of this protein. Nevertheless, one should bear in mind that some chemical modification studies suggest a more expanded structure for a-lactalbumin. Recent low angle x-ray scattering data for lysozyme and bovine a-lactalbumin suggest dissimilar shapes in solution (34), while Pessen et al. (35) concluded from the same measurements that the shapes of these two proteins are the same. The description of the model is taken from Browne et al. (33) and Kronman et al. (36), and from studies of the model of lysozyme in the laboratory of Dr. N. Sharon in the Biophysics Department. Of the 4 tryptophan residues of a-lactalbumin, tryptophan 26 can be regarded as being completely buried in a hydrophobic surrounding. Of the other 3 residues, tryptophan 118 appears to be the most exposed, with little hindrance from other amino acid side chains. The other 2 tryptophan residues, 60 and 104, are less exposed and lie in a cleft-like region of the molecule, homologous with the active site cleft of hen egg white lysozyme. Chemical modifications of a-lactalbumin with 2-hydroxy-5-nitrobenzyl bromide by Barman and co-workers (16, 30, 37) at pH 6 and 2.7 showed that tryptophan residues 26, 104, and 118 were modified, whereas tryptophan 60 remained intact. The same kind of modification with hen egg white lysozyme (a homologous protein) resulted in the modification of tryptophan residue 62 or 63, homologous to tryptophan 60 in a-lactalbumin (37). These authors concluded, therefore, that the conformations of bovine a-lactalbumin and hen egg white lysozyme are different (30).

Castellino and Hill (quoted in Kronman et al. (36)) showed that treatment of a-lactalbumin with N-bromosuccinimide resulted in oxidation of tryptophans 60 and 118 with a concomitant loss of the specifier protein activity. With lysozyme, the same modification caused oxidation of tryptophan 62 and the enzymatic activity was lost. With NPS-Cl, we showed that in lysozyme tryptophan 62 was the only sulfenylated residue (28), while in a-lactalbumin both tryptophans 60 and 118 were mod-
ified. These findings suggest that tryptophan 62 in lysozyme and its homologous tryptophan residue in α-lactalbumin, tryptophan 60, are available for sulfenylation. In α-lactalbumin another tryptophan, residue 118, is also available for modification with NPS-Cl, while the homologous tryptophan residue in lysozyme is unavailable for this modification.

In the kind of modification that we have employed, a 2-nitrophenylthioether group was attached to C-2 of the indole ring of the modified tryptophan residue. The same carbon atom is oxidized with N-bromosuccinimide and, indeed, similar results were obtained with NPS-Cl and N-bromosuccinimide modifications of lysozyme and α-lactalbumin.

A sulfenylated tryptophan residue in a protein molecule has some unique properties, such as the bulkiness of the NPS residue, the hydrophobic nature of the thiophenylether ring, and the electron-withdrawing properties of the nitrophenyl group. Considering all these factors we assume that the inability of NPS-Trp,-aLA and NPS-Trpns-αLA to participate in the lactose synthesis reaction could be due to modification of an essential tryptophan residue, minor conformational changes which led to weakening of the binding site of α-lactalbumin for galactosyltransferase, or physical blocking of these sites. The fact that the antigenic sites of native α-lactalbumin and NPS-αLA are almost identical indicates that no gross conformational changes have been caused by this modification.

Characterization of other sulfenylated α-lactalbumin derivatives which were formed during this modification reaction should enable us to learn more about the environment of the tryptophan residues of α-lactalbumin and their role in the biological activity of this protein.

Acknowledgments—We thank Dr. N. Sharon for access to the three-dimensional molecular model of hen egg white lysozyme and Dr. E. Maron for his cooperation in the performance of the immunological tests.

REFERENCES
Selective Sulfenylation of Tryptophan Residues in \( \alpha \)-Lactalbumin of Bovine Milk
Yoram Shechter, Abraham Patchornik and Yigal Burstein

*J. Biol. Chem.* 1974, 249:413-419.

Access the most updated version of this article at [http://www.jbc.org/content/249/2/413](http://www.jbc.org/content/249/2/413)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/249/2/413.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/249/2/413.full.html#ref-list-1](http://www.jbc.org/content/249/2/413.full.html#ref-list-1)