Selective Sulfenylation of Tryptophan Residues in \( \alpha \)-Lactalbumin of Bovine Milk*

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SUMMARY

Tryptophan residues of bovine \( \alpha \)-lactalbumin, one of the two protein components of lactose synthetase, were selectively sulfenylated with 2-nitrophenylsulfenyl chloride. When the reaction was performed in 0.1 M acetic acid for 1 hour, a modified protein containing 1.0 nitrophenylsulfenyl tryptophan residue per \( \alpha \)-lactalbumin molecule was formed and isolated in 42% yield. Upon reduction, carboxymethylation, and trypsin digestion, this sulfenylated \( \alpha \)-lactalbumin (NPS-\( \alpha \)-LA) yielded two yellow peptides. Amino acid composition of these peptides indicated that in NPS-\( \alpha \)-LA, tryptophan residues 60 and 118 were equally sulfenylated to the extent of 0.5 nitrophenylsulfenyl tryptophan per \( \alpha \)-lactalbumin. NPS-\( \alpha \)-LA cross-reacts with anti-\( \alpha \)-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 \( \alpha \)-lactalbumin.

Materials and Methods

Materials

Bovine \( \alpha \)-lactalbumin was isolated from raw skimmed milk by a modification of the method of Aschaffenburg 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 Brodbek 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 \( \alpha \)-lactalbumin) and an activity of 27 nmoles of galactose transferred per min per mg with glucose. This enzyme catalyzes the final step in the biosynthesis of lactose (1, 2): UDP-galactose + glucose \( \rightarrow \) lactose + UDP. This enzymatic reaction requires two proteins for significant activity, \( \alpha \)-lactalbumin (3-5) and galactosyltransferase (6). Galactosyltransferase alone will synthesize lactose only at high glucose concentrations (7, 8) but in the presence of \( \alpha \)-lactalbumin the apparent \( K_m \) for glucose is much lower and biosynthesis of lactose is much more effective. Denton and Ebner (14) have shown that upon iodination or nitration of \( \alpha \)-lactalbumin, tyrosine, 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 tyrosine residues. Tyrosinase oxidized 1 tryptophan residue in \( \alpha \)-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 \( \alpha \)-lactalbumin forms a complex with N-methylacetaminamide at neutral pH, 2 tryptophan residues form such complexes at pH 2, due to partial acid denaturation.

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

Materials and Methods

Materials

Proteins

Bovine \( \alpha \)-lactalbumin was isolated from raw skimmed milk by a modification of the method of Aschaffenburg 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 Brodbek 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 \( \alpha \)-lactalbumin). 1-Tosylamido-2-phenyl-ethyl chloromethyl ketone-treated trypsin was prepared by treating trypsin (lyophilized, twice crystallized, Lot TRL6295) with 2-thio-\( \beta \)-nitrophenyltryptophan residues 60 and 118 were equally sulfenylated to the extent of 0.5 nitrophenylsulfenyl tryptophan per \( \alpha \)-lactalbumin molecule.
Several proteins were precipitated with acetone-l-

Several enzymes were purified by gel filtration on a Sephadex G-25 column (2 × 15 cm) using 0.05 M ammonium bicarbonate. The reduced and alkylated proteins 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 SCM-aLA Derivatives—Reduced and alkylated derivatives of aLA (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 tryp-

topf were pooled and lyophilized. 4NPS(aLA was prepared

Several proteins 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. 4NPS-aLA was prepared according to the procedure of Scoffone et al. (17): to a solution of 1 μmole aLA 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 a-Lactalbumin—Concentrations of the covalently bound chromophore (NPS) were determined spectrophotometrically at 365 nm using $E_{280} = 4,000$ 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). aLA concentrations were determined spectrophotometrically at 280 nm, using $E_{280} = 29,100$ mole$^{-1}$ cm$^{-1}$.

Characterization of Sulfenylated Lysozyme Derivatives—Deter-

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 Beck-

Experimental Procedures Sulfenylation of a-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-aLA was prepared according to the procedure of Scoffone et al. (11): to a solution of 1 μmole aLA 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.

Reduction and Alkylation of Disulfide Bonds of a-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.

Trp assay Procedure—Activity of purified a-lactalbumin and its derivatives in promoting lactose synthesis in the presence of galactosyltransferase (A-protein) and glucose was assayed according to the method of Brobeck 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-Galactose, 0.4 μmole; α-glucose, 150 μmole; pyruvate kinase (a crude preparation, containing lactate dehydrogenase), 1 mg; and various amounts of galactosyltransferase and a-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 a-lactalbumin were compared under assay conditions in which linear responses could be obtained with respect to the concentration of the protein (20 to 80 μg per assay mix-

Immunological Procedure—To a fixed amount of iodinated

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. α-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 Beck-

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

α-Lactalbumin was sulfenylated with NPS-Cl in 0.1 M acetic acid at room temperature in the dark as described under “Methods.” The course of the sulfenylation reaction is presented in Fig. 1. As can be seen from this figure, sulfenylation reached a maximal value after 2 to 3 hours and leveled off when approximately 1.2 tryptophans were modified. A somewhat higher extent of sulfenylation (up to 1.5 NPS-Trp residues per α-lactalbumin) could be obtained by successive addition of NPS-Cl to the reaction mixture. In order to obtain a more homogenous product, we chose to stop the reaction after 1 hour by centrifuging excess reagent and purifying the modified protein by gel filtration on a Sephadex column. The protein fraction was pooled, lyophilized, and separated on a DEAE-cellulose column. Fig. 2 illustrates a typical separation of such a preparation which contained (Peaks a, a1, b, and b1 in B). The fractions corresponding to each protein peak were pooled, dialyzed against 0.05 M phosphate buffer-0.9% NaCl solution (pH 7.2). The precipitate was centrifuged, washed extensively, and its radioactive content was determined with a Packard model 3003 liquid scintillation spectrometer.

RESULTS

Sulfenylation of Tryptophan Residues in α-Lactalbumin

α-Lactalbumin was sulfenylated with NPS-Cl in 0.1 M acetic acid at room temperature in the dark as described under “Methods.” The course of the sulfenylation reaction is presented in Fig. 1. As can be seen from this figure, sulfenylation reached a maximal value after 2 to 3 hours and leveled off when approximately 1.2 tryptophans were modified. A somewhat higher extent of sulfenylation (up to 1.5 NPS-Trp residues per α-lactalbumin) could be obtained by successive addition of NPS-Cl to the reaction mixture. In order to obtain a more homogenous product, we chose to stop the reaction after 1 hour by centrifuging excess reagent and purifying the modified protein by gel filtration on a Sephadex column. The protein fraction was pooled, lyophilized, and separated on a DEAE-cellulose column. Fig. 2 illustrates a typical separation of such a preparation which contained 0.9 NPS-Trp residue per α-lactalbumin, and had about 60% biological activity in the lactose synthesis reaction. As can be seen from this figure, four distinct protein peaks were obtained (Peaks a, a1, b, and b1 in B). The fractions corresponding to each protein peak were pooled, dialyzed against 0.05 M ammonium bicarbonate, lyophilized, and purified by gel filtration.

Characterization of Tryptic Peptides Containing NPS-Trp in α-Lactalbumin

Amino acid analysis of the major protein fractions showed that sulfenylated α-lactalbumin eluted in Peak a (Fig. 2B) contained 0.8 NPS-Trp residue per α-lactalbumin molecule. This modified α-lactalbumin derivative had about 90% of the original biological activity of the protein in the lactose synthesis reaction. The protein which was eluted in Peak b (Fig. 2B) contained 1.0 NPS-Trp per α-lactalbumin molecule and had less than 5% of the original biological activity of the native protein. All further studies described in this report were performed with this derivative, which will be denoted as NPS1-αLA.

Tryptic Digestion of Sulfenylated α-Lactalbumin Derivatives

α-Lactalbumin was reduced with dithioerythritol in urea (pH 8.6) and the released sulphydryl groups were then carboxymethylated with 2-iodoacetic acid. NPS1-αLA and 4NPS-αLA were subjected to the same treatment. The reduced and carboxymethylated α-lactalbumin derivatives were digested with trypsin at pH 8.3 as described under “Methods.” The tryptic peptides of reduced and carboxymethylated α-lactalbumin, 4NPS-αLA and NPS1-αLA, were separated by gel filtration as illustrated in Fig. 3.

Characterization of Tryptic Peptides Containing NPS-Trp in 4NPS-αLA

Fig. 3B illustrates the separation of the tryptic peptides derived from 1.6 amoles of reduced and carboxymethylated 4NPS-αLA. Four yellow peaks could be identified, and will be referred to as I, II, III, and IV. The content of NPS-Trp in each fraction (as determined by its absorption at 365 nm, according to Scoffone et al. (17)) is presented in Table I. Peptides 3BII and 3BIV (Peaks III and IV in Fig. 3B) emerged pure from the column, as judged by paper chromatography and high voltage paper electrophoresis. On the basis of their amino acid compositions, which are listed in Table II, it was concluded that Peptide 3BII corresponded to residues 59 to 62, and Peptide 3BIV corresponded to residues 99 to 108 in α-lactalbumin. Peak II (Fig. 3B) contained several peptides, of which only one peptide was yellow. This yellow peptide was further purified by high voltage paper electrophoresis at pH 6.5. The amino acid composition of this peptide (Peptide 3BII) indicated that it corresponded to residues 115 to 122 in α-lactalbumin. The peptide mixture of Peak I (Fig. 3B) was further separated on a Sephadex G-50 column, and the Peptide 3BIA, corresponding to residues 3 Supplementary data are presented as a miniprint supplement at the end of this paper. Material published in miniprint form can be easily read with the aid of a large-field reading glass of a type readily available at most opticians. For the convenience of those who prefer to obtain supplementary material in the form of a 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 addressed to The Journal of Biological Chemistry, 9630 Rockville Pike, Bethesda, Maryland 20014 and must 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 \( \alpha \)-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 \( \alpha \)-lactalbumin (Table II) and resulted from incomplete tryptic cleavage at lysine 108.

Characterization of Tryptic Peptides Containing NPS-Trp in NPS\(_{1}\)-\( \alpha \)LA

Fig. 3C illustrates the separation of 1.6 \( \mu \)moles of tryptic peptides derived from reduced and carboxymethylated NPS\(_{1}\)-\( \alpha \)LA (Peak b in Fig. 2B). This modified protein contained 1.0 residue of NPS-Trp per \( \alpha \)-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 3BII 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 AI, AII, AIII, and AIV of Fig. 3A. Identification of the sulfenylated tryptophan residues of NPS\(_{1}\)-\( \alpha \)LA 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 \( \alpha \)-lactalbumin. Peptide 3CII, 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 \( \alpha \)-lactalbumin. The amino acid composition of these peptides is presented in Table II. From these results it seemed that in NPS\(_{1}\)-\( \alpha \)LA 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 Trp\(_{60}\) Trp\(_{60}\) Trp\(_{118}\) Trp\(_{118}\) was about 1:0.5:1:0.5, respectively, thus proving that in NPS\(_{1}\)-\( \alpha \)LA, 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 acid 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 NPS\(_{1}\)-\( \alpha \)LA

**Activity**—The activity of NPS\(_{1}\)-\( \alpha \)LA to promote lactose synthesis in the presence of galactosyltransferase (A-protein) and glucose was measured and compared with that of the native protein. NPS\(_{1}\)-\( \alpha \)LA had less than 5% of the activity of native \( \alpha \)-lactalbumin.

**Electrophoretic Mobility**—NPS\(_{1}\)-\( \alpha \)LA 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 NPS\(_{1}\)-\( \alpha \)LA**—The precipitin curve of the reaction of iodinated \( ^{125}I \)-NPS\(_{1}\)-\( \alpha \)LA with rabbit antiserum to \( \alpha \)-lactalbumin is presented in Fig. 7. For comparison, the
**FIG. 6 (left).** Disc gel electrophoresis of $\alpha$-lactalbumin derivatives. Left, native $\alpha$-lactalbumin; center, NPS-$\alpha$LA; right, a mixture of both. The electrophoresis was performed in 7% gels, pH 8.9, toward the anode.

**FIG. 7 (right).** Immunoassay of NPS-$\alpha$LA. To a solution of precipitin curve of native $\alpha$-lactalbumin is included. The curves are superimposable.

**DISCUSSION**

$\alpha$-Lactalbumin was sulfenylated with NPS-Cl in 0.1 M acetic acid (pH 2.9) and a monosulfenylated derivative (NPS-$\alpha$LA) was isolated in 42% yield. NPS-$\alpha$LA contained 1.0 NPS-tryptophan residue per $\alpha$-lactalbumin molecule and had almost no biological activity. NPS-$\alpha$LA 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-$\alpha$LA followed by isolation and identification of the sulfenylated NPS-tryptophan residues indicated that NPS-$\alpha$LA was composed of a 1:1 mixture of two monosulfenylated $\alpha$-lactalbumin derivatives, namely NPS-Trp$_6$-$\alpha$LA and NPS-Trp$_{118}$-$\alpha$LA. Both derivatives were inactive in the lactose synthesis reaction, indicating that blocking either of these tryptophan residues prevented the proper interaction of $\alpha$-lactalbumin with galactosyl transferase in the presence of glucose. Bradshaw and Demelieu (30), found that the charge transfer probe N-methylnicotinamide interacts very weakly with $\alpha$-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 $\alpha$-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. Circular dichroism measurements by Robbins and Holmes (32) indicated that changes in the pH result in changes in the environment of certain tryptophan residues of $\alpha$-lactalbumin. Solvent perturbation studies by Robbins and Holmes (15) indicated that an average of 2 tryptophan residues in $\alpha$-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 $\alpha$-lactalbumin, the molecular model proposed by Browne et al. (33), which is based on the striking similarity between the amino acid sequences of bovine $\alpha$-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 $\alpha$-lactalbumin. Recent low angle x-ray scattering data for lysozyme and bovine $\alpha$-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 $\alpha$-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 $\alpha$-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 60, 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 $\alpha$-lactalbumin (37). These authors concluded, therefore, that the conformations of bovine $\alpha$-lactalbumin and hen egg white lysozyme are different (30).

Castellino and Hill (quoted in Kronman et al. (36)) showed that treatment of $\alpha$-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 $\alpha$-lactalbumin both tryptophans 60 and 118 were mod-

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28 µl of iodinated ($^{125}$I) NPS-$\alpha$LA (2.5 nmoles; 2.3 X $10^8$ cpm) (○) or $\alpha$LA (2.5 nmoles; 3 X $10^8$ cpm) (●), increasing amounts of rabbit antiserum to $\alpha$-lactalbumin were added. The volume of the incubation mixture was 0.6 ml. The ordinate represents per cent of antigen in complex with anti-$\alpha$-lactalbumin antibodies.

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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-nitrophénylthioether 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 αLA and NPS-TrpαLA 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 NPSαLA 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.

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