Sulfenylation of Tryptophan-62 in Hen Egg-White Lysozyme*

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ABSTRACT: A single tryptophan residue in hen egg-white lysozyme was selectively sulfenylated by 2-nitrophenylsulfenyl chloride in aqueous solution at pH 3.5. The monosulfenylated lysozyme derivative (1·NPS-lysozyme) was purified by gel filtration and by ion-exchange chromatography. Upon reduction, carboxymethylation, and trypsin digestion, 1·NPS-lysozyme yielded only one yellow peptide. Amino acid composition and sequence analysis of this peptide showed that tryptophan-62 was the only sulfenylated residue in 1·NPS-lysozyme. 1·NPS-lysozyme has the same electrophoretic mobility as that of the native enzyme, and cross-reacts with antilysozyme antibodies, but unlike the native enzyme it has no lytic activity on dead cells of *Micrococcus lysodeikticus*, and two of its peptide bonds are susceptible to digestion by trypsin.

Hen egg-white lysozyme contains six tryptophan residues located at sequence positions 28, 62, 63, 108, 111, and 123 (Canfield, 1963b; Jollès *et al.*, 1963). X-Ray studies of lysozyme and lysozyme–inhibitor complexes have indicated that three out of the six tryptophan residues (namely residues 62, 63, and 108) are located in the region of the binding site of saccharides to the enzyme (Phillips, 1966, 1967). This fact is further demonstrated by the spectral perturbation caused to these residues upon interaction of the enzyme with substrates and inhibitors. These perturbations are reflected by changes in the ultraviolet (uv) spectra (Hayashi *et al.*, 1964; Dahlquist *et al.*, 1966), circular dichroism (CD) (Glazer and Simmons, 1966, Teichberg *et al.*, 1970), and fluorescence emission spectra (Shinitzki *et al.*, 1966; Lehrer and Fasman, 1966, 1967; Teichberg and Sharon, 1970) of the tryptophan residues in lysozyme and certain lysozyme derivatives.

Chemical modifications of the tryptophan residues in hen egg-white lysozyme have indicated that one or more of these residues are associated with the lytic activity of this enzyme. Hayashi and coworkers (1965) have demonstrated that *N*-bromosuccinimide oxidizes tryptophan-62 to oxindolealanine (at pH 4.5) with concomitant decrease of the enzymatic activity of the enzyme. Iodine at pH 5.5 effects the selective oxidation of a different tryptophan residue (No. 108) to oxindolealanine and produces a completely inactive enzyme (Hartdegen and Rupley, 1967). Changes in the X-ray diffraction pattern of the lysozyme crystals, iodinated at pH 4.7 with 5 moles of iodine/mole of enzyme, show that both tryptophan-62 and tryptophan-108 have undergone oxidation whereas reaction with 1 mole of iodine per mole of enzyme leads to the single oxidation of tryptophan-108 (Blake, 1967). In contrast, treatment of an anhydrous formic acid solution of lysozyme with ozone led to the conversion of both tryptophan-108 and tryptophan-111 to *N*-formylkynurenine residues without loss of enzymic activity (Previero *et al.*, 1967). Photooxidation of lysozyme in the presence of methylene blue led possibly to the degradation of tryptophan-28, and most of the lytic activity of the enzyme was lost (Kravchenko and Lapuk, 1970). Alkylation of lysozyme with 2-hydroxy-5-nitrobenzyl bromide at pH 2.7 brought the modification of tryptophan-62 or tryptophan-63 as was shown by Barman (1970). Recently, Scoffone *et al.* (1968) have introduced the reagent 2-nitrobenzenesulfenyl chloride (NPS-Cl[1]) for the selective modification of tryptophan residues in proteins. The 2-thio(2-nitrophenyl)tryptophan thus obtained absorbs light at 365 nm with a molar extinction coefficient of 4000 in aqueous acetic acid.

In this paper we report the specific sulfenylation of tryptophan-62 in hen egg-white lysozyme by 2-nitrophenylsulfenyl chloride. This modified protein (1·NPS-lysozyme) has almost completely lost its lytic activity toward dead cells of *Micrococcus lysodeikticus*, some of its peptide bonds have become susceptible to trypsin, but its original electrophoretic mobility and its immunological properties remain unchanged.

Materials

Hen egg-white lysozyme (three-times crystallized, dialyzed, and lyophilized, lot no. 109B-8010) and Tris, reagent grade (lot. no. 14B-5300), were purchased from Sigma (St. Louis, Mo.). L-(1-Tosylamido-2-phenyl)ether chloromethyl ketone trypsin was prepared by treating trypsin (lyophilized, twice crystallized, lot no. TRL 6295) with L-(1-tosylamido-2-phenyl)ether chloromethyl ketone (both purchased from Worthington Biochemical Corp., Freehold, N. J.) according to Carpenter (1967). NPS-Cl was obtained from Eastman Organic Chemicals (Rochester, N. Y.). Dithioerythritol (lot no. K-5531) was a product of Cyclo Chemical Corp. (Los Angeles, Calif.). Analytical reagent grade urea, obtained from BDH Ltd. (Poole, England), was recrystallized from 95% ethanol; solutions of this compound were prepared immediately before use. PTH-tryptophan was a product of Mann Research Laboratory (New York, N. Y.). All other chemicals were of analytical grade.

PTH-NPS-tryptophan was synthetized by reacting PTH-tryptophan with an equimolar amount of NPS-Cl in glacial

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[1] Abbreviations used are: NPS-Cl, 2-nitrophenylsulfenyl chloride (*o*-nitrobenzenesulfenyl chloride); 1·NPS-lysozyme, lysozyme derivative in which Trp-62 was sulfenylated; 6·NPS-lysozyme, lysozyme derivative in which all six tryptophan residues were sulfenylated; 8CM-lysozyme, lysozyme derivative in which all four disulfide bonds were reduced and carboxymethylated.

acetic acid, at room temperature. After 6 hr, water was added and the precipitate was collected and used without further purification. This yellow compound migrated as a single spot when analyzed by thin-layer chromatography on silica gel plates in several solvent systems according to Jeppson and Sjöquist (1967).

Methods

Spectrophotometric measurements were made with a Cary 14 spectrophotometer. Quartz cells with 1-cm light path were used.

pH-Stat titrations were carried out in an all-glass thermostated reaction vessel under nitrogen, using a Radiometer type TTT11b automatic titrator and a PHM 26 type pH-meter in combination with an ABU12 autoburette and SBR2c titrigraph recorder. The electrodes were the G2222B glass electrode and the K4112 calomel electrode (Radiometer, Copenhagen).

Amino acid analyses were performed on the Beckman-Spinco Model 120C automatic amino acid analyzer after hydrolysis in constant-boiling HCl for 22 hr at 110° (Spackman *et al.*, 1958, Spackman, 1963). Analyses of tryptophan residues were performed spectrophotocolorimetrically according to Spies and Chambers (1949). 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-tryptophan at that particular wavelength (ϵ_{280} 15,000 in acetic acid, Scoffone *et al.*, 1966, 1968).

Acrylamide disc electrophoresis was performed on a Shandon apparatus, using 15% gels, pH 4.3 for 3 hr, according to Reisfeld *et al.* (1962).

High-voltage paper electrophoresis and descending paper chromatography were carried out according to Katz *et al.* (1959). The electrophoretic separations at pH 3.5 and at pH 6.5 were performed on Whatman No. 3MM filter paper, for 60 min at 3000 V unless otherwise stated.

Trypsin Digestion. Method A. Lysozyme derivatives (2.5 mg) were dissolved in 1 ml of 0.05 M NaCl adjusted to pH 8.0 with 10^{-2} M NaOH. To this solution 0.01 ml of L-(1-tosylamido-2-phenyl)ether chloromethyl ketone trypsin (3 mg/ml; in 0.05 M NaCl adjusted to pH 8.0) was added. The digestion was performed at 30° with continuous stirring under nitrogen. The reaction was followed by means of the pH-Stat as described above.

Method B. The procedure given is a slight modification of that of Canfield (1963a). Lysozyme derivatives (10 mg/ml) were dissolved in 0.1 M ammonium bicarbonate and the solution was brought to pH 8.5 by a few drops of concentrated NH_4OH. To this solution L-(1-tosylamido-2-phenyl)ether chloromethyl ketone trypsin (1 mg/ml; in water) was added in three portions up to a final concentration of 0.1 mg/ml. The digestion was performed at 37° with continuous shaking for 4 hr. Extent of digestion was determined by subjecting a portion of the tryptic digest to a further digest by carboxypeptidase B. The released C-terminal lysine and arginine residues were determined by the automatic amino acid analyzer.

Lysozyme lytic activity on dead cells of *Micrococcus lysodeikticus* was assayed according to Shugar (1952).

Dialysis was performed with preheated (80°, 3 days) Visking seamless cellulose tubing (Union Carbide, Inc.).

Immunological Assay. The immunological properties of 1·NPS-lysozyme were studied by measuring its capacity to inhibit the immunospecific inactivation of lysozyme–bacteriophage conjugate with antilysozyme antibodies according to Haimovich *et al.* (1970a,b) and Maron *et al.* (1970). According to this technique, goat antilysozyme (hen egg-white) antibodies (2×10^{-5} μg sample) were kept for 24 hr at 4° with different concentrations (10^{-8}–10^{-3} μg/ml) of native lysozyme or 1·NPS-lysozyme. The mixture was further incubated with lysozyme-coated bacteriophage T_4 (about 500 plaque-forming units/sample) for 2 hr, thus allowing the remaining free antibodies to inactivate the modified bacteriophage. The extent of inactivation was calculated by plating the incubation mixture and scoring for number of survivors. An antibody concentration of 1.9×10^5 μg/sample was required to inactivate 80% of the phage in the absence of inhibitor.

Sulfenylation of Lysozyme. To a solution of lysozyme (0.5 μmole/ml) in the appropriate buffer, excess solid NPS-Cl was added (usually 40 equiv) in the dark at room temperature, with constant stirring. After certain 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. The fractions containing the protein peak were pooled and lyophilized.

Determination of Extent of Sulfenylation of Lysozyme by NPS-Cl. Concentrations of the covalently bound chromophore (NPS) were determined spectrophotometrically at 365 nm using ϵ_{365} 4000 l. mole^{-1} cm^{-1} in 80% acetic acid according to Scoffone *et al.* (1968). Protein concentrations were determined by amino acid analysis of an acid hydrolysate of the protein solution, assuming 6 residues of lysine and 11 residues of arginine (Canfield, 1963a). Lysozyme concentrations were determined spectrophotometrically at 280 nm using ϵ_{280} 39,000 l. mole^{-1} cm^{-1} (Wetlaufer and Stahman, 1958; Fromageot and Schnek, 1950).

Purification of 1·NPS-lysozyme. A protein sample of 20 mg was separated on a Bio-Rex 70 column (200–400 mesh, 1.0×45 cm) equilibrated with 0.015 M borate buffer (pH 10). The elution was carried out with a linear gradient of NaCl (0.05–0.15 M) in the same buffer at a rate of 1 ml/min; fractions of 6 ml were collected. The protein peaks were pooled together, dialyzed against 0.1 M acetic acid, lyophilized, and rechromatographed on a Sephadex G-25 column.

Reduction and Alkylation of the Disulfide Bridges in Lysozyme Derivatives. The procedure was a slight modification of that of Sperling *et al.* (1969). Lysozyme derivative was dissolved in a solution of 1 M Tris-chloride buffer (pH 8.6)–8 M urea–10^{-3} M EDTA (previously deaerated by a stream of nitrogen), to a final concentration of 0.5 μmole/ml. To this solution dithioerythritol was added to a final concentration of 20 μmole/ml and the reduction was allowed to proceed for 30 min at room temperature, with constant stirring under nitrogen. Iodoacetic acid (50 μmole/ml, final concentration) was then added to the stirred reaction mixture, and after a further 20 min some dithioerythritol was added to destroy the excess of the alkylating agent, and the whole reaction mixture was dialyzed against 200 volumes of 0.2 M acetic acid. The protein precipitate was separated by centrifugation and washed three times with water, and the product was dried *in vacuo*.

Ion-Exchange Chromatography of a Tryptic Digest of Reduced and Carboxymethylated 1·NPS-lysozyme. The tryptic digest of 60 mg of reduced and carboxymethylated 1·NPS-lysozyme (in 5 ml of 0.2 M pyridine–acetate, pH 3.1) was loaded on a preparative Dowex 50-X4 column (0.9×60 cm), developed with pyridine–acetic acid buffer at a flow rate of 22.5 ml/hr. The effluent of the column was divided by a stream-splitting device with a ratio of 4:5. The effluent (10 ml/hr) passed through a Technicon peptide analyzer which was equipped with an additional colorimeter for measuring the

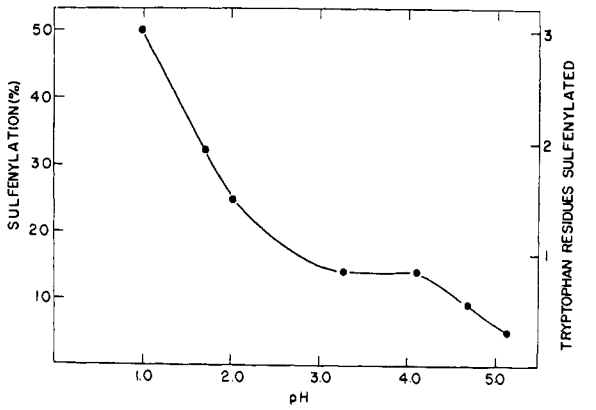

FIGURE 1: Extent of sulfenylation of tryptophan residues in hen egg-white lysozyme as a function of the pH. Sulfenylation was carried out at protein concentration of 0.5 μmole in 1 ml of 0.1 M buffered solutions (HCl–KCl at pH 1–2; sodium acetate at pH 3–5) with 20 μmoles of NPS-Cl for 5 hr.

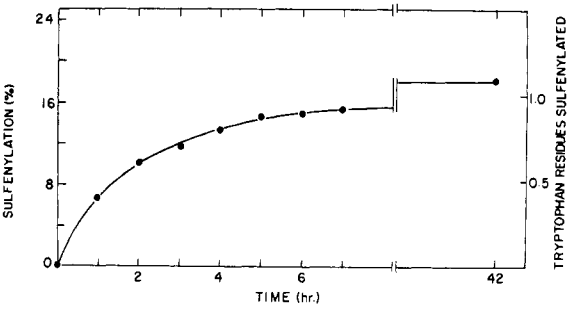

FIGURE 2: Course of sulfenylation of lysozyme by NPS-Cl in aqueous 0.1 M sodium acetate (pH 3.5). Sulfenylation was carried out at protein concentration of 0.5 μmole/ml with excess solid NPS-Cl (five portions of 10 μmoles/ml of solid NPS-Cl).

absorbancy at 365 nm. The remaining 12.5 ml/hr of the effluent was collected in 2.5-ml fractions (5 fractions/hr). The column was first eluted with 50 ml of 0.2 M pyridine–acetic acid buffer (pH 3.1), then with a linear gradient of pyridine–acetic acid which was composed of 250 ml of 0.2 M pyridine–acetic acid (pH 3.1) in the mixing chamber and 250 ml of 2 M pyridine–acetic acid (pH 6.5) as a limiting buffer. The elution was terminated with 50 ml of the limiting buffer.

Edman degradations of the peptides were carried out according to the procedure described by Konigsberg (1967). The peptide (1 μmole/ml) was dissolved in 50% pyridine (all solvents had been previously deaerated with nitrogen) and to it 0.1 ml of phenyl isothiocyanate in 50% pyridine was added. The carbamylation reaction was carried out at 45° for 60 min. The solvent was evaporated in a desiccator (previously heated *in vacuo* at 60° for 30 min) containing P_2O_5, H_2SO_4, and NaOH *in vacuo* and the residue was dissolved in 0.3 ml of trifluoroacetic acid. The cyclization reaction was performed at 45° for 30 min. The acid was evaporated and the residue dissolved in 0.6 ml of water and extracted several times with butyl acetate. The aqueous phase was evaporated, dried, and then redissolved in water for spectroscopic studies and amino acid analysis. The organic phase was concentrated and the PTH-amino-acids were analyzed by thin-layer chromatography on plates of silica gel, using heptane–1-butanol–75% formic acid (50:30:9, v/v) according to Jeppson and Sjöquist (1967).

Results

Sulfenylation of Tryptophan Residues in Lysozyme. The extent of sulfenylation of tryptophan residues in lysozyme by NPS-Cl in aqueous solutions was studied as a function of the pH. The reactions were carried out as described under methods for 5 hr.

It can be seen from Figure 1 that an average of approximately one tryptophan residue was sulfenylated when the reaction was carried out at a pH range of 2.5–4.0. When the pH was lower than 2, a higher extent of sulfenylation was observed, and a lower extent of sulfenylation was obtained at pH values higher than 4.0. Figure 2 represents the course of sulfenylation of lysozyme by NPS-Cl in aqueous solution at pH 3.5. Under these conditions sulfenylation reached its maximal value after about 4–5 hr and leveled off at approximately one modified tryptophan residue. Successive additions of solid NPS-Cl during the reaction did not increase the extent of sulfenylation of tryptophan residues in lysozyme. In the light of these experiments and in order to obtain a more homogeneous product, we chose to carry out the reaction in 0.1 M sodium acetate buffer (pH 3.5) with a 40 molar excess of the reagent, for 4 hr at room temperature. Excess of NPS-Cl was centrifuged down and the sulfenylation reaction mixture was applied on a Bio-Rex 70 column eluted with a NaCl gradient at pH 10. Figure 3 shows a typical purification of such a preparation which contained about 0.85 residue of NPS-tryptophan per lysozyme molecule. The first peak of the eluate (Figure 3) corresponded to the unreacted enzyme (as was demonstrated by a parallel control experiment); it had a typical spectrum of lysozyme with no absorbancy at 365 nm and had 100% lytic activity. The modified protein which was eluted in the second peak (Figure 3) absorbed light at 365 nm (as well as at 280 nm) and had less than 3% lytic activity on dead cells of *M. lysodeikticus*. The tubes corresponding to the second peak were pooled together, dialyzed against 0.1 M acetic acid, lyophilized, and rechromatographed on a Sephadex-G 25 column. Amino acid analysis

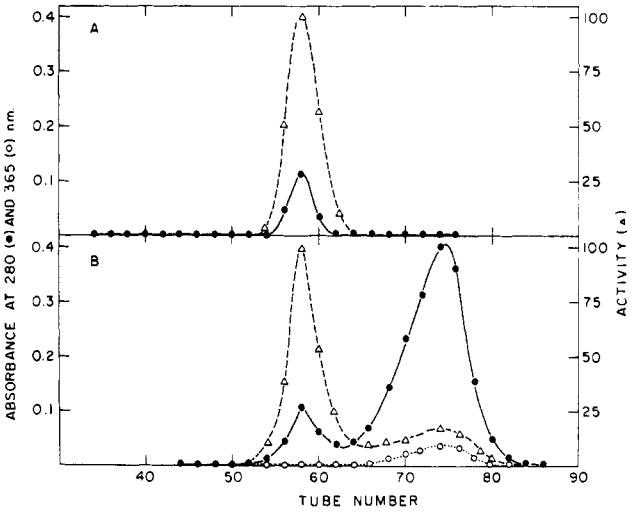

FIGURE 3: Ion-exchange chromatography of 1·NPS-lysozyme. Crude 1·NPS-lysozyme (20 mg) was applied on a Bio-Rex 70 column (1.0 × 45 cm). The elution was carried out with a linear gradient of NaCl (0.05–0.15 M) in 0.015 M sodium borate buffer (pH 10), at a rate of 1 ml/min, 6 ml/tube. The effluent fractions were assayed for their protein content (●), biological activity (△), and also absorbancy at 365 nm (○). (A) Native lysozyme; (B) crude 1·NPS-lysozyme.

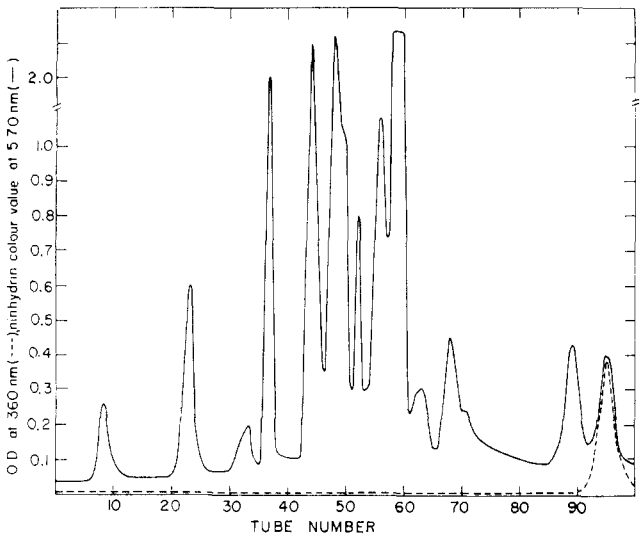

FIGURE 4: Ion-exchange chromatography of the tryptic digest of reduced and carboxymethylated 1·NPS-lysozyme. A tryptic digest of reduced and carboxymethylated 1·NPS-lysozyme (60 mg) was applied on a preparative Dowex 50-X4 column (0.9 × 60 cm) in a Technicon peptide analyzer, equipped with a stream divider splitter of a ratio of 4:5. The column was developed with 50 ml of 0.2 M pyridine acetate (pH 3.1), then with 500 ml of a linear gradient of pyridine acetate 0.2 M (pH 3.1) to 2 M (pH 6.5), and the elution was terminated with 50 ml of 2 M pyridine acetate (pH 6.5). The flow rate was 22.5 ml/hr and fractions of 2.5 ml (5 fractions/hr) were collected. Ninhydrin color value (—) and absorbance at 360 nm (---) were recorded.

TABLE I: Amino Acid Composition of 1·NPS-lysozyme.

Amino Acid	Lysozyme		1·NPS-lysozyme[a,b]
	Calcd[c]	Found[a,d]	
Aspartic acid	21	21.3	21.2
Threonine	7	6.8	7.1
Serine	10	9.1	9.1
Glutamic acid	5	4.9	4.7
Proline	2	2.0	1.8
Glycine	12	12.5	12.6
Alanine	12	*12.0*	*12.0*
Half-cystine	8	6.8	7.1
Valine	6	5.9	6.0
Methionine	2	1.9	2.1
Isoleucine	6	5.7	5.9
Leucine	8	7.7	7.5
Tyrosine	3	2.9	2.7
Phenylalanine	3	3.0	3.0
Lysine	6	5.9	6.1
Histidine	1	0.9	0.9
Arginine	11	11.0	10.7
Tryptophan	6	6.2	4.9
NPS-tryptophan[e]			1.0

[a] Not corrected for losses during acid hydrolysis. [b] Peak b in Figure 3. [c] According to Canfield (1963a) and Jolles *et al.* (1963). [d] Peak a in Figure 3. [e] Determined spectrophotometrically (Scoffone *et al.*, 1968).

of this modified protein showed that it contained only 1 NPS-tryptophan residue per molecule of modified lysozyme, as can be seen in Table I. This modified lysozyme was denoted by 1·NPS-lysozyme.

Identification of the Sulfenylated Tryptophan Residue in 1·NPS-lysozyme. 1·NPS-lysozyme was reduced by dithioerythritol in urea (pH 8.6) and the released sulfhydryl groups were then carboxymethylated by iodoacetic acid. The reduced and carboxymethylated protein was subjected to quantitative tryptic digest at pH 8.5 as described under Methods (method B). The whole digest was then loaded onto a Dowex 50 column and eluted with a linear gradient of pyridine acetate as described under Methods. Figure 4 illustrates the separation pattern of the tryptic digest of reduced and carboxymethylated 1·NPS-lysozyme.

As can be seen from this figure, only one peptide peak showed light absorption at 365 nm. This peptide, which had been isolated in 72% yield (based on its arginine content), proved to be a homogeneous one upon high-voltage paper electrophoresis at pH 3.5 and 6.5. The amino acid composition analysis of this peptide is presented in Table II (column T-9*). As this peptide proved to be highly aromatic, another way of purifying it was tried. The whole tryptic digest was loaded onto a Sephadex G-25 column and eluted with 0.2 M acetic acid. As can be seen from Figure 5, the mixture of the peptides was separated into two main fractions. The first fraction (peak a, Figure 5) had no absorption at 365 nm, while the second fraction (peak b, Figure 5) absorbed light at 365 nm. The tubes corresponding to each peak were pooled together and lyophilized. Each fraction, as well as the crude tryptic digest was subjected to high-voltage paper electrophoresis at pH 6.5. The results of this separation are presented in Figure 6.

As can be seen from this figure only one yellow spot could be detected in the crude tryptic digest of reduced and carboxymethylated 1·NPS-lysozyme (A in Figure 6). This yellow spot was the only missing spot in the leading fraction of the eluate of the gel filtration column (peak a, Figure 5), all other peptides were present (B in Figure 6). The retarded fraction of the gel filtration column (peak b, Figure 5) contained the missing yellow spot (C in Figure 6). This peptide was recovered in 84% yield (based on its arginine content) and was denoted T-9′ (corresponding to the nomenclature of Canfield (1963a). Peptide T-9′ proved to be a homogeneous one,

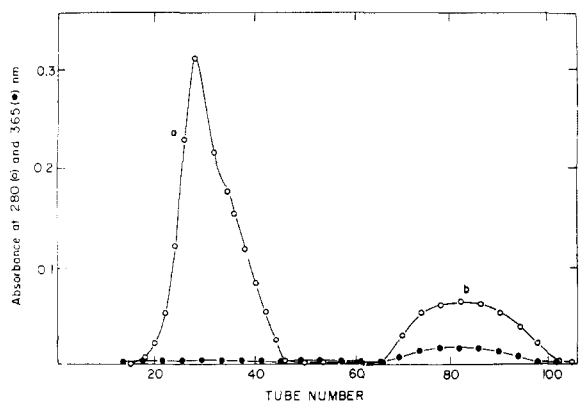

FIGURE 5: Gel filtration of the tryptic digest of reduced and carboxymethylated 1·NPS-lysozyme. A tryptic digest of 20 mg of reduced and carboxymethylated 1·NPS-lysozyme was applied on a Sephadex G-25 column (coarse, 1.0 × 130 cm) and eluted with 0.1 M acetic acid at a rate of 60 ml/hr. Fractions of 4 ml were collected. Each fraction was tested for its absorbancy of 280 (○) and 365 (●) nm.

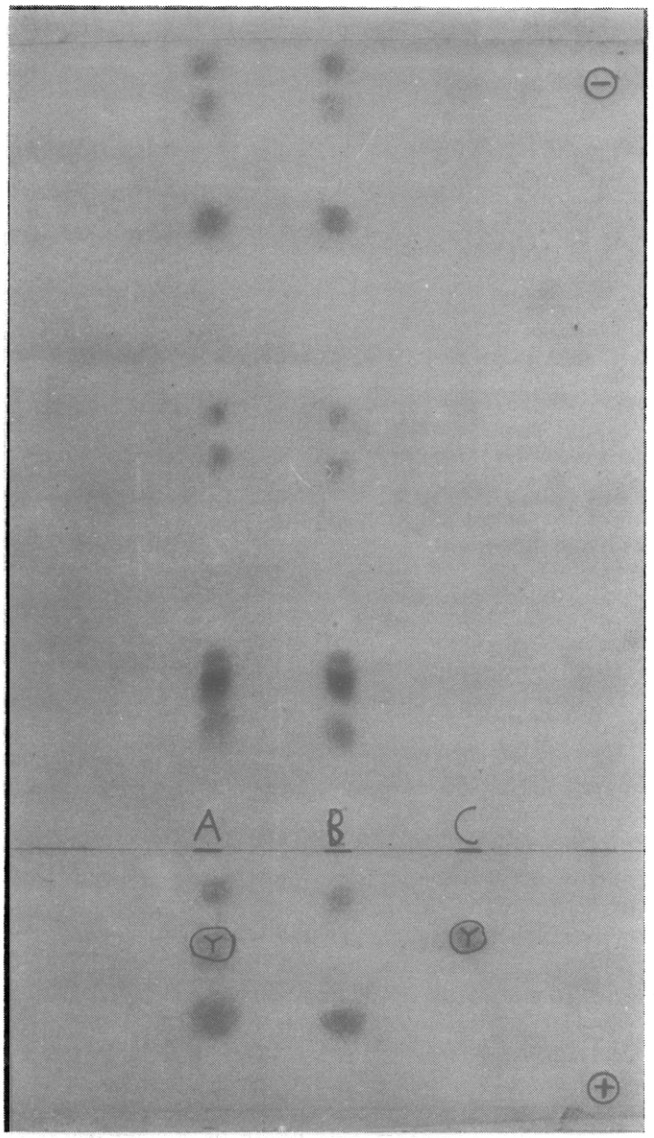

FIGURE 6: Electrophoretic pattern of the tryptic digest of 1·NPS-lysozyme. The digest was fractionated at pH 6.5, 40 V/cm, for 60 min. (A) The tryptic digest, (B) peak a in Figure 5, (C) peak b in Figure 5. The yellow spots were first marked (⊙) and then the electrophorogram was developed with ninhydrin–Cd reagent.

as was demonstrated by high-voltage paper electrophoresis at pH 3.5 and by descending paper chromatography in butanol–acetic acid–water, where only one yellow and ninhydrin-positive spot could be detected.

This peptide was subjected to acid hydrolysis and amino acid analysis, its tryptophan and NPS-tryptophan were determined spectrophotometrically as described under Methods. As can be seen from Table II (column T-9′), the amino acid composition of this peptide and of peptide T-9* corresponded to residues 62–68 in the hen egg-white lysozyme molecule.

Edman Degradations of Peptide T-9′. Peptide T-9′ contained two tryptophan residues in positions 1 and 2 at the N terminus of the peptide (corresponding to residues 62 and 63 in the lysozyme molecule). In order to determine the position of the modified tryptophan residue, two steps of Edman degradation were employed. The first step was carried out using 0.5 μmole of peptide T-9′. After extraction of the PTH derivative with butyl acetate, the remaining peptide E-1 was recovered in 76% (0.38 μmole), calculated on the basis of its

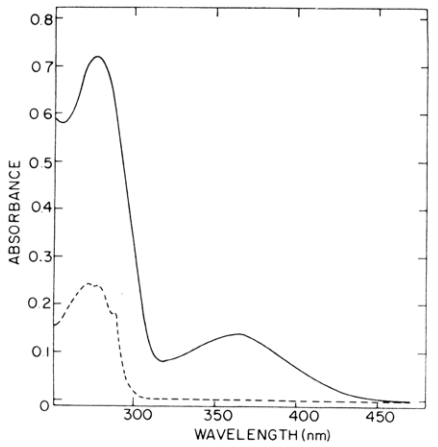

FIGURE 7: Ultraviolet absorption spectrum of peptide T-9′ (—) and peptide E-1 (---). Measurements were performed in 80% acetic acid (see text).

absorbancy at 280 nm and its arginine content. Peptide E-1 (0.23 μmole) was then subjected to a second step of Edman degradation. The peptide E-2, obtained in the second step of the Edman degradation, showed no absorption in the ultraviolet region (above 240 nm). The recovery of this peptide (E-2) was 68% (0.15 μmole) as calculated on the basis of its arginine content.

Figure 7 illustrates the absorption spectra of peptide T-9′ before and after the Edman degradation. The peptide corresponding to residues 62–68 in 1·NPS-lysozyme contained the characteristic absorption band at 365 nm owing to the presence of one NPS-tryptophan residue. After one step of the Edman degradation, namely residues 63–68 in the modified protein (peptide E-1), this peptide clearly showed the absorption spectrum of tryptophan, and had no absorption in the visible region (Figure 7). Its amino acid composition is given in Table II. The peptide E-2, obtained after the second step of the Edman degradation, showed no absorption spectrum in the ultraviolet region, and its amino acid composition is also shown in Table II.

TABLE II: Amino Acid Composition of Peptide T-9′ and Its Degradation Products.

	Peptide					
	T-9[a]					
Amino Acid	Calcd	Found	T-9*[b]	T-9′[c]	E-1[d]	E-2[e]
Tryptophan	2	1.90	0.95	1.00	0.95	0.0
NPS-tryptophan[f]			1.0	1.05	0.0	0.0
CM-cysteine	1	0.79	0.8	0.86	0.79	0.73
Aspartic acid	2	*2.00*	*2.00*	*2.00*	*2.00*	*2.00*
Glycine	1	1.05	1.10	1.00	1.05	1.02
Arginine	1	0.95	1.03	0.95	0.90	1.00

[a] Residues 62–68 in native lysozyme according to Canfield (1963a). [b] The "yellow peak" from the Dowex 50 column. [c] The yellow peak b from the Sephadex column. [d] Peptide T-9′ after one step of Edman degradation. [e] Peptide T-9′ after two steps of Edman degradation. [f] Determined spectrophotometrically (Scoffone *et al.*, 1968).

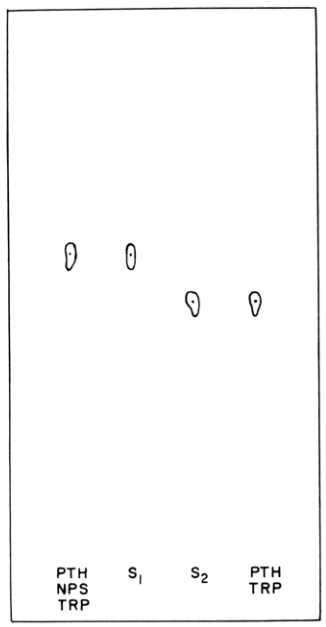

FIGURE 8: Thin-layer chromatography of the PTH-amino acids after Edman degradation. S_1: PTH-amino acid derived from peptide T-9′; S_2: PTH-amino acid derived from peptide El (see text). The separation was performed on silica gel plates using heptane–1-butanol–75% formic acid (50:30:9, v/v) as eluent.

The PTH-amino acids, obtained in these degradation reactions were analyzed by thin-layer chromatography on silica gel plates according to Jeppson and Sjöquist (1967). As shown in Figure 8, the PTH derivative of the first step had an R_F value corresponding to PTH-NPS-tryptophan, while the PTH derivative of the second step had an R_F value of PTH-tryptophan. All these results clearly indicate that Trp-62 was the only sulfenylated residue in 1·NPS-lysozyme.

Properties of 1·NPS-lysozyme. ULTRAVIOLET ABSORPTION SPECTRUM. Figure 9 illustrates the absorption spectrum of 1·NPS-lysozyme. For comparison, the absorption spectrum of the native protein is also presented. As can be seen, this modified protein showed the expected absorption spectrum, with maxima at 365 and 280 nm and with the molar extinction coefficients ϵ_{365} 4000; ϵ_{280} 49,500.

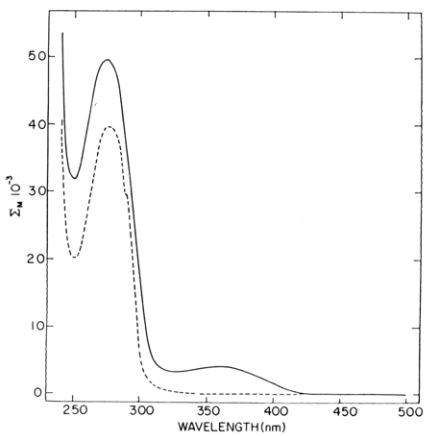

FIGURE 9: Ultraviolet absorption spectrum of 1·NPS-lysozyme (—) and native lysozyme (---). Measurements were performed in water at pH 7.0.

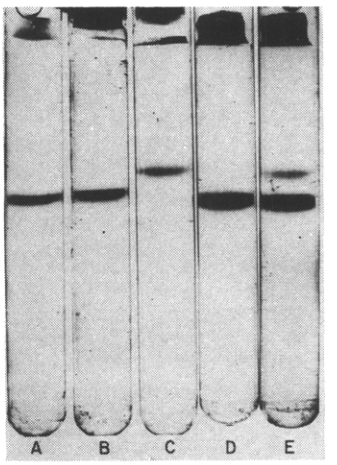

FIGURE 10: Acrylamide gel electrophoresis of sulfenylated derivatives of lysozyme. (A) native lysozyme, (B) 1·NPS-lysozyme, (C) 6·NPS-lysozyme, (D) 1·NPS-lysozyme + native lysozyme, (E) 1·NPS-lysozyme + 6·NPS-lysozyme + native lysozyme. The electrophoresis was performed in 15% gels, pH 4.3 toward the cathode.

ELECTROPHORETIC MOBILITY. 1·NPS-lysozyme migrated on acrylamide gel electrophoresis (pH 4.3) as a single band with a mobility similar to that of native lysozyme. For comparison the mobility of 6·NPS-lysozyme is also presented (Figure 10).

THE ENZYMATIC ACTIVITY of 1·NPS-lysozyme toward dead cells of *M. lysodeikticus* was measured and compared with that of the native enzyme. 1·NPS-lysozyme had less than 3% of the lytic activity of that of native lysozyme.

DIGESTION BY TRYPSIN. The susceptibility of 1·NPS-lysozyme to tryptic digest was measured with a pH-Stat at pH 8.0. Figure 11 shows the uptake of alkali as a function of time at pH 8.0 upon the digestion of 1·NPS-lysozyme, 6·NPS-lysozyme, 8CM-lysozyme, and native lysozyme by trypsin. The same titrations carried out in the absence of trypsin showed very low uptake of alkali, and the results presented in Figure 11 were corrected for this background uptake. Evidently, native lysozyme is hardly attacked by trypsin. 1·NPS-lysozyme was digested with an initial rate of about 10% of that of 8CM-lysozyme, and 6·NPS-lysozyme was digested with an initial rate of about 30% of that of 8CM-

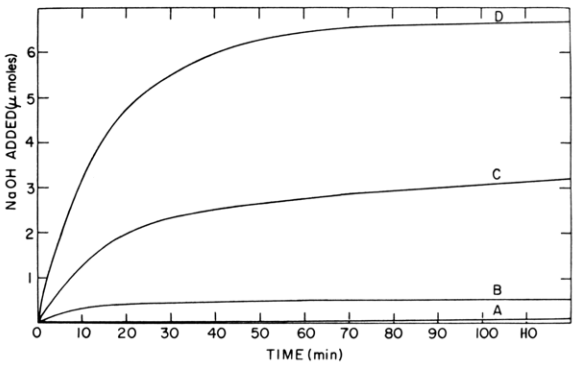

FIGURE 11: Digestion of native lysozyme (A), 1·NPS-lysozyme (B), 6·NPS-lysozyme (C), 8CM-lysozyme, and (D) by trypsin. The reaction was followed by recording the uptake of sodium hydroxide with time at pH 8.0 and 30°. The reaction mixture contained 2.5 mg of protein and 0.03 mg of trypsin in 1 ml of 0.05 M sodium chloride solution.

lysozyme. The digestion of 1·NPS-lysozyme tended to level off very quickly at an extent of proteolysis about 10% of that of 8CM-lysozyme, and about 20% of the proteolysis of 6·NPS-lysozyme (after about 2 hr).

IMMUNOLOGICAL PROPERTIES OF 1·NPS-LYSOZYME. Concentration of lysozyme which brought about 50% inhibition of the inactivation of lysozyme–bacteriophage conjugate with antilysozyme antibodies was 2.1×10^{-6} mg/ml in a specific immunological assay (see Methods). Under the same assay conditions, a concentration of 3.7×10^{-6} mg/ml of 1·NPS-lysozyme was needed in order to obtain the same extent of inhibition. The small differences in the inhibiting effect of 1·NPS-lysozyme and the native enzyme indicate that gross conformation of the native enzyme was retained in 1·NPS-lysozyme.

Discussion

The reagent 2-nitrophenylsulfenyl chloride was found to be most suitable for the sulfenylation of the indole nucleus of tryptophan residues in proteins, because of its high specificity and its relatively high stability in aqueous systems (Scoffone *et al.*, 1968). When we employed this reagent for the modification of lysozyme in aqueous buffered solutions, we found that at pH 3–4 about one (out of six) tryptophan residue was sulfenylated. At lower pH values, and especially at pH values lower than 2.0, the extent of sulfenylation was higher and less specific. This could be due to the exposure of other tryptophan residues as a consequence of conformational changes in lysozyme, caused by the destruction of hydrogen bonds at such acidic pH values (as shown by Ogasahara and Hamaguchi, 1967). Studies on the course of the sulfenylation reaction of lysozyme by NPS-Cl at pH 3.5 indicate that owing to the very low solubility of solid NPS-Cl in water, only a limited concentration of the reagent was achieved. The low concentration of NPS-Cl thus achieved rendered the reaction slow but very specific. Higher concentrations of NPS-Cl could be achieved by the dropwise addition of a solution of excess NPS-Cl in anhydrous dioxane or dimethylformamide to the reaction mixture. Under these conditions somewhat higher rates of reaction were obtained, but the reaction turned out to be a less specific one.

The monosulfenylated lysozyme derivative, 1·NPS-lysozyme, was purified by ion-exchange chromatography and was found to be a homogeneous product. The tryptic digest of 1·NPS-lysozyme contained only *one* yellow peptide. This yellow peptide was purified by different methods, such as ion-exchange chromatography, gel filtration, and high-voltage paper electrophoresis, and was isolated in relatively high yields (72–84%). This heptapeptide was identified as peptide 62–68 in the sequence of the lysozyme molecule. Edman degradations of this peptide clearly indicated that tryptophan-62 was the only sulfenylated residue in 1·NPS-lysozyme.

As for the biological properties of 1·NPS-lysozyme, it was found that this derivative had almost no lytic activity on dead cells of *M. lysodeikticus*. Its immunological properties were very similar to those of the native enzyme, thus indicating that the gross conformation of the native enzyme molecule was retained. Nevertheless, the susceptibility of two peptide bonds in 1·NPS-lysozyme to tryptic digest suggested that some conformational changes do occur.

Tryptophan-62 in hen egg-white lysozyme was found to be very sensitive toward chemical modifications. This residue could be selectively modified by *N*-bromosuccinimide (Hayashi *et al.*, 1965), by iodine (Blake, 1967), and by 2-hydroxy-5-nitrobenzyl bromide (Barman, 1970). All these modifications inactivated the enzyme, thus indicating the importance of this residue for its biological activity. X-Ray studies of crystals of lysozyme and lysozyme–inhibitor complexes (Phillips 1966, 1967) indicated that tryptophan-62 takes part in the binding of the saccharide moiety at subsite C in the cleft of the active site of the enzyme. Upon formation of the enzyme–substrate complex, the "left" side of the cleft moves in such a way as to narrow the cleft, with the indole side chain of tryptophan-62 moving by some 0.75 Å. Careful binding studies of saccharides to lysozyme have also indicated that subsite C contributes the lowest free energy of association of a saccharide to lysozyme (Chipman and Sharon, 1969). From all these data it is quite obvious that a modification of tryptophan-62 in lysozyme, in a way that prevents it from binding the substrate, inactivates the enzyme.

In the kind of modification that we have employed, a 2-nitrophenyl thioether group was attached to C-2 of the indole ring of tryptophan-62 in lysozyme. This kind of modification has some unique properties such as the bulkiness of the NPS residue, the hydrophobic nature of the thiophenyl ether ring, the electron-withdrawing properties of the nitrophenyl group, and the very high specificity of this modification toward tryptophan residues in proteins.

Considering all these factors, we assume that the inability of 1·NPS-lysozyme to hydrolyze its natural substrate could be due to blocking of its binding site, or to weakening or abolishing of some of the hydrogen bonds or the hydrophobic forces between the enzyme and its substrates. The limiting proteolysis of this derivative and the very great similarity of its immunological properties to those of the native enzyme indicate that only very small conformational changes had occurred upon sulfenylation of tryptophan-62 in lysozyme. The nature of these changes and the possibility of specifically modifying another tryptophan residue in lysozyme are being studied.

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