Sulfenyl Halides as Modifying Reagents for Polypeptides and Proteins. II. Modification of Cysteinyl Residues*

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ABSTRACT: Sulfenyl halides react selectively in acidic solvents with cysteine and tryptophan residues in polypeptides and proteins. Tryptophan is converted into a derivative with a thioether function at the 2 position of the indole nucleus. Cysteine is converted into an unsymmetrical disulfide. The halides which were studied included 2-nitro- (NPS-Cl), 4-nitro- (pNPS-Cl), and 2,4-dinitrophenylsulfenyl chloride (DNPS-Cl). By reduction of the mixed disulfides with β-mercaptoethanol, thioglycolic acid, or sodium borohydride, the original thiol group is easily restored. The unsymmetrical disulfides are stable under acidic conditions. In alkaline solution, however, they are decomposed, releasing the nitrothiophenol moiety quantitatively. The latter can be assayed by spectrophotometry, thus affording a direct measure of the free cysteine in a protein. The mildness of this reversible reaction with cysteine residues was checked with reduced ribonuclease (RNase). After reaction of the reduced enzyme with 2-nitro- or 4-nitrophenylsulfenyl chloride in 50% acetic acid, the NPS-RNase and pNPS-RNase were isolated and subjected to further reduction with β-mercaptoethanol in 8 M urea. After purification on Sephadex G-25 and air oxidation, the recovered enzyme retained 70–80% of its activity toward RNA.

In the preceding paper it was shown that sulfenyl halides are selective and mild reagents for tryptophan and cysteine (Scoffone *et al.*, 1968). The present paper describes the results of the reaction of sulfenyl halides with cysteine in model compounds and in proteins.

Sulfenyl halides react very rapidly and stoichiometrically with the thiol group of cysteine, leading to an unsymmetrical disulfide. The reaction, which occurs readily in acidic solvents (aqueous acetic acid), was applied to cysteine derivatives and to reduced glutathione, and the corresponding disulfides were isolated in good yield. This modification of cysteinyl residues is easily reversed by employing the reducing reagents (*e.g.*, β-mercaptoethanol, thioglycolic acid, and sodium borohydride) extensively used in protein chemistry for the reduction of disulfide bonds.

The suitability of sulfenyl halides in a reversible modification of SH groups was tested by treating the reduced ribonuclease (Anfinsen and Haber, 1961) with 2-nitro- (NPS-Cl)[1] and 4-nitrophenylsulfenyl chloride (pNPS-Cl). The NPS protein or pNPS protein was then further reduced with β-mercaptoethanol in 8 M urea. After purification on Sephadex G-25, air oxidation produces the enzyme without significant loss of activity, proving the mildness of the reaction conditions.

It was found that in acid solution the S–S bond of the mixed disulfides is stable, but it is easily cleaved in alkaline media, with the quantitative release of the nitrothiophenol moiety, allowing the determination of the free SH groups present in polypeptides and proteins.

Materials

NPS-Cl (Hubacher, 1943) and DNPS-Cl (Kharasch *et al.*, 1950) were obtained from Fluka AG, Basle (Switzerland), and were recrystallized from anhydrous ethyl ether (mp 75–76°) and chloroform–ethyl ether (mp 94–95°), respectively; pNPS-Cl prepared according to the literature (Zincke and Lenhardt, 1913) was recrystallized from anhydrous petroleum ether (mp 50–52°). L-Cysteine, L-cysteine methyl ester hydrochloride, *N*-acetyl-L-cysteine, reduced glutathione, β-mercaptoethanol, and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Fluka. Analytical reagent grade urea, obtained from Fluka, was recrystallized from 95% ethanol, and solutions of this compound were prepared immediately prior to use.

Bovine pancreatic ribonuclease (Worthington Biochemical Corp., recrystallized twice) contained, as shown by chromatography on IRC-50 according to Hirs *et al.* (1953), 90% of RNase A, the remainder representing RNase B and aggregates of RNase A. This product, which contained approximately 10% water, was used without further purification for the preparation of the NPS and pNPS derivatives, and for controls in all assays of enzymic activity and will be referred to as "native RNase." Concentrations of enzyme solutions were determined by ultraviolet absorption at 277.5 mμ using the extinction coefficient $E_{1\text{ cm}}^{0.1\%}$ 0.695 (Bernfield, 1965).

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[1] Abbreviations used: NPS, 2-nitrophenylsulfenyl; pNPS, 4-nitrophenylsulfenyl; DNPS, 2,4-dinitrophenylsulfenyl; DMF, dimethylformamide; NPS-RNase and pNPS-RNase, derivatives of reduced ribonuclease treated with 2-nitro- or 4-nitrophenylsulfenyl chloride. The amino acids, peptides, and peptides derivatives are of L configuration. The abbreviations are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature, *Biochemistry 5*, 1445 (1966).

Yeast ribonucleic acid in the form of the sodium salt was purchased from BDH Laboratory Chemical Division and further purified by exhaustive dialysis against 0.1 M NaCl and water, followed by lyophilization (Wellner *et al.*, 1963). Sephadex G-25, fine beads, was obtained from Pharmacia, Uppsala (Sweden).

Methods

Instrumentation. The instrumentation employed was described in the preceding paper (Scoffone *et al.*, 1968).

Reaction of Cysteine and Its Derivatives with NPS-Cl, pNPS-Cl, and DNPS-Cl (Table I). PROCEDURE A. To a solution of 50 mmoles of the cysteine derivatives in 15 ml of glacial acetic acid or 99% formic acid, 52 mmoles of NPS-Cl was added with stirring at room temperature. The mixture was shaken for 1 hr, filtered, and then concentrated *in vacuo* and crystallized by addition of ethyl ether. The precipitate was collected, washed several times with water, and dried *in vacuo* (10^{-2} torr) at 50° over P_2O_5.

The derivatives of *N*-acetyl-L-cysteine were dissolved in water with an equivalent amount of $NaHCO_3$ and precipitated again by acidification (pH 2) with 1 N HCl. The products were filtered, washed with water and ethyl ether, and dried.

The derivatives of cysteine methyl ester were redissolved in water and reprecipitated by adjusting the pH to 8–9 with 5% Na_2CO_3 solution. The *S*-NPS-cysteine methyl ester precipitates as an oil, and is extracted with ethyl ether. After drying over sodium sulfate, gaseous HCl was bubbled through the ethereal solution at 0° and the product consisting of pale yellow needles was collected and dried.

PROCEDURE B. To a solution of 0.495 g (16 mmoles) of reduced glutathione in 15 ml of 99% formic acid, NPS-Cl, pNPS-Cl, or DNPS-Cl (17 mmoles) was added at room temperature with stirring. The mixture was shaken for 1 hr and then filtered and poured into ethyl ether. The yellow precipitate was collected, washed with ethyl ether, and dried *in vacuo* over P_2O_5. The product was dissolved in 20 ml of water; spontaneous precipitation occurred. The precipitate was filtered, washed with water and ethyl ether, and dried *in vacuo* (10^{-2} torr) at 50° over P_2O_5.

Regeneration of Reduced Glutathione from Its S-NPS, S-pNPS, and S-DNPS Derivatives. To a solution of 20 μmoles of *S*-NPS-glutathione (or *S*-pNPS- and *S*-DNPS-glutathione) in 2 ml of 2% $NaHCO_3$, 0.1 ml of β-mercaptoethanol (or thioglykolic acid) was added. After 1 hr at 22–24° the reaction mixture was analyzed by thin-layer chromatography, using *n*-butyl alcohol–water–glacial acetic acid (3:1:1) as eluent. Reduced glutathione (R_F 0.18) was the only one ninhydrin-positive compound. The same results were obtained by reduction of the mixed disulfide with sodium borohydride (5 equiv) or with tin and 0.1 N HCl.

Relative Reactivity of Tryptophan and Cysteine toward NPS-Cl. To a solution of 1.02 g (0.5 mmole) of L-tryptophan and 0.62 g (0.5 mmole) of L-cysteine in 25 ml of 99% formic acid, 0.95 g (0.5 mmole) of NPS-Cl was added with vigorous stirring. The mixture was shaken at room temperature for 3 hr, and then filtered and poured into ethyl ether. The yellow precipitate was filtered, washed with ethyl ether, and air dried. Then the product was washed several times with water in order to remove the unreacted amino acids and dried *in vacuo* over P_2O_5 (1.1 g). Chromatographic analysis showed two yellow, ninhydrin-positive spots, identified as 2-NPS-tryptophan and *S*-NPS-cysteine. The compounds were separated by preparative thin-layer chromatography on SiO_2 using *n*-butyl alcohol–water–acetic acid (6:2:2). The spots were eluted with glacial acetic acid and, after appropriate dilution, analyzed spectrophotometrically. Assuming ε 3750 at 360 mμ for 2-NPS-tryptophan (Scoffone *et al.*, 1968) and ε 3050 at 365 mμ for *S*-NPS-cysteine (this paper), the mixture before separation contained 63% of NPS-tryptophan and 37% of *S*-NPS-cysteine.

Reaction of Reduced RNase with NPS-Cl and pNPS-Cl. Bovine pancreatic RNase (100 mg) was reduced with β-mercaptoethanol, as described by Anfinsen and Haber (1961). After precipitation and washing with acetone–1 N HCl (39:1) and ether, the reduced enzyme was dissolved to a concentration of 10 mg/ml in aqueous acetic acid, through which nitrogen had been bubbled for at least 15 min at room temperature. An amount of NPS-Cl or pNPS-Cl corresponding to a molar ratio of reagent to protein of 80:1 was first dissolved in glacial acetic acid (50 mg of reagent/ml of acid) and then added to the reduced RNase solution. The final concentration of solvent was approximately 50% aqueous acetic acid. The mixture was allowed to stand at room temperature for 1 hr. The protein was precipitated with acetone–1 N HCl (39:1) at −5°, separated by centrifugation, and washed several times with acetone and ether. A sample of the protein derivative was then further purified by gel filtration on Sephadex G-25 with 0.1 N acetic acid as eluent. The protein was located in the effluent by measuring the absorption at 358 and 328 mμ, for NPS-RNase and pNPS-RNase, respectively; then the effluent containing the protein was lyophilized.

Regeneration of Reduced RNase from Its NPS and pNPS Derivatives. The NPS-RNase or pNPS-RNase (40 mg) was dissolved in 1 ml of a freshly prepared 8 M solution of recrystallized urea and adjusted to pH 8.6 with 5% methylamine. β-Mercaptoethanol was added at a level of 1 μl/mg of NPS protein, the container was flushed with nitrogen, and the solution was allowed to stand for 4 hr at room temperature. After this time the entire solution was passed through a column of Sephadex G-25 with 0.1 N acetic acid as eluent.

Reoxidation of Reduced RNase. The protein in the column effluent, after appropriate dilution, was adjusted to pH 8.1 and allowed to stand at room temperature for 24 hr, at which time the enzyme's activity toward ribonucleic acid was measured.

Estimation of the Extent of Modification in pNPS-RNase. The pNPS protein (about 0.1 μmole) was dissolved in deaerated 0.1 N NaOH (10 ml). After standing 30 min at 22–24°, this solution was spectrophotometrically analyzed at 412 mμ and the concentration of 4-nitrothiophenol (ε 13,600 at 412 mμ) was determined. The extent of modification was calculated by comparing

TABLE I: Analytical Data and Specific Rotations of Cysteine and Cysteine Derivatives Treated with Sulfenyl Halides.

No.	Compound	Procedure[a]	Yield (%)[b]	Mp (°C) (not cor)	$[\alpha]_D^{20}$ (*c* 0.5, 80% HAc), deg	R_F[c]
1	H-Cys(NPS)-OH · H_2O	A	71	167 dec	−167.5	0.60
2	Ac-Cys(NPS)-OH · H_2O	A	80	150 dec	−49.2	0.75
3	Ac-Cys(pNPS)-OH · H_2O	A	53	132–133	−67.0	0.88
4	HCl · H-Cys(NPS)-OMe[d]	A	65	160 dec	+15.8	0.66
5	H-Cys(DNPS)-OMe	A	70	85 dec	+24.0[e]	0.78
6	H-γGlu-Cys(NPS)-Gly-OH · 2 H_2O	B	55	150 dec	−56.5	0.45
7	H-γGlu-Cys(pNPS)-Gly-OH · H_2O	B	68	191 dec	−64.0	0.62
8	H-γGlu-Cys(DNPS)-Gly-OH · 2 H_2O	B	72	186 dec	−51.6	0.58

[a] The preparation was achieved as described in the text. [b] Yields were calculated on analytically pure compounds. [c] The R_F values were determined by thin-layer chromatography (SiO_2) using the following solvent mixture: *n*-butyl.

this concentration with the amino acid content (Smyth *et al.*, 1963) in the pNPS protein hydrolysate (6 N HCl, 105°, 24 hr) obtained from an aliquot of the solution.

Assays of Enzymic Activity. The assay of enzymic activity of RNase was based on the method of Kunitz (1946), following the decrease of absorbance at 300 mμ.

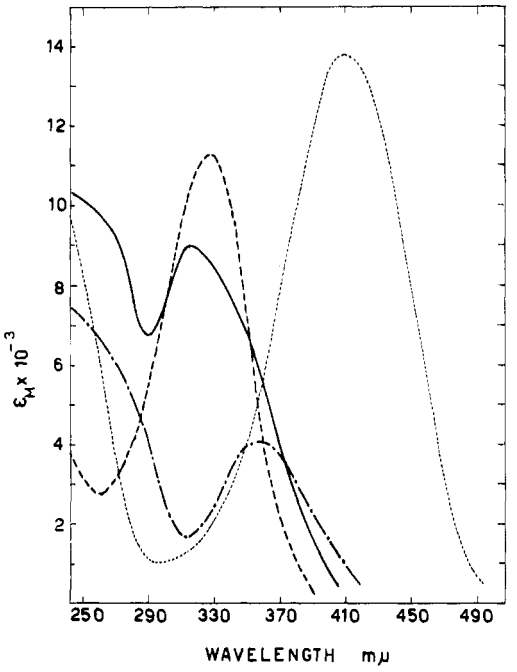

FIGURE 1: Absorption spectra of *S*-NPS (- · - · -), *S*-pNPS (- - -), and *S*-DNPS-glutathione (——) in 80% acetic acid and of *S*-pNPS-glutathione in 0.1 N NaOH (· · · ·).

Changes in optical density were recorded, using scale expansion. The substrate concentration was 1 mg/ml. All reactants and the cuvet holders were kept at 26 ± 0.1° by a water-circulation thermostat. Activity was calculated from the slopes of initial velocities.

All assays were performed in triplicate. Activities were calculated by comparison with a sample of native RNase of known concentration.

Results and Discussion

The selective reaction of sulfenyl halides with cysteine and tryptophan has already been discussed (Scoffone *et al.*, 1968). The formation of an unsymmetrical disulfide between sulfhydryl groups and sulfenyl halides is a known reaction used in the preparation of mixed disulfides (Parker and Kharasch, 1960; Sakakibara and Tani, 1956).

In an attempt to extend this reaction to proteins, the reactivity of sulfenyl halides with the thiol group of cysteine was studied in detail. The reaction occurs cleanly in glacial acetic acid or in 99% formic acid, as well in aqueous mixtures of these acids.

Cysteine, cysteine derivatives, and glutathione were allowed to react with NPS-Cl, pNPS-Cl, and DNPS-Cl. In each instance a clean reaction occurred and good yields of analytically pure compounds were obtained (Table I).

The relative reactivities of sulfenyl halides toward cysteine and tryptophan were examined by mixing equimolar proportions of these amino acids with NPS-Cl so that the final solution had a molar ratio of the two amino acids and the reagent of 1:1:1. The crude prod-

Formula (mol wt)	Calcd (%)				Found (%)			
	C	H	N	S	C	H	N	S
$C_9H_{10}N_2O_4S_2 \cdot H_2O$ (292.3)	36.94	4.10	9.57	21.93	37.49	3.98	9.47	22.50
$C_{11}H_{10}N_2O_4S_2 \cdot H_2O$ (316.2)	41.73	3.79	8.85	20.27	41.06	3.68	8.69	20.16
$C_{11}H_{10}N_2O_4S_2 \cdot H_2O$ (316.2)	41.73	3.79	8.85	20.57	41.45	3.51	8.66	20.05
$C_{10}H_{13}ClN_2O_4S_2$ (324.8)	36.94	4.00	8.62	19.74	37.14	4.14	8.44	19.82
$C_{10}H_{11}N_3O_6S_2$ (333.2)	36.00	3.30	12.60	19.24	36.19	3.08	12.71	18.90
$C_{16}H_{18}N_4O_7S_2 \cdot 2\ H_2O$ (478.5)	40.16	4.63	11.71	13.39	40.63	4.83	11.70	13.03
$C_{16}H_{18}N_4O_7S_2 \cdot H_2O$ (460.5)	41.69	4.34	12.16	13.92	41.36	4.35	12.04	13.78
$C_{16}H_{17}N_5O_9S_2 \cdot 2\ H_2O$ (523.5)	36.67	3.63	13.37	12.25	36.99	3.48	13.26	12.41

alcohol–water–glacial acetic acid (3:1:1). The compounds were detected by ninhydrin spray or using the hypochlorite–starch–KI test (Porn and Dutcher, 1956). [d] Calcd: Cl, 10.92. Found: Cl, 11.03. [e] *c* 0.5, DMF.

uct was separated by thin-layer chromatography. From the spectrophotometric data it was calculated that 63% of the tryptophan had reacted, compared to 37% of the cysteine.

The absorption spectra of *S*-NPS-, *S*-pNPS-, and *S*-DNPS-glutathione are shown in Figure 1; in Table II the maximum wavelengths of absorption and the molar absorptivities are reported.

As expected the thiol group of cysteine is easily restored by reduction of the disulfide bridge with β-mercaptoethanol, thioglycolic acid, sodium borohydride, or other reducing agents, widely employed in protein chemistry. For this purpose *S*-NPS-glutathione was dissolved in 2% $NaHCO_3$ and treated with 50 equiv of one of the above thiols, or with sodium borohydride; the reaction mixture was then analyzed by thin-layer chromatography. Reduced glutathione was the only ninhydrin-positive compound.

Because of the reversibility of the reaction and the

TABLE II: Wavelengths of Maximum Absorption (λ_{max}) and Molar Absorptivities (ϵ) in 80% Acetic Acid of Cysteine and Cysteine Derivatives Treated with Sulfenyl Halides.

No.	Compound	λ_{max} (mμ)	$\epsilon \times 10^{-3}$
1	H-Cys(NPS)-OH · H_2O	356	3.05
2	Ac-Cys(NPS)-OH · H_2O	358	4.00
3	Ac-Cys(pNPS)-OH · H_2O	328	10.5
4	HCl · H-Cys(NPS)-OMe[a]	353	3.15
5	H-γGlu-Cys(NPS)-Gly-OH · $2H_2O$	358	4.00
6	H-γGlu-Cys(pNPS)-Gly-OH · H_2O	328	11.2
7	H-γGlu-Cys(DNPS)-Gly-OH · $2H_2O$	315	9.05

[a] The molar absorptivity was measured in methanol.

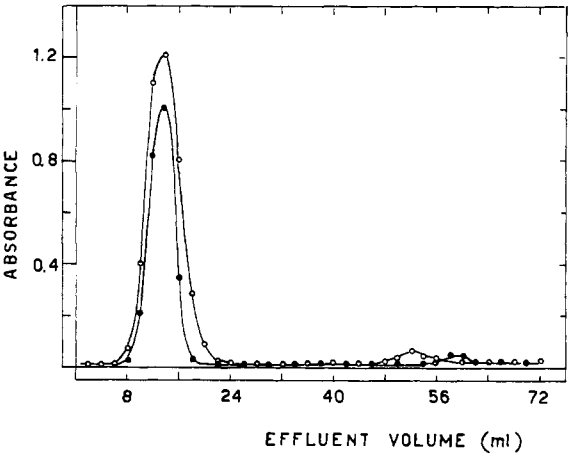

FIGURE 2: Gel filtration of NPS-RNase (○—○) and pNPS-RNase (●—●). The sulfenylation of the enzyme was performed as described in the text. The samples (20 mg) were then each passed over a Sephadex G-25 column (1 × 65 cm) at a flow rate of 0.2 ml/min, with 0.1 M acetic acid as eluent. Aliquots of the effluent were removed and after appropriate dilution analyzed spectrophotometrically at 358 and 328 mμ (NPS-RNase and pNPS-RNase, respectively).

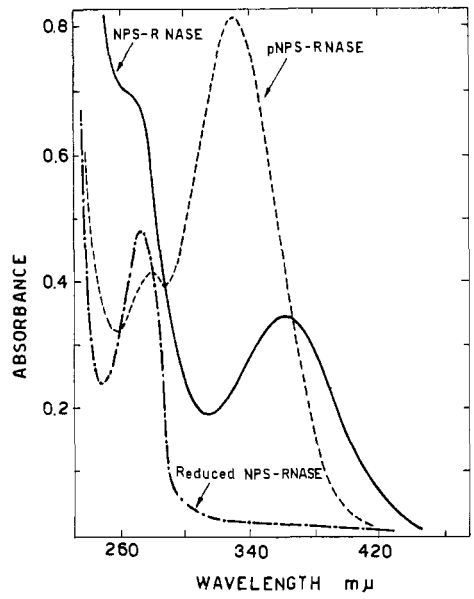

FIGURE 3: Absorption spectra of NPS-RNase (0.25 mg/ml), of pNPS-RNase (0.2 mg/ml), and of reduced RNase obtained from NPS-RNase by reduction in 8 M urea with β-mercaptoethanol and purification on Sephadex G-25. All samples were dissolved in 0.1 N acetic acid.

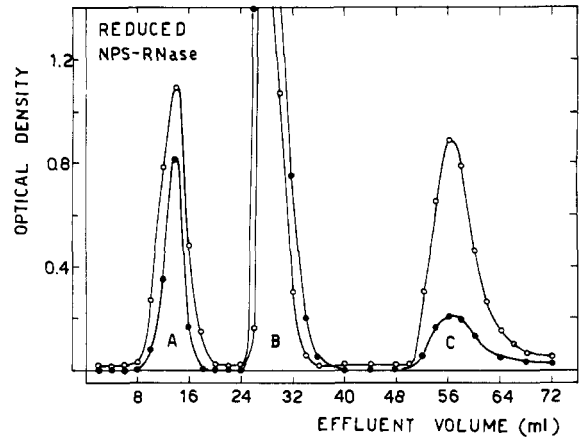

FIGURE 4: Regeneration of reduced RNase from NPS-RNase. The reaction was performed as described in the text and then the entire solution was applied to a 1 × 65 cm column of Sephadex G-25 which had previously been equilibrated with 0.1 M acetic acid, at a flow rate of 0.2 ml/min. Estimation of the SH content (●—●) in aliquots of the effluent was determined with Ellmann's (1959) reagent; in addition the absorbance at 275 mμ was measured (○—○). The bulk of the protein (A) emerged completely separated from reagents (urea and β-mercaptoethanol) (B) as well the *o*-nitrothiophenol (C), which was released by reduction of the mixed disulfide of NPS-RNase. The thiol was identified from their absorption spectra by comparison with known sample.

mild conditions required for the formation of the mixed disulfides, the reaction was applied to reduced RNase, in which tryptophan is not present. It was carried out in 50% acetic acid (see Experimental Section). The NPS and pNPS proteins, further purified on a Sephadex G-25 column (Figure 2), show absorption maxima at 358 and 328 mμ, respectively (Figure 3).

Treatment of pNPS-RNase in 0.1 N NaOH (see further) released *p*-nitrothiophenol (ε 13,600 at 412 mμ; Ellman, 1958). It was calculated that 7.8 SH groups in reduced RNase had reacted (theoretically eight residues). After reduction of the mixed disulfides with β-mercaptoethanol, purification on Sephadex G-25 (Figure 4), and air oxidation according to the procedure of Anfinsen and Haber (1961), the enzymic activity of recovered RNase toward RNA was restored to the extent of 70–80%.

Since it is known that aromatic mixed disulfides are not stable compounds and rearrange quickly at high pH, the stability of the mixed disulfides of cysteine in acidic and in basic solutions was studied.

None of the compounds prepared was stable under the conditions commonly used for the hydrolysis of proteins. Reduced glutathione in which the SH group was treated with NPS-Cl, showed after hydrolysis an amino acid composition (Table III) very similar to that of oxidized glutathione, indicating that a disulfide-bond

TABLE III: Amino Acid Analysis of *S*-NPS-glutathione.[a]

Amino Acid	Calcd (μmoles)	Found (μmoles)
Glutamic acid	1.0	1.00
Glycine	1.0	1.00
Cystine	0.5	0.42
Cysteic acid		0.01

[a] The hydrolysis was performed in 6 N HCl *in vacuo* at 105° for 20 hr. A further ninhydrin-positive compound, approximately 0.01 μmole, appeared in the chromatogram after the cystine peak. The analysis was performed accordingly to Spackman *et al.* (1958).

TABLE IV: Behavior of *S*-NPS-cysteine Methyl Ester Hydrochloride in Various Solvents.[a]

Expt No.	Solvent	Time; Mp (°C)	Remarks
1	80% HAc	60 hr; 22–24	Stable
2	80% HAc	30 min; 100	Stable
3	80% HAc	2 hr; 100	Not stable
4	10% HAc	48 hr; 22–24	Stable
5	10% HAc	15 min; 100	Stable
6	10% HAc	1 hr; 100	Not stable
7	1% $NaHCO_3$	1 hr; 22–24	Not stable
8	1% Na_2CO_3	5 min; 22–24	Not stable
9	0.1 N NaOH	5 min; 22–24	Not stable

[a] The compound (10 mg) was dissolved in 5 ml of the indicated solvents. The reaction mixture was then analyzed by thin-layer chromatography (SiO_2) using the following solvent mixture: ethyl acetate–pyridine–water–glacial acetic acid (60:20:14:6). The spots were developed by spraying with ninhydrin.

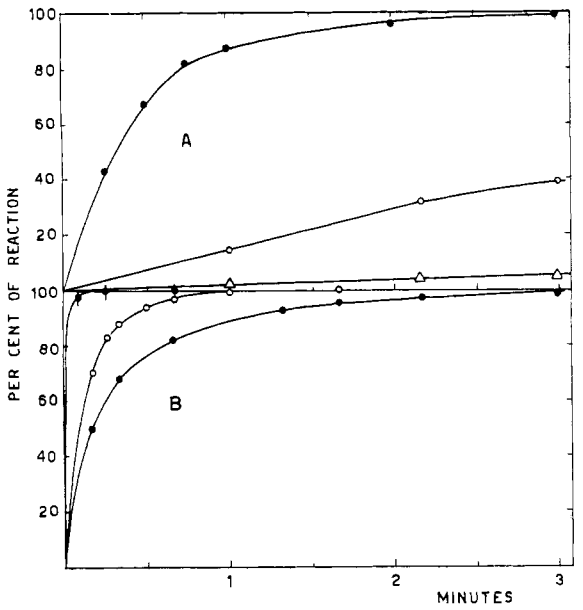

FIGURE 5: Cleavage of the S–S bond in *N*-acetyl-*S*-pNPS-cysteine (A) and in *S*-pNPS-glutathione (B) in different alkaline media. The compound was dissolved in water (0.4 mg/ml), and 0.1 ml of this solution was removed and added to a 2.9 ml of the alkali solution in a cuvet. The increase in absorbance at 412 mμ was recorded on a spectrophotometer, using scale expansion. All solutions through which nitrogen was passed for at least 15 min were thermostated at 24 ± 0.1° as was the cuvet holder, by a circulating-water thermostat. The per cent of reaction was calculated from the maximum value of absorption obtained. In each case, within experimental error, a practically quantitative release of *p*-nitrothiophenol (ε 13,500–13,600) was observed. (A) (●) in 1 N NaOH, (○) in 0.1 N NaOH, and (Δ) in 5% Na_2CO_3. (B) (◐) in 0.1 N NaOH, (○) in 5% Na_2CO_3, and (●) in 2% Na_2CO_3.

interchange occurred under the strongly acidic conditions (6 N HCl) which were used. The 2-nitrophenyl disulfide precipitates and shifts the equilibrium in the direction of the formation of cystine.

However, the unsymmetric disulfides are stable in mildly acidic solution, whereas they are cleaved rapidly in alkaline media (Table IV). As stated by Parker and Kharasch (1960) the cleavage of mixed disulfides in alkaline media occurs with the formation of the more stable thiol and of a sulfenic acid, which further rearrange. A possible alternative route to the formation of the thiol is *via* a β elimination (known to occur in alkali-treated cystinyl compounds) producing a dehydroalanine residue and a persulfide ion, which further decomposes to a thiol and free sulfur (Tarbell and Harnish, 1951).

By using the *S*-pNPS derivatives of *N*-acetylcysteine and glutathione, the cleavage of the mixed disulfide was followed conveniently by spectrophotometry, since the *p*-nitrothiophenol released absorbs at 412 mμ, while the starting materials absorb at 328 mμ (Figure 1).

The rate of cleavage depends on the structure of the compound carrying the mixed-disulfide function. A much higher rate of cleavage was observed in *S*-pNPS-glutathione then in *N*-acetyl-*S*-pNPS-cysteine (Figure 5). It is noteworthy that the release of the p-nitrothiophenol moiety is quantitative (Figure 6), provided that

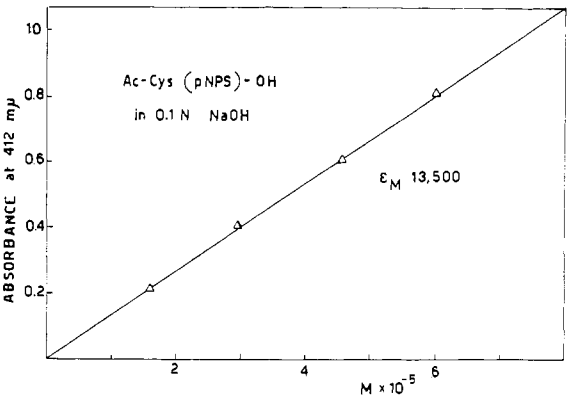

FIGURE 6: Relation between concentration and color development at 412 mμ in 0.1 N NaOH of *N*-acetyl-*S*-pNPS-cysteine. The compound was dissolved in 0.1 N NaOH, and, after standing at 22–24° for 10 min, the optical density at 412 mμ was measured.

deaerated solutions are employed. This makes possible the quantitative determination of the reacted free SH in a protein, as shown with reduced RNase.

In studies of the sulfhydryl disulfide relationships in proteins, SH groups are generally stabilized by reaction with suitable alkylating reagents such as iodoacetic acid, iodoacetamide, or *N*-ethylmaleimide. However in spite of care in the maintenance of proper pH values and times of reaction, the experiences of various laboratories have indicated that alkylation of groups other than SH may occur to a small but significant extent (Gundlach *et al.*, 1959) (*e.g.*, methionine sulfur to the sulfonium derivative, lysine ε-amino groups to the *N*-alkyl derivative). A reversible protection of the SH groups may be achieved through *p*-mercuribenzoate reaction (Anfinsen and Haber, 1961), or by a disulfide-exchange reaction (Stracher, 1964). The SH group is restored by the action of reducing agents.

The stoichiometric reaction between sulfenyl halides and SH groups in proteins offers a better technique for a reversible protection of these groups. However, it is necessary to work in acidic media, in which they are stable, rather than in alkaline solution in which they decompose (see Table IV). By observing this precaution, the way is opened for the use with proteins of a new and broad class of reagents containing both aliphatic and aromatic groups of various kinds.

Cleavage of disulfide bonds is necessary in order to expose buried residues in the polypeptide chain to the action of chemical reagents or to obtain quantitative cleavage by proteolytic enzymes. The reversible protection of SH groups by reaction with sulfenyl halides make possible, *inter alia*, a study of the ability of reduced enzymes to re-form enzymatically active material after chemical modification of single residues in the peptide chain or after limited selective cleavage with proteolytic enzymes.

Furthermore, the reactions of sulfenyl halides with SH groups are of analytical interest, since they are quantitative and stoichiometric. The addition of alkali to the mixed disulfides releases the various nitrothiophenols quantitatively and permits direct analysis of

the free SH groups in proteins. The most desirable of the reagents from this point of view is *p*-nitrophenylsulfenyl chloride.

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References

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.
Bernfield, M. R. (1965), *J. Biol. Chem. 240*, 4753.
Ellman, G. L. (1958), *Arch. Biochem. Biophys. 74*, 443.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Gundlach, H. G., Stein, W. H., and Moore, S. (1959), *J. Biol. Chem. 234*, 1754.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1953), *J. Biol. Chem. 200*, 493.
Hubacher, M. H. (1943), Organic Syntheses, Coll. Vol. 2, New York, N. Y., Wiley, p 445.
Kharasch, N., Gleason, G. I., and Buess, C. M. (1950), *J. Am. Chem. Soc. 72*, 1796.
Kunitz, M. (1946), *J. Biol. Chem. 164*, 563.
Parker, A. J., and Kharasch, N. (1960), *J. Am. Chem. Soc. 82*, 3071.
Porn, S. C., and Dutcher, J. T. (1956), *Anal. Chem. 38*, 836.
Sakakibara, S., and Tani, H. (1956), *Bull. Chem. Soc. Japan 29*, 85.
Scoffone, E., Fontana, A., and Rocchi, R. (1968), *Biochemistry 7*, 971 (this issue; preceding paper).
Smyth, D. G., Stein, W. H., and Moore, S. (1963), *J. Biol. Chem. 238*, 227.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stracher, A. (1964), *J. Biol. Chem. 239*, 1118.
Tarbell, D. S., and Harnish, D. P. (1951), *Chem. Rev. 49*, 11.
Wellner, D., Silman, H. I., and Sela, M. (1963), *J. Biol. Chem. 238*, 1324.
Zincke, Th., and Lenhardt, S. (1913), *Ann. 400*, 2.

Nonheme Iron Proteins. IV. Structural Studies of *Micrococcus aerogenes* Rubredoxin*

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ABSTRACT: The *S*-β-aminoethylcysteinyl derivative of *Micrococcus aerogenes* rubredoxin was digested with trypsin and chymotrypsin in separate experiments. The fragments were purified and their sequences were determined by standard procedures. From the sequences of the individual peptides and the overlaps between them, the total amino acid sequence of the *M. aerogenes* rubredoxin was deduced. The stability of the chelate structure of this protein in dilute acids, 8 M urea, 50% ethanol, and 2.5 and 5 M guanidine hydrochloride was determined with respect to time. These studies and the resistance of the native protein to proteolytic digestion suggest a compact and stable confirmation for the native protein.

The nonheme iron protein, rubredoxin, was first crystallized from extracts of *Clostridium pasteurianum* by Lovenberg and Sobel (1965). Similar proteins have also been purified from several other anaerobic bacteria (Stadtman, 1965; Mayhew and Peel, 1966; Le Gall and Dragoni, 1966; Lovenberg, 1966; Bachmayer *et al.*, 1967a). In addition, Peterson *et al.* (1966) have isolated a rubredoxin-like protein from an aerobic bacterium, *Pseudomonas fluorescens*, and showed that this protein was necessary for ω hydroxylation of certain fatty acids.

All the rubredoxins isolated thus far have been reported to contain 1 g-atom of nonheme iron/mole of protein but no inorganic sulfide. The molecular weight of rubredoxin is approximately 6000 (Lovenberg and Sobel, 1965).

The present report describes the determination of the complete amino acid sequence of *Micrococcus aerogenes* rubredoxin. Observations on the stability of rubredoxin, the action of proteases on the native protein, and discussion concerning the possible iron ligands are included. Short communications of parts of the

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