Sulfenyl Halides as Modifying Reagents for Polypeptides and Proteins. III. Azobenzene-2-sulfenyl Bromide, a Selective Reagent for Cysteinyl Residues*

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ABSTRACT: Azobenzene-2-sulfenyl bromide (Burawoy, A., and Vellins, C. E. (1954), *J. Chem. Soc.*, 90) is found to react selectively with cysteinyl residues in polypeptides and proteins giving unsymmetrical disulfides. The reagent is soluble in water and the reaction occurs selectively in buffer solutions close to neutrality (pH 5). Several model compounds were prepared using cysteine derivatives and glutathione and their physical and chemical properties were investigated. The thiol function is easily restored by using reducing agents such as β-mercaptoethanol, thioglycolic acid, or sodium borohydride. The mixed disulfides are stable in acidic solution, but are decomposed in alkaline media. The basicity of the azo group, determined spectrophotometrically, was found to be much less in the azobenzene-2-sulfenyl derivative of reduced lysozyme than in the simple model compounds.

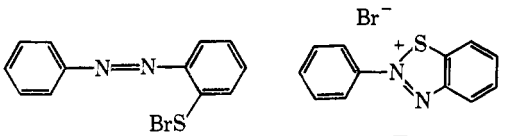
That the protein structure affects the basicity of the azo group was confirmed by the fact that the ionization equilibrium of azobenzene-2-sulfenyllysozyme is equal to that of azobenzene-2-sulfenylcysteine in 8 M urea solutions.

Specific chemical reagents for amino acid residues in polypeptides and proteins are of great help in establishing the participation of amino acid side chains in the biological functions of proteins. In recent years, great attention has been focused on selective modification of particular amino acid residues and even more methods are available to biochemists (Witkop, 1961; Timasheff and Gorbunoff, 1967).

The cysteine residue undoubtedly has the distinction of receiving more attention than any other reactive group of the enzyme protein molecule (Cecil and McPhee, 1959; Boyer, 1959). A large number of reactions (oxidation, alkylation, and mercaptide formation) leading to the modification of this residue in proteins have been studied. The use of *p*-mercuribenzoate (Boyer, 1954) is particularly important in structure–activity studies in enzyme catalysis as well in analytical work. However there are numerous examples of the binding of mercury by protein groups other than thiol residues, although at low concentrations of the reagent, the reaction with SH appears to be the most significant. For this reason in the use of mercurials for enzyme studies attention needs to be given to their possible lack of specificity (Boyer, 1959).

We have recently described (Scoffone *et al.*, 1968; Fontana *et al.*, 1968) the selective reaction of sulfenyl halides with tryptophan and cysteine residues in proteins. In acidic solution tryptophan is attacked at the 2 position of the indole nucleus to give a thioether, whereas cysteine is converted into a mixed disulfide. With the aim of producing in proteins varied modifications leading to changes in charge, size, or other physicochemical properties, we are examining other examples of this wide class of reagents.

This paper deals with the selective reaction of ABS-Br[1] (I) with the cysteinyl residue. The reagent,



I II

chosen mainly for its unusual solubility and great stability in water, properties which are related to its salt-like structure (II) (Burawoy and Vellins, 1954; Burawoy *et al.*, 1954), reacts selectively and stoichiometrically only with cysteine in buffer solutions close to neutrality (pH 5). Tryptophan and tryptophan peptides, as well as lysozyme, a protein containing tryptophan but not cysteine, were recovered unchanged after reaction with ABS-Br in buffer solution at pH 5 or in glacial acetic acid.

Several Cys-ABS derivatives were synthesized and their chemical and physical properties are reported. The basicity of the azo group in the ABS derivatives of cys-

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[1] Abbreviations used: ABS-Br, azobenzene-2-sulfenyl bromide; Cys-ABS, *S*-(azobenzene-2-sulfenyl)cysteinyl residue; ABS-lysozyme, reaction product of ABS-Br with the corresponding reduced protein; the amino acids have the L configuration; the abbreviations for amino acids and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, *Biochemistry 5*, 1455 (1966).

teine and of reduced lysozyme was studied spectrophotometrically and found to be influenced by the protein environment.

Experimental Section

Materials. ABS-Br was prepared as described by Burawoy and Vellins (1954). Cysteine, glutathione, *N*-acetylcysteine, cysteine methyl ester hydrochloride, and tryptophan were all obtained from Fluka AG, Basel (Switzerland), and were used without further purification. Sodium borohydride, thioglycolic acid, β-mercaptoethanol, and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) (Ellman, 1959) were reagent grade products (Fluka). Urea (Fluka) was recrystallized from 95% ethanol and only fresh solutions of this compound were used. The tryptophan-containing peptides used in this work were previously described (Veronese *et al.*, 1967). Salt-free egg-white lysozyme (Lot LYSF GJA) was obtained from Worthington Biochemical Corp. and used without further purification. Dehydrated cells of *Micrococcus lysodeikticus* were obtained from Difco Laboratories, Inc. Sephadex G-25 (fine beads) was purchased from Pharmacia, Uppsala, Sweden.

Methods. Spectra were obtained with a Perkin-Elmer Hitachi Model 139 self-recording spectrophotometer, while absorption at single wavelengths was determined with a Perkin-Elmer Hitachi Model 125 spectrophotometer. Rotatory powers were measured with a Perkin-Elmer Model 141 polarimeter; concentrations are given in g/100 ml of solvent. The melting points were determined using the Tottoli apparatus (Büchi, Flawil, Switzerland) and are uncorrected. The pH values were determined with a Beckman expanded-scale pH meter.

Thin-layer chromatography (SiO_2) was performed using the following solvent mixture, 1-butanol–water–glacial acetic acid (3:1:1). The compounds were in turn detected by ninhydrin spray and the hypochlorite–starch–KI test (Porn and Dutcher, 1956). The tryptophan derivatives were also detected by the Ehrlich reaction.

Lysozyme activity was determined by measuring the rate of change of turbidity of a substrate suspension (0.15 mg of dehydrated cells of *M. lysodeikticus*/ml in 0.066 M phosphate buffer at pH 6.2 containing approximately 5 μg of lysozyme/ml. The change in turbidity was recorded automatically at 540 mμ. From the slopes of the curves obtained the lysozyme activity was determined.

Selectivity of the Reaction. Reaction of ABS-Br with tryptophan derivatives. The peptide (*N*-carbobenzoxy-Leu-Trp-OMe, *N*-carbobenzoxy-Phe-Trp-OMe) was dissolved in glacial acetic acid and treated with 3 equiv of ABS-Br. After 2 hr at room temperature the reaction mixture was analyzed by thin-layer chromatography. The tryptophan peptide was found to be unchanged. Also tryptophan methyl ester hydrochloride and *N*-carbobenzoxytryptophan in either acetic acid or 0.1 M acetate buffer solution of pH 5 were found unchanged after reaction with ABS-Br for several hours at 22–24°.

Reaction with Lysozyme. The protein (1 μmole) was dissolved in 2 ml of 0.1 M acetate buffer solution of pH 5 and 40 equiv of ABS-Br dissolved in the same buffer was added. After 1 hr at 22–24° the protein was precipitated with acetone–1 N HCl (39:1) at −5°, separated by centrifugation, washed several times with acetone and ethyl ether, and dried *in vacuo* (10^{-2} torr) over P_2O_5. The sample of the protein was still fully active toward *M. lysodeikticus* and its ultraviolet spectrum was unchanged.

Reaction of ABS-Br with cysteine and cysteine derivatives (Table I). To a solution of 10 mmoles of cysteine or cysteine derivative in 30–50 ml of acetate buffer (0.2 M, pH 5, previously deaerated by means of a stream of nitrogen), 10 mmoles of ABS-Br dissolved in 50 ml of the same buffer was added at room temperature. The precipitated product was collected by filtration, carefully washed with water, and then dried *in vacuo* over P_2O_5. The ABS derivative of cysteine was further recrystallized from acetone.

Reaction of reduced lysozyme with ABS-Br. The protein (40 mg) was reduced with β-mercaptoethanol (20 μl) in 2 ml of a freshly prepared 8 M solution of recrystallized urea and the solution was adjusted to pH 8.6 with 5% methylamine (Anfinsen and Haber, 1961). After standing for 1 hr at 22–24°, the protein was precipitated with acetone–1 N HCl (39:1) at −5°, separated by centrifugation, and washed with acetone. The protein was then dissolved in 1.5 ml of H_2O and 0.5 ml of acetic acid. The ABS-Br (35 mg) was dissolved in 50% acetic acid (1 ml) and added to the solution of the reduced enzyme. After standing for 30 min at 22–24°, the entire solution was applied to a Sephadex G-25 column (90 × 1 cm) which was equilibrated with 0.2 M acetic acid. The column was then eluted with the same eluent at a flow rate of 0.37 ml/min. In the effluent the ABS-protein was located by spectrophotometric analysis at 323 mμ and emerged completely separated from excess reagent. The ABS-protein was then recovered by lyophilization.

Regeneration of Reduced Lysozyme from Its ABS Derivative. The ABS-lysozyme (10 mg) was dissolved in 2 ml of 8 M urea adjusted to pH 8.6 with 5% methylamine. β-Mercaptoethanol was added (10 μl), 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 (90 × 1 cm) of Sephadex G-25 using 0.2 M acetic acid as eluent. The reduced protein in the effluent was located by using Ellman's (1959) reagent.

Reoxidation of the Reduced Protein. The effluent containing the reduced protein was adjusted to pH 8.2 and, after appropriate dilution, the reoxidation was carried out in presence of β-mercaptoethanol (molar ratio of thiol to protein sulfhydryl groups about 50:1) as described by Epstein and Goldberger (1963). The protein concentration in the effluent was determined spectrophotometrically at 280 mμ using the extinction coefficient ε 39,000.

Regeneration of Reduced Glutathione from Its ABS Derivative. The ABS-glutathione (10 mg) was dissolved in 2 ml of 2% $NaHCO_3$ containing 30 μl of β-mercaptoethanol or thioglycolic acid. After 2 hr at 22–24° the

reaction mixture was analyzed by thin-layer chromatography (SiO_2) using *n*-butyl alcohol–water–glacial acetic acid (3:1:1) as eluent. Reduced glutathione was the only ninhydrin-positive spot. Analogous results were obtained by reducing the ABS-glutathione by means of sodium borohydride.

The pK_a of the azo group of ABS-cysteine was determined spectrophotometrically from changes in the visible absorption spectrum with pH following a standard procedure (Albert and Serjeant, 1962). The acidic species has an absorption peak near 353 mμ (ϵ 16,700), the conjugate base at about 323 mμ (ϵ 14,000). The absorption of the acidic and basic forms of ABS-cysteine was determined in 0.1 N HCl and pH 6.5 phosphate buffer, respectively. A solution (0.25 ml) of ABS-cysteine prepared by dissolving 50 mg in 50 ml of methanol was added to 10 ml of appropriate buffer and the optical density was measured. The buffers used were 0.1 N HCl, 0.1 M citrate, and phosphate.

Estimation of the Extent of Modification in ABS-lysozyme. The ABS-protein was dissolved in 0.1 N HCl (2 mg/ml) an aliquot (0.3 ml) was added to 2.7 ml of 8 M urea (pH 1) and this solution was analyzed spectrophotometrically at 353 mμ in order to determine the concentration of the ABS residues (ϵ 16,700 at 353 mμ). The extent of modification was calculated by comparing this concentration with the amino acid content in the ABS-protein hydrolysate (6 N HCl, 105°, 24 hr) obtained from an aliquot of the original solution in 0.1 N HCl.

Results and Discussion

Reaction of ABS-Br with Model Compounds. Our attempts to obtain modification of tryptophan or tryptophan derivatives (Z-Leu-Trp-OMe, Z-Phe-Trp-OMe, Z-Trp, and Trp-OMe-HCl) by reaction with ABS-Br in aqueous buffers or in glacial acetic acid were unsuccessful. The tryptophan derivatives were found unchanged (thin-layer chromatography assay) after reaction for several hours at room temperature. The lack of reactivity of ABS-Br toward the indole nucleus of tryptophan can be explained in terms of the "sulfenamide nature" of the reagent, since its true structure is that of a 2-phenylbenzo-1-thia-2,3-diazolium ion, as depicted in formula II (Burawoy *et al.*, 1954). As expected on the basis of this sulfenamide structure all attempts to obtain substituted sulfenamides by reaction of amino acids or of their alkyl esters with the reagent in aqueous or basic organic solutions was unsuccessful. On the other hand, the reactivity toward cysteine was expected, since sulfenamides are known to react with thiols. In fact we had earlier employed this reaction for the removal of protective sulfenyl residues from amino groups in peptide synthesis (Fontana *et al.*, 1966a,b).

The reaction of ABS-Br with the thiol group of cysteine occurs very rapidly in both acidic and neutral solution. In each case high yields of analytically pure compounds were obtained. In Table I are reported yields, melting points, rotatory powers, chromatographic behavior, and analytical data for ABS-cysteine and related derivatives.

Since the disulfides obtained are easily reduced by

TABLE I: Analytical Data and Specific Rotations of Cysteine and Cysteine Derivatives Treated with ABS-Br.

Compound	Yield (%)[a]	Mp (°C) (not cor)	$[\alpha]_D^{20}$ (deg)	R_F[b]	Formula (mol wt)	Calcd (%)				Found (%)			
						C	H	N	S	C	H	N	S
H-Cys-ABS-OH	87	180 dec	−117[c]	0.55	$C_{15}H_{15}N_3O_2S_2$ (333.43)	54.02	4.58	12.56	19.17	53.75	4.60	12.63	19.07
Ac-Cys-ABS-OH	85	140–142	−29.6[d]	0.90	$C_{17}H_{17}N_3O_3S_2$ (375.48)	54.38	4.58	11.16	17.03	54.17	4.60	11.19	17.00
H-Cys-ABS-OMe	95	125–127	+35.9[c]	0.77	$C_{16}H_{17}N_3O_2S_2$ (347.45)	55.30	4.96	12.05	18.40	55.37	5.30	12.15	18.50
H-γ-Glu-Cys-ABS-Gly-OH	80	183–184	−18.0[d]	0.75	$C_{22}H_{25}N_5O_6S_2$ (519.58)	50.81	4.81	13.47	12.34	50.85	5.00	13.40	12.53

[a] The preparation was carried out as described in the text. Yields were calculated on analytically pure compounds. [b] The R_F value was determined as described in the text. [c] 0.5, in glacial acetic acid. [d] 0.5, in 80% acetic acid.

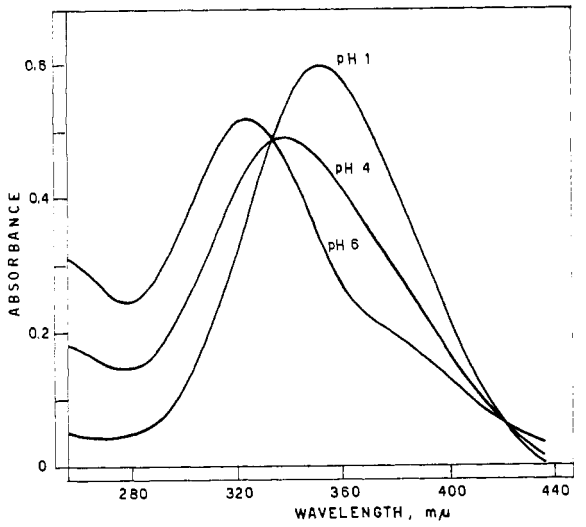

FIGURE 1: Effect of pH on the absorption spectra of ABS-cysteine (3.6×10^{-5} M). The compound was dissolved in methanol (1 mg/ml) and an aliquot of the solution was added to the buffer.

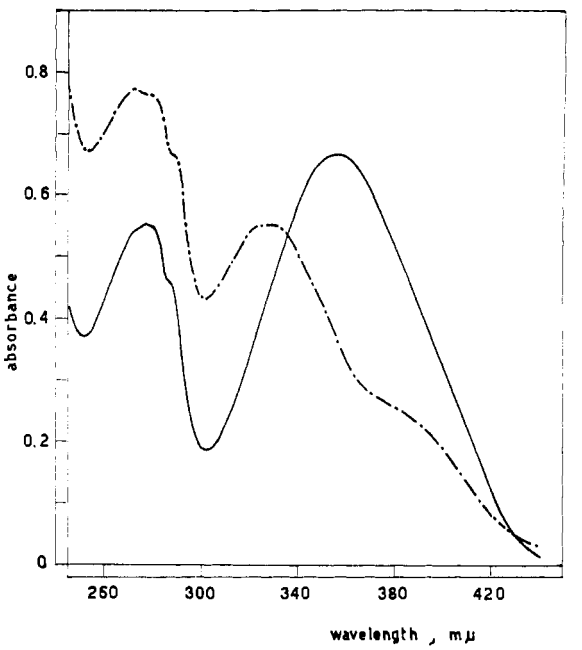

FIGURE 2: Absorption spectra of ABS-lysozyme (0.2 mg/ml) in 0.1 N HCl (—·—·—) and in 8 M urea, pH 1 (——). The number of ABS groups per molecule of enzyme in this sample was 3.3 (see text).

the use of thiols or sodium borohydride, the modification of cysteine is easily reversed. As an example when ABS-glutathione was incubated with β-mercaptoethanol, thioglycolic acid, or sodium borohydride in 2% sodium bicarbonate solution, reduced glutathione was obtained, as determined by thin-layer chromatography (ninhydrin test).

As previously reported (Parker and Kharasch, 1960) aromatic–aliphatic disulfides are stable compounds. For example, (2-nitrophenyl)ethyl disulfide and related derivatives underwent no cleavage when heated for several hours in aqueous methanol or acetone at reflux temperature. On the other hand, rapid cleavage took place in alkaline solution.

As expected, the Cys-ABS derivatives prepared in the course of the present work are decomposed rapidly in alkaline media, but are stable under acidic conditions. Thus when ABS-glutathione was dissolved in aqueous buffers and the solutions were allowed to stand at room temperature for 3 hr, evidence of decomposition was obtained only in the case of buffers of pH greater than 7 (thin-layer chromatographic assay). However the ABS derivatives were found to be less stable than the corresponding 2-nitrophenylsulfenyl derivatives (Fontana *et al.*, 1968) either when heated in aqueous acetic acid or when allowed to stand in organic solvents for several days at room temperature. These results indicate that only freshly prepared acidic solutions of ABS derivatives should be employed.

Reaction of ABS-Br with Lysozyme. The reaction of ABS-Br with cysteine residues was also applied to proteins. In order to confirm the selectivity of the reaction observed with simple tryptophan derivatives, lysozyme, a protein containing six tryptophan and no cysteine residues (Canfield, 1963), was treated with 40 equiv of the reagent in 0.1 M acetate buffer of pH 5. The recovered enzyme was shown to be unchanged on the basis of its ultraviolet spectrum and the fact that it was fully active toward *M. lysodeikticus*. On the other hand, reaction occurred when the protein was first reduced with β-mercaptoethanol in 8 M urea (Anfinsen and Haber, 1961) and then treated with ABS-Br. When the fully reduced protein was used the reaction gave an insoluble material. In practice therefore the protein was only partially reduced by the use of short reaction times and less reducing agent, prior to reaction with ABS-Br. The sample of ABS-lysozyme obtained was dissolved in 8 M urea (pH 1) and from the absorption at 353 mμ the number of ABS groups per molecule of enzyme was found to be 3.3.

The ABS-lysozyme was then further reduced with β-mercaptoethanol in 8 M urea; after purification on Sephadex G-25, the reduced enzyme no longer showed absorption in the visible region of the spectrum (absorption due to the ABS group) and after air oxidation at pH 8.2 at 25° in presence of β-mercaptoethanol (Epstein and Goldberger, 1963), the lytic activity toward *M. lysodeikticus* of the recovered enzyme was 70–75%. This degree of lytic activity compares favorably with that (65–85%) already obtained in the reoxidation of reduced lysozyme under the same experimental conditions (Epstein and Goldberger, 1963). These results confirm the high selectivity and the mildness of the reaction conditions required to introduce and remove the ABS groups.

Ionization Equilibria of the Azo Group. The acidity constant of the azo group attached to cysteine was studied spectrophotometrically since the acidic species has an absorption peak near 353 mμ (ε 16,700) and the conjugate base at about 323 mμ (ε 14,000). The effect of pH on the absorption spectrum of ABS-cysteine is shown in Figure 1. From the optical titration curve the pK_a of the azo group was found to be 3.8. This value of pK_a is

relatively high compared with those of other azobenzene derivatives (Jaffé and Gardner, 1958). We offer no explanation for this difference at the present time.

When the ABS residue was attached to the protein, the acidity constant of the azo group was markedly different. In fact ABS-lysozyme dissolved in solutions of pH 1 showed the spectrum of the conjugate base rather than that of the acidic species (Figure 2). The shift to higher wavelengths, corresponding to protonation of the azo group, occurs only at pH 0 and for obtaining full protonation high concentrations of strong acid (2–3 M HCl) are required. But under these conditions the quantities of acid required markedly contribute to the ionic strength; in addition the disulfide linkage is not stable in strongly acidic solution and exchange reactions may occur (Benesch and Benesch, 1958). Thus it was not possible to determine exactly the pK_a of the azo indicator covalently bound to the protein.

It is particularly interesting that in aqueous urea (5–8 M) at pH 1 the spectra of the ABS-protein and ABS-cysteine are similar (Figures 1 and 2), showing that the ionization equilibrium of the azo group in both compounds is the same.

The fact that the basicity of the azo group of the ABS residue is greatly different depending upon whether it is attached to a protein or to a small molecule shows that the protein enivronment has a very profound effect on the ionization of this group. These results must be correlated with the numerous investigations of proton equilibria of acid–base groups on proteins and in particular to the results of Klotz and Ayers (1957) on the basicity of the *p*-dimethylaminobenzeneazophenylmercuric group. These authors found that when the residue was attached to cysteine the pK_a of this group was much higher than when it was attached to bovine serum albumin.

In line with a general viewpoint (Neméthy and Scheraga, 1962; Neméthy, 1967), our results can be explained in terms of an icelike model of the water in the immediate environment of the protein. This also can explain the effect of urea in restoring the basicity of the azo group in the protein to the strength it shows in the small molecule. Because of the strong hydrogen-bonding characteristics of urea the frozen structure of the water envelope around the protein is broken and transformed into one more nearly like that of the bulk aqueous environment (Frank and Evans, 1945).

In view of the fact that the ionization of the ABS group is influenced by the protein structure, an interesting application might be its use as a reporter group for conformational studies. However a limitation inherent in the method is the great instability of the disulfide linkage in alkaline media thus precluding in some instances the possibility of studying the properties of the modified enzymes over the complete range of pH.

The analytical interest of the reaction in titrating the free SH groups in proteins is under investigation.

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