Interaction of Phosphorothioate with the Disulfide Bonds of Ribonuclease and Lysozyme*

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Disulfide bonds are undoubtedly of great importance in stabilizing the native conformations of many proteins. For studies of the covalent structure of proteins it is desirable, as a first step, to cleave the disulfide bonds (1). This has been carried out either by oxidation (1-3) or by reduction followed by alkylation of the sulfhydryl groups formed. Iodoacetic acid (or iodoacetamide) has been widely used for the alkylation step, and has the advantage that in its radioactive form it can serve as a marker for the sulfhydryl groups with which it reacts.

The reductive cleavage of disulfide bridges in biologically active proteins may result in the loss of their activities. These activities may, in some cases, be recovered upon the spontaneous reoxidation of the reduced proteins to their native forms (5-18). In studies on the correlation of structure with biological function, it is disadvantageous to block the sulfhydryl groups in an irreversible manner.

Looking for a reagent that would quantitatively cleave disulfide bonds, react with and stabilize protein sulfhydryl groups, be obtained easily with a radioactive label, and be removable from the protein under relatively mild conditions, we have tested trisodium phosphorothioate (monothiophosphate) (19). This reagent has recently been shown to act as a reducing agent, to cleave the disulfide bonds of cystine and glutathione, and to activate the enzymes papain and ficin.

In the study presented here, it is shown that the disulfide bonds of bovine pancreatic ribonuclease and egg white lysozyme are cleaved by phosphorothioate. The stoichiometry of the reaction, the stability of the phosphorothiolated enzymes, and the removal of phosphorothioate are also described.

EXPERIMENTAL PROCEDURE

Materials—Five times recrystallized RNase (Lot R233-263) was used as purchased from Sigma Chemical Company, St. Louis, Missouri. Twice recrystallized egg white lysozyme was a gift of Dr. William Harrington, The Johns Hopkins University. β-Mercaptoethanol was purchased from Organic Research Chemicals, Ltd., Poyle Estate, Bucks, England. Analytical reagent grade urea, obtained from The British Drug Houses, Ltd., Poole, England, was recrystallized from 95% ethanol; solutions were prepared immediately before use. RNA was prepared by the method of Crestfield, Smith, and Allen (21). Sephadex G-25 was purchased from Pharmacia, Uppsala, Sweden. Iodoacetic acid was obtained from Eastman Organic Chemicals, Rochester, New York. Sodium p-hydroxymercuribenzoate was purchased from Sigma Chemical Company. Phosphorus trichloride, labeled with 35P, and radioactive sulfur (35S) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. Tris (Sigma 7-9) was purchased from Sigma Chemical Company.

Reduction of RNase—RNase was reduced with β-mercaptopethanol in 8 M urea at pH 8.5, and the reduced protein was separated from these reagents by gel filtration according to the method previously reported and known to yield the protein with a content of 8 sulfhydryl groups (9).

Additional studies were performed to determine the extent of reduction of RNase by β-mercaptopethanol in the absence of urea. Two reaction mixtures were prepared, each containing 2 μmoles of RNase dissolved in 2 ml of 0.1 M Tris-chloride buffer, pH 8.5. To one of the reaction mixtures 5 μl of β-mercaptoethanol were added (yielding a 4.5-fold molar excess of reducing agent over half-cystine residues); to the other, 100 μl were added (yielding a 90-fold molar excess). After standing at room temperature for 24 hours, the two reaction mixtures were passed through columns of Sephadex G-25, and the fractions corresponding to the protein peaks were assayed for sulfhydryl content.

Reoxidation of Reduced RNase—Before the initiation of reoxidation, solutions of reduced RNase were kept at pH 3.0 and at 4°, conditions known to prevent the spontaneous reoxidation of the reduced enzyme. Reoxidation was accomplished by diluting a solution of reduced enzyme with 0.1 M Tris-chloride buffer, pH 8.5, to yield a final protein concentration of 0.05 mg per ml and a final pH of 8.2. After 16 hours at room temperature, the reoxidized protein was assayed for its RNase activity and for its content of sulfhydryl groups.

Carboxymethylation of Reduced RNase—Reduced RNase was carboxymethylated by reaction with iodoacetic acid at pH 8.2, as described by Sela, White, and Anfinsen (4). The reduced and alkylated protein was precipitated by the addition of 20 volumes of acid-acetone (49 parts acetone:1 part 1.0 N hydrochloric acid), dissolved in a small volume of 0.1 M acetic acid, subjected to gel filtration on Sephadex G-25, and lyophilized.

Preparation of Trisodium Phosphorothioate—Trisodium phosphorothioate (monothiophosphate) (19) was prepared by the modification of Åkerfeldt (20) from thiophosphoryl chloride (19, 22) and sodium hydroxide. Two radioactive preparations were synthesized, one labeled with 35S (specific activity, 49 μc per
Analytical Methods—Protein concentrations of solutions of RNase and lysozyme were calculated from their absorbance at 280 m\(\mu\), and the known extinction coefficients for the native and reduced forms of the two enzymes. The calculation of the concentrations of solutions of phosphorothio derivatives of RNase and lysozyme was based on the known extinction coefficients of the reduced forms of the two proteins. For estimation of the concentration of the phosphorothioate in solution, 0.1 to 0.5 ml was plated on planchets, dried, and counted for 1 to 5 minutes in a Nuclear-Chicago gas flow counter, equipped with an automatic sample changer and digital recorder. Concentrations were then calculated by comparison with known standards, prepared, plated, and counted together with the unknown solutions. For amino acid analyses, reduced and alkylated RNase was subjected to hydrolysis with 6 \text{n} HCl for 18 hours at 110\(^\circ\). A Spinco model 120B automatic amino acid analyzer (23) was used for all determinations. The optical densities of solutions were measured with a Beckman DU spectrophotometer in quartz cuvettes with a light path of 10 mm. All final adjustments of pH were made with a Radiometer TTTla autotitrator. Dialyses were performed with tubing that had been heated to diminish its permeability to RNase (24). All dialyses were carried out against 100 volumes of dialysis fluid, with six changes in 36 hours. For gel filtration, Sephadex G-25 was equilibrated with 0.1 M acetic acid in columns, 1.5 to 2 \times 30 to 50 cm. The filtrations were performed with the same solvent.

Titration of Sulfhydryl Groups—The sulfhydryl groups of proteins were assayed by titration with \(p\)-hydroxymercuribenzoate at pH 7.0 by a modification of the method of Boyer (25). The modification involved the stepwise addition of aliquots of a solution of the reagent, adjusted to pH 7.0, to a single protein solution in 0.1 M phosphate buffer, pH 7.0. The change in absorbance at 250 m\(\mu\) was measured after each addition. A blank, in which there was no protein, was treated in an identical manner. This modified method allowed the estimation of the content of sulfhydryl groups in as little as 0.03 \(\mu\)mole of protein. The results obtained by this procedure with native and reduced RNase were identical with those obtained by the original method of Boyer.

Assay for RNase Activity—RNase activity was assayed by digestion of yeast RNA (21), followed by precipitation with uranyl acetate-perchloric acid solution (26). Between 1 and 100 \(\mu\)g of enzyme were assayed in duplicate along with 1 \(\mu\)g of native RNase and the appropriate blanks (from which enzyme was omitted). The results were expressed in terms of the percentage of activity displayed by the native enzyme.

Reaction of Phosphorothioate with RNase, with Reduced and Alkylated RNase, and with Lysozyme—Trisodium phosphorothioate (100 \(\mu\)mole, comprised of radioactive and nonradioactive material in a ratio yielding mixtures of constant specific activity), was dissolved in 2.0 ml of 0.1 M Tris-chloride buffer, pH 8.5, containing 0.96 g of urea. Protein, 2 \(\mu\)mole (containing 16 \(\mu\)eq of cysteine sulfur), was dissolved in this solution. Thus, the final reaction medium contained a 10-fold excess of phosphorothioate over cysteine sulfur, was at pH 9.0, and was 8 \text{M} in urea and 0.1 M in Tris-chloride. The reaction medium was kept at room temperature for 16 hours and then subjected to gel filtration on Sephadex G-25. Routinely, 3-ml fractions were collected, and an aliquot was removed from each fraction for measurement of protein concentration and radioactivity.

Removal of Phosphorothioate Groups from RNase—The removal of phosphorothio groups from the phosphorothiolated RNase was attempted in the following ways: (a) dialysis against 0.1 M acetic acid; (b) gel filtration on Sephadex G-25; (c) exposure for 60 minutes to a solution of 8 \text{M} urea at pH 3.0; (d) dialysis against 0.1 M Tris chloride buffer 0.003 M \(\beta\)-mercaptoethanol, pH 8.5; and (e) exposure for 10 minutes to \(\beta\)-mercaptoethanol (25 \(\mu\)mole per \(\mu\)mole of half-cysteine residues) at pH 3.0.

Results

Characterization of Phosphorothio Derivatives of RNase and Lysozyme—Reaction of phosphorothioate with RNase, under the conditions stated above, routinely yielded a product with 8.0 (±0.5) moles of phosphorothioate per mole of protein. This product was termed PS-RNase. No significant difference was noted in the results with \(^{35}\text{S}\)-phosphorothioate from those with \(^{32}\text{P}\)-phosphorothioate. The pattern obtained upon gel filtration of the reaction mixture in a typical experiment is shown in Fig. 1. A constant ratio of radioactivity to protein was found in the material represented by approximately the first two-thirds of the protein peak. In order to avoid inhomogeneity, only the protein represented by the first half of the peak was used for further investigation.

![Fig. 1. Gel filtration of a reaction mixture containing phosphorothioate and RNase, on a column (70 × 1.4 cm) of Sephadex G-25. RNase, 2 \(\mu\)moles, was dissolved in 2.0 ml of a solution of 8 \text{M} urea-0.1 M Tris-chloride buffer containing 160 \(\mu\)moles of phosphorothioate. The reaction mixture, with a final pH of 9.0, was kept at room temperature for 16 hours and then subjected to gel filtration. The effluent fractions were assayed for their content of protein (■) and radioactivity (○). Free phosphorothioate was found to emerge at the known "salt volume" of the column.](http://www.jbc.org/)

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Titration of PS-RNase with p-hydroxymercuribenzoate failed to disclose the presence of any sulfhydryl groups. PS-RNase was found to lack RNase activity, even when assayed in an amount 100 times greater than that required to give a reliable value for the native enzyme.

Reaction of RNase with phosphorothioate at pH 6.7 gave the same result as that obtained under the standard conditions described above.

Reaction of phosphorothioate with lysozyme yielded a result similar to that obtained with RNase, namely that the product contained 7.8 moles of the reagent per mole of lysozyme.

Lack of Reactivity of Reduced and Alkylated RNase toward Phosphorothioate—Under the conditions used in the above experiments no reaction between reduced and alkylated RNase (containing 8 carboxymethylcysteine residues per molecule of protein as determined by amino acid analysis) and phosphorothioate could be demonstrated. The pattern obtained upon gel filtration of a typical reaction mixture is shown in Fig. 2. Insignificant amounts of radioactivity were found in the fractions containing protein, indicating that no (less than 0.01) phosphorothio groups were bound to the reduced and alkylated RNase.

Stability of PS-RNase—The molar ratio of phosphorothioate to protein was not changed by (a) dialysis against 0.1 M acetic acid, (b) gel filtration on Sephadex G-25, or (c) gel filtration on Sephadex G-25 60 minutes after exposure to 8 M urea at pH 3.0 (Fig. 3A). However, gel filtration after exposure of PS-RNase at pH 3.0 to 25 μmoles of β-mercaptoethanol per μmole of half-cysteine residues for 10 minutes yielded a protein devoid of phosphorothioate groups, as shown in Fig. 3B. When the fractions corresponding to the first half of the protein peak were analyzed for protein content and radioactivity, they were found to contain less than 0.1 mole of phosphorothioate per mole of protein.

Dialysis of PS-RNase against 0.1 M Tris-chloride buffer-0.003 M β-mercaptoethanol, pH 8.5, caused precipitation of the protein. The contents of the dialysis bag were centrifuged, and the supernatant fluid, which was devoid of protein, was discarded. The residue was dissolved in 8 M urea-0.1 M acetic acid and was found to contain all of the original protein but no phosphorothio groups.
Sulfhydryl Content after Removal of Phosphorothioate Groups from PS-RNase—After removal of phosphorothioate groups from PS-RNase by exposure to $\beta$-mercaptoethanol at pH 3.0, the protein was titrated with p-hydroxymercuribenzoate. The titration indicated the presence of 8.0 (±0.2) sulfhydryl groups.

Reoxidation of Product Obtained by Removal of Phosphorothioate Groups from PS-RNase—The RNase derivative from which the phosphorothioate groups had been removed, and which had been shown to contain 8 sulfhydryl groups, was reoxidized by the method described above. The reoxidized protein displayed RNase activity equivalent to 24% of that of the native enzyme, and contained no sulfhydryl groups. At this stage, the reasons for the low recovery are not clear.

Sulfhydryl Content of RNase after Incubation with $\beta$-Mercaptoethanol in Absence of Urea When RNase had been incubated for 16 hours at room temperature with 4.5 $\mu$moles of $\beta$-mercaptoethanol per $\mu$ mole of half-cystine residues, no sulfhydryl groups could be detected in the protein after gel filtration. When the molar ratio of $\beta$-mercaptoethanol to half-cystine residues had been increased to 90, the sulfhydryl content of the protein after filtration was found to be 4.9 groups per molecule.

**DISCUSSION**

Under the conditions chosen in the present study for reaction of phosphorothioate with RNase and lysozyme, 8 moles of the reagent were bound per mole of protein. The fact that the results obtained with phosphorothioate labeled with $^{32}$P were identical with those obtained with the $^{35}$S-labeled reagent indicates that both the phosphorus and the sulfur atoms of the reagent become bonded to the protein. The sulfhydryl group of the reagent must be involved in the reaction with protein, since no sulfhydryl groups could be titrated in the PS-RNase.

The covalent nature of the bond between phosphorothioate groups and protein is suggested by the stability of the PS-RNase in 8 m urea and upon dialysis or gel filtration in 0.1 m acetic acid. The hypothesis that the sites in the protein to which phosphorothioate is bonded are the sulfur atoms of the cysteine residues is substantiated by several observations. The stoichiometry of the reactions with RNase and lysozyme indicates eight reactive sites on both proteins, and the only obvious candidates for such sites are the 8 half-cystine residues present in each of these two enzymes (26–29). Phosphorothioate is quantitatively removed from PS-RNase by an excess of the sulfhydryl reagent. The covalent nature of the bond between phosphorothioate and RNase is reversible. After removal of phosphorothioate groups from PS-RNase, the reduced protein (with 8 sulfhydryl groups) can be reoxidized with a recovery of 24% of the original enzymic activity of the RNase. This extent of reactivation seems rather low, and additional experiments will be required to determine the maximum possible recovery.

It appears from this study that phosphorothioate may be a useful reagent in studies of the correlation between the structure and the biological function of proteins. Its advantageous features include quantitative cleavage of disulfide bonds under mild conditions; simple assay (inorganic phosphate or radioactivity); stable blocking of cysteine sulfur, at least at low pH values; and convenient, quantitative removal from protein under mild conditions, yielding the protein in its reduced form. It seems likely that a reagent of this type may also be useful in the future in certain stages of the chemical synthesis of proteins.

**SUMMARY**

In 8 m urea at pH 9, trisodium phosphorothioate (monothio-phosphate) quantitatively cleaves the disulfide bonds of ribonuclease and lysozyme to yield products that contain 8 phosphorothioate groups per molecule. These groups are quantitatively removed from ribonuclease by a small amount of $\beta$-mercaptoethanol to yield a protein containing 8 sulfhydryl groups. In the presence of atmospheric oxygen, the reagent reacts specifically with and stabilizes sulfhydryl groups of proteins. Trisodium phosphorothioate may be obtained labeled with either $^{32}$P or $^{35}$S, or both, and the labeled form is readily assayed. Thus, trisodium phosphorothioate appears to be a useful reagent for the reversible cleavage of disulfide bonds and labeling of sulfhydryl groups in proteins.

**REFERENCES**

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