trans-3,4-Dihydroxy-1-selenolane Oxide: A New Reagent for Rapid and Quantitative Formation of Disulfide Bonds in Polypeptides

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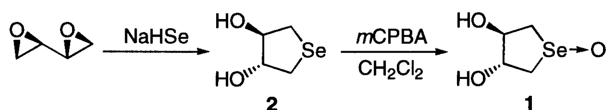
The title compound was found to be a powerful quantitative reagent for the formation of the disulfide bonds in polypeptides in a wide range of pH in the oxidative regeneration experiments of bovine pancreatic ribonuclease A (RNase A).

Redox reactions of the disulfide (SS) bond[1] are involved in many biologically important processes, such as in defense systems against active oxygen species and in the stability and the folding of protein structures. To control the redox states, small sulfur reagents [e.g., glutathione (GSH)[2] and dithiothreitol (DTT)[3]] are employed in vivo and in vitro. However, such reagents are only effective under limited pH conditions, hence it is important to develop more versatile redox reagents.[4] In this paper, a new redox reagent (**1**) that contains enhanced redox-active selenium instead of sulfur has been developed. According to the oxidative regeneration experiments of RNase A having four intramolecular SS bonds, it appeared that **1** is a rapid and quantitative reagent for the formation of the SS bonds in polypeptides in a wide range of pH (at least from pH 3 to 9).

A new selenium compound **1**[5] was synthesized according to Scheme 1. 1,3-Butadiene diepoxide was used as the starting material by mimicking the synthesis of *trans*-1,2-dithiane-4,5-diol (oxidized DTT).[6] Selenolane oxide **1** obtained as stable colorless crystals was soluble in water and methanol but insoluble in dichloromethane. By the reactions with GSH or DTT in various buffer solutions of pH 3 to 9, **1** was quantitatively transformed to the reduced form (**2**),[7] suggesting that the oxidizing power of **1** exceeds by far that of the corresponding sulfur reagents.



Scheme 1.

Extreme oxidizing ability of **1** was demonstrated by the regeneration experiments of RNase A at various pH's. SS-coupled regeneration pathways of RNase A starting from its fully reduced form (R) have been well studied by Scheraga's group at pH 8.0 and 25 °C using oxidized DTT as an oxidizing agent.[8] Under such weakly basic conditions, a pseudo steady state (R ↔ 1S ↔ 2S ↔ 3S ↔ 4S, where 1S, 2S, 3S, and 4S correspond to the ensembles of partially folded intermediates with 1, 2, 3, and 4 SS bonds, respectively) attains within ~1 h. The native form of RNase A is then slowly produced mainly from 3S intermediates. We applied **1**, instead of oxidized DTT, as an oxidizing agent in similar regeneration experiments.

According to the literature method,[8] fully reduced RNase A (R), which has 8 cysteine SH residues along the chain, was isolated in a degassed 0.1 M acetic acid (pH 3.1) or 0.1 M Tris buffer solution (pH = 7.0, 8.0 or 9.0). To the solution, stoichiometric amounts of **1** dissolved in water were added at room temperature (25 ± 1 °C). After 1 min, the regeneration reactions were stopped by addition of aqueous 2-aminoethyl methanethiosulfonate (AEMTS),[9] which quickly blocked free SH residues of the intermediates to prevent further oxidation and SS reshuffling. The resulting folding intermediates were then desalted with a Sephadex G25 column and were analyzed by a cation-exchange HPLC method.[10] Figure 1 shows the series of HPLC charts obtained for the experiments in 0.1 M acetic acid.

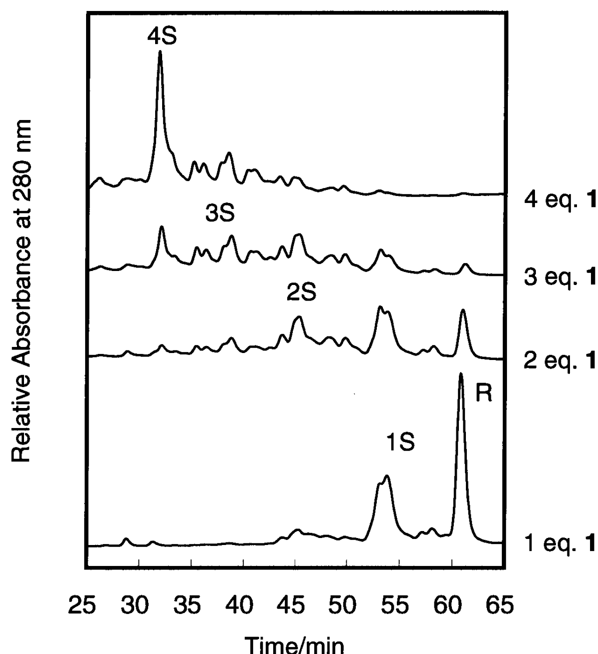


Figure 1. Series of chromatograms for the regeneration of RNase A using **1** as an oxidizing agent in 0.1 M AcOH (pH 3.1) at room temperature. The initial concentration of RNase A (R) was 20 μM. Regeneration time was 1 min.

Since the blocking of each SH residue by AEMTS introduces one positive charge in the polypeptide chain,[9] the folding intermediates with a different number of SS bonds could be separated by the cation-exchange column according to their net charges. Therefore, the groups of intermediates observed in Figure 1 were reasonably assigned to 4S, 3S, 2S, and 1S from the earlier eluants.[8] It is clearly seen that **1** almost quantitatively reacted with R to produce 1S, 2S, 3S, and 4S intermediates as the amounts of **1** increased. Similar results were obtained for the experiments at higher pH's, although the peak profiles of

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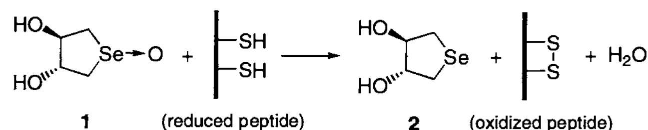
1S, 2S, 3S, and 4S were different from those shown in Figure 1. Consequently, it was found that **1** is a powerful quantitative reagent for the formation of the peptide SS bonds in a wide range of pH (Scheme 2), although the quantitative formation of **2** could not be detected experimentally under the regeneration conditions.



Scheme 2.

The HPLC chromatogram for the regeneration of RNase A with 4 molar equivalent **1** (the top chart in Figure 1) shows that there are still some amounts of 3S in the solution, suggesting that, strictly speaking, the reaction of Scheme 2 is not quantitative. However, since the regeneration intermediates of RNase A are considerably disordered,[8] some of the 3S intermediates may have the two remaining SH residues spatially separated. This would prohibit the formation of the fourth SS bond significantly. The other possibility is that some by-products might be formed during the regeneration experiments. The 4S intermediates may also consist of a number of structures with various SS bond combinations. To refold them back to the native structure, reduction followed by reshuffling of the SS bonds is necessary.

By comparing the oxidizing ability of **1** for the peptide SS bonds with that of oxidized DTT, two important features of **1** are obvious. First, the reaction of **1** with peptide SH residues is rapid and almost quantitative. Even in the presence of excess amounts of **2**, the backward reaction did not proceed in 0.1 M acetic acid. Therefore, the SS bond formation may be irreversible under the conditions. This is in sharp contrast with oxidized DTT, which reacts with peptide SH residues slowly and reversibly.[8] Second, the reaction of **1** with peptide SH residues (Scheme 2) rapidly proceeds not only under basic conditions but also under acidic ones. This feature is important because the reaction of oxidized DTT with peptide SH residues does not proceed at low pH.[3]

Barany et al. recently developed novel solid-phase reagents for facile formation of peptide SS bonds:[4] the polymer support enabled efficient formation of peptide SS bonds at pH 2.7 to 6.6 although the active site of the reagents includes an SS functional group like oxidized DTT. However, the rate of SS bond formation was much faster for **1** than that for Barany's reagents. The widely applicable pH range of **1** as well as its speed in the formation of the SS bonds will be useful for controlling the redox states of proteins under various pH conditions.

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5 *trans*-3,4-Dihydroxy-1-selenolane oxide (**1**). To the CH_2Cl_2 solution of **2**[7] (0.33 mmol), the CH_2Cl_2 solution of *m*-chloroperbenzoic acid (0.33 mmol) was added. Precipitated white crystals were collected by filtration and were purified by recrystallization from MeOH. Yield, 69%. Colorless cryst. mp 124–125 °C. 500 MHz ^{1}H NMR (in MeOH-d_4), δ 2.87 (d, 1H), 3.08 (dd, 1H), 3.58 (dd, 1H), 3.78 (dd, 1H), 4.63 (m, 1H), 4.72 (dd, 1H), 4.80 (s, 2H). 125.65 MHz ^{13}C NMR, δ 55.0, 58.0, 79.1, 79.5. 95.35 MHz ^{77}Se NMR, δ 941.8. Anal. Found: C, 26.16; H, 4.17%. Calcd for $C_4H_8O_3Se$: C, 26.24; H, 4.41%.

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7 *trans*-3,4-Dihydroxy-1-selenolane (**2**). To the freshly prepared aqueous solution of NaHSe[11] (19 mmol), 1,3-butadiene diepoxide (16 mmol) was added. The mixture was stirred for 10 min at room temperature under nitrogen atmosphere and then overnight under air. The resulting mixture was filtered. **2** was obtained from the filtrate by continuous extraction with ether and then by silica gel column chromatography (ether) or recrystallization from $CHCl_3$. Yield, 55%. Colorless cryst mp 79–80 °C. 500 MHz ^{1}H NMR (in $CDCl_3$), δ 2.10 (d, 2H), 2.87 (dd, 2H), 3.16 (dd, 2H), 4.27 (m, 2H). 125.65 MHz ^{13}C NMR, δ 26.8, 78.9. 95.35 MHz ^{77}Se NMR, δ 65.6. Anal. Found: C, 28.55; H, 4.70. Calcd for $C_4H_8O_2Se$: C, 28.76; H, 4.83%.

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10 A Hewlett Packard HP1100 Series HPLC system equipped with a 5-mL injection loop and a TOSOH TSK-gel SP-5PW strong cation exchange column (7.5 × 75 mm) was employed. The system was equilibrated with a 90:10 mixture solution of buffer A (25 mM HEPES, 1 mM EDTA, pH 7.0) and buffer B (buffer A with 0.5 M Na_2SO_4) at the flow rate of 0.5 mL/min. After sample injection, a salt gradient was applied by changing the ratio of buffer B from 10 to 45% in 50 min.

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