

DISULFIDE GROUPS

Disulfide bonds in proteins are subject to cleavage by mild reducing agents (Section 8-1), by a few oxidizing agents (Section 8-6), or by nucleophilic displacement (Section 8-2). Of the procedures for cleavage, those involving reduction are probably the mildest and most specific. Many smaller proteins when reduced retain the capacity to correctly refold and reoxidize under appropriate conditions. Complete reduction usually requires the presence of urea or another denaturant; limited and selective cleavage of a few disulfide bonds can frequently be obtained in the absence of such agents. Procedures have been described for limited reduction of disulfide bonds in proteins using dithiothreitol (Bewley and Li, 1969; Liu and MeiEnhofer, 1968; Sperling et al., 1969), sodium borohydride (Light and Sinha, 1967; Kress and Laskowski, 1967), sodium phosphorothioate (Neumann et al., 1967), and β -mercaptoethanol (Shapira and Arnon, 1969). Complete reduction of disulfide bonds is usually accomplished in 6 *M* guanidine/HCl or 8 *M* urea using a procedure such as that described by Anfinsen and Haber (1961) for the reduction and reoxidation of pancreatic ribonuclease.

Reduction. In a typical experiment, 350 mg of ribonuclease were dissolved in 10 ml of a freshly prepared 8 *M* solution of recrystallized urea, adjusted to pH 8.6 with 5% methylamine. Mercaptoethanol was added at a level of 1 μ l per mg of protein, the container was flushed with nitrogen, and the solution was allowed to stand for 4 1/2 hours at room temperature. Methylamine was employed for neutralization to insure the decomposition of any thioglycollides that might be present in the reducing agent. After this period, the pH was adjusted to 3.5 with glacial acetic acid and the entire solution was applied to a column of Sephadex G-25, equilibrated with 0.1 *M* acetic acid. The column was developed with the same solvent. Titrations with *p*-mercuribenzoate (Sections 10-2 and A-2) can be used to quantitate the extent of reaction. The same procedure has also been applied to trypsinogen, chymotrypsinogen, and lysozyme, giving fully reduced products that were soluble in the dilute acetic acid solvent.

Conversion of sulfhydryl groups in reduced ribonuclease to disulfide bonds is extremely slow at the hydrogen-ion concentration of the acetic acid solution employed for the Sephadex columns. At room temperature and pH 3.0, a sample of protein containing 7.8 sulfhydryl groups per mole contained, after 24, 72, and 97 hours of exposure to atmospheric oxygen, respectively, 7.5, 6.9, and 4.5 sulfhydryl groups per mole. At icebox temperatures, the sulfhydryl group content is undiminished for two to three days.

Reoxidation. High yields of enzymatically active material and the absence of precipitation is achieved when oxidation is carried out at low protein concentrations and when conditions of surface denaturation (e.g., bubbling with air) are avoided. Efficient oxidation occurs when dilute solutions, adjusted to pH 8.0 to 8.5, are allowed simply to stand in open vessels at room temperature for approximately 20 hours. Under these

conditions, the yields of regenerated, active ribonuclease have been uniformly between 80% and 100%.

Sulfite. Sulfite reacts with disulfide bonds of proteins cleaving them and giving 1 equivalent each of S-sulfonate and a sulfhydryl group. In a dissociating medium (i.e., in high concentrations of urea or guanidine/HCl), and in the presence of an oxidizing agent, the reaction can proceed until all half-cystine residues are converted into S-sulfonates. Complete sulfitolysis can be obtained by stepwise addition of an oxidant such as iodosobenzoate (Section 8-4) or more often in a continuous fashion using cupric ion as the oxidative agent (Swan, 1957). The procedure taken from Cole (1967) is as follows:

To 1 g of protein in 50 ml of water, add 0.5 M NH₄OH to pH 9.0. Add to this 1.0 mmole of CuSO₄ dissolved in 40 ml of water adjusted to pH 9.0, followed by 10 ml of 0.7 M Na₂SO₃. Allow to stand for 2 hours at room temperature and then dialyze against 0.1 M sodium citrate pH 7.0 at 4°C. When all the cupric ion has been removed, dialyze against water to remove other electrolytes. While such a procedure gives complete cleavage of the disulfide bonds in α-lactalbumin and β-lactoglobulin most proteins will require 8 M urea or another denaturing medium in place of water.

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