Reagents for Rapid Reduction of Disulfide Bonds in Proteins

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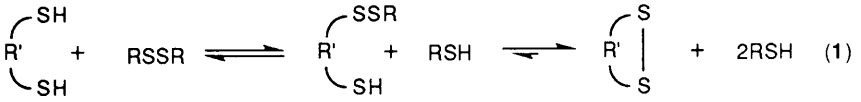
I. Introduction

Disulfide-reducing reagents are routinely used in biochemical manipulations for (i) reducing the native disulfide bonds in proteins and (ii) maintaining the essential thiol groups in proteins by preventing their oxidation to the disulfide state. Dithiothreitol (DTT) is the most popular disulfide-reducing reagent (1). DTT is, however, slow in reducing disulfides at pH 7-8. The value of pK_a of the thiol groups in DTT is high (9.2) and therefore at pH 7 only a small fraction (~1%) of thiol groups in DTT are present in the reactive thiolate form.

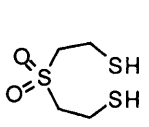
We have developed several new dithiol reagents for rapid reduction of disulfide groups (2-6). These dithiol reagents reduce disulfide bonds by the mechanism of thiol-disulfide interchange (Eq 1).



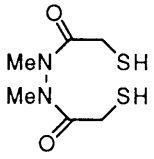
The design of these dithiol reagents is based on two requirements: (i) a low value of pK_a (~7 to 8) of their thiol groups and (ii) a high reduction potential. The reactivity of a thiol is influenced

both by its fraction present in the thiolate form and by the nucleophilicity of the thiolate anion. A thiol group of low pK_a has a significant fraction present in the reactive thiolate form, but the nucleophilicity of its thiolate anion is lower than it is for a thiol of higher pK_a. The overall effect is that the apparent rate of thiol-disulfide interchange is maximum for a thiol whose pK_a value is approximately equal to the pH of the solution (3). A dithiol reagent whose thiol groups have pK_a values of ~7 to 8 and which has a high reduction potential is therefore expected to reduce disulfide bonds rapidly at pH 7-8.

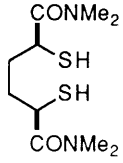
We have developed several new reagents [N,N'-dimethyl-N,N'-bis(mercaptoacetyl)hydrazine (DMH), bis(2-mercaptoethyl)sulfone (BMS) and *meso*-2,5-dimercapto-N,N,N',N'-tetramethyladipamide (DTA)] whose thiol groups have pK_a values of ~7.8 (2-6). Based on Brønsted correlations these reagents are expected to reduce disulfide groups at pH 7 faster than DTT by a factor of ~5 (3,5). This report focuses on the comparison of reactivities of BMS, DMH, and DTT toward disulfide groups in several proteins under nondenaturing conditions at pH 7.



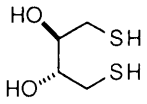
BMS



DMH



DTA



DTT

II. Materials and Methods

BMS and DTA are available from US Biochemical Corporation. The synthesis of BMS, DMH and DTA are straightforward from readily available materials (4-6). Papain-S-SCH_3 was prepared as described before (7). Trypsinogen (bovine pancreas) and α-chymotrypsinogen A (bovine pancreas) were purchased from

Sigma. The murine monoclonal antibody anti-B4 (IgG_1) was purified from hybridoma culture supernatants.

BMS, DMH and DTA are solids at room temperature. We recommend that their stock solutions (~10 mM) in phosphate buffer (50 mM sodium phosphate, pH 7, 1 mM in EDTA) be prepared fresh by brief sonication to ensure complete solubilization. These solutions can be assayed for thiol groups by Ellman's assay (8).

A. Reduction of Papain-S-SCH_3 Using BMS, DMH and DTT

Samples of papain-S-SCH_3 (0.042 mg/mL, 1.8 μM) in deoxygenated 50 mM sodium phosphate buffer (pH 7, 2 mM in EDTA) were reduced using dithiol reagent (25 μM; BMS, DMH or DTT) at 23°C. At several time intervals (1-, 6-, 11-, 16-, and 21-min), aliquots (200 μL) of the reaction mixture were added to substrate (800 μL of 3.4 mM N-benzoyl-L-arginine-*p*-nitroanilide in 50 mM bis-tris buffer, pH 6.3, containing 1 mM EDTA and 5% v/v DMSO) and the rates of increase in absorbance at 410 nm were measured. The concentration of dithiol was in excess over that of papain-S-SCH_3, and was therefore assumed to be constant during the course of reduction; the kinetics is therefore pseudounimolecular. For the reduction by DTT, the apparent rate constant (k_{app}) was calculated from the plot of -ln[{(maximum regenerated papain activity)-(regenerated papain activity)}/(maximum regenerated papain activity)] *vs* time, for which slope = k_{app}[Dithiol]. For the reductions using BMS and DMH, the regenerated papain activity was measured at 1 min in four separate experiments, and k_{app} was calculated using the rate equation: -ln[{(maximum regenerated papain activity)-(regenerated papain activity)}/(maximum regenerated papain activity)] = k_{app}[Dithiol]t.

B. Reduction of Trypsinogen Using Dithiol

Samples of trypsinogen (5 mg/mL, 0.21 mM) in 50 mM sodium phosphate buffer (pH 7, 1 mM in EDTA) on ice

(0°C) were reduced using dithiol (0.5 mM; BMS, DMH, DTT). At 10-, 20-, 30-, and 200-min time intervals, aliquots (200 μL) of the reaction mixture were purified by gel-filtration, and were analyzed for thiol content using Ellman's assay and for protein concentration by measuring absorbance at 280 nm (2). Under these conditions a maximum of 0.6 disulfide residue was reduced per trypsinogen molecule. Assuming pseudounimolecular kinetics, the apparent rate constant (k_{app}) was calculated from the plot for -ln([remaining disulfide]/[maximum reducible disulfide]) *vs* time, for which slope = k_{app}[Dithiol].

C. Reduction of α-Chymotrypsinogen A Using Dithiol

Samples of α-Chymotrypsinogen A (6.8 mg/mL, 0.27 mM) in 50 mM sodium phosphate buffer (pH 7, 1 mM in EDTA) at room temperature were reduced using 4.8 mM dithiol. Under these reaction conditions a maximum of 0.75 disulfide residue per α-Chymotrypsinogen A molecule was reduced (2). The analysis for reduction of α-Chymotrypsinogen A was similar to that for trypsinogen.

D. SDS-PAGE Analysis of Reduction of Immunoglobulin by Dithiol

Samples of a murine immunoglobulin (IgG_1, 6.3 mg/mL) in 50 mM sodium phosphate buffer (pH 7, 0.5 mM in EDTA) were reduced using dithiol (BMS, DMH, DTT; 4.8 mM). At several time intervals, aliquots (25 μL) of the reaction mixture were quenched using iodoacetamide (250 μL of a 0.3 M iodoacetamide solution in 50 mM sodium phosphate buffer, pH 7, 1 mM in EDTA), and analyzed by 4-12% gradient SDS-PAGE under nonreducing conditions (2).

Table I. Comparisons of Rate Constants for Reduction of Disulfide Bonds in Proteins Using Dithiol Reagents (DTT, BMS, DMH)[1]

Protein	Reduction Conditions	k_{DTT}	$\frac{k_{BMS}}{k_{DTT}}$	$\frac{k_{DMH}}{k_{DTT}}$
Trypsinogen	pH 7, 0°C	8 M^{-1} min^{-1}	7.7	6.6
α-Chymotrypsinogen A	pH 7, 28°C	12 M^{-1} min^{-1}	2.3	-
	pH 7, 26°C	9 M^{-1} min^{-1}	-	2.3
Papain-S-SCH_3	pH 7, 23°C	2700 M^{-1} min^{-1}	10	25

[1]Rate constants (k) are apparent rate constants based on total dithiol concentration. The calculations of rate constants are described in Methods section. The rate constants for trypsinogen and α-chymotrypsinogen A are from reference 2.

III. Results and Discussion

Table I shows a comparison of the apparent rate constants for the reduction of disulfide bonds in proteins using BMS, DMH and DTT. BMS and DMH reduce the disulfide bonds in proteins at pH 7 significantly faster than does DTT.

The disulfide bond in trypsinogen is reduced more rapidly using BMS and DMH than using DTT by a factor of ~7 (Table I). The rate of reduction of trypsinogen by BMS is ~20% faster than by DMH (Figure 1). A maximum of 0.6 disulfide residues were reduced (i.e. 1.2 thiol residues were formed) per trypsinogen molecule under these reaction conditions. A selective cleavage of 179-203 disulfide bond in trypsinogen has been reported under similar conditions of reduction (0.5 mM dithioerythritol, 0°C, pH 8.5; Ref. 9).

The disulfide bond in α-chymotrypsinogen A is reduced about 2.3-fold faster using BMS and DMH than by DTT (Table I). A maximum of 0.75 disulfide group per α-chymotrypsinogen A molecule was reduced under the reduction conditions. The apparent rate constant for the reduction of disulfide bond in

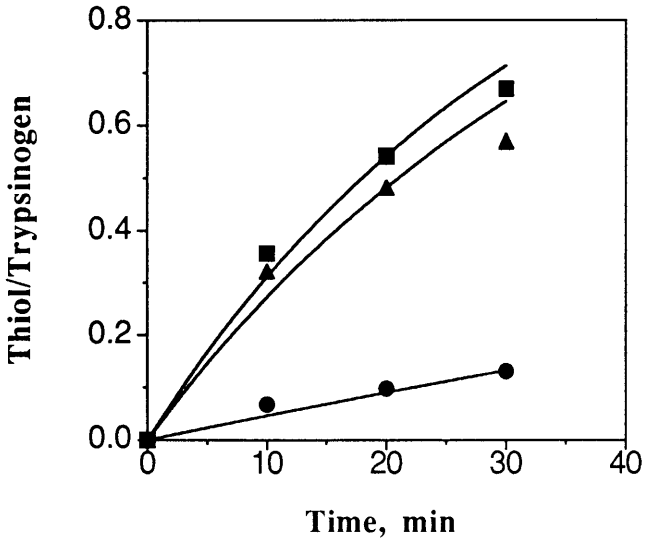


Figure 1. Reduction of Trypsinogen using dithiols [DTT (●), BMS (■), and DMH (▲)]. Trypsinogen (5 mg/mL, 0.21 mM) in 50 mM sodium phosphate buffer (pH 7.0, 1 mM in EDTA) was reduced using dithiol (0.5 mM) at 0°C. The curves plotted are based on the values of apparent rate constants shown in Table I.

α-chymotrypsinogen A by DTT at 26°C is similar to that for reduction of trypsinogen at 0°C (Table I). It is therefore predicted that the rate of cleavage of disulfide bond in α-chymotrypsinogen A would be significanly slower than that for trypsinogen at the same temperature. The 191-220 disulfide bond in α-chymotrypsinogen A is reported to be less accessible than the analogous 179-203 disulfide bond in trypsinogen (9).

The reactive disulfide bond in papain-S-SCH_3 is reduced especially rapidly by DMH (Figure 2, Table I). The rates of reduction of papain-$SSCH_3$ using DMH and BMS are faster than that using DTT by factors of 25 and 10 respectively (Table I). The thiol group in papain has a low pK_a (~4) and is essential for its activity. The inactive mixed disulfide of papain (papain-S-SCH_3) is reactivated completely within 5 min using small concentrations of DMH and BMS (Figure 2).

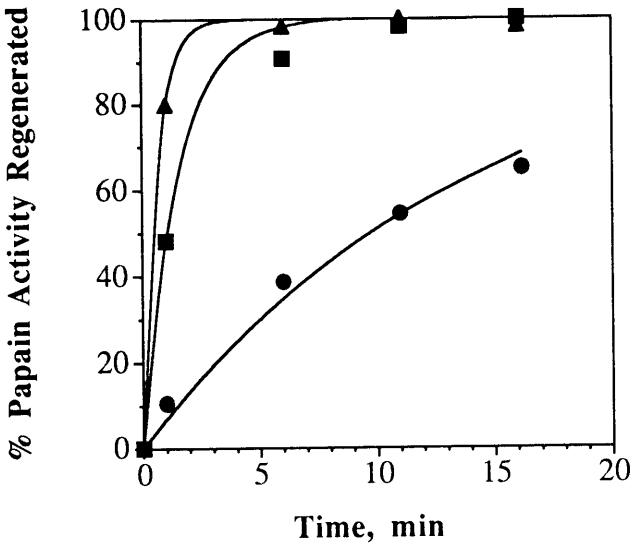


Figure 2. Regeneration of activity of papain from papain-S-SCH_3 using dithiols [DTT (●), BMS (■), and DMH (▲)]. Papain-S-SCH_3 (0.042 mg/mL, 1.8 μM) in 50 mM sodium phosphate buffer (pH 7, 2 mM in EDTA) at 23°C was reduced using dithiol (25 μM). At several time intervals aliquots of reaction mixtures were added to substrate solution and the activities of papain were measured. The curves plotted are based on the values of apparent rate constants shown in Table 1.

The disulfide bonds in immunoglobulin (IgG_1) are reduced ~5-fold faster using DMH and BMS than using DTT (2). Murine IgG_1 contains two heavy chains and two light chains; the two heavy chains are linked to each other by two disulfide bonds, and each heavy chain is linked to a light chain by a disulfide bond (10). SDS-PAGE analysis of iodoacetamide-quenched reaction mixtures of IgG_1 and dithiols shows that the immunoglobulin molecule is cleaved significantly faster using DMH and BMS than using DTT (2).

IV. Conclusions

Both BMS and DMH reduce disulfide bonds in proteins at pH 7 faster than does DTT by a factor of ~5-7 in

nondenaturing conditions. Although the typical rate enhancements expected from using BMS and DMH over that using DTT are ~5 based on Brønsted correlations, variations are seen for some proteins: the relatively less accessible disulfide bond in α-chymotrypsinogen A is reduced 2.3-fold faster using BMS and DMH than using DTT; the highly reactive disulfide bond in papain-S-SCH_3 is reduced faster using DMH than using DTT by a factor of 25. The values of equilibrium constants for the reduction of bis(2-hydroxyethyl) disulfide (Eq 1) for BMS, DMH and DTT are 60 M, 2 M and 180 M respectively (4,5,11). BMS is therefore more reducing than DMH and slightly less reducing than DTT. All these dithiols (BMS, DMH, DTT) have significantly high reduction potentials and reduce noncyclic disulfides completely. Although both BMS and DMH reduce disulfides at similar rates, we recommend the use of BMS because it is commercially available, it is odorless and it has a high reduction potential.

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