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Reagents for Rapid Reduction of Native Disulfide Bonds in Proteins

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Bis(2-mercaptoethyl)sulfone (BMS) and *N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH) reduce native disulfide bonds in proteins at pH 7 significantly faster than does dithiothreitol (DTT). The accessible disulfide bonds in immunoglobulin and trypsinogen are reduced under nondenaturing conditions at pH 7 faster using BMS and DMH than using DTT by a factor of ~5 to 7. The relatively less accessible disulfide bond in α-chymotrypsinogen A is also reduced faster using BMS and DMH than using DTT by a factor of 2.3. Although both BMS and DMH reduce disulfides at similar rates, we recommend BMS because it is commercially available and has superior physical characteristics and a higher reduction potential than DMH.

INTRODUCTION

Reagents that reduce disulfide groups are useful for the purpose of (i) reducing native disulfide groups in proteins and (ii) maintaining thiol groups in solution by preventing their oxidation to the disulfide state (*1–3*). Dithiothreitol (DTT) is the most popular reagent used presently in biochemistry for the reduction of disulfide groups (*4*). DTT is, however, slow in reducing disulfide groups at pH 7 to 8, because of its high value of pK_a (9.2; Ref. *5*). The fraction of DTT present in the reactive thiolate form is only 0.6% at pH 7 and only 6% at pH 8.

The primary considerations in designing a dithiol reagent that can reduce disulfide bonds rapidly are its reactivity and its reduction potential. The reactivity of a thiol toward a disulfide is influenced both by its fraction present in the reactive thiolate form and by the nucleophilicity of the thiolate anion. A thiol of low pK_a has a relatively large fraction of total thiol present as the reactive thiolate anion. The nucleophilicity of the thiolate anion is, however, lower for a thiol of lower pK_a than it is for one with high pK_a. In the reduction of a disulfide at a given pH, the maximum apparent rate of thiol–disulfide interchange is observed for a thiol whose value of pK_a is approximately equal to the value of pH of the solution (*6, 7*). The design of a reagent that can reduce disulfide bonds rapidly at pH 7–8

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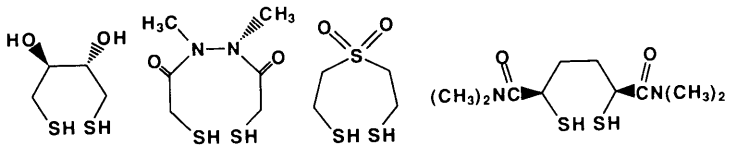

DTT DMH BMS DTA

therefore requires a dithiol group whose pK_a is ~7–8 and which has a sufficiently high redox potential to reduce protein disulfides quantitatively.

We have previously reported the synthesis of several dithiols [*N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH); meso-2,5-dimercapto-*N,N,N',N'*,-tetramethyladipamide (DTA); bis(2-mercaptoethyl) sulfone (BMS); Refs. *8–10*] that have lower values of pK_a (~7.8) than DTT (9.2). Based on a Brønsted correlation obtained using kinetic data from an extensive series of thiol–disulfide interchange reactions, we expected the apparent rates of reduction of a disulfide by these dithiols (DMH, DTA, and BMS) to be faster than that by DTT by a factor of ~5 at pH 7 (*5, 8*). The observed rates of reduction of small organic disulfides [bis(2-hydroxyethyl) disulfide and oxidized glutathione] are faster using DMH and DTA by a factor of ~7 than those using DTT (*8, 9*). The kinetics of reduction of disulfide groups using BMS has not been studied previously. The rates of reduction of semisynthetic disulfide groups in enzymes (creatine kinase-*S*-*S*-glutathione, papain-*S*-SCH_3, and ficin-*S*-SCH_3) using dithiol reagents decrease in the order DMH > DTA > DTT (*8, 9*). The rate of reduction of the semisynthetic disulfide groups in proteins using DTA is slower than expected (although it is faster than that with DTT), perhaps due to the steric hindrance in the attack of the secondary thiol group of DTA on the disulfide groups in these proteins. A direct examination of the efficacy of these reagents in reducing the native disulfide bonds in proteins is therefore needed for selection of the most useful reducing reagent.

In this study we have compared the rates of reduction of *native* disulfide groups in proteins using BMS, DMH, and DTT. The proteins chosen for this study—immunoglobulin (IgG_1), trypsinogen, and α-chymotrypsinogen A—have accessible disulfide groups that can be reduced in nondenaturing conditions (*11*).

MATERIALS AND METHODS

Materials. 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 by affinity chromatography on Protein A followed by ion-exchange chromatography. Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)] was purchased from Aldrich. DTT was from Fisher Biotech. DMH and BMS were synthesized as described (*8, 10*). BMS and DTA are available from US Biochemical Corporation (Cleveland, OH). Sephadex G-25 (fine) was purchased from Pharmacia. Precast 4–12 and 12% polyacrylamide gels were purchased from Novex.

SDS–PAGE analysis of reduction of immunoglobulin by DTT, BMS, and DMH. Samples of a murine IgG_1 antibody (6.3 mg/ml; M_r 160,000) were incubated with dithiol (4.8 mM; DTT, BMS, or DMH) at 22°C in 50 mM sodium phosphate buffer, pH 7.0, containing NaCl (25 mM) and EDTA (0.5 mM). Aliquots (25 μl) of the reaction mixture were removed at 3-min, 6-min, 11-min, and 1-h intervals and were quenched using iodoacetamide (250 μl of a 0.3 M iodoacetamide solution in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA). SDS–PAGE of the quenched reaction mixtures was performed under nonreducing conditions on a 4–12% gradient gel using Coomassie blue for staining (Fig. 1).

Reduction of trypsinogen using DTT, BMS, and DMH. Samples containing trypsinogen (5 mg/ml) were reduced with dithiol (0.5 mM; DTT, BMS, or DMH) in 50 mM sodium phosphate buffer, pH 7, containing EDTA (1 mM) on ice (0°C). At 10-, 20-, 30-, and 200-min intervals, aliquots (200 μl) of the reaction mixture were gel filtered on a 2-ml Sephadex (fine) column using 100 mM sodium acetate buffer, pH 4.5, containing EDTA (0.5 mM). The eluted fraction containing protein was assayed for protein concentration by measuring absorbance at 280 nm ($\varepsilon_{280\ nm}$ = 1.39 mg^{-1} ml cm^{-1}; $M_{r[trypsinogen]}$ 24,000; Ref. *12*) and for thiol concentration using Ellman's assay (*13, 14*). In a typical assay, 450 μl of the eluted sample was added to 550 μl of a 500 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM), and the absorbance at 280 nm was measured; Ellman's reagent (10 μl of a 100 mM solution in DMSO) was then added and the absorbance at 412 nm was measured.

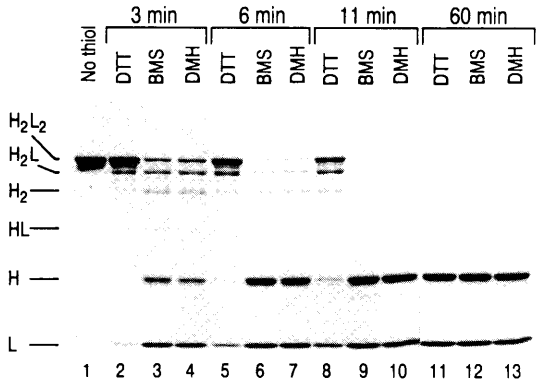

FIG. 1. Reduction of the disulfide bonds in Immunoglobulin (IgG_1) using dithiols (DTT, BMS, and DMH). A sample of murine IgG_1 (6.3 mg/ml; M_r 160,000) was reduced using dithiol (4.8 mM) at 22°C in 50 mM sodium phosphate buffer, pH 7.0, containing EDTA (0.5 mM). Aliquots of the reaction mixture were quenched at several time intervals using iodoacetamide; the products were separated on a 4–12% gradient SDS–PAGE under nonreducing conditions and were stained using Coomassie blue. Lane 1 contains unreduced IgG_1. Lanes 2–4 contain samples of IgG_1 reduced with DTT, BMS, and DMH, respectively, for 3 min. Lanes 5–7 contain samples of IgG_1 reduced with DTT, BMS, and DMH, respectively, for 6 min. Lanes 8–10 contain samples of IgG_1 reduced with DTT, BMS, and DMH, respectively, for 11 min. Lanes 11–13 contain samples of IgG_1 reduced with DTT, BMS, and DMH, respectively, for 60 min. All lanes contain 5.7 μg protein.

Samples of α-chymotrypsinogen A (6.8 mg/ml) were reduced with dithiol (4.8 mM; DTT, BMS, or DMH) in 50 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM) at room temperature. At 5-min, 10-min, 15-min, and 3-h intervals, aliquots were gel filtered and analyzed as described above ($\varepsilon_{280\ nm}$ = 2.0 mg^{-1} ml cm^{-1}; $M_{r[chymotrypsinogen]}$ 25,000; Ref. *15*).

RESULTS AND DISCUSSION

The rates of reduction of native disulfide bonds in immunoglobulin, trypsinogen, and α-chymotrypsinogen A were compared using DTT, DMH, and BMS under nondenaturing conditions at pH 7. The observed rate constants for the reduction of trypsinogen and α-chymotrypsinogen A are listed in Table 1. The relative rates of reduction of the disulfide bonds in immunoglobulin and trypsinogen using dithiols are shown in Figs. 1 and 2, respectively.

The rate of reduction of disulfide bonds in immunoglobulin (IgG_1) using DMH and BMS is significantly faster than that using DTT (Fig. 1). Immunoglobulin

TABLE 1

Comparison of Observed Rate Constants for Reduction of Disulfide Bonds in Proteins Using Dithiols (DTT, BMS, and DMH)

Protein	Reduction Conditions	k_{app} (M^{-1} min^{-1})[a]		
		DTT	BMS	DMH
Trypsinogen	0 °C, pH 7.0[b]	8	60	51
α-Chymotrypsinogen A	28 °C, pH 7.0[c]	12	29	-
	26 °C, pH 7.0[c]	9	-	21

[a] The observed rate constant (k_{app}) was determined from the linear plot of −ln [(remaining disulfide)/(maximum reducible disulfide)] vs time, for which slope = k_{app}(dithiol). The kinetics was assumed as pseudounimolecular because the concentration of the dithiol was in significant excess over that of the protein disulfide, and the decrease in concentration of the dithiol during the course of reduction was less than 10% of its initial concentration. k_{app} is based on total thiol concentration.

[b] Trypsinogen (5 mg/ml, 0.21 mM; M_r 24,000) was reduced using 0.5 mM dithiol at 0°C in 50 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM). Under these reaction conditions, ~0.6 disulfide residue per trypsinogen molecule was reduced after 200 min.

[c] α-Chymotrypsinogen A (6.8 mg/ml, 0.27 mM; M_r 25,000) was reduced using 4.8 mM dithiol at room temperature in 50 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM). Under these reaction conditions, ~0.75 disulfide residue per chymotrypsinogen was reduced after 3 h.

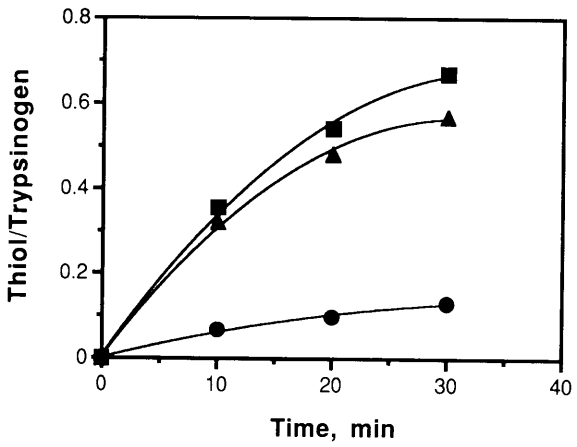

FIG. 2. Reduction of trypsinogen using dithiols [DTT (●), BMS (■), and DMH (▲)]. Trypsinogen (5 mg/ml) was reduced using dithiol (0.5 mM) at 0°C in 50 mM sodium phosphate buffer, pH 7.0, containing EDTA (1 mM). Aliquots of the reaction mixture were gel filtered at several time intervals and were analyzed for protein concentration (based on absorbance at 280 nm) and for thiol content (based on Ellman's assay). The values of average thiol residue per protein molecule were plotted vs time. The rate constants were calculated as described in the note to Table 1.

(H_2L_2) has two heavy and two light chains of M_r 60,000 and 23,000, respectively. The two heavy chains (H) are connected to each other by two disulfide bonds, and each heavy chain is linked to a light chain (L) by a single disulfide bond (*16*). The sequential reduction of the disulfide bonds in immunoglobulin by dithiol results in formation of cleavage products H_2L, H_2, HL, H, and L. The extent of reduction of immunoglobulin after 60 min using DTT is similar to that obtained in 11 min using DMH or BMS (Fig. 1). The reduction of the disulfide bonds in immunoglobulin using either DMH or BMS is faster than that using DTT by a factor of ~5.

Although both DMH and BMS reduce the disulfide bonds in immunoglobulin rapidly, the reduction is more complete using BMS than using DMH: traces of H_2 and HL bands are observed after 11 min and 60 min in the reduction using DMH but not in the reduction using BMS. The complete cleavage of the H_2 and HL molecules requires dithiols (such as DTT and BMS) of higher redox potential than that of DMH, presumably because of the noncovalent interactions between the two heavy chains and between each heavy and light chain. The values of the equilibrium constants for the reduction of bis(2-hydroxyethyl) disulfide using DTT, BMS, and DMH are 180, 63, and 2.5 M, respectively; both DTT and BMS are therefore more reducing than DMH (*17*).

The observed rate constants for reduction of trypsinogen using BMS and DMH are higher than that using DTT by a factor of ~6–7 (Table 1, Fig. 2). A plot of the thiol residues generated per trypsinogen molecule vs time (Fig. 2) shows that the rate of reduction by BMS was ~20% higher than that by DMH. After reduction

of trypsinogen using BMS (0.5 mM; 0°C, pH 7) for 200 min, ~1.2 thiol residues were formed (i.e., ~0.6 disulfide residues were reduced) per trypsinogen molecule. Trypsinogen has six disulfide residues, and a selective cleavage of the 179–203 disulfide bond in trypsinogen has been reported under similar conditions of reduction (0.5 mM dithioerythritol, 0°C, pH 8.5; Ref. *18*). A second disulfide bond at residues 122–189 is reported to be cleaved at higher concentrations of the reducing dithiol (10 mM dithioerythritol, 0°C, pH 8.5; Ref. *18*). Our SDS–PAGE analysis of the iodoacetamide-quenched reaction mixtures of trypsinogen and BMS, using a 12% gel, shows no shift in the mobility of the trypsinogen band after limited reduction of a single disulfide bond (0.5 mM BMS, 0°C, 200 min); a shift to a lower mobility is, however, observed after reduction of two disulfide bonds (5 mM BMS, 23°C, 20 min). On complete reduction of the disulfide bonds (with β-mercaptoethanol, SDS–PAGE under reducing conditions) the mobility of the trypsinogen band is lowered even further than that after reduction of two disulfide bonds (data not shown).

The observed rate constants for the reduction of disulfide bond in α-chymotrypsinogen A using BMS and DMH are higher than that using DTT by a factor of 2.3 (Table 1). A maximum of ~0.75 disulfide residues per chymotrypsinogen molecule are reduced after 3 h (4.8 mM BMS, pH 7.0, room temperature). α-Chymotrypsinogen A contains five disulfide bonds, but the selective cleavage of only one disulfide bond at residues 191–220 is reported under similar conditions (10 mM dithioerythritol, pH 8.5, 0°C; Ref. *18*). Our SDS–PAGE analysis of the iodoacetamide-quenched reaction mixtures of chymotrypsinogen and dithiols, using a 12% gel, shows a small gradual shift of the chymotrypsinogen band toward lower mobility with increase in the time of reduction (data not shown).

Despite 40% amino acid homology between trypsinogen and α-chymotrypsinogen A, their disulfide bonds differ in reactivity toward disulfide-reducing reagents (dithioerythritol and sodium borohydride): the 191–220 disulfide bond in chymotrypsinogen is reported to be less accessible than the analogous 179–203 disulfide bond in trypsinogen; the 136–201 disulfide bond in chymotrypsinogen is unreactive, although the analogous 122–189 bond in trypsinogen can be reduced under nondenaturing conditions (*18*). We observe that the rate constant for reduction of the disulfide bond in chymotrypsinogen by DTT at *room temperature* is similar to that of trypsinogen at *0°C* (Table 1). The rate of reduction of chymotrypsinogen is therefore predicted to be significantly slower than that for trypsinogen at the same temperature. The rate of cleavage of the disulfide bond in chymotrypsinogen increases by a factor of 2.3 in going from DTT to BMS or DMH, whereas the corresponding increase for trypsinogen is ~7 (Table 1). We do not understand the correlation between the lower accessibility of the disulfide bond in chymotrypsinogen and the smaller than expected rate enhancement in its reduction in going from DTT to BMS (or to DMH).

CONCLUSIONS

Both BMS and DMH reduce the native disulfide groups in proteins faster than does DTT by a factor of ~5–7 in nondenaturing conditions. We believe that the

disulfide groups in proteins can be rapidly and selectively reduced using low concentrations of BMS and DMH at low pH (~7), and the reduced thiol groups can be modified in the same reaction mixture using a small excess of a modifying reagent. We recommend the use of BMS as a reducing agent for four reasons: (i) BMS is commercially available and is less expensive to synthesize than DMH; (ii) BMS has no odor, in contrast to both DTT and DMH, which have faint odors; (iii) BMS is a crystalline solid, in contrast to DMH that is a low-melting solid; (iv) BMS ($\varepsilon_0 = -0.313$ V) has a higher reduction potential than DMH ($\varepsilon_0 = -0.272$ V; Ref. *17*), and this reducing potential may be required for complete reduction of some disulfide bonds in protein that are stabilized by multiple noncovalent interactions.

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