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A Reagent for Reduction of Disulfide Bonds in Proteins That Reduces Disulfide Bonds Faster Than Does Dithiothreitol

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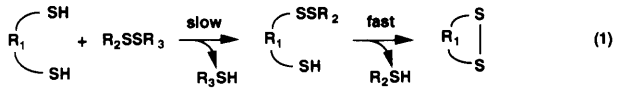
We have synthesized a new reagent—*N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH)—for the reduction of disulfide bonds in proteins. DMH reduces disulfide bonds 7 times faster than does dithiothreitol (DTT) in water at pH 7. DMH reduces mixed disulfides of cysteine proteases (papain and ficin) especially rapdily (30 times faster than DTT). DMH (ϵ° = −0.300 V) reduces noncyclic disulfides completely, although it is less strongly reducing than DTT (ϵ° = −0.356 V).

Introduction

This report describes the synthesis of *N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH) and the use of this reagent for reduction of disulfide bonds in water at pH ~7. Disulfide-reducing reagents are used in biochemistry for a number of purposes, especially in reduction of cystine groups in proteins and in maintaining essential thiol groups in reduced state.[1-3] The requirements for an optimal reducing reagent for cystine groups in proteins are as follows: (i) a reduction potential higher than the cystine group(s) to be reduced; (ii) a pK_a for thiol groups close to the pH of the solution in which the protein is to be manipulated; (iii) convenient physical properties (a crystalline solid with low odor and adequate solubility in water).[4,5]

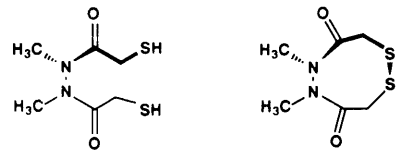
Thiol–disulfide interchange involves the nucleophilic attack of thiolate anion along the S–S bond axis of the disulfide.[5-9] The pK_a of simple alkanethiols in water is ~9–10; for these thiols, only a small fraction (1–0.1%) is present as thiolate at pH 7. The apparent rate of thiol–disulfide interchange is maximum when the pK_a of the thiol is close to the pH of the solution.[5,6] α,ω-Dithiols are more strongly reducing than monothiols, because the intramolecular reaction (the second step in eq 1) is faster



(1)

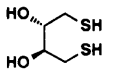
than the corresponding intermolecular reaction for monothiols. Among α,ω-dithiols, the dithiols that form cyclic six-membered disulfide are the most reducing, reflecting a balance between the thermodynamic stability of the CSSC dihedral angle and the entropy of formation of the ring from the acyclic reduced form.[6,10,11]

Dithiothreitol (DTT)[4] and β-mercaptoethanol (ME) are the favorite disulfide-reducing reagents in biochemistry.[1] DTT reduces noncyclic disulfides to thiols completely, and intermediate mixed disulfides are absent in any significant concentration (eq 1).[4] DTT has, however, some shortcomings: the value of its first thiol pK_a is 9.2,[6] and it is therefore relatively slow as a reducing reagent at pH ~7. It is expensive.[12] Its ability to chelate metals and generate H_2O_2 on exposure to air can cause problems.[13] Because mercaptoethanol is inexpensive, it can be used in large amounts (0.1–0.7 M) in biochemical manipulations (for example, in conjunction with SDS gel electrophoresis).[1] The pK_a of mercaptoethanol is 9.6.[6] The disadvantages of mercaptoethanol are that it is a weak reducing reagent and is foul-smelling. Because it is weakly reducing, it often generates complex reaction mixtures containing mixed disulfides.[6]

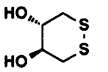


DMH

DMH^{ox}



DTT



DTT^{ox}

(1) Jocelyn, P. C. *Methods Enzymol.* **1987**, *143*, 246–256.
(2) Gilbert, H. F. *Adv. Enzymol.* **1990**, *63*, 69–172.
(3) Jocelyn, P. C. *Biochemistry of the SH Group*; Academic: London, 1972. Huxtable, R. J. *Biochemistry of Sulfur*; Plenum: New York, 1986.
(4) Cleland, W. W. *Biochemistry* **1964**, *3*, 480–482.
(5) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, *42*, 332–338.
(6) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, 102, 2011–2026.
(7) Shaked, Z.; Szajewski, R. P.; Whitesides, G. M. *Biochemistry* **1980**, *19*, 4156–4166.
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(9) Houk, J.; Singh, R.; Whitesides, G. M. *Methods Enzymol.* **1987**, *143*, 129–140.
(10) Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825–6836.
(11) Burns, J. A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1990**, *112*, 6296–6303.
(12) The cost of dithiothreitol (DTT) is $10/g (FW 154).
(13) Trotta, P. P.; Pinkus, L. M.; Meister, A. *J. Biol. Chem.* **1974**, *249*, 1915–1921. Costa, M.; Pecci, L.; Pensa, B.; Cannella, C. *Biochem. Biophys. Res. Commun.* **1977**, *78*, 596–603.

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Table I. Physical Characteristics of DMH, DTT, and ME

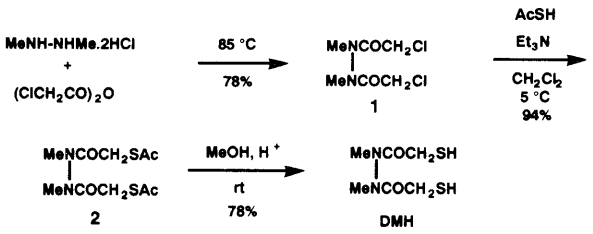
	DMH		DTT		ME	
	red	ox	red	ox	red	ox
mp (°C)	38	155	42	132		
pK_a	7.6, 8.9		9.2, 10.1		9.6	
$\epsilon°$ (V)	−0.300		−0.356		−0.209	
K^a	1200 M		10^5 M		1	
$K^{app\,b}$ (M^{-1} s^{-1})	67		10			
odor	weak	no	weak	no	strong	no
solubility[c] (mM)	250	23				

[a] The values of the equilibrium constant (K) are for the reduction of ME^{ox} by DMH and by DTT (see Table II). [b] The apparent rate constants (k^{app}) are for the reduction of the mixed disulfide of creatine phosphokinase and glutathione (see Table III). [c] The solubilities were determined in D_2O buffer (pD 7, 50 mM in phosphate, 25 °C). ME and DTT are very soluble in water.

We designed the new reagent DMH for use in reduction of S–S bonds in water at pH ~7 with the following expectations: (i) The first pK_a of its thiol groups would be ~7, and the rate at which the thiol would act as a nucleophile in thiol–disulfide interchange would be rapid. (ii) The steric bulk of the methyl groups on adjacent nitrogen atoms would force the two amide moieties into approximately perpendicular planes and thereby allow the formation of cyclic eight-membered disulfide having a thermodynamically stable CSSC dihedral angle of ~90°. (iii) The rigidity of the two sterically constrained amide units would limit the degrees of rotational freedom that would have to be frozen on going from dithiol to cyclic disulfide, and thus give a high reduction potential. We also anticipated (although we have not so far demonstrated experimentally) that the substituents at nitrogen could be changed to make the reagent more water soluble and to prepare chiral or polymer-linked reagents.

Results and Discussion

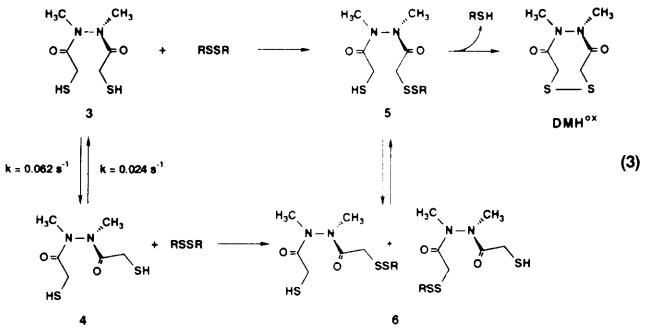
Synthesis. The synthesis of DMH from *N,N'*-dimethylhydrazine dihydrochloride is straightforward (eq 2). The steps involve commercially available reagents, mild reaction conditions, easy separations, and good yields. The most expensive reagent in the synthesis is *N,N'*-dimethylhydrazine dihydrochloride.[14]



(2)

Physical Characteristics. Table I compares the physical characteristics of DMH, DTT, and ME. DMH and DMH^{ox} are white, crystalline solids. DMH^{ox} is stable to heat and does not polymerize on heating at 155 °C for 20 min, in contrast to strained disulfides which polymerize easily on heating and some of which are polymeric at room temperature.[15] DMH^{ox} shows no UV absorption in 320–400-nm region, in contrast to the strained cyclic five-membered disulfides (λ_{max} = 330 nm).[16] We infer that DMH^{ox} is relatively unstrained. The aqueous solubility for DMH^{ox} is lower than for DTT^{ox} and for ME^{ox}; the low solubility of DMH^{ox} may be useful for its convenient extraction from the reaction mixture of a water-soluble disulfide and DMH.

We infer that DMH exists in two conformations from its ^1H NMR spectrum. The assignments of the two conformations—symmetrical (**3**, eq 3) and unsymmetrical (**4**, eq 3)—are described in the Experimental Section. In



(3)

the reduction of disulfides (RSSR) by DMH, the intermediate (mixed disulfide of DMH and RSH) can cyclize directly to form DMH^{ox} for the symmetrical conformation (**5**) only. The intermediate mixed disulfide from the unsymmetrical conformation (**6**) must isomerize to the symmetrical form (**5**) before it can cyclize to form DMH^{ox}. Using dynamic NMR, we have calculated that the value of the intramolecular rate constant for the interchange of the unsymmetrical to symmetrical form at 25 °C is 0.024 s^{-1} (see the Experimental Section). At 10 mM concentration of RSSR, the pseudounimolecular intermolecular rate constant for the conversion of **4** to **6** is calculated to be ~0.005 s^{-1}.[17] The rate-determining step in the reduction of disulfides by DMH in dilute solutions is therefore the intermolecular thiol–disulfide interchange, and not the intramolecular isomerization of the unsymmetrical mixed disulfide (**6**) into the symmetrical form (**5**).

Equilibrium Constants for Thiol–Disulfide Interchange of DMH with Cyclic and Noncyclic Disulfides. Table II lists the equilibrium constants for thiol–disulfide interchange reactions between DMH and several disulfides. DMH reduces noncyclic disulfides completely; DMH oxidizes completely to DMH^{ox} on reaction with excess dithiodiglycolic acid, and no mixed disulfide is seen (Figure 1). The reduction potential of DMH is comparable to that for dihydrolipoic acid, although it is less than that for dithiothreitol. DMH is more reducing than 1,6-hexanedithiol probably because fewer degrees of rotational freedom are lost on going from the dithiol to the cyclic disulfide.

Kinetics of Reduction of Disulfides by DMH. DMH reduces disulfides faster than does DTT in water at pH 7 (Table III). The rate of reduction of ME^{ox} by DMH is 7-fold higher than that by DTT. The factor of 7 is approximately that expected from the Brønsted equation because the pK_a of thiol in DMH is lower than that in DTT.[18] As model protein disulfides, we prepared mixed disulfides of enzymes that are active in thiol form.[19] We

(14) The cost of 1,2-dimethylhydrazine dihydrochloride is $4/g (FW 133).

(15) Houk, J.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 91–102.

(16) Barltrop, J. A.; Hayes, P. M.; Calvin, M. *J. Am. Chem. Soc.* **1954**, *76*, 4348–4367. Schmidt, U.; Grafen, P.; Altland, K.; Goedde, H. W. *Adv. Enzymol.* **1969**, *32*, 423–469. Bergson, G. *Arkiv. Kemi* **1962**, *18*, 409–434.

(17) The apparent second-order rate constant for the reaction of DMH and ME^{ox} in D_2O at pD 7 is 0.5 M^{-1} s^{-1}. Assuming that the concentration of the disulfide (ME^{ox}) is 0.01 M, the pseudounimolecular rate constant for the conversion of **4** to **6** is calculated to be 0.005 s^{-1} (=0.5 M^{-1} s^{-1} × 0.01 M); for a smaller concentration (<0.01 M) of the disulfide, the rate constant would be smaller than 0.005 s^{-1}.

(18) Using the Brønsted equation $\log k = 7.0 + 0.5pK_a^{nuc} - 0.27pK_a^{c} - 0.73pK_a^{lg}$ and the equation $k = k^{app}(1 + 10^{pK_a-pH})$,[6] we derive $\log k^{app}_{DMH} - \log k^{app}_{DTT} = 0.5(pK_a^{DMH} - pK_a^{DTT}) - \log(1 + 10^{pK_a^{DTT}-pH}) + \log(1 + 10^{pK_a^{DMH}-pH})$. Using $pK_a^{DMH} = 7.6$, $pK_a^{DTT} = 9.2$, and pH = 7.0, we get $k^{app}_{DMH}/k^{app}_{DTT} = 5.1$.

Table II. Equilibrium Constants of Thiol–Disulfide Interchange of DMH with Cyclic and Noncyclic Disulfides in Water at pH 7

disulfide	ring size	K_{eq}	$\epsilon°$ (V)[a]
bis(2-hydroxyethyl) disulfide (ME^{ox})		1200 M[b]	−0.209
1,2-dithiocane	8	10[c]	−0.269
lipoic acid	5	0.18[c]	−0.320
trans-4,5-dihydroxy-1,2-dithiane (DTT^{ox})	6	0.014[c]	−0.356

[a] All values of $\epsilon°$ are relative to that of mercaptoethanol (−0.209 V) as reference.[10] [b] K_{eq} = ([RSH]^2[DMH^{ox}])/([RSSR][DMH]). This value of K_{eq} is estimated from $\epsilon°$ for DMH = −0.300 V. [c] K_{eq} = ([HSRSH][DMH^{ox}])/([$\overline{SRS}$][DMH]). All values of K_{eq} were determined by ^{1}H NMR spectroscopy. The K_{eq} for lipoic acid was also determined by UV spectroscopy. These values of K_{eq} are unitless. 1,2-Dithiocane is $\overline{S(CH_2)_6S}$.

Table III. Comparison of Rate Constants for Reduction of Disulfides by DMH and DTT in Water at pH 7

disulfide	k^{app} (M^{-1} s^{-1})[a,b] DMH	DTT	k_{DMH}/k_{DTT}
$HOCH_2CH_2$-S-S-CH_2CH_2OH (ME^{ox})	0.52	0.075	7
creatine kinase-S–S-glutathione	67	10	7
papain-S-S-CH_3	1400	44	30
ficin-S-S-CH_3	690	21	30
deoxyribonuclease I	1.3	0.29	4.5

[a] The rate constants are for 25 °C. [b] The error in apparent rate constant (k^{app}) is ±10%; the values are an average of two experiments.

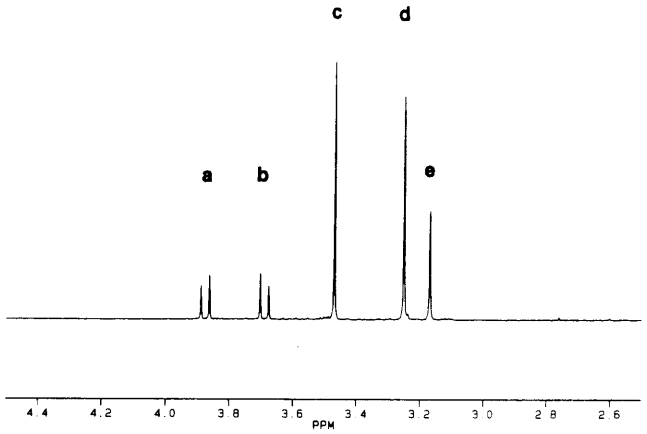


Figure 1. ^{1}H NMR spectrum (500 MHz) of a mixture initially containing DMH (8.5 mM) and excess dithiodiglycolic acid ($^-OOCCH_2SSCH_2COO^-$, 20 mM) in D_2O buffer at pD 7. Peak assignments: (a, b) CH_2 (DMH^{ox}); (d) CH_3 (DMH^{ox}); (c) CH_2 ($^-OOCCH_2SSCH_2COO^-$); (e) CH_2 ($^-OOCCH_2SH$).

studied the rates of reduction of the mixed disulfides by DMH and by DTT from the regeneration of the enzymatic activity.[7] The rate of regeneration of the active thiol group of creatine kinase (from creatine kinase-S–S-glutathione) by DMH is also faster by a factor of 7 than that by DTT (Figure 2). DMH regenerates the active thiol groups of papain and ficin (by reduction of papain-S-S-CH_3 and ficin-S-S-CH_3) faster than does DTT by a factor of 30 (Figure 2). The rate of reduction of papain-S-S-CH_3 and ficin-S-S-CH_3 by DMH is anomalously high relative to that by DTT and may result from the higher affinity of DMH to the enzymes.

We studied the loss of activity of deoxyribonuclease I (DNaseI) by cleavage of its native disulfide bonds.[7,20,21]

(19) Smith, D. J.; Maggio, E. T.; Kenyon, G. L. *Biochemistry* **1975**, *14*, 766–771.

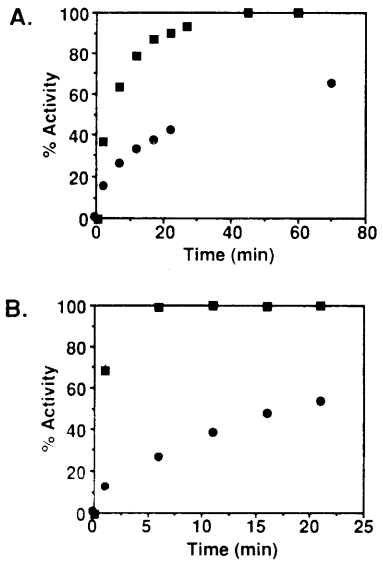


Figure 2. Regeneration of essential thiol groups in enzymes containing mixed disulfides: (A) reduction of creatine kinase-S–S-glutathione by DMH (■) and by DTT (●); (B) reduction of ficin-S-S-CH_3 by DMH (■) and by DTT (●).

DMH inactivates DNaseI 4.5 times faster than does DTT. The cleavage of the disulfide bond may not be the only rate-determining step in the inactivation of DNaseI, because the conformational changes after the cleavage of the disulfide bond may also contribute to the overall rate of loss of activity.

Conclusions

DMH reduces disulfide bonds in water at pH 7 faster than does DTT. Although DMH is a weaker reducing agent than is DTT, it reduces noncyclic disulfides completely. Like DTT it does not give mixed disulfides. The synthesis of DMH is straightforward from *N,N'*-dimethylhydrazine dihydrochloride. The most expensive reagent in the synthesis of DMH is *N,N'*-dimethylhydrazine. The availability of a cheaper and improved synthesis of *N,N'*-dialkylhydrazine would reduce significantly the overall cost of DMH.[22]

DMH would be useful in biochemical manipulations at pH ≤7 when the rate of reduction is important: Its rate of reaction is approximately 7 times faster than DTT and mercaptoethanol. DMH may be especially useful in regeneration of activities of cysteine proteases: The thiol groups of papain and ficin are regenerated from mixed disulfides 30 times faster by DMH than by DTT. DMH is less reducing than DTT and may not completely reduce some cyclic disulfides. The low solubility of DMH^{ox} may be useful for convenient extraction with ethyl acetate in the reduction of ionic nonprotein disulfides.

Experimental Section

General Procedures. *N,N'*-Dimethylhydrazine dihydrochloride, dithiothreitol, *trans*-4,5-dihydroxy-1,2-dithiane (DTT^{ox}), lipoic acid, methylmethanethiosulfonate, and hexane-1,6-dithiol

(20) Price, P. A.; Stein, W. H.; Moore, S. *J. Biol. Chem.* **1969**, *244*, 929–932.

(21) Lysozyme did not lose any activity over 3 h on incubation with 10 mM DMH or DTT.

(22) The traditional synthesis of *N,N'*-dimethylhydrazine involves benzoylation of hydrazine, followed by methylation and acidic hydrolysis: Hatt, H. H. *Organic Syntheses*; Wiley: New York, 1943; Collect. Vol. 2 pp 208–211. A more recent and possibly cheaper synthesis is by electrochemical reduction of nitromethane: Iversen, P. E. *Chem. Ber.* **1971**, *104*, 2195–2198.

were purchased from Aldrich. Chloroacetic anhydride and thiolacetic acid were purchased from Fluka. Papain (EC 3.4.4.10, papaya latex) was purchased from Boehringer Mannheim. Creatine kinase (EC 2.7.3.2, rabbit muscle), ficin (EC 3.4.4.12, fig latex), deoxyribonuclease I (EC 3.1.21.1, bovine pancreas), DNA (calf thymus, highly polymerized), and *N*-benzoyl-L-arginyl-*p*-nitroanilide were purchased from Sigma.

***N,N'*-Dimethyl-*N,N'*-bis(chloroacetyl)hydrazine (1).**[23] To a 500-mL round-bottomed flask equipped with a stir bar were added, *N,N'*-dimethylhydrazine dihydrochloride (10.15 g, 76.3 mmol) and chloroacetic anhydride (71.8 g, 420 mmol). The flask was stoppered with a rubber septum, and a needle connected to a bubbler was pierced through the septum. *Caution: N,N'-Dimethylhydrazine dihydrochloride is a carcinogen, and this reaction should be done in a well-ventillated hood.* The flask was heated to 85 °C in 20 min, and the flask was kept at 85 °C for 20 min. The molten reaction mixture became clear. The solution was cooled to room temperature and poured dropwise into water (1.2 L) in an ice bath. Methylene chloride (400 mL) was added to the aqueous suspension. Sodium hydroxide (40% aqueous solution) was added dropwise to the water/methylene chloride suspension in an ice bath with stirring until the pH of the aqueous layer was 7. The methylene chloride layer was separated, washed with brine solution (100 mL), dried ($MgSO_4$), and concentrated at reduced pressure to yield a white solid (14.0 g, 86%). The crude white solid was recrystallized with ethyl acetate to yield a white crystalline solid (12.7 g, 78%): mp 117–118 °C; ^{1}H NMR ($CDCl_3$) (two conformers were seen in 1.4:1 ratio) δ 4.11 (s, 4 H, CH_2Cl), 3.24 (s, 6 H, CH_3N) (major conformation), 4.02 (m, CH_2Cl), 3.43 (s, CH_3N), 3.13 (s, CH_3N) (minor conformation). Anal. Calcd for $C_6H_{10}Cl_2N_2O_2$: C, 33.82; H, 4.73; N, 13.15. Found: C, 34.02; H, 4.77; N, 13.15.

***N,N'*-Dimethyl-*N,N'*-bis[(acetylthio)acetyl]hydrazine (2).** To a solution of *N,N'*-dimethyl-*N,N'*-bis(chloroacetyl)hydrazine (1.30 g, 6.10 mmol) in methylene chloride (100 mL) in an ice bath was added thiolacetic acid (1.30 mL, 18.2 mmol) followed by triethylamine (2.40 mL, 17.3 mmol). The solution was stirred in ice bath for 30 min, washed with water (100 mL), with cold 10% aqueous $NaHCO_3$ solution, and finally with brine solution (100 mL), dried ($MgSO_4$), and concentrated at reduced pressure to yield a pale viscous liquid (2.06 g). The crude oil was purified by flash chromatography on a silica gel column with ethyl acetate to yield a pale liquid (R_f 0.48) that solidified at room temperature (1.68 g, 94%): mp 74 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) (two conformers were seen in 2.6:1 ratio) δ 3.95, 3.91, 3.74, 3.70 (AB quartet, J = 16.3 Hz, 4 H, CH_2), 3.26 (s, 6 H, CH_3N), 2.39 (s, 6 H, CH_3CO) (major conformer); 3.47 (s, CH_3N), 3.09 (s, CH_3N), 2.42 (s, CH_3CO), 2.36 (s, CH_3CO) (minor conformer). Anal. Calcd for $C_{10}H_{16}N_2O_4S_2$: C, 41.08; H, 5.52; N, 9.58. Found: C, 41.30; H, 5.54; N, 9.47.

***N,N'*-Dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH).** To a solution of *N,N'*-dimethyl-*N,N'*-bis[(acetylthio)-acetyl]hydrazine (1.00 g, 3.42 mmol) in methanol (20 mL) was added concentrated HCl (0.2 mL of a 37 wt % aqueous solution), and the solution was stirred at room temperature for 80 h. The reaction mixture was concentrated at reduced pressure and in vacuo to yield a colorless liquid (0.73 g). For further purification, the crude oil was dissolved in methanol (6 mL), and the solution was cooled in a dry ice/acetone bath; the supernatant was decanted, and the white precipitate was dried in vacuo (0.56 g, 78%): mp 37–38 °C. Anal. Calcd for $C_6H_{12}N_2O_2S_2$: C, 34.60; H, 5.81; N, 13.45. Found: C, 34.15; H, 5.98; N, 13.28.

DMH^{ox}. To a solution of crude *N,N'*-dimethyl-*N,N'*-bis-(mercaptoacetyl)hydrazine (1.08 g, 5.2 mmol) in ethyl acetate (150 mL) in an ice bath was added cold $KHCO_3$ solution (50 mL of a 10% aqueous solution). A solution of iodine (1.32 g, 5.2 mmol) in ethyl acetate (50 mL) was added dropwise with stirring until the reaction mixture turned brown. An aqueous solution of sodium thiosulfate was added to quench the excess iodine. The ethyl acetate layer was separated, and the aqueous layer was extracted once more with ethyl acetate (50 mL). The combined ethyl acetate extract was dried (Na_2SO_4) and concentrated at reduced pressure to yield a white crystalline solid (0.89 g, 83%). The crude product was recrystallized with ethyl acetate: mp 155 °C; ^{1}H NMR (CD_3CN, 300 MHz) δ 3.76, 3.72, 3.54, 3.50 (AB quartet, J = 12.8 Hz, 4 H), 3.12 (s, 6 H). Anal. Calcd for $C_6H_{10}N_2O_2S_2$: C, 34.94; H, 4.89; N, 13.58. Found: C, 34.83; H, 4.81; N, 13.41.

Papain-S–S-CH_3. Papain-S–S-CH_3 was prepared by a modification of the reported procedure of Shaked et al.[7] Methylmethanethiosulfonate (5.0 μL, 4.9 × 10^{-5} mol) was dissolved in 15 mL of aqueous buffer (0.1 M in phosphate, 2 mM in EDTA, pH 7) to prepare a stock solution (3.2 mM). To 1.5 mL of the stock solution of methylmethanethiosulfonate was added KCl (0.011 g, 0.15 mmol); the solution was deoxygenated by bubbling argon through it for 15 min, and papain (0.5 mL of a suspension purchased from Boehringer Mannheim, ~5 mg of protein, MW 21 000, ~150 U) was added. The solution was kept at room temperature for 12 h under argon. The excess methylmethanethiosulfonate was removed by size-exclusion chromatography on a Sephadex G-25 column with aqueous buffer (0.1 M in phosphate, 0.1 M in KCl, 0.5 mM in EDTA, pH 7.0). The concentration of Papain-S–S-CH_3 in the chromatographed fraction was 2 × 10^{-5} M using $\epsilon^{1\%}_{278}$ = 25.

Ficin-S–S-CH_3. From a solution of methylmethanethiosulfonate (4 μL, 39 μmol) in 3.5 mL of deoxygenated aqueous buffer (pH 7, 0.1 M in phosphate, 2 mM in EDTA), a 1.75-mL aliquot was added to ficin solution (0.25 mL of a suspension in 2 M NaCl, 0.03 M in cysteine, pH 5, ~6 mg of protein, MW 26 000), and the mixture was stirred under argon for 2 h. The solution was concentrated and was washed with aqueous buffer (8 × 0.5 mL, pH 7, 0.1 M in phosphate, 2 mM in EDTA) by centrifugation over a Centricon microconcentrator (Amicon, MW cutoff 10 000) to remove excess methylmethanethiosulfonate. The final volume of ficin-S–S-CH_3 solution was 0.35 mL.

Creatine Kinase-S–S-Glutathione.[24] In a Centricon microconcentrator (Amicon, MW cutoff 10 000) was taken creatine kinase (1 mg of lyophilized powder from rabbit muscle, MW 81 000) in 1 mL of aqueous buffer (pH 8, 0.1 M in Tris, 2 mM in EDTA), and oxidized glutathione (0.0092 g, 15 μmol) was added. The solution was ~12 μM in creatine kinase and ~15 mM in oxidized glutathione. The solution was kept at room temperature for 2 h, centrifuged, and washed with aqueous buffer (5 × 0.5 mL, pH 8, 0.1 M in Tris, 2 mM in EDTA) in order to remove excess oxidized glutathione.

General Methods for Deoxygenation of Aqueous Reaction Mixtures in Kinetic and Equilibrium Studies. All flasks and NMR tubes were stoppered with rubber septa and were purged with argon. All transfers were done with use of gas-tight syringes. The D_2O buffer (pD 7.0, 50 mM in phosphate),[25] or DMSO-d_6, was deoxygenated by bubbling argon through it for 1 h.

Kinetics of Reduction of Papain-S–S-CH_3 (or Ficin-S–S-CH_3) by DMH and by DTT. Papain-S–S-CH_3 (0.26 mL of the chromatographed fraction) was diluted with 2.34 mL of deoxygenated aqueous buffer (pH 7, 0.1 M in imidazole, 2 mM in EDTA), and the enzyme solution was added to two flasks (1.2 mL in each). DTT or DMH (6 μL of a 5 mM solution) was added to the flask containing the enzyme solution, and the stopwatch was started. The initial concentrations in the reaction mixture were $[\text{papain-S-S-CH}_3]_{init}$ = 2 × 10^{-6} M and $[DTT]_{init}$ = 2.5 × 10^{-5} M. From the reaction mixture, aliquots (0.2 mL) were withdrawn at various time intervals and were added to a cuvette containing the substrate solution (0.8 mL of a 1.25 mM solution of *N*-benzoyl-L-arginyl-*p*-nitroanilide in aqueous buffer, pH 6, 0.1 M in imidazole, 2 mM in EDTA), and the rate of change of absorbance at 410 nm was recorded.[26] The values of concentrations in the cuvette were [papain-SH] + [papain-S-S-CH_3] = 0.4 μM and [*N*-benzoyl-L-arginyl-*p*-nitroanilide] = 1.0 mM. DTT

(23) The preparation of 1 was similar to that of *N,N'*-dimethyl-*N,N'*-diacetylhydrazine from *N,N'*-dimethylhydrazine dihydrochloride: Hinman, R. L. *J. Am. Chem. Soc.* **1956**, *78*, 1645–1649.

(24) The procedure was analogous to the preparation of mixed disulfide of fructose-6-phosphate kinase and glutathione: Walters, D. W.; Gilbert, H. F. *J. Biol. Chem.* **1986**, *261*, 15372–15377.

(25) The pH meter reading in the D_2O buffer was corrected (pD = pH meter reading + 0.4): Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188–190.

(26) For assays of papain and ficin see: Mole, J. E.; Horton, H. R. *Biochemistry* **1973**, *12*, 816–822. Nakayama, S.; Watanabe, T.; Takahashi, K.; Hoshino, M.; Yoshida, M. *J. Biochem. (Tokyo)* **1987**, *102*, 531–535, and references therein.

was present in excess over papain-S-S-CH_3 in the regeneration mixture, and the concentration of DTT was therefore assumed to be constant during the reaction. The apparent rate (k^{app}) was calculated from the slope for the plot of $-\ln \{([papain]_{total} - [papain]_{SH})/[papain]_{total}\}$ vs time, for which the slope = $k^{app}[DTT]$. The activation curves were biphasic, and the plots for DMH and DTT were compared after the initial burst, i.e., for activities greater than ~15 % of the total activity. At the end of experiment, the thiol contents of DTT and DMH stock solutions were verified by Ellman's assay.[27] Ficin was assayed with the same substrate as above by a similar procedure. The enzyme solution of ficin-S-S-CH_3 was prepared by dissolving 0.05 mL of the stock solution of ficin-S-S-CH_3 in 2.55 mL of deoxygenated buffer (pH 7, 0.1 M in imidazole, 2 mM in EDTA).

Kinetics of Reduction of Creatine Kinase-S-S-Glutathione by DMH and by DTT. The solution of the mixed disulfide of creatine kinase and glutathione (10 μL) was diluted with 2.5 mL of deoxygenated aqueous buffer (pH 7, 0.1 M in imidazole, 2 mM in EDTA), and the diluted solution was added to two flasks (1.0 mL in each). To the flask containing the enzyme solution was added DTT or DMH (5 μL of a 5 mM solution in pH 6 aqueous imidazole buffer), and the stopwatch was started. From the reaction mixture, aliquots (50 μL) were withdrawn and were added to 950 μL of assay solution (pH 6, 0.1 M in imidazole, 2 mM in EDTA), and the rate of increase in absorbance at 340 nm was recorded. The assay solution contained Mg^{2+} (10 mM), ADP (2 mM), D-glucose (20 mM), $NADP^+$ (2 mM), phosphocreatine (30 mM), hexokinase (50 U), and glucose-6-phosphate dehydrogenase (35 U).[28] The apparent rate constant was determined in the same manner as described for papain.

Kinetics of Inactivation of Deoxyribonuclease I (DNaseI) by DMH and by DTT. The assay was similar to that described by Price et al.[20] The solution of DNA in acetate buffer containing Mg^{2+} was prepared according to the Sigma protocol.

Kinetics of Reduction of Bis(2-hydroxyethyl) Disulfide (ME^{ox}) by DMH and DTT by ^1H NMR Spectroscopy. Stock solutions (20 mM) of DMH (0.0040 g in 0.96 mL of D_2O buffer), DTT (0.0046 g in 1.50 mL of D_2O buffer), and bis(2-hydroxyethyl) disulfide (0.0060 g in 1.94 mL of D_2O buffer) were prepared in D_2O buffer (pD 7.0, 50 mM in phosphate). In a NMR tube containing ME^{ox} (0.25 mL) was added DMH or DTT (0.25 mL), and the stopwatch was started. The reaction was quenched after 2 min (or 4 min) by addition of DCl (30 μL of a 12 wt % solution in D_2O). The extent of reaction was determined from integration of the ^1H NMR peak areas. The initial values of concentration in the reaction mixture were [DMH] = $[ME^{ox}] = c_{init}$ = 10 mM. The apparent rate constant (k^{app}) was calculated by $k^{app} = [(1/c_{final}) - (1/c_{init})]/t$.

Determination of the Equilibrium Constant of DMH with DTT^{ox} by ^1H NMR Spectroscopy. To a flask containing DTT^{ox} (0.0017 g, 11 μmol) was added a stock solution of DMH (1.0 mL of a 11 mM solution in D_2O buffer) that had been estimated previously by Ellman's assay. The equilibrium mixture was transferred to an NMR tube with a cannula under argon. The top of the NMR tube was sealed with paraffin wax, and the ^1H NMR spectra were recorded after 29 h. The amounts of DMH and DMH^{ox} at equilibrium were estimated by integration of the areas of CH_3N peaks, and the amounts of DTT and DTT^{ox} at equilibrium were estimated by integration of the areas of CH_2S peaks. A similar procedure was used for determining the equilibrium constant of DMH with lipoic acid.

Determination of the Equilibrium Constant of DMH with Lipoic Acid by UV Spectroscopy. A solution of lipoic acid (0.0024 g, 1.2×10^{-5} mol) in 3 mL of deoxygenated aqueous phosphate buffer (pH 7.0, 2 mM in EDTA, 0.1 M in phosphate) was added to a flask containing DMH (0.0030 g, 1.4×10^{-5} mol). The equilibrium mixture was transferred to a cuvette, and the absorbance at 330 nm was recorded. Extinction coefficient ϵ_{330} = 147 M^{-1} cm^{-1} was used for calculating the concentration of the cyclic five-membered lipoic acid at equilibrium.[6]

Determination of the Equilibrium Constant of DMH^{ox} with Hexane-1,6-dithiol by ^1H NMR Spectroscopy. In a flask containing DMH^{ox} (0.0052 g, 2.5×10^{-5} mol) and hexane-1,6-dithiol (0.0038 g, 2.5×10^{-5} mol) was added deoxygenated DMSO-d_6 (0.5 mL). In another flask containing potassium *tert*-butoxide (0.0159 g, 0.142 mmol) was added DMSO-d_6 (0.45 mL) to prepare a stock solution of potassium *tert*-butoxide (0.315 M). To an NMR tube were added DMSO-d_6 (0.25 mL), the solution of DMH^{ox} and hexane-1,6-dithiol in DMSO-d_6 (0.25 mL, 1.25×10^{-5} mol of each), and the stock solution of potassium *tert*-butoxide in DMSO-d_6 (3 μL of a 0.315 M solution, 1×10^{-6} mol). The top of the NMR tube was sealed with paraffin wax, and the ^1H NMR spectra were recorded after 30 min and 2 h. The values of initial concentration in the NMR tube were $[DMH^{ox}]$ = 25 mM, [hexane-1,6-dithiol] = 25 mM, and [potassium *tert*-butoxide] = 2 mM. The equilibrium concentrations were determined by integration of the ^1H NMR peak areas. The same value of K_{eq} (=10) was obtained from spectra recorded after 30 min and after 2 h.

Determination of the Rate of Conformational Interchange of DMH in Water by Dynamic ^1H NMR Spectroscopy. To an NMR tube containing DMH (0.0022 g, 1.1×10^{-5} mol) was added deoxygenated D_2O buffer (0.5 mL), and the top of the NMR tube was sealed with Apiezon W cement. The ^1H NMR spectra (500 and 250 MHz) of the DMH solution (21 mM) were recorded at various temperatures in the range 45–73 °C. The NMR temperature values were calibrated against a sample of ethylene glycol.[29] The ^1H NMR spectra were simulated with the program DNMR4[30] on a VAX 8600 for various rate constants (k) and were visually compared with the experimental spectra for the best fit. Only two conformations of DMH were observed in the ^1H NMR spectrum: a symmetrical conformation (**3**) (*E,E*) showed one CH_3N peak at 3.23 ppm; an unsymmetrical conformation (**4**) (*E,Z*) showed two CH_3N peaks at 3.43 and 3.10 ppm (eq 3). The symmetrical conformation was presumed to be **3** because the chemical shift of the CH_3N peak (3.23 ppm) was similar to that of the CH_3N peak (3.25 ppm) of DMH^{ox}. Another conformation (*Z,Z*), possible for DMH, was not observed.[31] For DNMR simulation, the population ratio of **3** to **4** was 0.275:0.725, T_2^* = 0.126 s (500 MHz), and T_2^* = 0.160 s (250 MHz).[32] The plot of log k vs $1/T$ was a straight line (R^2 = 0.996 for seven points). The extrapolated values of rate constant at 25 °C were 0.062 s^{-1} for **3** going to **4** and 0.024 s^{-1} for **4** going to **3**. Similar values for rate constants of conformational interchange were observed for *N,N'*-dimethyl-*N,N'*-bis(5-hydroxy-3-thiapentanoyl)hydrazide ($(HOCH_2CH_2SCH_2CONMe)_2$). We infer that the rate constants for the isomerization of **5** and **6** (eq 3) would be similar to those for the isomerization of **3** and **4**. The population ratio **3**:**4** in CD_3CN was 0.45:0.55. The symmetrical form (**3**) has a lower dipole moment than the unsymmetrical form (**4**) and should be more favored in a solvent of low dielectric constant than in a solvent of higher dielectric constant.

Determination of the pK_a of DMH. The spectroscopic method of Benesch and Benesch[33] was used for determining the pK_a of the thiol groups in DMH. The absorbance due to thiolate for solutions of DMH (0.26 mM) in various buffers in the pH range 5–10 was measured at 238 nm. The dissociation curves of the two thiols overlap; the values of first and second pK_a are estimated as 7.6 and 8.9, respectively.

Determination of the Solubilities of DMH and DMH^{ox} in D_2O Buffer. A saturated solution of DMH (or DMH^{ox}) was

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prepared in 1 mL of deoxygenated D_2O buffer (pD 7, 50 mM in phosphate as buffer, 20 mM in 2,3-butynediol as ^1H NMR standard). The solubility of DMH (or DMH^{ox}) was determined by integration of the ^1H NMR peak areas of CH_3N peaks for DMH (or DMH^{ox}) and of CH_2OH peaks for 2,3-butynediol ($HOCH_2C{-}CCH_2OH$).

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Registry No. 1, 89580-95-0; 2, 131760-66-2; DMH, 131760-67-3; DMH^{ox}, 131760-68-4; DTT, 27565-41-9; DTT^{ox}, 86023-22-5; ME^{ox}, 1892-29-1; DNaseI, 9003-98-9; MeNHNHMe·2HCl, 306-37-6; $(ClCH_2CO)_2O$, 541-88-8; AcSH, 507-09-5; $HS(CH_2)_6SH$, 1191-43-1; lipoic acid, 62-46-4.