CHAPTER **13**

Thiol–disulfide interchange

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I. INTRODUCTION

Thiol–disulfide interchange is the reaction of a thiol (RSH) with a disulfide (R′SSR′), with formation of a new disulfide (RSSR′) and a thiol (R′SH) derived from the original disulfide (equation 1). The reaction is unique in organic chemistry: although it involves the cleavage and formation of a strong covalent bond (the S—S bond; bond energy *ca* 60 kcal mol^{-1}), it occurs reversibly at room temperature in water at physiological pH (*ca* 7)[1–4]. The reaction is moderately fast: a typical value of the observed rate constant is *ca* 10 M^{-1} min^{-1} at pH 7 and room temperature[5]. The half-life for the reaction is *ca* 2 h for mM concentrations of thiol and disulfide in aqueous solution at pH 7, and for alkanethiols with normal values of pK_a (*ca* 9–10). The yield of the reaction is quantitative if side-reactions—such as air oxidation of thiol to disulfide, and cleavage of disulfide bonds at high pH—are prevented[1].

$$\mathrm{RSH + R'SSR' \rightleftharpoons RSSR' + HSR'} \tag{1}$$

Thiol–disulfide interchange is an S_N2 reaction. Thiolate anion (RS$^-$) is the active nucleophile and the reaction can be stopped if the solution is made acidic[1]. The reaction is overall second-order: first-order each in thiolate and in disulfide (equations 2–4)[6–20]. In this chapter, we will distinguish between *thiol*–disulfide interchange (the overall observed process, equation 1), and *thiolate*–disulfide interchange (the reaction of thiolate anion with disulfide, equation 3). The two processes differ according to the extent to which thiol is dissociated to thiolate anion under the reaction conditions.

$$\mathrm{RSH \rightleftharpoons RS^- + H^+} \quad (\mathrm{p}K_a^{\mathrm{RSH}}) \tag{2}$$

$$\mathrm{RS^- + R'SSR' \rightleftharpoons RSSR' + {}^-SR'} \tag{3}$$

$$\mathrm{H^+ + {}^-SR' \rightleftharpoons HSR'} \quad (\mathrm{p}K_a^{\mathrm{R'SH}}) \tag{4}$$

Thiolate–disulfide interchange is base catalyzed[21–25] and involves the backside nucleophilic attack of thiolate anion along the S—S bond axis of the disulfide[26]. It shows less sensitivity to solvent than most S_N2 reactions involving oxygen and nitrogen nucleophiles[15]. The rates of thiolate–disulfide interchange in polar aprotic solvents (DMSO, DMF) are faster by a factor of approximately 10^3 than rates in polar protic solvents (water, methanol). For comparison, the rate enhancement for oxyanions (which are more highly solvated than thiolates)[15] on moving from protic to polar aprotic solvents is 10^6–10^7.

Biologically important molecules containing the thiol or the disulfide group are widely distributed in nature[1,2], and the unique position of thiols and disulfides in biochemistry has been reviewed in several excellent books and articles[1–3]. The thiol-containing amino acid—cysteine—is present in proteins and peptides; examples include glutathione[27] and trypanothione[28–30]. Several cofactors—coenzyme A, dihydrolipoamide, coenzyme M ($^-O_3SCH_2CH_2SH$)[31]—contain the essential thiol functionality[1,2]. The disulfide bonds

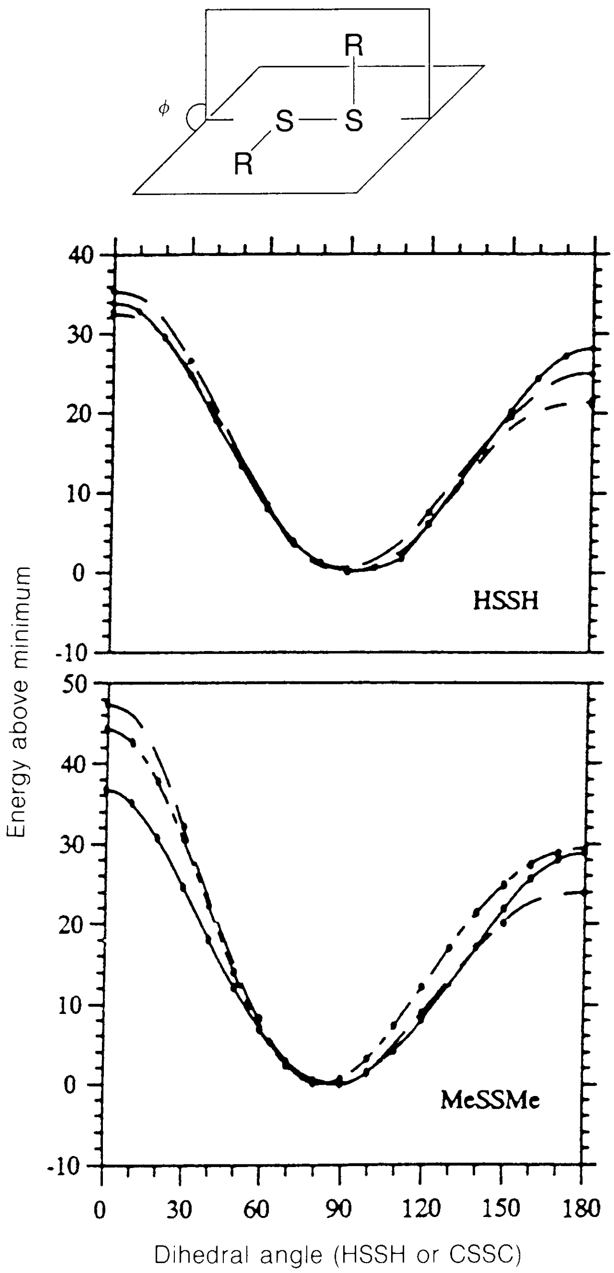


FIGURE 1. Relative energy (kJ mol^{-1}) of HSSH and MeSSMe as a function of the dihedral angle (ϕ, RSSR): (—) MM2 (85); (— — —) 6–31G* [M. Aida and C. Nagata, *Theor. Chim. Acta*, **70**, 73 (1986)]; (- -) SCF and MP2 [C. J. Marsden and B. J. Smith, *J. Phys. Chem.*, **92**, 347 (1988)]; (— - —) OPLS [W L. Jorgensen, *J. Phys. Chem.*, **90**, 6379 (1986)]. Reprinted with permission from Reference 16. Copyright (1990) American Chemical Society

between cysteine residues are important tertiary and quarternary structural elements in proteins (especially extracellular proteins) such as immunoglobulins, enzymes, hormones, procollagen and albumin[1-3]. The cleavage of the disulfide bond(s) of many proteins (e.g. deoxyribonuclease I) results in loss of activity[32]. Thiol–disulfide interchange may play a role in metabolic regulation of enzymatic activities[3,33,34]. The activities of several chloroplast enzymes such as fructose-1,6-biphosphatase, NADP-malate dehydrogenase, sedoheptulose-1,7-biphosphatase, NADP-glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase are enhanced by reduction of their specific disulfide bonds by photogenerated reducing equivalents transferred via ferredoxin and thioredoxin[3,35-37]. The cleavage of disulfide bonds of β-adrenergic and other cell surface receptors by thiols activates the receptor in a manner similar to binding of agonist[38,39].

The thiol functional group is essential for activity of many enzymes[1,2] such as thiol proteases (papain, ficin, bromelain)[40], β-ketoacylthiolase[41-43], enolase[44], creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase and adenylate kinase[1-3,11]. These enzymes are inactive in mixed disulfide form, and can be reactivated using strongly reducing thiols (e.g. dithiothreitol, *N,N'*-dimethyl-*N,N'*-bis(mercapto-acetyl)hydrazine)[11,18]. A thiolate anion may be involved in methanogenesis by 2e/1e redox coupling with a Ni cofactor in methyl coenzyme M reductase[31]. The reactive thiol functionality is masked as a trisulfide in the potent DNA-cleavage agents—calichemycin and esperimicin[45-48]. The rate-determining step in the unmasking of these DNA-cleavage agents is the cleavage of the trisulfide by a thiol (e.g. glutathione)[48]. The thiolate anion formed undergoes intramolecular Michael-type attack on an enone system, which in turn facilitates the ring closure of the enediyne moiety to form the reactive aromatic diradical.

Thiolate anion is a strong nucleophile and a good leaving group because of its high polarizability and low degree of solvation. The thiol group is less strongly hydrogen-bonded, and the thiolate anion is less solvated, than the alkoxide anion. The value of pK_a of the SH group of butanethiol in DMSO (*ca* 17) is 7 units higher than in water (*ca* 10); the corresponding pK_a value for the OH group of butanol is 12 units higher in DMSO (*ca* 28) than in water (*ca* 16)[49-51].

The optimum CSSC dihedral angle in the disulfide bond is *ca* 90° (Figure 1). The energy barrier to rotation around CSSC bond is *ca* 7.5 kcal mol^{-1} [52]. Five-membered cyclic disulfides are strained and have a CSSC dihedral angle of *ca* 30° [53]. Sulfur shows concatenation: molecular sulfur S_8 exists as an eight-membered ring; organosulfur compounds containing tri- and polysulfide linkages (RS_nR) are found in nature and have been characterized[54,55].

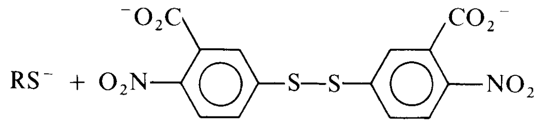
This chapter focuses on the physical-organic aspects of the thiol–disulfide interchange reaction. The biochemical aspects of the reaction are only lightly touched upon here, and we recommend References 1–3 to the reader for a detailed review of the biochemistry.

II. METHODS USED IN FOLLOWING THIOL–DISULFIDE INTERCHANGE

A. Spectroscopic (UV, NMR) Assays

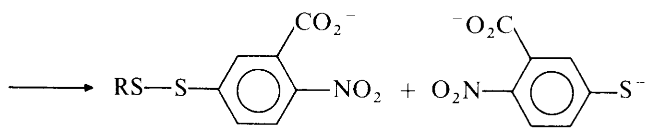
The kinetics of thiol–disulfide interchange reactions involving formation of a chromophoric thiolate are conveniently followed by UV spectroscopy. The reaction of thiolates with excess Ellman's reagent [EllS-SEll, 5,5'-Dithiobis(2-nitrobenzoic acid)] is used for quantitative estimation of thiol by measuring the absorption due to Ellman's thiolate ($EllS^-$) at 412 nm (equation 5)[56-58]. Reactions of thiols with 4,4'-dipyridyl disulfide and 2,2'-dipyridyl disulfide generate chromophoric thiols: 4-thiopyridone ($\varepsilon_{324\,nm} = 19600\ M^{-1}\,cm^{-1}$) and 2-thiopyridone ($\varepsilon_{343\,nm} = 8080\ M^{-1}\,cm^{-1}$) respectively[59-62].

The kinetics and equilibria of reactions involving cyclic five- and six-membered disulfides can be followed at 330 nm and 290 (or 310 nm) respectively. Five-membered



Ellman's Reagent
EllS-SEll

(5)



EllS$^-$
$\varepsilon_{412nm} = 13600\ M^{-1}\ cm^{-1}$

cyclic disulfides absorb in the UV region at 330 nm ($\varepsilon = 147\ M^{-1}\ cm^{-1}$) and six-membered disulfides absorb at 290 nm ($\varepsilon = 290\ M^{-1}\ cm^{-1}$) or 310 nm ($\varepsilon = 110\ M^{-1}\ cm^{-1}$)[5,53,63–65].

^{1}H NMR spectroscopy can be used to determine the position of equilibria in thiol–disulfide interchange reactions[10,13,18], and to follow the kinetics of the reactions (either in the reacting system or after quenching with acid)[18,66–68]. This method is useful where the methylene protons α and β to the sulfur in the reactants and products differ in chemical shift and can be integrated accurately. Dynamic ^{1}H NMR lineshape analysis[15,17] and spin-transfer methods[9,69] have been used to determine the rate constants of degenerate intermolecular thiolate–disulfide interchange reactions: $RS^- + RSSR = RSSR + RS^-$. Analysis of ^{1}H NMR lineshapes, where the resonances are exchange-broadened, is useful for determining the rates of fast degenerate intermolecular interchange reactions between thiolates and disulfides (k_{s^-} *ca* 10–$10^7\ M^{-1}\ s^{-1}$)[15,17]. The spin-transfer method between pairs of exchanging protons and carbons (α to sulfur) is applicable for slower rates (k_{s^-} *ca* 2–60 $M^{-1}\ s^{-1}$)[9,69].

B. Enzymatic Assays

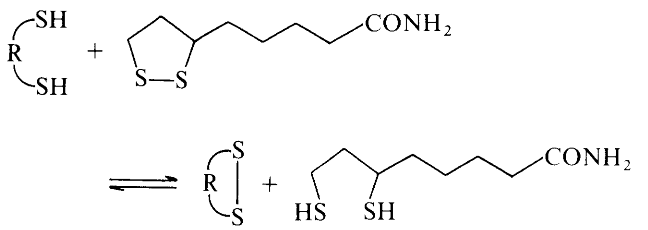
The kinetics of reduction of oxidized glutathione (GSSG) by thiols is conveniently followed[5] using a fast enzymatic reaction involving glyoxalase-I. The glutathione (GSH) that is released is converted to S-lactoyl glutathione by reaction with methylglyoxal in the presence of glyoxalase-I (GX-I), and the appearance of S-lactoyl glutathione is followed spectrophotometrically at 240 nm ($\varepsilon = 3370\ M^{-1}\ cm^{-1}$) (equations 6–8)[5]. The rates of reactions involving aminothiols cannot be determined by this method because they react rapidly with methylglyoxal and form species that absorb strongly at 240 nm and thus interfere with the spectroscopic measurement[5]. This assay is subject to errors due to oxidation of thiol groups if air is not carefully excluded.

$$RS^- + GS{-}SG \rightleftharpoons RS{-}SG + GS^- \quad (6)$$

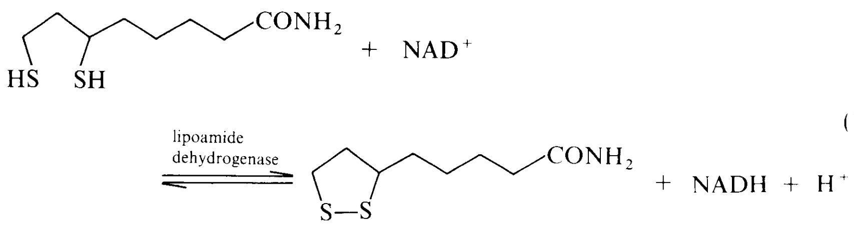
$$GS^- + CH_3COCHO \rightleftharpoons GS{-}CH(OH){-}COCH_3 \quad (7)$$

$$GS{-}CH(OH){-}COCH_3 + GX\text{-}I \rightleftharpoons GS{-}CO{-}CH(OH)CH_3 + GX\text{-}I \quad (8)$$

The equilibria of thiol–disulfide interchange reactions between α,ω-dithiols and lipoamide can be determined conveniently by the addition of lipoamide dehydrogenase and NAD$^+$. NADH is produced; this compound is coveniently monitored at 340 nm[5,70]



(9)



(10)

(equations 9 and 10). The equilibrium constants for α,ω-dithiol and lipoamide are then calculated from the measured equilibrium constant of the α,ω-dithiol with respect to NAD^+ and from the standard value of the equilibrium constant for lipoamide and NADH.

C. Assays Based on Chromatography

The kinetics and equilibrium constants of thiol–disulfide interchange reactions involving cysteine derivatives or peptides containing cysteine have been determined by HPLC[71] and gel-filtration chromatography[72] on the reaction mixtures after quenching with acid. The equilibrium products of the reaction of glutathione and cystine have been separated by ion-exchange chromatography[73] or by electrophoresis of the ^{35}S-labeled compounds[74]. Gas chromatography of the equilibrium mixture of alkanethiols and disulfides has been used to estimate equilibrium constants[75].

III. MECHANISM

A. Products

Thiol–disulfide interchange of a monothiol (RSH) with a disulfide (R′SSR′) involves multiple equilibria (equations 1 and 11): the reaction products include all possible thiols (RSH and R′SH), symmetrical disulfides (RSSR, R′SSR′) and mixed or unsymmetrical disulfides (RSSR′).

$$\text{RSH} + \text{RS—SR}' \rightleftharpoons \text{RS—SR} + \text{R}'\text{SH} \qquad (11)$$

B. Dependence on Solution pH, and on the pK_a Values of Thiols

Because the thiol–disulfide interchange reaction requires thiolate anion, the observed rate of reaction (and, in systems in which the participating thiols have different values of pK_a, the observed position of equilibrium) depends upon the pH of the solution and the extent of ionization of the various thiols. For a thiol of pK_a 10, only 0.1% of thiol is present as thiolate at pH 7, and 1% of thiol is in thiolate form at pH 8; the observed rate constant of the thiol–disulfide interchange at pH 8 is therefore 10 times faster than that at pH 7. The thiolate can be generated by addition of base, e.g. potassium *t*-butoxide, in polar aprotic solvents (DMSO, DMF)[15]. Thiol is a much weaker nucleophile than thiolate, and direct reaction between *thiol* and disulfide has not been observed. The reaction of thiolate with disulfide is effectively quenched by addition of acid and conversion of RS^- to RSH.

C. Kinetics

1. Rate law

The thiol–disulfide interchange reaction is overall second-order: first-order in thiolate and in disulfide[6,7]. For a representative reaction of a thiolate (RS^-) with Ellman's disulfide (EllS-SEll, equation 5) the rate laws are given by equations 12 and 13. The rate constant k_{RS^-} derived from equation 12, based on the concentration of the thiolate, is independent of pH; the observed rate constant k_{obsd}, based on total concentration of thiol (equation 13), depends upon pH. The value of the rate constant k_{RS^-} is useful for interpretations of reactivity (such as Brønsted correlations of rates or equilibrium constants with values of pK_a of thiols). The calculation of the observed rate constant k_{obsd} at the pH of reaction is straightforward from equation 13 using the value of the total thiol present in solution, $[RSH]_{total} = [RS^-] + [RSH]$, and is useful for predicting rates at the same pH. The two rate constants k_{obsd} and k_{RS^-} are related to each other by equation 14 and can be interconverted using the values of the pK_a of thiol and the pH of solution[5,6]. Table 1 lists the values of rate constants for representative thiol–disulfide interchange reactions in water.

$$(d[EllS^-]/dt) = k_{RS^-}[RS^-][EllS\text{-}SEll] \tag{12}$$

$$(d[EllS^-]/dt) = k_{obsd}[RSH]_{total}[EllS\text{-}SEll] \tag{13}$$

$$k_{RS^-} = k_{obsd}(1 + 10^{pK_a - pH}) \tag{14}$$

TABLE 1. Representative rate constants of thiol–disulfide interchange reactions in water

Reactants						
Thiol	Disulfide	pK_a (thiol)	k_{RS^-} ($M^{-1}\,min^{-1}$)	k_{obsd}[a] ($M^{-1}\,min^{-1}$)	Temp (°C)	Ref.
Mercaptoethanol	Oxidized glutathione	9.6	3.4×10^3	8.7	30	5
3-Mercaptopropanoic acid	Oxidized glutathione	10.6	1.2×10^4	3.2	30	5
Mercaptoethanol	Ellman's disulfide	9.6	1.5×10^7	3.7×10^4	30	6
Propanethiol	Ellman's disulfide	10.5	6.4×10^7	2.0×10^4	25	7
Thiophenol	Ellman's disulfide	6.6	1.3×10^6	9.6×10^5	30	6
Dithiothreitol	Papain-S-SCH_3	9.2	5.3×10^5	3.3×10^3	30	11
Dithiothreitol	Oxidized mercaptoethanol	9.2	3.7×10^2	2.3[b]	25	18

[a]Values of k_{obsd} are at pH 7. [b]The value of the rate constant is corrected statistically for the presence of two thiol groups on dithiothreitol.

2. Brønsted relation

The log of the rate constants (k_{RS^-}) of thiolate–disulfide interchange reactions follows a Brønsted correlation with the values of pK_a of the thiols. The Brønsted plot for the reaction of thiolates with oxidized glutathione[5] has a Brønsted coefficient (slope), β_{nuc}, of 0.5. The Brønsted coefficients are also *ca* 0.4–0.5 for the reaction of thiolates with Ellman's disulfide[6,7]. Alkyl and aryl thiolates show separate correlation lines with Ellman's disulfide[7], but show a similar slope, $\beta_{nuc} = 0.5$. An aromatic thiolate reacts with Ellman's reagent[7] or the mixed disulfide EllS-$SCH_2CH_2CH_2OH$[76] at a rate faster by a factor of 6 than an aliphatic thiolate of the same pK_a. The higher reactivity of aromatic thiolates in comparison to aliphatic thiolates is probably due to greater softness (and weaker solvation) of the former[7,8]. Brønsted correlations have also been reported for the reaction of thiolates with 4,4′-dipyridyl disulfide[59] and 2,2′-dipyridyl disulfide[60].

A Brønsted correlation (equation 15) for thiol–disulfide interchange (equation 16) has been assembled empirically by systematic examination of the influence of pK_a^{RSH} for the nucleophilic (nuc), central (c) and leaving group (lg) thiols on the rate of the reaction. Equations 17 and 18 represent different types of fits to data (k_{RS^-} is in units of $M^{-1}\,min^{-1}$)[5]. Equation 17 represents the best fit to all the available data, although it is suspect because the values of β_{nuc} and β_{lg} are not obviously compatible with a transition state with charge symmetrically distributed between the terminal sulfur atoms. The data included in the correlation are taken from a range of studies and are not necessarily directly comparable. Several different sets of Brønsted coefficients give similar fits to the observed data (equations 17 and 18); this observation suggests that the Brønsted coefficients are not sharply defined. We recommend equation 18 for general use based on the symmetry of the values of β_{nuc} and β_{lg}. The value of β_c for the central thiol has been estimated as *ca* -0.3 to -0.4 from a limited set of data for reactions of RS^- with EllS-SEll, RS-SEll, and $HOCH_2CH_2CH_2S$-SEll[76].

$$\log k_{RS^-} = C + \beta_{nuc} pK_a^{nuc} + \beta_c pK_a^{c} + \beta_{lg} pK_a^{lg} \qquad (15)$$

$$R^{nuc}S^- + R^cS{-}SR^{lg} \xrightarrow{k_{RS^-}} R^{nuc}S{-}SR^c + {}^-SR^{lg} \qquad (16)$$

$$\log k_{RS^-} = 7.0 + 0.50\,pK_a^{nuc} - 0.27\,pK_a^{c} - 0.73\,pK_a^{lg} \qquad (17)$$

$$\log k_{RS^-} = 6.3 + 0.59\,pK_a^{nuc} - 0.40\,pK_a^{c} - 0.59\,pK_a^{lg} \qquad (18)$$

The data on which equations 17 and 18 are based cover a range of different (and perhaps not directly comparable) thiol and disulfide structures. A study of the Brønsted coefficients of the nucleophilic, central and leaving group thiols using a carefully limited and consistent set of thiols and disulfides would be useful mechanistically. In the absence of unambiguously interpretable data, these correlations (equations 17 and 18) should be considered as kinetic models for thiolate–disulfide interchange reactions. Although they do not have an unimpeachable mechanistic foundation, they are useful in predicting rate constants (k_{RS^-}) in water. The value of k_{RS^-} can be converted to the observed rate constant (k_{obsd}) at the pH of reaction using equation 14.

The optimum value of pK_a of the nucleophilic thiol for the maximum observed rate (k_{obsd}) of thiol–disulfide interchange at a given pH can be predicted by a Brønsted correlation (equation 19)[5,6]. The optimum value of pK_a of the nucleophilic thiol (based on the assumption of $\beta_{nuc} = 0.50$) is therefore the value of the pH of the reaction mixture; at pH 7 a nucleophilic thiol of $pK_a = 7$ will show the maximum observed rate of thiol–disulfide interchange (Figure 2). This prediction closely matches the observed rates of thiol–disulfide interchange[5,6] and is useful in designing reagents that reduce disulfide bonds rapidly at pH 7[18,20]. For a thiol of $pK_a \gg pH$, only a small fraction of the total thiol is present as thiolate; for a thiol of $pK_a \ll pH$, thiol is present as thiolate, but its

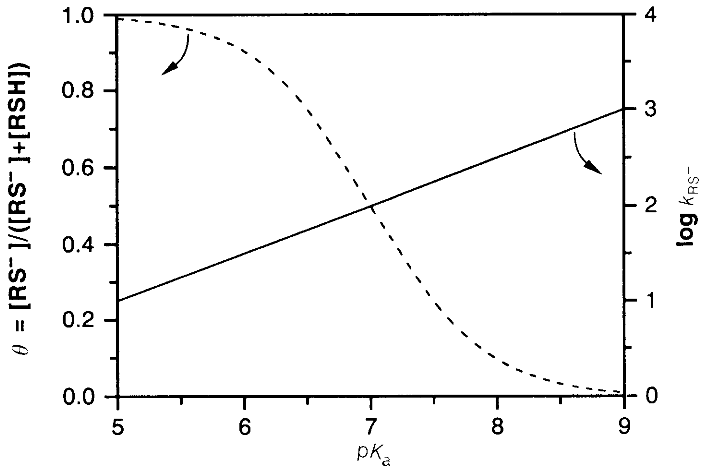


FIGURE 2. Comparison of plots of the log of rate constant of the thiolate–disulfide interchange reaction ($\log k_{RS^-}$, —) vs pK_a of a nucleophilic thiol, and of the degree of dissociation at pH 7 (θ, ----) vs pK_a of thiol. The values of rate constant (k_{RS^-}) are calculated using equation 17, assuming the value of β_{nuc} as 0.5, and the value of pK_a of both the central and leaving group thiols as 8.5. The observed rate constant (k_{obsd}) in terms of the total concentration of thiol and thiolate in solution is given by $k_{obsd} = \theta k_{RS^-}$

nucleophilicity is low. A thiol of $pK_a = pH$ balances the proportion of thiol present as thiolate and adequate nucleophilicity of the thiolate.

$$pH = pK_a + \log[(1 - \beta)/\beta] \tag{19}$$

3. Substituent effects

a. Steric. The steric effect is most pronounced when all three thiols in the transition state are fully substituted at the carbon α to sulfur. It is significantly large even when two of the three adjacent thiols in the transition state are fully substituted at the carbon α to sulfur. The rate constant for reaction of *t*-butyl thiolate with bis(*t*-butyl) disulfide ($k_{s^-} = 10^{-7}\,M^{-1}\,s^{-1}$) in butanol is *ca* 10^6-fold slower than that for 1-butyl thiolate with bis(1-butyl) disulfide ($k_{s^-} = 0.26\,M^{-1}\,s^{-1}$)[24]. The observed rate constant for the reaction of penicillamine ($^-OOC—CH(NH_3^+)—C(CH_3)_2SH$) with the mixed disulfide of penicillamine and glutathione at pH 7.4 ($k_{obsd} = 0.00047\,M^{-1}\,min^{-1}$) is *ca* 10^5-fold slower than that of penicillamine with glutathione disulfide which contains no substitution α to sulfur ($k_{obsd} = 37\,M^{-1}\,min^{-1}$)[66]. The observed rate constant for the reaction of glutathione with penicillamine disulfide ($0.012\,M^{-1}\,min^{-1}$) is also *ca* 2000-fold lower than for glutathione with mixed penicillamine-glutathione disulfide ($27\,M^{-1}\,min^{-1}$)[66]. The equilibrium constant for the formation of bis(*t*-butyl) disulfide is small in the reaction of *t*-butyl thiol with mixed 1-butyl *t*-butyl disulfide, i.e. the formation of bis(*t*-butyl) disulfide is disfavored[75,77].

Steric effects are small for alkyl substitution at carbon β to sulfur: the rate constant for degenerate thiolate–disulfide interchange of neopentyl thiolate with its disulfide is only threefold lower than that of 1-butyl thiolate with its disulfide[15]. The reaction of thiol group of the Bovine serum albumin (BSA) with Ellman's disulfide is anomalously slower, by a factor of 14, than that with cystamine ($^+H_3NCH_2CH_2SSCH_2CH_2NH_3^+$)[78].

The thiol groups in some proteins appear to be relatively inaccessible, possibly due to a combination of steric effect and other factors such as local hydrophobicity or charge–charge repulsion.

b. Acidity. The rate constants of thiolate–disulfide interchange reactions vary significantly with the acidities of the substrate thiols: The reaction of mercaptoethanol with Ellman's disulfide ($k_{RS^-} = 1.5 \times 10^7\ M^{-1}\ min^{-1}$)[6] is significantly faster than that of mercaptoethanol with glutathione disulfide ($k_{RS^-} = 3.4 \times 10^3\ M^{-1}\ min^{-1}$)[5] in water; the relevant values of pK_a are 4.5 for EllSH and 8.7 for GSH. Brønsted correlations (equations 15–18) describe the effect of acidities (pK_a) of the nucleophilic, central and leaving group thiols on the rate constants of thiolate–disulfide interchange reactions. The rate constants for thiolate–disulfide interchange (k_{RS^-}) are larger for increasing values of pK_a for nucleophilic thiols, and for decreasing pK_a values for central and leaving group thiols. The rate constant should be affected more by a change in the pK_a values of the nucleophilic and leaving group thiols than that for the central thiol, because the Brønsted coefficients are larger for the nucleophilic and leaving group thiols than for the central thiol[5].

A mixed disulfide (R′SSR″) may have two constituent thiols of different acidities (p$K_a^{R'SH} >$ p$K_a^{R''SH}$). The cleavage of the mixed disulfide R′SSR″ by a nucleophilic thiolate RS^- occurs favourably with release of the more acidic thiol (R″SH), and the less acidic R′S group is retained in the new mixed disulfide (RSSR′)[76].

c. Charge. The rates of thiol–disulfide interchange reactions in aqueous solutions with charged substituents vary by as much as a factor of 2.5 from the predicted rate constants based on structure–reactivity correlations with uncharged substituents[79]. The deviations from predicted values based on uncharged substituents are the greatest when the charge is on the central group, and the deviations decrease with increasing distance of the charge from the reactive site[79]; e.g., both the rates of reactions of $^-O_2CCH_2CH_2S^-$ with the mixed disulfides $^-O_2CCH_2CH_2SSC_6H_4NO_2$-*p* and $^-O_2CCH_2CH_2CH_2SSC_6H_4$-$NO_2$-*p* are lower than the predicted values based on the Brønsted correlation with uncharged substituents, but the deviation is lower with $^-O_2CCH_2CH_2CH_2SSC_6H_4$-$NO_2$-*p*[79]. A similar effect of the negative charge on R groups is seen in the rate constants for degenerate RS^-/RSSR interchange reactions[9]. Thiolates without charged substituents, such as mercaptoethanol thiolate, react 25 times faster with a positively charged analog of Ellman's disulfide than with the negatively charged Ellman's disulfide; this ratio decreases to *ca* 1 to 3.5 for a thiolate with a positively charged substituent three bonds from sulfur (cysteine ethyl ester), and increases to 120 for a thiolate with a negatively charged substituent ($^-O_2CCH_2S^-$)[80].

The electrostatic influence of the local cysteine environments in peptides has been observed in thiol–disulfide interchange reactions[71,81]. The rate constants in water for the reaction of the negatively charged Ellman's disulfide and a peptide containing cysteine with two positive neighbors, one positive and one neutral neighbor, or two neutral neighbors are 130,000, 3350 and 370 $M^{-1}\,s^{-1}$ respectively at pH 7 and 20 mM ionic strength[81]. Electrostatic contributions totaling a factor of 2000 ($\Delta G = 4.3$ kcal mol^{-1}) have been estimated for the fastest and the slowest thiol–disulfide interchange reactions of small charged substrates in 50% methanol–water mixture; these contributions to the free energy comprise +3.0 kcal mol^{-1} from attraction and −1.3 kcal mol^{-1} from repulsion[71].

d. Hydrogen bonding. The rates of thiolate–disulfide interchange in polar aprotic solvents are not significantly affected by groups capable of intramolecular hydrogen bonding[15]. The rate constant for the degenerate thiolate–disulfide interchange reaction

TABLE 2. Comparison of rate constants for degenerate thiolate–disulfide interchange ($RS^- + RSSR \rightleftharpoons RSSR + {}^-SR$) in polar protic and polar aprotic solvents

RS^-	M^+	Solvent	$10^{-3}k^{a,b}$ ($M^{-1}s^{-1}$) (297 K)	$\Delta G^‡$ (kcal mol^{-1}) (297 K)	$\Delta H^‡$ (kcal mol^{-1})	$\Delta S^‡$ (cal K^{-1} mol^{-1})
$HOCH_2CH_2S^-$	Na^+	D_2O	0.0077	16.2	13	−10
	K^+	D_2O	0.0095	16.1	13	−11
	K^+	CD_3OD	0.0040	16.6	13	−12
	K^+	DMF-d_7	20	11.5	8	−13
	K^+	DMSO-d_6	21	11.5		
$CH_3CH_2CH_2CH_2S^-$	Na^+	DMF-d_7	43	11.1		
	K^+	DMSO-d_6	54	11.0		
$CH_3C(CH_3)_2CH_2S^-$	K^+	DMF-d_7	15	11.7		
	K^+	DMSO-d_6	16	11.7		
$HOC(CH_3)_2CH_2S^-$	K^+	DMSO-d_6	1.1	13.2	10	−10
$HOCH_2C(CH_3)_2CH_2S^-$	K^+	DMSO-d_6	0.67	13.5	9	−16

[a]Uncertainties are: k, $\pm 10\%$; $\Delta G^‡$, ± 0.1 kcal mol^{-1}; $\Delta H^‡$, ± 1 kcal mol^{-1}; $\Delta S^‡$, ± 2 cal K^{-1} mol^{-1}.
[b]Rate constants were inferred from visual comparison of the simulated ^{1}H NMR line shapes with the experimental line shapes. The values for CD_3OD are unpublished observations of R. Singh and G. M. Whitesides; all other values are from reference 15.

of 2-hydroxyethanethiolate is only twofold lower than that of 1-butanethiolate. In sterically hindered thiolates, introduction of a hydroxy group either β or γ to the C—S bond slows the interchange by approximately a factor of 15 in DMSO (Table 2). A *gem*-dimethyl effect and weaker solvation of the hydroxyl group in the sterically hindered substrate may result in greater intramolecular hydrogen bonding than in the sterically unhindered 2-hydroxyethanethiolate[15].

e. Reactions involving cyclic disulfides. The rate constant for degenerate intermolecular thiolate–disulfide interchange involving cyclic five-membered disulfides (1,2-dithiolane) is higher than that involving cyclic six-membered disulfides (1,2-dithiane) by a factor of *ca* 650 ($\Delta\Delta G^‡$ *ca* 3.8 kcal mol^{-1})[17]. The rate constants for the cyclic six- and seven-membered disulfides are similar to those for noncyclic disulfides[17]. The ring strain of 1,2-dithiolane (estimated by calorimetry) is higher than that of 1,2-dithiane by 3.7 kcal mol^{-1} [82]. The agreement of the value of $\Delta\Delta G^‡$ (3.8 kcal mol^{-1}) from kinetics and the value of ring strain (3.7 kcal mol^{-1}) from calorimetry suggests that the ring strain in the cyclic five-membered disulfide is completely released in the transition state[17]. In the transition state, the S—S bond is expected to be longer than in the ground state of disulfide, and the CSS angle at the central carbon is energetically most favorable at *ca* 90° [83,84]. This geometry expected for the transition state is better matched by the structure of the ground state of the cyclic five-membered disulfide than that of cystine, based on X-ray crystallographic structural parameters[17].

4. Solvent effects

The rates of thiolate–disulfide interchange reactions are larger in polar aprotic solvents (DMSO, DMF) than in polar protic solvents (water, methanol) by a factor of *ca* 10^3 (Table 2)[15]. The nature of the counter ions (Na^+, K^+), or addition of 18-crown-6 to the reaction involving potassium alkanethiolate, has no effect on the rate of thiolate–disulfide interchange in DMSO[15].

The transition state is expected to have a more delocalized negative charge and therefore to be less influenced by solvation than the ground state thiolate. The higher rates of thiolate–disulfide interchange in polar aprotic solvents (DMSO, DMF) than in polar protic solvents (water, methanol) may be explained by a smaller destabilization of the transition state than that of the ground state thiolate, in going from polar protic solvents to polar aprotic solvents (Figure 3)[15]. The log of the rate constant depends linearly on the solvent composition in mixtures of water and DMSO (Figure 4)[15,17].

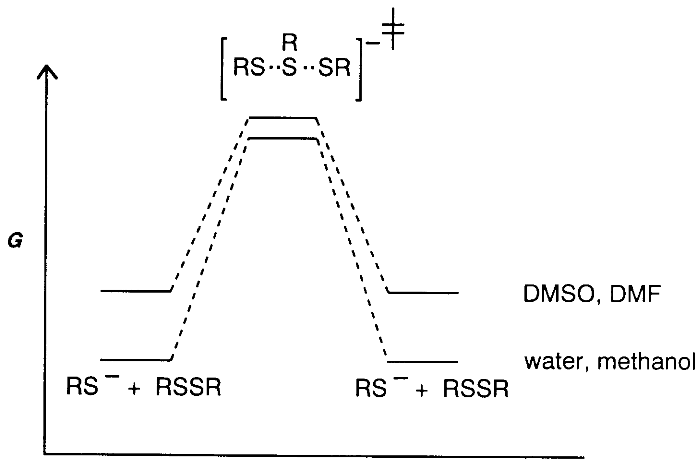


FIGURE 3. Hypothetical plot of free energy vs reaction coordinate for thiolate–disulfide interchange reaction in polar protic solvents (water, methanol) and in polar aprotic solvents (DMSO, DMF)

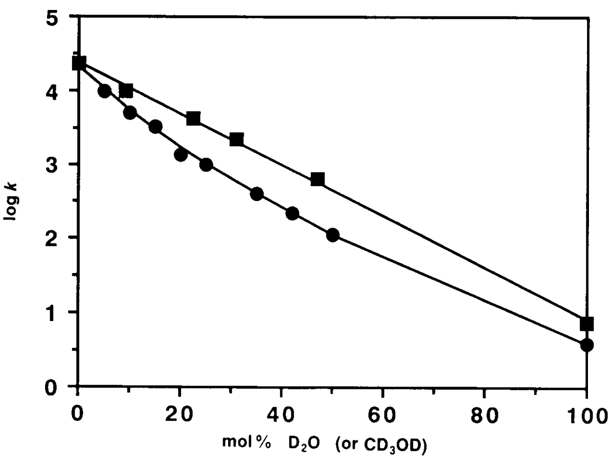
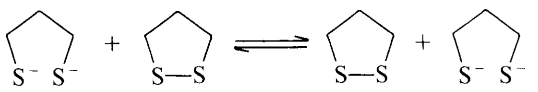


FIGURE 4. Effect of addition of D_2O (■) or CD_3OD (●) on log of rate constants (k) of thiolate–disulfide interchange of potassium 2-hydroxyethanethiolate and bis(2-hydroxyethyl) disulfide in DMSO-d_6. The values of rate constants are for 297 K and are in $M^{-1}s^{-1}$

The corresponding plot for methanol–DMSO mixture, although not linear, also shows a gradual decrease in the log of the rate constant with an increasing mole fraction of methanol (Figure 4)[85]. The absence of a sharp drop in rate on addition of small mole fractions of water or methanol to DMSO suggests the absence of specific solvation of thiolate by polar protic solvents. In going from polar protic to polar aprotic solvents, the increase of approximately 10^3 in rate of reaction involving thiolate anion (RS^-) is less than that (10^6–10^7) involving S_N2 reactions of alkoxide anion (RO^-)[15]. The alkoxide anions are more solvated in water than are thiolate anions[86,87].

The rate of thiolate–disulfide interchange of 1,3-propanedithiolate and 1,2-dithiolane (cyclic five-membered disulfide) is extremely fast in DMSO (k_{RS} *ca* $10^8\ M^{-1}s^{-1}$) and only *ca* 10^2 slower than the diffusion limit[17] (equation 20). This large rate arises from two factors: (i) the ground state of 1,2-dithiolane is destabilized relative to the transition state because of ring strain, and (ii) the thiolate is relatively more destabilized in DMSO than is the transition state with its more delocalized charge[17].


(20)

A comparison of the strengths of the $RS^-\cdot\cdot HOR$ complexes and $RO^-\cdot\cdot HOR$ complexes by pulsed high-pressure mass spectrometry shows that complexes incorporating alkoxides are more stable by 2–7 kcal mol^{-1} than those incorporating thiolates[88]. The weak contributions of ionic hydrogen bond to solvation in $RS^-(H_2O)_n$ complexes are effectively dissipated within the first 2–3 solvent molecules ($n = 2$–3)[88].

5. *Gas-phase studies*

The reaction of ethanethiolate ($C_2H_5S^-$) with dimethyl disulfide (CH_3SSCH_3) in the gas phase occurs exclusively with thiolate–disulfide interchange; this reaction yields methanethiolate (CH_3S^-) and mixed ethyl methyl disulfide ($CH_3SSC_2H_5$)[89]. A possible side reaction, the carbon-centered substitution to yield CH_3SS^- and $CH_3SC_2H_5$, is not observed[89]. The value of the rate constant for the reaction of ethanethiolate with dimethyl disulfide in the gas phase is estimated as $3 \times 10^9\ M^{-1}s^{-1}$. Comparison of this rate constant with the collisional rate constant suggests that the reaction occurs with a probability of 0.003 per collision[89].

6. *Catalysis*

A number of species—aromatic thiols, nonthiol nucleophiles and cations—have been surveyed as potential catalysts for thiol–disulfide interchange in water[15]; catalysis is observed only with selenols[15,19], and even with these species the magnitudes of the catalysis are not large.

Selenols are only effective as catalysts for thiol–disulfide interchange reactions involving strongly reducing dithiols[19]. The observed rate of reduction of bis(2-hydroxyethyl) disulfide by dithiothreitol in water at pH 7 is enhanced by a factor of 15 in the presence of 5 mol% 2-aminoethaneselenol. This catalytic activity of selenols is probably due to a combination of the low pK_a (*ca* 5.5 to 7) (and hence significantly high concentration of RSe^- at pH 7) for selenols, and weak solvation and high polarizability (and hence high nucleophilicity) of the selenolate anion. The precursors of selenols, diselenides (RSeSeR) and selenocyanates (RSeCN) can also be conveniently used to catalyze the thiol–disulfide interchange reactions involving strongly reducing dithiols[19]. Thiol–disulfide interchange reactions involving monothiols are not catalyzed by selenols, because these disulfides oxidize the selenols to diselenides. Strongly reducing dithiols at even moderate concentrations can reduce diselenides to selenols, and therefore in the thiol–disulfide interchange reactions involving strongly reducing dithiols, the selenol remains in the reduced (and catalytic) state[19].

7. Comparison with selenolate–diselenide interchange

The observed rate of selenolate–diselenide interchange for selenocysteamine and selenocystamine ($k_{obsd} = 1.65 \times 10^7\,M^{-1}\,s^{-1}$)[69] in water at pH 7 is faster by a factor of 10^7 than the corresponding thiol–disulfide interchange reaction of cysteamine and cystamine ($k_{obsd} = 1.4\,M^{-1}\,s^{-1}$), possibly due to (i) better nucleophilicity and better leaving group ability of selenolate than for thiolate, and (ii) low pK_a of selenols (*ca* 5.5 to 7) and therefore high concentration of the nucleophilic selenolate anion at pH 7[69]. In this system, the absolute rate constant (k_{RSe^-}) for the selenolate–diselenide interchange is higher than that of the structurally analogous thiolate–disulfide interchange (k_{RS^-}) by 2.4×10^5.

D. Transition State Structure

A study of crystal structures of compounds containing divalent sulfur (Y—S—Z; Y,Z $\neq$ H) shows that nonbonded contacts of nucleophiles are directed along the extension of one of the covalent bonds to sulfur[26]. According to the frontier-orbital model, the HOMO of the nucleophile interacts preferentially with the LUMO (σ^*) orbital of S—Y or S—Z. Attractive nonbonded interactions may represent the incipient stages of chemical reactions[26]. The preferred attack of the thiolate nucleophile on the disulfide (S—S) bond is therefore along the extension of the S—S bond.

In the transition state the negative charge must be delocalized over the three sulfur atoms. The transition state is qualitatively pictured as having greater negative charge at the terminal sulfurs than at the central sulfur, based on the value of the Brønsted coefficients: $\beta_{nuc} = \beta_{lg} \approx 0.5$ (by symmetry); $\beta_c \approx -0.3$ to -0.4[5–8]. The absence of curvature in the Brønsted plots for attack of thiolate anions having a range of pK_a values on a single disulfide suggests that the transition state structure does not change with changes in structure of the thiolate anions or the disulfide groups for these thiol–disulfide interchange reactions[5–7]. Superposition of plots of $\log k_{RS^-}$ (rate constant) vs $\log K_s$ (equilibrium constant) for a series of thiol–disulfide interchange reactions, varying in equilibrium constant by a factor of approximately 10^{21}, shows gradual curvature of the type expected on the basis of the Hammond postulate[8]. Although these data indicate a change in transition state structure[8], factors other than Hammond postulate behavior, such as solvation, can cause curvature in Brønsted plots. Although thiolate anions are not as strongly solvated as alkoxide anions, interpretations suggesting a change in structure of the transition state from a curved Brønsted plot should be treated with caution[50,90,91].

The value of $\Delta S^\ddagger$ for thiol–disulfide interchange in polar protic (water, methanol) and polar aprotic (DMSO, DMF) solvents is *ca* -10 to $-16\,cal\,K^{-1}\,mol^{-1}$ (Table 2)[15]. This value is less than that expected for complete localization of two particles in the transition state, and suggests that the decrease in entropy in the transition state relative to two particles in the ground state is partially compensated either by release of solvent molecules attached to the thiolate in the ground state[15], or by a relatively loose transition-state structure (with two weak, partial S··S bonds) or both.

E. Theoretical Calculations on Thiol–Disulfide Interchange

An *ab initio* MO study on the thiolate–disulfide interchange reaction indicates that the reaction is a typical S_N2 reaction and proceeds via a single transition state with little conformational distortion[92]. The charge distribution in the transition state is calculated to be higher on the two terminal sulfurs and lower on the central sulfur, in agreement with the experimental results based on Brønsted coefficients[92]. The geometry of the transition state has been suggested to be a trigonal bipyramidal configuration at the central sulfur with the nucleophilic and leaving sulfurs in apical positions[83]. The participation of d orbitals is not essential in stabilization of the transition state[83].

$$\begin{array}{ccc} 0.4- & 0.2- & 0.4- \\ \mathrm{RS}\cdots & \mathrm{S} & \cdots\mathrm{SR} \\ & \mathrm{R} & \end{array}$$

F. Mechanistic Uncertainties

The geometry of the transition state is unclear: the relative dispositions of the alkyl groups on the three sulfur atoms in the transition state are not known. The symmetry of the transition state with respect to the nucleophilic and leaving group thiols is still ambiguous, although microscopic reversibility would indicate a symmetrical structure if there is a single transition state. Unsymmetrical transition states connected by a symmetrical intermediate are possible, but seem unlikely. A more complete characterization of the Brønsted coefficients, and appropriate calculations, will both be useful in understanding this issue. The transition state seems to be less solvated than the ground state thiolate, but the degree of solvation of the transition state is not known. Resolving the question of solvation may be useful in designing strategies for catalysis of thiol–disulfide interchange. Strategies for catalysis based on desolvation and destabilization of the ground state thiolates seem unlikely to produce large effects. A more plausible strategy (although one that represents a difficult problem in molecular design) will be to stabilize the transition state of the catalyzed reaction, perhaps by appropriate charge–charge interactions in the charge-delocalized transition state.

IV. EQUILIBRIUM IN THIOL–DISULFIDE INTERCHANGE REACTIONS

A. Equilibria Involving Monothiols

In the equilibria involving a monothiol (RSH) and a disulfide (R′SSR′) (equations 1 and 11), the distribution of species is nearly random or statistical if the pK_a values of the thiols (RSH and R′SH) are similar, i.e.

$$K_1 = \{([\mathrm{RSSR'}][\mathrm{R'SH}])/([\mathrm{RSH}][\mathrm{R'SSR'}])\} \approx 2$$

and

$$K_2 = \{([\mathrm{RSSR}][\mathrm{R'SH}])/([\mathrm{RSH}][\mathrm{RSSR'}])\} \approx 0.5$$

The experimental values of equilibrium constants for the interchange involving glutathione (RSH) and cystine (R′SSR′) in water at pH 7 are similar to those expected from random distribution, $K_1 = 3.7$ and $K_2 = 0.79$[23,73,93].

The equilibrium constants for thiol–disulfide interchange reactions for a series of monothiols and disulfides, at values of pH in which the equilibrium concentration of thiolate anion is small, are relatively insensitive to changes in substituents (except for sterically hindered structures with alkyl substituents at carbon α to sulfur)[68]. The formation of bis(*t*-butyl) disulfides is disfavored in equilibria involving *t*-butyl thiol and mixed *t*-butyl 1-butyl disulfide[75,77,94], and the formation of penicillamine disulfide is disfavored in equilibria involving penicillamine and mixed penicillamine cysteine disulfide[68].

The equilibrium constant for the interchange of a monothiol (RSH) with a disulfide

(R′SSR′) is pH dependent if the values of pK_a of the thiols (RSH and R′SH) are different. In general, the equilibrium mixture favours the most stable thiolate: the equilibrium is, in effect, driven to one side by the free energy of ionization of the most acidic thiol.

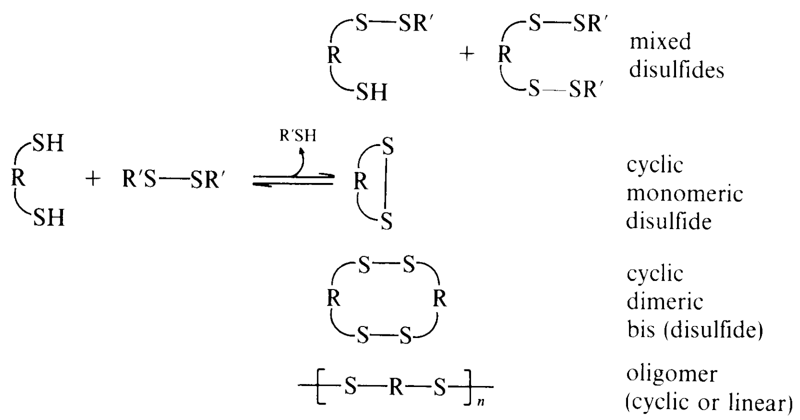
At a value of pH between the values of pK_a of RSH and R′SH, the formation of the thiolate corresponding to the thiol of lower pK_a is preferred[5]. The equilibrium of the thiol–disulfide interchange reaction involving mercaptoethanol ($pK_a = 9.6$) and Ellman's disulfide (pK_a of EllSH *ca* 4.5) in aqueous buffer at pH 7–8 is shifted entirely toward the formation of Ellman's thiolate and the disulfide of mercaptoethanol. The amount of Ellman's thiolate is approximately quantitatively equal to that of initial mercaptoethanol, and hence the utility of Ellman's assay.

The values of the equilibrium constant (K^{obsd}) of thiol–disulfide interchange in aqueous medium can be dissected into K^{SH} (defined for thiols) and K^{S^-} (defined for thiolates)[5]. The equilibrium constant K^{SH} shows no obvious correlation with the values of pK_a, but is influenced by steric effects. The plot of the log of the equilibrium constant K^{S^-} vs $2(pK_a^{RSH} - pK_a^{R'SH})$ is linear (slope *ca* 1.2)[5]. K^{S^-} is therefore strongly influenced by the acidities of the participating thiols[5].

Electrostatic effects on equilibria of thiol–disulfide interchange reactions are small in magnitude, but occur in the expected direction. The formation of mixed disulfide with unlike charges on the two component thiols is favored, and the formation of mixed disulfide with like charges on the two component thiols is disfavored[72]. In the equilibrium involving *N*-acetylcysteine (**A**, bearing one negative charge on the cysteine carboxylate) and the 85–114 peptide fragment of Kunitz soybean trypsin inhibitor (**B**, bearing one positive charge on the *N*-terminal leucine residue next to cysteine), the proportions of disulfides **A**—**B**, **A**—**A** and **B**—**B** in water at pH 7 and low ionic strength (20 mM) are 72%, 10% and 18% respectively, and at high ionic strength (1 M) are 61%, 15% and 24% respectively. The expected statistical distributions are 50%, 25% and 25% respectively. The electrostatic effect at low ionic strength (20 mM) favors the formation of **A**—**B**, and disfavors the formation of **A**—**A** more than that of **B**—**B**, because the two negative charges on **A**—**A** are closer to each other than are the positive charges on **B**—**B**. At high ionic strengths the electrostatic effects are shielded and the observed distribution is similar to that statistically expected[72].

B. Equilibria Involving α,ω-Dithiols

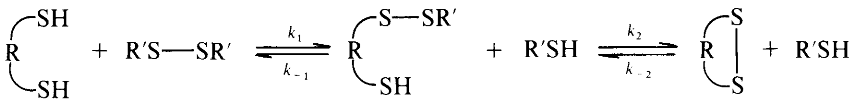
Thiol–disulfide interchange of α,ω-dithiols (HS—R—SH) with a disulfide (R′SSR′) can generate a variety of products ranging from cyclic monomeric disulfide, cyclic dimeric



(21)

bis(disulfide) to oligomeric disulfide (equation 21). The product distribution depends on the nature of R, and on the concentrations of the dithiol and the disulfide.

Cyclic monomeric disulfides are the major products for the thiol–disulfide interchange reactions of 1,3-dithiols to 1,6-dithiols in which the two thiol groups are separated by three to six atoms (equations 22 and 23). The formation of the cyclic monomeric disulfide occurs via the intramolecular thiol–disulfide interchange reaction of the intermediate mixed disulfide (k_2, equation 22); this reaction is significantly faster than the corresponding intermolecular reaction. High effective concentration (EC, see below and also Table 3, for footnote *c*) favors the formation of the cyclic monomeric disulfide[17].



(22)

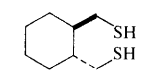
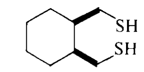
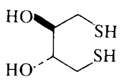
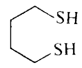
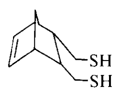
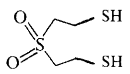
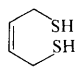
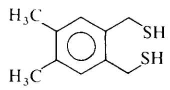
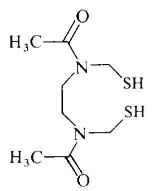
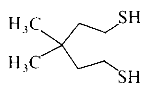
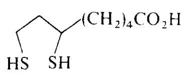
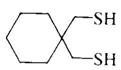
$$K_{eq} = ([\overline{SRS}][R'SH]^2)/([HS—R—SH][R'SSR']) \qquad (23)$$

The stability of the cyclic disulfide is an important factor in the overall equilibrium. Cyclic six-membered disulfides have a CSSC dihedral angle of *ca* 60° and are more stable than cyclic five-membered disulfides, which have a CSSC dihedral angle of *ca* 30°[14]. The ring strain in cyclic five-membered disulfides has been estimated as 3.7 kcal mol^{-1} higher than that for the cyclic six-membered disulfides[82]. It has been estimated that the ring cleavage (k_{-2}, equation 22) of cyclic five-membered disulfides is faster by a factor of *ca* 600 than that of cyclic six-membered disulfides[17]. On the other hand, the formation of the cyclic five-membered disulfide (k_2, equation 22) is faster by a factor of *ca* 20 than that of the cyclic six-membered disulfide, based on values of kinetic effective concentrations for analogous reactions[17]. The overall result is that K_{eq} for formation of a six-membered disulfide from the corresponding dithiol is more favorable than that for a five-membered disulfide from its dithiol by a factor of *ca* 30[13,17].

The reducing ability of α,ω-dithiols depends on two factors: (i) the stability of the monomeric cyclic disulfide, and (ii) the kinetic effective concentration for the intramolecular ring-closure step (k_2, equation 22). 1,4-Alkanedithiols that form strain-free cyclic six-membered disulfides are the most reducing (K_{eq} *ca* 10–10^3 M, equation 23); 1,3- and 1,5-alkanedithiols that form five- and seven-membered rings respectively are *ca* 10-fold less reducing (Table 3). Rings smaller than six-membered are less favored primarily for enthalpic reasons (ring strain, including angle strain in the CSSC group). Rings larger than six-membered are less favored because of conformational entropy (low kinetic effective concentration for the intramolecular ring-closure)[13,16]. In 1,8-dithiols, the effective concentration for intramolecular ring-closure is sufficiently low that intermolecular oligomeric disulfide formation becomes competitive with cyclic monomeric disulfide formation[13]. The reduction potentials of dithiols that form oligomeric products are similar to those for monothiols[5,13]. 1,2-Dithiols form cyclic bis(disulfide) dimers in relatively dilute solution (*ca* 1 mM), but polymerize at higher concentrations[13].

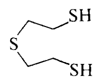
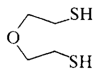
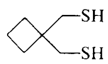
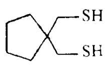
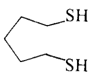
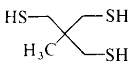
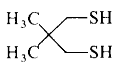
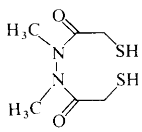
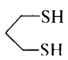
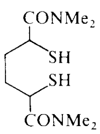
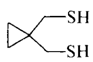
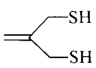
Molecular mechanics calculations of equilibria of thiol–disulfide interchange reactions involving α,ω-dithiols with 1,2-dithiane correlate well with experimental results, but do not give the absolute values of energies[16]. The empirical relationship between calculated differences in strain energy (ΔSE) and the experimental values of ΔG is: ΔG *ca* 0.4ΔSE. Why the molecular mechanics calculations overestimate strain is not known. This correlation may be a useful guide for designing α,ω-dithiols of appropriate reduction potential.

TABLE 3. Equilibrium constants for thiol–disulfide interchange

Structure	$K(\mathrm{ME}^{ox})$	$\varepsilon_0(\mathrm{V})$[a]	Eq. against[b]	References
Dithiols that form cyclic monomers[c]				
	1500 M	−0.354	DTT	*d,e*
	670 M	−0.344	DTT	*d,e*
	180 M	−0.327	Lip	*d,e*
	77 M	−0.316	DTT	*d,e*
	65 M	−0.314	DTT	*d,e*
	63 M	−0.313	DTT	*f*
	44 M	−0.309	DTT	*d,e*
	19 M	−0.298	DTT	*e,g*
	15 M	−0.295	DTT	*f*
	14 M	−0.294	DTT	*d,e*
	8.6 M	−0.288	ME, DTT	*d,e*
	8.0 M	−0.287	DTT	*d,e*

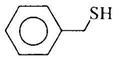
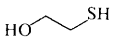
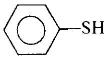
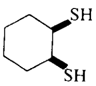
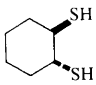
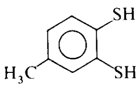
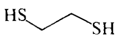
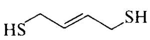
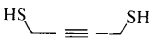
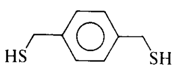
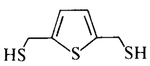
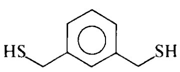
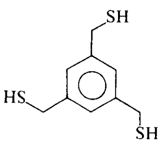
(continued)

TABLE 3. (*continued*)

Structure	$K(ME^{ox})$	$\varepsilon_0(V)$[a]	Eq. against[b]	References
	6.7 M	−0.285	DTT	*d,e*
	6.1 M	−0.284	DTT	*d,e*
	4.4 M	−0.279	DTT	*d,e*
	3.6 M	−0.277	DTT	*d,e*
	3.6 M	−0.277	DTT	*d,e*
	3.1 M	−0.275	DTT	*d,e*
	2.9 M	−0.274	DTT	*d,e*
	2.5 M	−0.272	DTT	*h*
	2.3 M	−0.271	ME, DTT	*d,e*
	1.8 M	−0.269	DTT	*i*
	1.2 M	−0.263	DTT	*d,e*
	0.67 M	−0.255	DTT	*d,e*
6,6′-sucrose disulfide	0.30 M	−0.245	ME	*j*
$HS(CH_2)_6SH$	0.21 M	−0.240	DTT	*d,e*

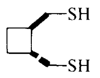
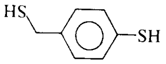
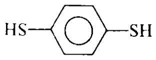
(*continued*)

TABLE 3. (*continued*)

Structure	$K(ME^{ox})$	$\varepsilon_0(V)^a$	Eq. against[b]	References
Monothiols that form dimers				
	2.6	−0.272	ME	*d*
$CH_3(CH_2)_6SH$	1.1	−0.261	ME	*d*
	1.0	−0.260	ME	*d*
	0.31	−0.245	ME	*g*
Dithiols that form cyclic dimers				
	0.40 M	−0.254	ME	*d,e*
	0.38 M	−0.254	ME	*d,e*
	0.32 M	−0.253	ME	*e,g*
	0.035 M	−0.239	ME	*d,e*
Dithiols that form polymers				
	4.8	−0.280	ME	*d*
	4.0	−0.278	ME	*d*
	3.4	−0.276	ME	*g*
	3.1	−0.275	ME	*d*
	3.0	−0.275	ME	*g*
	2.8	−0.274	ME	*k*

(*continued*)

TABLE 3. (*continued*)

Structure	$K(ME^{ox})$	$\varepsilon_0(V)^a$	Eq. against^b	References
	1.8	−0.268	ME	*d*
$HS(CH_2)_8SH$	1.7	−0.267	ME	*d*
$HS(CH_2)_7SH$	1.4	−0.265	ME	*d*
	1.3	−0.264	ME	*g*
	0.20	−0.240	ME	*g*

[a]$\varepsilon_0(V)$ values vs standard hydrogen electrode at pH 7.0 and 25 °C. All $\varepsilon_0(V)$ values are calculated using the $\varepsilon_0(V)$ values for lipoic acid [−0.288 V, D. R. Sanadi, M. Langley and R. L. Searls, *J. Biol. Chem.*, **234**, 178 (1959) and C. V. Massey, *Biochem. Biophys. Acta*, **37**, 314 (1960)] and the K_{eq} value between lipoic acid and the compound of interest.
[b]Abbreviations: DTT, dithiothreitot; Lip, lipoic acid; ME, 2-mercaptoethand.
[c]The value of $K(ME^{ox})$ for this group of compounds is sometimes called the effective concentration (EC).
[d]Equilibrations were carried out at 25 °C, in a 1/1 mixture of d_4-methanol/phosphate buffer (50 mM, pH 7.0) in D_2O, see Reference 13.
[e]The equilibrium constants (*K*) in the Houk and Whitesides paper (13) were systematically in correct by a factor of 10^3 (originating in error in manipulation of units during the original calculations) and have been adjusted accordingly. The values of equilibrium constants, which were obtained from equilibrium with DTT, have also been readjusted by a factor of approximately 2 so as to obtain a similar value to that reported in this paper.
[f]Equilibrations were carried out at 25 °C in a 1/1 mixture of d_4-methanol/phosphate buffer (50 mM, pH 7.0) in D_2O, see G. V. Lamoureux and G. M. Whitesides, *J. Org. Chem.*, **58**, 633 (1993).
[g]Equilibrations were carried out in d_4-methanol with 0.02 mM sodium methylate added, see Reference 13.
[h]Equilibrations were carried out in D_2O (pD 7.0, 50 mM phosphate), see Reference 18.
[i]Equilibrations were carried out in D_2O (pD 7.0, 50 mM phosphate), see Reference 20.
[j]Equilibrations were carried out in D_2O (pD 7.0, 50 mM phosphate), see W. J. Less and G. M. Whitesides, *J. Org. Chem.*, **58**, 642 (1993).
[k]Equilibrations were carried out in d_6-benzene with 0.02 mM tetramethylguanidine added, see Reference 13.

The equilibrium constant for the thiol–disulfide interchange of an α,ω-dithiol with a disulfide (equations 22 and 23) has also been termed as the 'effective concentration' (EC)[95,96]. The equilibrium expression for effective concentration is a measure of the propensity of thiols to form the cyclic disulfide[96]. The EC has also been interpreted in terms of the *proximity* of these thiol groups in the ground state (that is, as a kind of local concentration), and thus used to infer information about conformation. Considering that the value of the equilibrium constant is very strongly influenced by strain in the CSSC group and by ring strain (for cyclic disulfides), its interpretation in terms of 'proximity' and 'concentration' must be evaluated with the possibility of contributions from these terms in mind[16]. If the EC is used (and interpreted) just as an equilibrium constant, but as one with an easily remembered reference value (EC *ca* 1–10 M for an α,ω-dithiol forming a strain-free cyclic disulfide) it has the virtue of being easy to remember and to interpret.

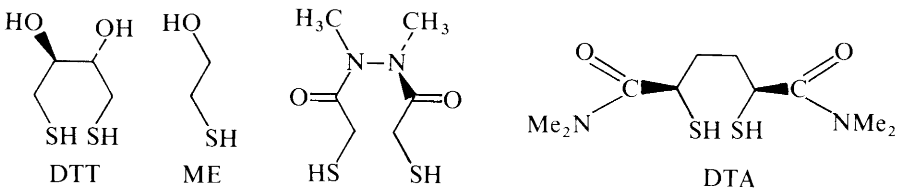
The equilibrium expression for effective concentration (EC = K_{eq}, equations 22 and 23) involves a ring-closure step (k_2) in the forward reaction (k_2 is a measure of the kinetic effective concentration), and a ring-cleavage reaction (k_{-2}) in the reverse direction. The ring-cleavage reaction, k_{-2}, is faster for a more strained cyclic disulfide than for an unstrained one, and correspondingly the value of the equilibrium expression for effective concentration (EC = K_{eq}) is lower for the more strained cyclic disulfide.

In cases involving cyclic disulfides with ring strain, the trends of the equilibrium expression for effective concentration (equilibrium EC) may be different than the trends of the kinetic values for effective concentration (kinetic EC). In a comparison of the formation of cyclic five-membered disulfide and cyclic six-membered disulfide, the equilibrium EC (related to K_{eq}, equations 22 and 23) is favored for the formation of the six-membered disulfide by a factor of 30 over the five-membered disulfide, whereas the kinetic EC (related to k_2, equation 22) is higher for the ring-closure reaction for formation of the five-membered disulfide than that of the six-membered disulfide by a factor of 20. The value of the equilibrium EC is easier to determine than that of the kinetic EC. As we have indicated, however, the equilibrium EC is a direct measure of proximity only when there is no strain in the disulfides or dithiols, and it is perhaps most useful as a measure of proximity when these other factors (e.g. ring strain reflecting an unfavorable CSSC dihedral angle; terms destabilizing the dithiol relative to disulfide) are absent or can be independently estimated[16].

V. APPLICATIONS OF THIOL–DISULFIDE INTERCHANGE IN BIOCHEMISTRY

The subject of the thiol–disulfide interchange reaction is an important one in biochemistry, and has been discussed extensively elsewhere[1–3]. Here we will only outline some of the issues.

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 their reduced state[97]. α,ω-Dithiols, such as dithiothreitol (DTT)[70], *N,N'*-dimethyl-*N,N'*-bis(mercaptoacetyl)hydrazine (DMH)[18] and *meso*-2,5-dimercapto-*N,N,N',N'*-tetramethyladipamide (DTA)[20], have higher reduction potential than cystine groups in proteins, and are useful disulfide-reducing reagents because they react specifically with the cystine disulfide to be reduced without any unwanted side-reaction with the protein. The value of the first pK_a of DTT is 9.2[5], and it is therefore relatively slow as a reducing reagent at pH 7. DMH and DTA (pK_a *ca* 8) reduce small organic disulfides and disulfide bonds in proteins *ca* 7 times faster than does DTT in water at pH 7[18,20]. Mercaptoethanol (ME, pK_a *ca* 9.6) is inexpensive and is used in large amounts (0.1–0.7 M) in biochemical manipulations, for example in conjunction with SDS gel-electrophoresis[97]. Mercaptoethanol is weakly reducing and it often generates complex reaction mixtures containing mixed disulfides[5].



DTT ME DMH DTA

The cyclic five-membered disulfide—lipoamide—is a cofactor of the pyruvate dehydrogenase complex[98,99]. The rate of ring opening of this cyclic five-membered disulfide by thiolate–disulfide interchange is faster by a factor of *ca* 10^3 than that involving cyclic six- or seven-membered disulfides[17]. The evolutionary selection of lipoamide as a cofactor in pyruvate dehydrogenase complex may reflect the fast rate of ring opening of the cyclic five-membered ring by nucleophiles and the resulting ability of the lipoamides to maintain a high flux through the pyruvate dehydrogenase complex[17].

The values of pK_a of thiol groups in proteins have been measured kinetically from the Bronsted correlation of thiol–disulfide interchange reactions[11]. The pK_a of the active-site thiol in papain is estimated as *ca* 4 at pH 6, and *ca* 8.4 at pH 9[11]. At low pH (*ca* 6) the proximate positively charged group increases the acidity of the active-site thiol in papain. The pK_a of the thiol group of reduced lysozyme is *ca* 11. These values of pK_a, although semiquantitative, are useful for comparison with the values of pK_a determined by other methods[11].

The redox equilibria between the cystine-bridged cyclic disulfide structures in proteins and their corresponding reduced open-chain α,ω-dithiol forms have been measured for several proteins[3]. The value of the equilibrium constant (or equilibrium expression for effective concentration, equilibrium EC) for the thiol disulfide interchange reaction of a protein α,ω-dithiol can be a useful measure of proximity of the two thiol groups in the protein if there is no ring strain associated with the corresponding cyclic disulfide. A high value of the equilibrium EC suggests that the two thiol groups are nearby spatially, are limited in mobility and can form a CSSC group with little or no angle strain[16,95,96].

The disulfide bonds in proteins are formed after translation[100,101]. The pathway of sequential disulfide bond formation has been studied for bovine pancreatic trypsin inhibitor (BPTI)[102,103] and for ribonuclease A[104]. In the case of BPTI, interpretations of different sets of data have led to different conclusions[102,103]. The conclusion of Weissman and Kim—that all well-populated folding intermediates in the oxidative folding of BPTI contain only native disulfide bonds—is still being actively debated[105,106].

The inclusion bodies, obtained from the expression of eukaryotic proteins in genetically engineered *E. coli*, may contain protein with unformed and mismatched disulfide bonds[107,108]. The conversion of the 'wrongly' disulfide-connected protein to the 'correctly' disulfide-connected protein is a major problem in biotechnology. The general approach is to reduce the 'wrongly' disulfide-connected protein completely and to oxidize it gradually with a redox buffer containing a mixture of thiol and disulfide[109]. Protein–disulfide isomerase has been proposed as catalyst for the thiol–disulfide interchange involving proteins[110]. Its low catalytic activity and absence of specificity make its biological role uncertain[111,112]. Thioredoxin has a cysteine of low pK_a and it reacts with disulfides rapidly at pH 7. Thioredoxin is redox-coupled to NADPH via the enzyme thioredoxin reductase, and may be of metabolic significance in thiol–disulfide interchange reactions[113–115].

VI. CONCLUDING REMARKS

Thiol–disulfide interchange is a reversible S_N2 reaction that involves cleavage and formation of a covalent S–S bond. The active nucleophile is the thiolate anion (RS^-); the thiol (RSH) is not active. The rates of reaction of thiolate anions with disulfides show a Brønsted correlation with the values of pK_a of thiols. The value of the Brønsted coefficient for the nucleophilic thiol (*ca* 0.5) is well studied, but a more complete analysis of the Brønsted coefficients for the central and leaving group thiols would be a useful step toward a better understanding of the structure of the transition state.

The rate constant of the thiolate–disulfide interchange reaction (k_{RS^-}, based on the concentration of thiolate anion) is influenced by factors such as pK_a of thiol and CSSC dihedral angle in the disulfide. The rate constant (k_{RS^-}) increases with increasing values of pK_a of the thiols because of the increasing nucleophilicity of the thiolate anions. The observed rate constant of reaction (k_{obsd}), however, is optimum for the value of pK_a of the thiol equal to the pH of the solution. The most stable CSSC dihedral angle in the disulfide is *ca* 90°. Cyclic five-membered disulfides, with CSSC dihedral angle of *ca* 30°, are strained, and are cleaved *ca* 10^3 times faster than the less-strained cyclic six-membered disulfide. An improved theoretical conformational analysis of the ground state of cyclic disulfides—in terms of the bond angles, bond lengths, and the CSSC and CCSS dihedral angles—would be useful to predict the ring strains and rates of thiol—disulfide interchange reactions involving cyclic disulfides.

Thiolate–disulfide interchange reactions are faster in polar aprotic solvents such as DMSO and DMF than in water. The rate enhancement in going from water to polar aprotic solvents is lower than for reactions of alkoxide anions. The thiolate–disulfide interchange involving strained cyclic five-membered disulfide is extremely fast (k_{RS} *ca* $10^8\ M^{-1}\,s^{-1}$) in polar aprotic solvents.

Disulfide bonds are present in proteins and are formed from the cysteine thiols after translation. The physical-organic study of several biochemical issues related to the thiol–disulfide interchange—the mode of formation of the 'correct' disulfide bonds, the degree of stability imparted to the protein by the disulfide bond, the strain in the large-ring protein disulfides, the role of thiol–disulfide interchange in regulation of protein activity and the design of reagents that can efficiently reduce disulfide bonds—would be important and useful.

VII. ACKNOWLEDGMENTS

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