Equilibrium Constants for Thiol-Disulfide Interchange Reactions: A Coherent, Corrected Set[1]

Watson J. Lees[2] and George M. Whitesides*

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

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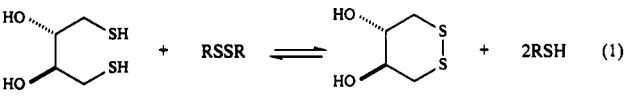
Equilibrium constants (K_{eq}) for the thiol-disulfide interchange reactions between dithiothreitol (DTT) and lipoic acid (14.2 ± 0.7), lipoic acid (Lip) and mercaptoethanol (13.3 M ± 1.0 M), and mercaptoethanol (ME) and glutathione (GSH or GSSG) (1.20 ± 0.10) were measured in D_2O at pD 7.0 by ^1H NMR spectroscopy. Two of these equilibrium constants [DTT and Lip (21.3 ± 0.9), and Lip and ME (8.6 ● 0.7)] were also measured in D_2O/CD_3OD. These values are compared with those obtained by other methods. A coherent set of values for the equilibrium constants between DTT or ME and thiols having a range of structures was assembled (Table III). The recommended value for the equilibrium constant between DTT and GSH is 210 M (K_{eq} = $[DTT^{ox}][GSH]^2/([DTT^{red}][GSSG])$).

Introduction

The thiol-disulfide interchange reaction is important to a number of subjects in biochemistry:[3,4] renaturing of proteins with correct cystine connectivity,[5] understanding mechanisms of action of enzymes and multienzyme complexes such as pyruvate dehydrogenase,[3,6,7] studying conformations of biomolecules,[8-12] stabilizing proteins in solution,[13,14] tracing refolding pathways for proteins,[15-24] and maintaining redox potentials in assay systems. The reducing strength of thiols in thiol-disulfide interchange is usually measured relative to a reference thiol/disulfide pair in an equilibrium system, rather than by an absolute measurement (e.g. electrochemical measurement of redox potential). The literature now contains a number of discordant values for the equilibrium constants of the thiol-disulfide interchange reactions most often used as reference systems: that is, those involving glutathione (GSH), mercaptoethanol (ME), lipoic acid (Lip), or dithiothreitol (DTT). The differences in these values are now being resolved through the effort of several groups,[25,26] and a coherent set of reference values for equilibrium constants is emerging. Some of the literature values (including some from our group) are wrong. It is apparent how the errors in certain of these values arose; other values are in disagreement with the corrected consensus values, but the source of the disagreement is not obvious. This paper reports new determinations of the equilibrium constants involving DTT and ME, DTT and GSH, and ME and GSH, obtained using ^1H NMR spectroscopy. It compares these values with values published elsewhere. It also assembles a number of equilibrium constants from the literature, corrects errors in them, and presents a coherent set of values.

Results and Discussion

Method. The principal objective of this work was the determination of reliable values for the two equilibria represented by eqs 1-2. We have determined these values



$$K_{eq} = \frac{[DTT^{ox}][RSH]^2}{[DTT^{red}][RSSR]} \quad RSH = ME \text{ or } GSH \quad (2)$$

by ^1H NMR spectroscopy, using one or two relays; that is, we determined values of equilibrium constants between DTT and Lip, between Lip and ME, and between ME and GSH. The equilibria were approached from both directions, and in each instance gave similar values (Table I). Figure 1 shows representative spectra.

Sources of Error. We measured integrals representing peaks from each of the four species (two oxidized species

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Table I. K_{eq} between Lipoic Acid (Lip) and Mercaptoethanol (ME) or Dithiothreitol (DTT) and between Glutathione (GSH) and Mercaptoethanol (ME)

equilibrating species		approximate		trial					
A	B	ratio [A]:[B]	solvent	1[a]	2[a]	3[b]	4[b]	5[b]	average[e]
DTT	Lip	1:1	D_2O	14.0	14.1	15.2	13.7	13.9[c]	14.2 ± 0.7
DTT	Lip	1:1	CD_3OD/D_2O	20.7	21.4	22.3	21.5	20.5[c]	21.3 ± 0.9
Lip	ME	1:20	D_2O	14.8 M	12.7 M	13.1 M	13.0 M	13.0 M[d]	13.3 ± 1.0 M
Lip	ME	1:20	CD_3OD/D_2O	9.1 M	8.1 M	7.7 M	9.0 M	8.9 M[d]	8.6 ± 0.7 M
GSH	ME	1:1	D_2O	1.23	1.15	1.27	1.15		1.20 ● 0.10

[a] The equilibrium was approched from the direction of oxidized A. [b] The equilibrium was approached from the direction of oxidized B. [c] The ratio of concentrations of A:B was changed to 1:2. [d] The ratio of concentrations of A:B was changed to 1:40. [e] The error is calculated at the 95% confidence limit.

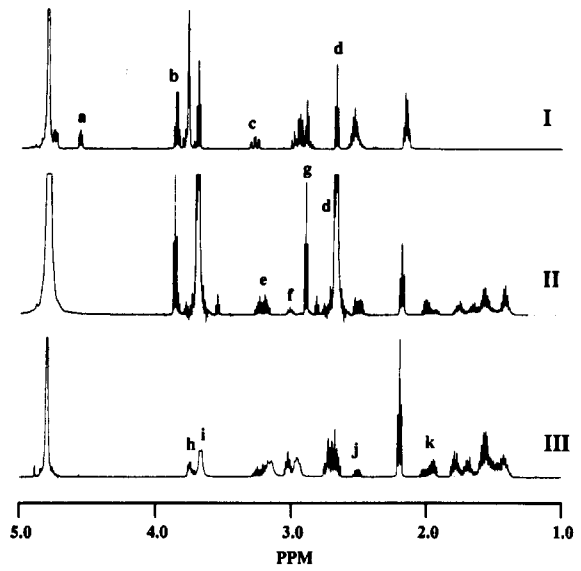

Figure 1. ^1H NMR spectrum of the equilibria between ME and GSSG (I), ME and Lip (II), and Lip and DTT (III); a is GSH, b is ME^{ox}, c is GSSG, d is ME^{red}, e is Lip^{ox}, f is Lip^{red}, g is ME^{ox}, h is DTT^{red}, i is DTT^{ox}, j is Lip^{ox}, and k is Lip^{red} + Lip^{ox}. The peaks denoted in each spectrum are those used to determine the equilibrium constants using procedures described in detail in the Experimental Section.

and two reduced species) to eliminate errors due to adventitious air oxidation and due to uncertainties in weighing materials and measuring volumes of solution. In some instances, where it was not possible to identify a complete nonoverlapping set of resonances for a species, the integral of the species of interest was determined by subtracting one integral from another or by integrating the portion of the resonance that was observable (see the Experimental Section). To minimize the uncertainties introduced by subtracting integrals, we judiciously chose the initial concentrations of the equilibrating species.[27] As a check on the consistency of the integration, the summation of the integrals of one compound in its oxidized and reduced state (i.e. Lip^{ox} + Lip^{red}) was compared to the summation of the integrals of the other compound in its oxidized and reduced state (i.e. ME^{ox} + ME^{red}). This ratio was within ±15% of the ratio calculated using the initial weights of each compound and the number of protons contributing to each integral. These residual discrepancies between the summation of the integrals and the weights could arise from errors in weighing, absorption of water by the starting materials, errors in syringe volumes, or errors in the integration. It was essential to allow a sufficient delay time between pulses to avoid marginal differential saturation of the signals being integrated: we used delays of 40 s. The precision of the measurement of the equilibrium constant was, in the worst case, ±8% at the 95% confidence level. The error in the accuracy of the equilibrium constant measurement is slightly greater than the precision due to systematic errors in the integrals.

Equilibrium Constants. Table II summarizes values of equilibrium constants obtained by us and by others.[16,25,26,28-30] Our values obtained by ^1H NMR spectroscopy in D_2O are in good agreement with the values of Chau and Nelson[25] and of Rothwarf and Scheraga[26] obtained in H_2O which are based on HPLC analysis. We expect the values of K_{eq} (eq 1) obtained in D_2O and H_2O to be very similar.[31]

In the HPLC measurements, the equilibrium mixture between DTT and GSH was quenched with acid or methyl methanethiosulfonate (MMTS) and loaded onto a C-18 HPLC column. The eluents were detected by a UV detector and a disulfide detection system. Using the extinction coefficients of the compounds at 210 nm and the relative area of the peaks, Rothwarf and Scheraga[26] calculated the equilibrium constant between the thiol and disulfide. In this method, the quenching of the reaction mixture is critical and potentially problematic; exchange that takes place during this step may cause a systematic error in the final equilibrium constant. Historically the quenching of thiol–disulfide reactions has been difficult,[15] but with MMTS, a kinetically fast quenching reagent, the systematic error in the above system is less than 5%.[26,32]

The values of Houk and Whitesides[28] have been corrected for a consistent error of 10^3 in the original paper. (This error resulted from a mistake in manipulating units; M was used instead of mM in a key calculation.) This

(27) For the equilibrations of DTT and Lip, and GSSG and ME, the ratios of initial concentrations were slightly greater than 1. In the case of DTT and Lip, this would minimize the [Lip^{ox}] substracted from [DTT^{red}] (see the Experimental Section). In the case of GSSG and ME, this would allow the result to be double checked by integrating ([GSSG] + [GSME]) and ([ME^{ox}] + [GSME]) and showing that they were approximately equivalent; therefore K_{eq} = ([GSH]/[ME^{red}])^2.

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(31) The pK_a of a thiol is approximately 0.5 units higher in D_2O than in H_2O. Hence, when the pH of the equilibrium mixture is close to the pK_a of the thiol, the resulting NMR spectra in D_2O and H_2O could be considerably different. The value of K_{eq} for eq 1 should, however, be constant regardless of pH (only the protonated forms of the thiol are involved in the equilibrium expression). The lowest pK_a (H_2O) of the thiols used in this paper is 8.7 (glutathione). See: Keire, D. A.; Strauss, E.; Guo, W.; Noszal, B.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 123–127.

(32) We expect the systematic error using this assay to be greatest with 1,3-dithiols since 1,3-dithiols form disulfides with 5-membered rings at a faster rate (20 times) than 1,4-dithiols form disulfides with 6-membered rings. See: Singh, R.; Whitesides, G. M. *J. Am. Chem. Soc.* **1990**, *112*, 6304–6309.

Table II. Values of Equilibrium Constants for the Reaction of Mono- or Dithiols with Disulfides

RSH or HSR'SH	R''SSR'' or SR'''S	K_{eq}[a]	assay: method	assay: conditions	source
		Current Values (Published 1991–92)			
DTT	GSSG	200 M	HPLC	pH 7.0, 0.1 M Tris, 50 mM P_i, 0.2 M KCl	Chau and Nelson[25]
DTT	GSSG	190 M	HPLC	pH 7.0, 0.1 M P_i	Rothwarf and Scheraga[26]
DTT	GSSG	230 M	NMR	pD 7.0, 0.1 M P_i	this work, derived constant[b]
DTT	Lip^{ox}	14.2	NMR	pD 7.0, 0.1 M P_i	this work
Lip^{red}	Me^{ox}	13.3 M	NMR	pD 7.0, 0.1 M P_i	this work
DTT	Me^{ox}	190 M	NMR	pD 7.0, 0.1 M P_i	this work, derived constant[b]
Me^{red}	GSSG	1.20	NMR	pD 7.0, 0.1 M P_i	this work
		Previous Values (Published Prior to 1991)			
DTT	cystine	13000 M	enzymatic	pH 7.0, 0.2 M P_i	Cleland[30]
DTT	GSSG	1160 M (390 M)[d]	electrophoretic	pH 8.7, 0.1 M Tris, 0.2 M KCl	Creighton and Goldberg, derived constant[16,b]
DTT	GSSG	8800 M	enzymatic	pH 7.0, 20–50 mM P_i	Szajewski and Whitesides[29]
DTT	lipoamide	15	enzymatic	pH 7.0, 20–50 mM P_i	Szajewski and Whitesides[29]
Me^{red}	GSSG	1.2	enzymatic	pH 7.0, 20–50 mM P_i	Szajewski and Whitsides[29]
DTT	ME^{ox}	94 M	NMR	CD_3OD/D_2O (pD 7.0, 50 mM P_i)	Houk and Whitesides[28] (corrected)[c]

[a] $K_{eq} = [SR'S][HSR'''SH]/[HSR'SH][SR'''S]$ or $K_{eq} = [SR'S][R''SH]^2/[HSR'SH][R''SSR'']$ or $K_{eq} = [RSSR][R''SH]^2/([RSH]^2\text{-}[R''SSR''])$. [b] These values were not measured directly and required multiplying two or three values of other equilibrium constants, i.e. K_{eq}(DTT, ME) equals K_{eq}(DTT, Lip) multiplied by K_{eq}(Lip, ME). [c] The values in this paper have been multiplied by a factor of 10^{-3} to eliminate an error in manipulating units. [d] Corrected to pH 7.0 using a pK_a(GSH) value of 8.7 and a pK_a(DTT) value of 9.2.[26]

value of 94 M for the equilibrium between DTT and ME differs from the value of 180 M obtained in this paper by approximately a factor of 2. The value for the equilibrium constant between DTT and Lip obtained by Houk and Whitesides (1/(0.048 ± 0.002) or 20.8 ± 0.8) is indistinguishable from the value obtained in this work (21.3 ± 0.9), but the value for the equilibrium constant between ME and Lip (3.5 ± 1.0 M) is different from the value obtained in this work (8.6 ± 0.7 M). This difference could be due to an underestimation of the error involved in calculating the concentration of Lip^{ox} from the concentration of ME^{ox} at equilibrium and the initial concentration of lipoic acid, or due to adventitious oxidation of the mercaptoethanol (ca. 0.4% would be required).

The results of Szajewski and Whitesides[29] and Cleland[30] were obtained by enzymatic assays, in which the concentrations of thiols were coupled through lipoamide and lipoamide dehydrogenase to the reduction of NAD^+. Although the value of K_{eq} between DTT and glutathione or cystine from these two similar assays are in reasonable agreement, we conclude, based on their disagreement with the more direct NMR and HPLC assays, that both are incorrect. The values for the equilibrium constants between DTT and lipoamide, and between GSH and ME obtained in the lipoamide/lipoamide dehydrogenase assay,[29] are indistinguishable from the values obtained in this work by NMR with DTT and lipoic acid (Lip), and GSH and ME. This observation suggests that the error originates in coupling the reduction of Lip^{ox} to the oxidation of GSH or ME. These enzymatic assays are complicated, and we have not traced the source of the systematic error in them. One suggestion is that the lipoamide dehydrogenase may be inactivated or inhibited before the reaction has reached equilibrium.[26]

The values of K_{eq} determined by Creighton and Goldenberg[16] were based on the ratios of the rates of reaction of DTT^{ox} or DTT^{red} and GSSG or GSH with reduced or oxidized bovine pancreatic trypsin inhibitor (BPTI). We adjusted the value of the equilibrium constant between DTT and glutathione obtained by Creighton and Goldenberg at pH 8.7 (K_{eq} = 1160 M), using values of the pK_a of DTT (9.2) and GSH (8.7), so that it could be compared directly with the values of the equilibrium constant obtained at pH 7.0 using the NMR and HPLC methods. After adjustment, the equilibrium constant obtained by equilibration with BPTI (K_{eq} = 390 M) is within a factor of 2 of the results obtained by methods based on NMR spectroscopy and HPLC. This residual difference results from possible errors related to quenching of the thiol–disulfide interchange reaction with iodoacetate,[15] or other unidentified experimental errors.

The recent results using HPLC[25,26] and the NMR spectroscopic assays described here provide the most accurate values of the K_{eq} between DTT and GSSG now available. The precision of either type of assay for a single measurement is ±10% at the 95% confidence level. Due to the similarities in precision, each technique should be as accuarte as the other for a single set of measurements. The NMR spectroscopic assay requires three separate sets of measurements to generate a value of K_{eq} between DTT and GSSG (K_{eq} between DTT and Lip, K_{eq} between Lip and ME, K_{eq} between ME and GSSG). Because three separate measurements are required, the resulting value is inherently slightly less accurate (±13% based on propagating errors) than the value of K_{eq} obtained from the HPLC assay, which requires only one set of measurements (K_{eq} between DTT and GSSG), provided that the systematic errors in both types of assays are equal.

The NMR spectroscopic assay has, however, several advantages relative to the HPLC assay: it measures the equilibrium in situ, without quenching of the reaction mixture; it does not rely on the calculation of extinction coefficients; and it is less susceptible to errors due to oxidation durign manipulations. This assay also has several disadvantages: it has a smaller dynamic range of K_{eq} values for a single set of measurements than does the HPLC method; to be useful, it must have at least one distinguishable resonance for each species.

In summary, the three best values now available for the equilibrium constant between GSSG and DTT are 200 M from Chau and Nelson; 190 M from Rothwarf and Scheraga; 230 M from this work. There is still a 20% difference between the high and the low values, but there is no obvious basis for choosing among them, or for

believing that one is intrinsically more accurate than the others. We intend to use the average of these three values, K_{eq} = 210 M, in our future and present work.

Collected Values from the Literature. Table III collects a number of values for equilibrium constants from the literature, corrects errors in these values, and adjusts them (if needed) to the consensus values of equilibrium constants for reference reactions. The majority of these values are from Houk and Whitesides.[28] Many of the values in that paper were systematically too large by 10^3 because of an error made in manipulating units. The values in Table III have been corrected for this error. The reference value for K_{eq} (DTT, ME^{ox}) in D_2O/methanol-d_4 used in the work of Houk and Whitesides was 94 M. The value inferred from the current studies for this constant was 180 M in D_2O/methanol-d_4 [K_{eq} (DTT, Lip) × K_{eq} (Lip, ME^{ox}), 21.3 × 8.6 M = 183 M]. In water, the value is also 180 M as determined by taking the average value of 210 M determined in water for K_{eq} (DTT, GSSG) and dividing by the equilibrium constant between glutathione

Table III. Equilibrium Constants for Thiol/Disulfide Interchange

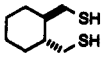
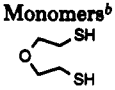
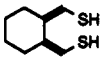
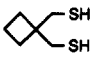
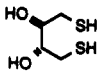
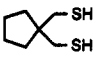
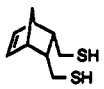
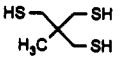
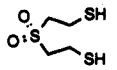
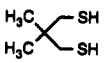
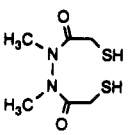
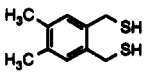
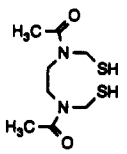
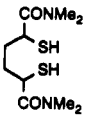
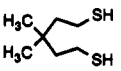
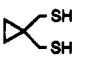
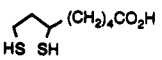
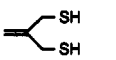
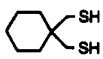
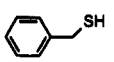
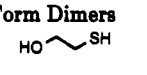
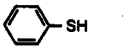
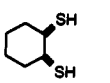
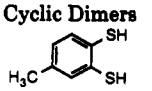
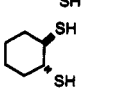
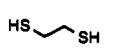
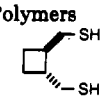
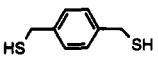
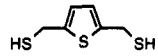
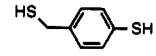
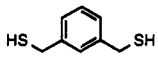
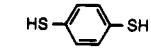
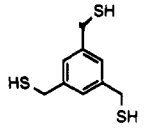
structure	K(ME^{ox})	ϵ_0(V)[a]	eq against	ref	structure	K(ME^{ox})	ϵ_0(V)[a]	eq against	ref
					Dithiols That Form Cyclic Monomers[b]				
	1500 M	−0.354	DTT	*c, d*		6.1 M	−0.284	DTT	*c, d*
	670 M	−0.344	DTT	*c, d*		4.4 M	−0.279	DTT	*c, d*
	180 M	−0.327	Lip	*c, d*		3.6 M	−0.277	DTT	*c, d*
	77 M	−0.316	DTT	*c, d*		3.6 M	−0.277	DTT	*c, d*
	65 M	−0.314	DTT	*c, d*		3.1 M	−0.275	DTT	*c, d*
	63 M	−0.313	DTT	*e*		2.9 M	−0.274	DTT	*c, d*
	44 M	−0.309	DTT	*c, d*		2.5 M	−0.272	DTT	*g*
	19 M	−0.298	DTT	*d, f*		2.3 M	−0.271	ME, DTT	*c, d*
	15 M	−0.295	DTT	*e*		1.8 M	−0.269	DTT	*h*
	14 M	−0.294	DTT	*c, d*		1.2 M	−0.263	DTT	*c, d*
	8.6 M	−0.288	ME, DTT	*c, d*		0.67 M	−0.255	DTT	*c, d*
	8.0 M	−0.287	DTT	*c, d*	6,6'-sucrose disulfide	0.30 M	−0.245	ME	*i*
	6.7 M	−0.285	DTT	*c, d*	$HS(CH_2)_6SH$	0.21 M	−0.240	DTT	*c, d*
					Monothiols That Form Dimers				
	2.6	−0.272	ME	*c*		1.0	−0.260	ME	*c*
$CH_3(CH_2)_6SH$	1.1	−0.261	ME	*c*		0.31	−0.245	ME	*f*
					Dithiols That Form Cyclic Dimers				
	0.40 M	−0.254	ME	*c, d*		0.32 M	−0.253	ME	*d, f*
	0.38 M	−0.254	ME	*c, d*		0.035 M	−0.239	ME	*c, d*

Table III (Continued)

structure	$K(ME^{ox})$	ϵ_0(V)[a]	eq against	ref	structure	$K(ME^{ox})$	ϵ_0(V)[a]	eq against	ref
Dithiols That Form Polymers									
	4.8	−0.280	ME	c		1.8	−0.268	ME	c
	4.0	−0.278	ME	c	$HS(CH_2)_8SH$	1.7	−0.267	ME	c
	3.4	−0.276	ME	f	$HS(CH_2)_7SH$	1.4	−0.265	ME	c
	3.1	−0.275	ME	c		1.3	−0.264	ME	f
	3.0	−0.275	ME	f		0.20	−0.240	ME	f
	2.8	−0.274	ME	j					
Other Biological Reducing Agents									
NADH		−0.320		k	NADPH		−0.324		k

[a] ϵ_0 (V) values vs standard hydrogen electrode at pH 7.0 and 25 °C. All ϵ_0 (V) values are calculated using the ϵ_0 (V) value for lipoic acid (−0.288 V, see: Sanadi, D. R.; Langley, M.; Searls, R. L. *J. Biol. Chem.* **1959**, *234*, 178. Massey, V. *Biochim. Biophys. Acta* **1960**, *37*, 314 and the K_{eq} value between lipoic acid and the compound of interest. [b] The value of $K(ME^{ox})$ for this group of compounds is sometimes called the effective concentration (EC). [c] Equilibrations were carried out at 25 °C, in a 1/1 mixture of methanol-d_4/phosphate buffer (50 mM, pD 7.0) in D_2O, see: Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825. [d] The equilibrium constants (K) in the Houk and Whitesides paper were systematically incorrect by a factor of 10^3 (originating in an error in manipulation of units during the original calculations) and have been adjusted accordingly. The values of equilibrium constants, which were obtained from equilibrations with DTT, have also been readjusted by a factor of ca. 2 so as to obtain a similar value to that reported in this paper. [e] Equilibrations were carried out at 25 °C in a 1/1 mixture of methanol-d_4/phosphate buffer (50 mM, pD 7.0) in D_2O; see: Lamoureux, G. V.; Whitesides, G. M. *J. Org. Chem.*, in press. [f] Equilibrations were carried out in methanol-d_4 with 0.02 mM sodium methylate added; see: Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825. [g] Equilibrations were carried out in D_2O (pD 7.0, 50 mM phosphate); see: Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2332. [h] Equilibrations were carried out in D_2O (pD 7.0, 50 mM phosphate); see: Lees, W. J.; Singh, R.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 7328. [i] Equilibrations were carried out in D_2O (pD 7.0, 50 mM phosphate), see: Lees, W. J.; Whitesides, G. M. *J. Am. Chem. Soc.*, in press. [j] Equilibrations were carried out in benzene-d_6 with 0.02 mM tetramethylguanidine added; see: Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825. [k] Loach, P. A. in *CRC Handbook of Biochemistry*, 2nd ed.; Sober, H. A., Ed.; The Chemical Rubber Company: Cleveland, 1970; p J-39.

and mercaptoethanol in water (1.2). Hence we further adjusted the reference value of K_{eq} (DTT, ME^{ox}) for the data of Houk and Whitesides to 180 M.

The Formal Reduction Potential of Glutathione Disulfide. We estimated the formal reduction potential of glutathione disulfide ($E^{o\prime}$(GSSG), pD 7.0, 0.10 M $NaPO_4$) to be −0.252 V in D_2O, using K_{eq} (Lip, ME^{ox}) and K_{eq} (ME, GSSG) in D_2O (Table I), and $E^{o\prime}$ for lipoic acid (−0.288 V).[29] Due to solvent effects, the value of K_{eq} (Lip, ME^{ox}) in D_2O (13.3 M) is different from the value of K_{eq} (Lip, ME^{ox}) in D_2O/CD_3OD (8.6 M). This difference in the values of K_{eq} (Lip, ME^{ox}) in D_2O/CD_3OD and in D_2O would result in different values of $E^{o\prime}$(GSSG) in these solvents. Since K_{eq} (ME, GSSG) was only measured in D_2O (Table I) and not in D_2O/CD_3OD, we have not included a value for $E^{o\prime}$(GSSG) in D_2O/CD_3OD in Table III (most of the equilibrium constants in Table III were measured in D_2O/CD_3OD).

Choice of $E^{o\prime}$ for NAD^+ and for Lipoic Acid. A number of different values have been reported for the $E^{o\prime}$ value of NAD^+.[33] Of these values, the most commonly referenced, but not necessarily the most accurate, is the value of Burton and Wilson (−0.320 V at 25 °C).[29,30,34-37] Another value that is less commonly used is that of Rodkey (−0.311 V at 25 °C).[38,39] To ease the comparison of the results obtained in this paper with previous values, we chose to use the value of −0.320 V for $E^{o\prime}$ (NAD^+).

The value of K_{eq} between lipoic acid (and/or lipoamide) and NADH has been measured by several groups,[29,33,40,41] and the consensus value for this equilibrium constant is 0.086 (pH = 7.0 and 25 °C; this value of K_{eq} is for lipoamide, but lipoamide and lipoic acid are stated to have very similar if not equal values of $E^{o\prime}$ and thus of K_{eq}).[33,40] The calculated value of $E^{o\prime}$(lipoic acid) is thus −0.288 V. We used this value of −0.288 V for $E^{o\prime}$ of lipoic acid as a standard to calculate the other values of $E^{o\prime}$ listed in Table III ($E^{o\prime}$(compound of interest) = $E^{o\prime}$(lipoic acid) − 0.029 58 log (K_{eq} (compound of interest, Lip)). The measurements of $E^{o\prime}$(lipoic acid) were done in H_2O and solvent effects have not been taken into consideration. The systematic error in these values of $E^{o\prime}$ could be on the order of 0.01 V due to the uncertainties mentioned above.

Conclusion

The values obtained from the HPLC assay[25,26] and from the NMR assay for the K_{eq} between DTT and GSSG are

(33) Clarke, W. M. *Oxidation-Reduction Potentials of Organic Systems*; Williams and Wilkins: Baltimore, 1960; pp 483-496.
(34) Burton, K.; Wilson, T. H. *Biochem. J.* **1953**, *54*, 86-94.
(35) Loach, P. A. In *CRC Handbook of Biochemistry*, 2nd ed.; Sober, H. A., Ed.; The Chemical Rubber Company: Cleveland, 1970; p J-39.
(36) Walsh, C. T. *Enzymatic Reaction Mechanisms*; W. H. Freeman and Co.: New York, 1979; p 313.
(37) Jocelyn, P. C. *Eur. J. Biochem.* **1967**, *2*, 327-331.
(38) Rodkey, F. L. *J. Biol. Chem.* **1955**, *213*, 777-786.
(39) Rodkey, F. L. *J. Biol. Chem.* **1959**, *234*, 188-190.
(40) Sanadi, D. R.; Langley, M.; Searls, R. L. *J. Biol. Chem.* **1959**, *234*, 178-182.
(41) Massey, V. *Biochim. Biophys. Acta* **1960**, *37*, 314-326.

200 M ± 10%,[25] 190 M ± 9%,[26] and 230 M ± 13%. The similarity of these three numbers measured by two completely independent methods provides strong support that they are accurate indicators of this important equilibrium constant. We suggest that the value of K_{eq} (DTT and GSSG) be considered to be the average of these three values: 210 M.

Experimental Section

General. Measurements of equilibrium constants were carried out under an atmosphere of argon. Deuterated solvents were obtained from Cambridge Isotopes Limited. Other chemicals were obtained from Sigma Chemical Co. or Aldrich Chemical Co. Mercaptoethanol was further purified by distillation. ^{1}H NMR spectra were recorded with a 90° pulse width, 64 scans, and a 40-s receiver delay (5 times the value of T_1 of the slowest relaxing resonances (reduced mercaptoethanol)).

Thiol Equilibrations. General. Deuterated phosphate buffer was prepared by dissolving phosphoric acid (10 mmol, 1.19 g, 85% D_3PO_4 in D_2O) in ca. 50 mL of D_2O, adjusting the pD to 7.0[42] with NaOD (30% solution in D_2O), and diluting with D_2O to a final volume of 100 mL. The deuterated phosphate buffer was then deoxygenated by bubbling argon through the solution for 2 h.

Equilibrium experiments were carried out in 5-mm NMR tubes, which had been sealed with a septum and flushed with argon before the addition of the solutions. The tubes were stored under an atmosphere of argon for 24–48 h, before an NMR spectrum was acquired.

Example of a Procedure for Measuring the Equilibrium Experiment. Lipoic acid (oxidized or reduced, ca. 13 mg) was added to a 5-mL flask. The flask was sealed with a septum and flushed with argon for 10 min. Phosphate buffer (3.0 mL) was then added and the mixture sonicated for 10 min to dissolve the lipoic acid. The sonicated lipoic acid solution (2.0 mL) was added to a 5-mL flask containing dithiothreitol (reduced or oxidized, ca. 6 mg) under an atmosphere of argon. After swirling for 3 min, 0.5 mL of this solution was added to each of two NMR tubes, one containing 0.5 mL of CD_3OD and the other containing 0.5 mL of phosphate buffer.

The equilibrium experiments were repeated twice from each direction in each solvent. The experiment in one instance was also performed with half as much oxidized dithiothreitol as the general procedure (see Table I).

Integration of ^{1}H NMR Signals. To determine the K_{eq} between DTT and lipoic acid in D_2O, we determined the relative concentration of each species using the areas of the following NMR resonances: k[Lip^{ox}] = (area of Lip^{ox} resonance at 2.51 ppm); k[Lip^{red}] = ((area of Lip resonance at 2.05–1.90 ppm)/2 − [Lip^{ox}]); k[DTT^{ox}] = ((area of DTT^{ox} resonance at 3.68 ppm)/2); k[DTT^{red}] = ((area of DTT^{red} resonance at 3.75 ppm)/2 − k[Lip^{ox}]/2), where k is the proportionality constant linking the area obtained from the NMR integral to the concentration of the species in solution. The correction due to k[Lip^{ox}] or k[Lip^{ox}]/2 were always less than 35% of the final value of k[Lip^{red}] or k[DTT^{red}], respectively.

To determine the K_{eq} between DTT and lipoic acid in CD_3OD/D_2O, we determined the relative concentration of each species using the areas of the following NMR resonances: k[Lip^{ox}] = area of Lip^{ox} resonance at 2.49 ppm; k[Lip^{red}] = ((area of Lip resonance at 1.98–1.87 ppm)/2 − k[Lip^{ox}]); k[DTT^{ox}] = ((area of DTT^{ox} resonance at 3.59 ppm)/2); k[DTT^{red}] = ((area of DTT^{red} resonance at 3.71 ppm)/2).

To determine the K_{eq} between ME and lipoic acid in D_2O, we determined the relative concentration of each species using the areas of the following NMR resonances: k[ME^{red}] = ((area of ME^{red} resonance at 2.70 ppm)/2); k[ME^{ox}] = ((area of ME^{ox} resonance at 2.92 ppm)/4); k[Lip^{ox}] = ((area of Lip^{ox} resonance at 3.25 ppm)/2); k[Lip^{red}] = (area of Lip^{red} resonance at 3.04 ppm). The concentration of ME^{red} at equilibrium was determined from the ratio of k[ME^{red}] to k[ME^{ox}] and the sum of the initial concentrations ([ME^{red}] + [ME^{ox}]).

To determine the K_{eq} between ME and lipoic acid in CD_3OD/D_2O, we determined the relative concentration of each species using the areas of the following NMR resonances: k[ME^{red}] = ((area of ME^{red} resonance at 2.63 ppm)/2); k[ME^{ox}] = ((area of ME^{ox} resonance at 2.86 ppm)/4); k[Lip^{ox}] = ((area of Lip^{ox} resonance at 3.25 ppm)/2); k[Lip^{red}] = ((area of Lip resonance at 1.92 ppm) − k[Lip^{ox}]). The ratio of [Lip^{red}]/[Lip^{ox}] was about 1.0 except when twice the normal concentration of ME was used. The concentration of ME^{red} at equilibrium was determined from the ratio of k[ME^{red}] to k[ME^{ox}] and the sum of the initial concentrations ([ME^{red}] + [ME^{ox}]).

To determine the K_{eq} between ME and GSH in D_2O, we determined the relative concentration of each species using the areas of the following NMR resonances: k[ME^{red}] = ((area of ME^{red} resonance at 2.70 ppm)/2); k[ME^{ox}] = (area of downfield peak of ME^{ox} resonance at 3.87 ppm); k[GSH] = (area of GSH resonance at 4.58 ppm); k[GSSG] = (1.10(area of the two downfield peaks of GSSG resonance at 3.31 ppm)). The factor of 1.1 was introduced to counteract the leaning of the peak towards higher field. The factor of 1.1 was derived from an NMR spectrum of GSSG taken under identical conditions.

Calculation of K_{eq}. The K_{eq} values were calculated using standard equations and the relative concentrations obtained from the NMR integrals.

Acknowledgment. We would like to thank Rajeeva Singh and Guy Lamoureux for their helpful discussions, Jan Houk and Richard Szajewski for their willingness to look over past results, Prof. W. W. Cleland (University of Wisconsin—Madison) and Prof. J. W. Nelson (Louisiana State University) for their helpful comments, and Prof. H. Scheraga (Cornell) for kindly providing us with a preprint of ref 26 and for pointing out some of the problems with ref 29.

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