Oxidation/Reduction Potential of Glutathione

Kevin K. Millis, Kim H. Weaver, and Dallas L. Rabenstein*

Department of Chemistry, University of California, Riverside, California 92521

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Introduction

Although the importance of the glutathione/glutathione disulfide (GSH/GSSG) redox system in biological oxidation/reduction reactions has long been recognized,[1] there still is uncertainty as to its half-cell potential.[2-7] To illustrate, literature values ranging from −0.17 to −0.27 V are listed in Table I. Direct measurement of the half-cell potential for the GSH/GSSG system, and other thiol/disulfide redox systems, by standard electrochemical methods is not possible due to formation of stable metal–thiolate complexes at electrode surfaces.[8] Thus, the half-cell potentials of GSH and other thiol-containing molecules are determined indirectly by measurement of equilibrium constants for their reaction with redox systems of known half-cell potential.[8]

The first three values and the fifth value listed in Table I for $E^{o'}_{GSSG/GSH}$ were determined indirectly by measurement of the equilibrium constant for reaction of the GSH/GSSG redox system with the nicotinamide adenine dinucleotide phosphate (NADPH/NADP$^+$) redox system.[2-4,6] The fourth and sixth values were determined

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+ \quad (1)$$

indirectly relative to the nicotinamide adenine dinucleotide (NADH/NAD$^+$) redox system.[5,7] The large range covered by the values listed in Table I is probably due in part at least to limitations of the experimental procedures used, since equilibrium constants were determined by direct measurement of the concentration of only one or two of the species involved in the equilibrium reactions.[2-7]

In view of the fundamental importance of the GSH/GSSG redox system in biology, and its use as the reference system for determination of redox potentials for other thiol/disulfide systems, we have redetermined $E^{o'}_{GSSG/GSH}$ relative to the half-cell potential for the NADPH/NADP$^+$ redox system by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Compared to the spectrophotometric methods used previously,[2-7] ^{1}H NMR has the advantage that the concentrations of all four species involved in the equilibrium reaction can be determined directly. Values are reported for $E^{o'}_{GSSG/GSH}$ at pH (pD) 7.0 in H_2O and D_2O solutions.

Table I. Half-Cell Potential of the Glutathione/Glutathione Disulfide Redox System

K[a]	$E^{o'}_{GSSG/GSH}$[b]	condns	ref
1.2×10^5[c]	−0.17	pH 7.0	2
7.0×10^4[c]	−0.16	pH 7.4, 20 °C, 0.1 M phosphate	3
1.55×10^2[c]	−0.25	pH 6.8, 25 °C, 0.167 M phosphate	4
9.8×10^2[d]	−0.24	pH 7.4, 40 °C, 0.125 M phosphate	5
51[c]	−0.28	pH 7.0, 38 °C, I = 0.25 M	6
6.7×10^3[d]	−0.205	pH 7.0, 30 °C	7
$1.39(\pm 0.21) \times 10^2$[d]	−0.263[e]	pH 7.07, 25 °C, 0.10 M phosphate	this work
$1.20(\pm 0.15) \times 10^2$[d]	−0.262[e]	pD 6.97, 25 °C, 0.10 M phosphate	this work
	−0.252	pD 7.0, 0.10 M phosphate	17

[a] Conditional equilibrium constant as defined by eq 2 or the analogous equilibrium constant for the reaction with NADH. [b] Volts vs the standard hydrogen electrode. [c] Equilibrium constant for the reaction given in eq 1. [d] Equilibrium constant for the reaction of GSH/GSSG with the NAD$^+$/NADPH redox system. [e] Calculated using $E^{o'}_{NADP/NADPH} = -0.324$ V at pH 7.0 and 25 °C. See ref 11 for values calculated using a different value for $E^{o'}_{NADP/NADPH}$.

Results

The concentrations of GSH, GSSG, NADP$^+$, and NADPH in equilibrium solutions were determined by ^{1}H NMR. To illustrate, the ^{1}H NMR spectrum of an equilibrium mixture prepared by reaction of 60 mM GSH with 30 mM NADP$^+$ in 0.1 M phosphate buffered H_2O solution, pH 7.07, 25 °C, is presented in Figure 1. Glutathione reductase was added to catalyze the reaction.[9] It is clear from the spectrum that the equilibrium for the reaction given by eq 1 lies far to the right, with the formation of only small amounts of GSSG and NADPH. The resonances used to determine the equilibrium concentrations of GSH, GSSG, NADP$^+$, and NADPH are identified in Figure 1 and assigned in the figure legend. The equilibrium constant (eq 2) calculated using these concentrations is a conditional constant, as indicated by the subscript, which incorporates the pH (pD) of the solution. The formal half-cell potential, $E^{o'}_{GSSG/GSH}$, was calculated from the equilibrium constant using eq 3.

$$K_c = \frac{[GSH]^2[NADP^+]}{[GSSG][NADPH]} \quad (2)$$

$$E^{o'}_{GSSG/GSH} = E^{o'}_{NADP^+/NADPH} + \frac{RT}{nF} \ln K_c \quad (3)$$

An average value of 139 ± 21 M was determined from 17 measurements (n = 17) of the equilibrium constant in H_2O solution at pH 7.07 and 25 °C. A value of −0.263 ±

(1) Meister, A. In *Glutathione: Chemical, Biochemical and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1989; Part A, pp 1–48.
(2) Rall, T. W.; Lehninger, A. L. *J. Biol. Chem.* **1952**, *194*, 119–130. The value reported by Rall and Lehninger for $E^{o'}_{GSSG/GSH}$ was corrected by Rost and Rapoport[5] using a more accurate value for $E^{o'}_{NADP/NADPH}$.
(3) Mapson, L. W.; Isherwood, F. A. *Biochem. J.* **1963**, *86*, 173–191. The value listed in Table I for $E^{o'}_{GSSG/GSH}$ was calculated by Rost and Rapoport[5] using the value reported by Mapson and Isherwood for the equilibrium constant.
(4) Scott, E. M.; Duncan, I. W.; Ekstrand, V. *J. Biol. Chem.* **1963**, *238*, 3928–3933.
(5) Rost, J.; Rapoport, S. *Nature* **1964**, *201*, 185.
(6) Veech, R. L.; Eggleston, L. V.; Krebs, H. A. *Biochem. J.* **1969**, *115*, 609–619. The value listed in Table I for $E^{o'}_{GSSG/GSH}$ was calculated using the value reported by Veech et al. for the equilibrium constant and $E^{o'}_{NADP/NADPH} = -0.324$ V.
(7) Szajewski, R. P.; Whitsides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011–2026.
(8) Jocelyn, P. C. *Biochemistry of the SH Group*; Academic Press: New York, 1972; p 55.
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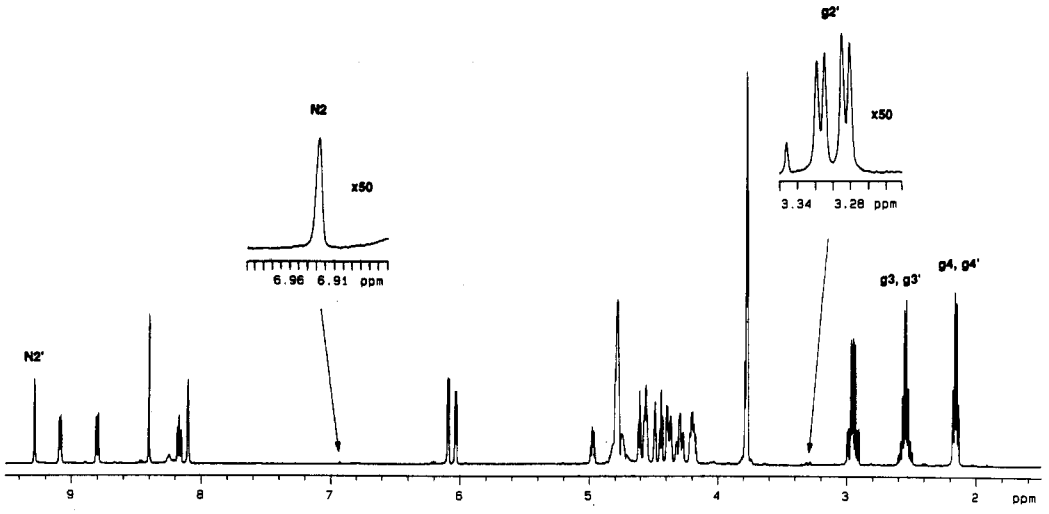

Figure 1. The 500-MHz ^{1}H NMR spectrum of an equilibrium reaction mixture of NADPH, NADP$^+$, GSH, and GSSG in 0.1 M aqueous phosphate solution, pH 7.07, 25 °C. The initial concentrations of GSH and NADP$^+$ were 60 and 30 mM, respectively. The H_2O resonance at 4.77 ppm was suppressed by selective presaturation for 9 s prior to the nonselective observation pulse. The resonances used to obtain equilibrium concentrations are identified; N2 and N2′ are resonances for the proton on C2 of the nicotinamide ring of NADPH (N2) and NADP$^+$ (N2′), g2′ is one half of the AB part of the ABX pattern for the β-CH_2 protons of the cysteinyl residues of GSSG, and g3, g3′ and g4, g4′ are for the δ-CH_2 and β-CH_2 protons, respectively, of the glutamyl residues of GSH and GSSG. The expansions of the g2′ and N2 resonances are plotted with a vertical scale 50X that was used to plot the entire spectrum.

0.002 V is calculated for $E^{o'}_{GSSG/GSH}$ using this equilibrium constant and a value of −0.326 V for the reference potential $E^{o'}_{NADP/NADPH}$.[10-12] An average value of 120 ± 15 M (n = 29) was determined for the equilibrium constant in D_2O solution at pD 6.97 and 25 °C, from which a value of −0.262 ± 0.001 V was calculated for $E^{o'}_{GSSG/GSH}$ using a value of −0.323 V for $E^{o'}_{NADP/NADPH}$.[13] The uncertainties of ±0.001 or ±0.002 V represent the precision of the measurements; however, the actual uncertainty in the $E^{o'}_{GSSG/GSH}$ values may be as large as ±0.01 V due to uncertainty in the reference value used for $E^{o'}_{NADP/NADPH}$.[11]

Discussion

The values determined in this work for $E^{o'}_{GSSG/GSH}$ are based on measurement of the equilibrium constant for the reaction given in eq 1. The first five values listed in Table I were also calculated from equilibrium constants for this reaction[2-4,6] or the analogous reaction with the NADH/NAD$^+$ redox system.[5] However, the concentrations of only one or two of the species involved in the equilibrium reaction could be determined directly with the experimental methods used to determine the first five equilibrium constants in Table I. For example, the first two equilibrium constants were determined by measuring the concentration of NADPH by spectrophotometry.[2,3] The equilibrium concentration of GSSG was then assumed to equal the concentration of NADPH produced by the reaction, and the concentrations of GSH and NADP$^+$ were calculated from these concentrations and the initial concentrations. Since the equilibrium for the reaction (eq 1) lies far to the right (Figure 1), the equilibrium concentration of GSSG produced by the reaction of GSH with NADP$^+$ is small, and thus the values calculated for the equilibrium constant and $E^{o'}_{GSSG/GSH}$ would be in error if there were appreciable amounts of GSSG initially present in the GSH.[14] The third and fourth values listed in Table I were also determined spectrophotometrically; however, experimental procedures were used which would take into account any GSSG initially present in the GSH.[4,5] The fifth value reported in Table I was also determined by measuring the concentration of NADPH by spectrophotometry; however, details of the experimental procedure were not reported.[6]

The sixth value in Table I was obtained indirectly by a procedure which involved determination of the equilibrium position of two simultaneous redox reactions: the reaction of the GSH/GSSG redox system with the reduced lipoamide/oxidized lipoamide (lipo^{red}/lipo^{ox}) redox system and the reaction of the lipo^{red}/lipo^{ox} redox system with the NADH/NAD$^+$ redox system.[7] The concentration of NADH was determined by spectrophotometry, and then the concentrations of the five other species in the two simultaneous equilibria were calculated using this concentration. Calculation of $E^{o'}_{GSSG/GSH}$ required the use of literature values for both the lipo^{red}/lipo^{ox} and NADH/NAD$^+$ redox potentials.

Although direct comparisons are not possible due to small differences in solution conditions, the values de-

(10) The value of $E^{o'}_{NADP/NADPH}$ = −0.326 V vs the standard hydrogen electrode at pH 7.07 was calculated from $E^{o'}_{NADP/NADPH}$ = −0.324 V at pH 7.0 and 25 °C[11] using a pH dependence of −0.0301 V/pH unit.

(11) Several values have been reported for $E^{o'}_{NADP/NADPH}$ (Clark, W. M. *Oxidation-Reduction Potentials of Organic Systems*; Williams and Wilkins: Baltimore, 1960; pp 495–496). The value of $E^{o'}_{NADP/NADPH}$ = −0.324 V is the most widely used; however, according to Clark, $E^{o'}_{NADP/NADPH}$ = −0.317 ± 0.002 V at pH 7.0 and 30 °C (reported by: Rodkey, F. L.; Donovan, J. A. *J. Biol. Chem.* **1959**, *234*, 677–680) might be more accurate. Using the value reported by Rodkey and Donovan, a temperature dependence of −0.0013 V/°C, a pH dependence of −0.0301 V/pH unit, and the equilibrium constant determined in this work, $E^{o'}_{GSSG/GSH}$ is calculated to be −0.250 ± 0.002 V at pH 7.07 and 25 °C.

(12) Rodkey, F. L. *J. Biol. Chem.* **1955**, *213*, 777–786.

(13) The value of $E^{o'}_{NADP/NADPH}$ = −0.323 V vs the standard hydrogen electrode at pD 6.97 was calculated from $E^{o'}_{NADP/NADPH}$ = −0.324 V at pH 7.0 and 25 °C since no values have been reported for $E^{o'}_{NADP/NADPH}$ in D_2O solution. As discussed in ref 11, a less commonly used but possibly more accurate value for $E^{o'}_{NADP/NADPH}$ is −0.317 V at pH 7.0 and 30 °C. Using this value, a temperature dependence of −0.0013 V/°C and a pH dependence of −0.0301 V/pH unit, $E^{o'}_{GSSG/GSH}$ in pD 6.97 solution is calculated to be −0.250 ■ 0.001 V.

(14) We typically find up to 0.3% GSSG in the GSH; others report 0.5–1.0% GSSG.[5]

Table II. Half-Cell Potentials of Thiol/Disulfide Redox Systems[a-c]

thiol	$E^{\circ\prime}_{RSSR/RSH}$, V	thiol	$E^{\circ\prime}_{RSSR/RSH}$, V
captopril	−0.287 ± 0.001	homocysteine	−0.256 ± 0.005
N,N-dimethyl-cysteamine	−0.271 ± 0.003	β-mercapto-ethanol	−0.253 ± 0.003
glutathione	−0.262 ± 0.001	cysteine	−0.245 ± 0.001
cysteamine	−0.260 ± 0.001	penicillamine	−0.243
3-mercaptopro-pionic acid	−0.257 ± 0.004	2-amino-2-methyl-1-propane thiol	−0.221 ± 0.002
coenzyme A	−0.256 ± 0.003		

[a] Relative to the standard hydrogen electrode. Calculated using $E^{\circ\prime}_{GSSG/GSH} = -0.262$ V. [b] pD 7.0, 25 °C. [c] The uncertainties represent the precision of the measurement; the actual uncertainty may be as large as ±0.01 V due to uncertainty in the reference value for $E^{\circ\prime}_{NADP/NADPH}$, as discussed in the text.

termined in the present work for $E^{\circ\prime}_{GSSG/GSH}$ are in good agreement with those reported by Scott et al.[4] and by Rost and Rapoport.[5] As discussed above, these would also appear to be the most reliable values listed in Table I since they were determined by procedures which would account for any GSSG initially present in the GSH.

Recently, half-cell potentials were determined for a group of thiol-containing biological molecules and related molecules by measuring equilibrium constants for their thiol/disulfide exchange reactions with, in most cases, the GSH/GSSG system.[15] Values were calculated for

$$2RSH + GSSG \rightleftharpoons RSSR + 2GSH \qquad (4)$$

$E^{\circ\prime}_{RSSR/RSH}$ from the equilibrium constants using the value reported by Szajewski and Whitesides[7] for $E^{\circ\prime}_{GSSG/GSH}$. We have recalculated the half-cell potentials for these thiol-containing molecules relative to the value determined in this work for $E^{\circ\prime}_{GSSG/GSH}$. The results are reported in Table II.

Experimental Section

NADP$^+$, glutathione, and glutathione reductase were obtained from Sigma Chemical Co. glycine from Aldrich Chemical Co., and D_2O, DCl (35% in D_2O), and NaOD (40% in D_2O) from Isotech Inc.

^{1}H NMR measurements were made at 500 MHz. T_1 values were estimated by the inversion-recovery method for the analytical resonances (Figure 1) used to quantitate the equilibrium mixtures using separate solutions of GSH, GSSG, NADPH, and NADP$^+$. ^{1}H NMR spectra were measured for the equilibrium mixtures using relaxation delays 4.5 times the longest T_1 of the analytical resonances together with a flip angle of 84°. The HOD or H_2O resonances were suppressed by selective presaturation. Relative resonance intensities for the analytical resonances were determined by the cut and weigh method[16] or with the standard software on the Varian VXR-500S spectrometer.

Solutions were prepared in sodium phosphate buffered H_2O or D_2O solution (0.125 M buffer; pH 7.0 or pD 7.0). Standard solutions of GSH and NADP$^+$ were prepared in a nitrogen atmosphere in a Plas Labs, Inc. Model 885-AC anaerobic chamber or a glovebag. The nitrogen gas was passed through a column of BASF copper catalyst R3-11 to remove traces of oxygen. The concentrations of the standard solutions, including the concentration of GSSG initially present in the GSH solutions, were determined by ^{1}H NMR, using glycine as an internal intensity standard. On the basis of the concentrations determined for the standard solutions, appropriate volumes of the standard solutions together with 7–10 units of glutathione reductase were combined directly in NMR tubes in the anaerobic chamber or the glovebag. In some cases, glycine was added to the reaction mixture as an internal intensity standard and the equilibrium concentrations were determined from the relative resonance intensities. The NMR tubes were capped, the caps were wrapped with parafilm to further exclude oxygen, and then the tubes were covered to minimize light-induced degradation of NADPH. The pH or pD of the solutions was measured directly in the NMR tubes in the anaerobic chamber or glovebag with an Ingold microelectrode, which was calibrated using pH 4.00 and 7.00 aqueous buffers. pH meter readings for D_2O solutions were converted to pD values using the equation pD = pH + 0.40.

The initial concentrations of GSH and NADP$^+$ used in the experiments conducted in H_2O solution were, respectively: 60 and 30 mM (n = 4), 38 and 19 mM (n = 1), ~85 and 12 mM (n = 6), and ~42 and ~30 mM (n = 6). Those used in the experiments conducted in D_2O were, respectively: 42 and 17 mM (n = 2), 53 and 20 mM (n = 3), ~80 and ~10 mM (n = 12), ~38 and ~25 mM (n = 6), and ~44 and ~20 mM (n = 6). To establish that equilibrium was achieved before NMR spectra were measured, spectra were measured as a function of time for 200 min after the GSH, NADP$^+$, and enzyme solutions were combined in two separate experiments. It was found that equilibrium had been achieved by the time the first spectrum was measured (20 min after mixing). All spectra used to calculate equilibrium constants were measured at least 30 min after the reactants were combined. To establish that the same equilibrium constant is obtained when equilibrium is approached from the other direction, NMR spectra were measured for equilibrium solutions prepared by combining standard solutions of GSH, NADP$^+$, and enzyme. Then GSSG standard solution was added to the NMR tube to increase the GSSG concentration by a factor of two to four times the equilibrium concentration, and the system was allowed to return to equilibrium by the reverse reaction. Values of 145 ± 15 (n = 6) and 142 ± 30 (n = 6) were obtained for the equilibrium constant in H_2O from spectra measured before and after the addition of GSSG. The corresponding values for D_2O solution are 127 ± 17 (n = 12) and 117 ± 13 (n = 12).

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(16) Harris, W. E.; Kratochvil, B. *Chemical Separations and Measurements: Background and Procedures for Modern Analysis*; W. B. Saunders Golden Series: Philadelphia, 1974; p 197.

(17) After this paper was submitted, a value of −0.252 V was reported by: Lees, W. J.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 642–647. This value was determined relative to the $E^{\circ\prime}$ value for lipoic acid using equilibrium constants measured by NMR for the lipoic acid/oxidized mercaptoethanol and mercaptoethanol/glutathione disulfide redox reactions.