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Short communication

Direct spectroelectrochemical titration of glutathione

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Abstract

Previous potentiometric attempts to determine the formal potential ($E^{0\prime}$) of key intracellular redox buffer glutathione resulted in contradictory values. We have developed a spectroelectrochemical method using direct reduction on metal oxide electrodes. Disulfide absorbance at 258 nm was used to titrate glutathione in the thin layer cell reversibly. At conditions close to physiological ([GSH]=0.001–0.005 mol/l, pH=7.34; I=0.1 mol/l; T=298.15 K), we have measured glutathione $E^{0\prime}$ =−0.22±0.02 V (NHE), corroborating the results of equilibrium measurements.
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Keywords: Glutathione; Formal potential; Spectroelectrochemistry; Metal oxide electrode; Redox equilibrium

1. Introduction

Glutathione (γ-Glutamylcysteinylglycine) is a key endogenous tripeptide that provides a large pool of reducing equivalents, and is considered to be the main intracellular redox buffer [1]. It is present in millimolar concentrations and is involved in antioxidant defense, regulation of cell proliferation, deoxyribonucleotide synthesis, and cell–cell signaling. Glutathione physiological functions derive from side chain sulfhydryl residue in cysteine (GSH) which can be oxidized to disulfide (GSSG) by $NADP^+$ in a reaction catalyzed by glutathione reductase:

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

We are interested in the formal potential $E^{0\prime}$ for the biochemical redox reaction:

$$2GSH = GSSG + 2H^+ + 2e^- \quad (2)$$

Previous attempts to determine $E^{0\prime}$ were based on two broad methodologies: potentiometric titration [2–4] and equilibrium constant measurements of the reaction (1) [5–7].

Early potentiometric titration of GSH [2] using I_2, KIO_3 and $K_2Cr_2O_7$ as GSH oxidants resulted in $E^{0\prime}$ (pH=7) values ranging from 0.07 to 0.08 V vs. NHE. Although only oxidative titration was found to be experimentally feasible the reaction was treated as reversible [3,4]. Later studies were focused on measuring the equilibrium constant for the reaction (1) and calculating glutathione formal potential using various NADP/NADPH formal potential values ($E^{0\prime}$ =−0.28–0.32 V) [5–7]. The fact that the position of equilibrium of reaction (1) lies far to the left makes the equilibrium measurement a difficult one [7,8]. This resulted in the GSSG/GSH formal potential values ranging from −0.23 [5] to −0.28 V [7]. These values are on average 0.3 V more negative compared to potentiometric values that suggests novel approaches to glutathione formal potential measurements should be explored. Accurate measurements of $E^{0\prime}$ are critical to determine the direction of initial electron flow in the various physiological glutathione–redox partner combinations and, more generally, provide added insight about the cell redox environment [1]. We have reexamined the thermodynamic charge transfer equilibrium of GSSG/GSH using a direct redox titration in a thin layer spectroelectrochemical cell. This approach was tested previously with several redox

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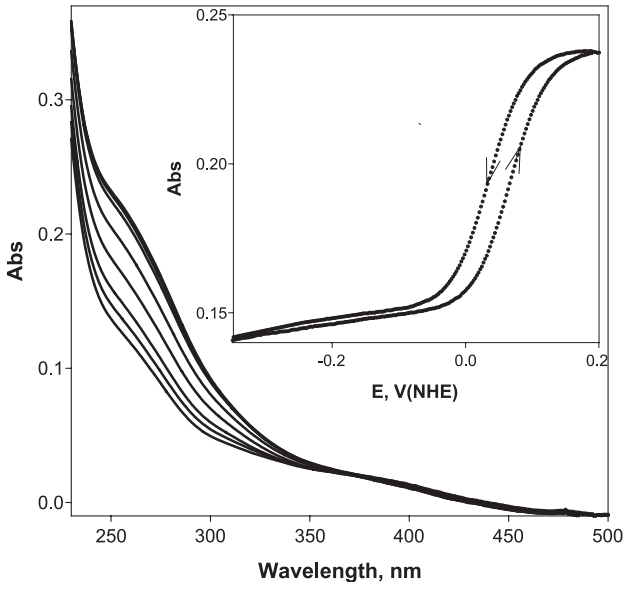

Fig. 1. Glutathione absorbance during the reversible redox cycle in a thin layer spectroelectrochemical cell. Optical path-length—5 mm. Insert shows absorbance at 258 nm recorded during the 0.1 mV/s cyclic potential scan. The average absorbance values from the cathodic and anodic scans were used to estimate glutathione oxidation degree.

proteins [9,10] and allows reliable Nernst titration of the GSSG/GSH solution while approaching redox equilibrium from both sides, thereby assuring the thermodynamic reversibility.

2. Experimental

Both reduced and oxidized glutathione were purchased from Sigma[1] (Sigma, St. Louis, MO). Solutions were prepared in 0.1 M phosphate buffer (0.0998 mol/l K_2HPO_4+0.0138 mol/l H_3PO_4, pH=7.34), which contained 0.001–0.005 mol/l oxidized or reduced glutathione. $E^{0\prime}$ was quantified by spectroelectrochemical titration using a thin layer cell and Chem2000 fiber optic spectrometer (Ocean Optics, Dunedin, Fl) [10]. Nano-crystalline Sb-doped tin oxide electrodes, prepared according to [11], were mounted to form a 0.2 mm gap and served as a working electrode. A gel containing 5 nm diameter Sb-doped SnO_2 nanocrystals (Alfa, Ward Hill, MA) was spread on conductive In–Sn oxide covered glass slides (Aldrich, Milwaukee, WI). After drying in air for 0.5 h, the gel film was fired at 450 °C for 20 h with subsequent cooling to room temperature. These metal oxide electrodes are resistant to fouling and have sufficient charge transfer rate to glutathione to maintain redox equilibrium within the 0.2 mm solution layer. The airtight quartz cell also contained Pt wire counter electrode, Ag/AgCl reference (Microelectrodes, Bedford, NH) and provisions for solution and Ar gas delivery. Before titration solutions were poised at the initial potential at least 2 h while purging with oxygen-free water saturated Ar gas. A gas blanket was maintained in the cell headspace during the potential scans. Solution absorbances were measured along the electrode gap thus avoiding the strong absorption of metal oxide electrodes in the UV. In addition, optical path-length increased to 5 mm, providing significantly higher S/N ratio, compared to the perpendicular sampling geometry. Average solution absorbances during the cathodic and anodic scans were used to calculate reaction (2) quotient. Both fully reduced and oxidized glutathione were used as initial states to ensure reaction reversibility.

Cell was calibrated using Phenosafranine as an internal standard, monitored at 500 nm (E^0=−0.252 V vs. NHE at pH=7) [12].

3. Results and discussion

Both oxidized and reduced glutathione are transparent in the visible but have a weak 258 nm disulfide absorption band [13], which partially overlaps the prominent amide absorption at 220 nm (Fig. 1).

This band varies monotonically between fully oxidized and fully reduced glutathione states and was used to monitor the oxidation degree x during the roundtrip potential scans between 0.2 and −0.4 V at 0.1 mV/s:

$$x = \frac{A - A_{red}}{A_{ox} - A},$$

where A—absorbance at 258 nm, A_{red}—GSH absorbance, A_{ox}—GSSG absorbance. Glutathione concentrations during

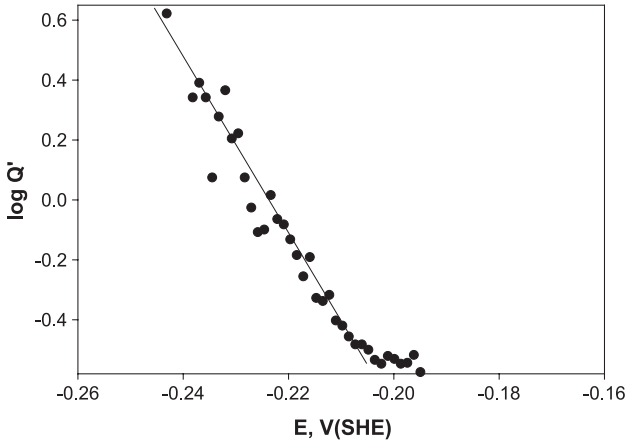

Fig. 2. Glutathione Nernst plot obtained from the 258 nm absorbance data at pH=7.34; T=298.15 K; [GSH]=0.003 mol/l. Apparent formal potential value $E^{0\prime}$ was obtained as the intersection with abscissa drawn at log Q' =0.

[1] Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Table 1
Literature values of $E^{0\prime}$ adjusted to pH=7.34 and T=298.15 K

Method of measurement	E, V (SHE)	Reference
Potentiometric titration	0.07	2
Potentiometric titration	0.08	3
Reaction with thiolate anions	−0.23	5
Reaction with NADP	−0.26	6
Reaction with NADP	−0.28	7
Spectroelectrochemical titration	−0.22	this study

the redox titration were calculated from x and initial concentration $[GSSG]_0$:

$$[GSSG] = x[GSSG]_0 \text{ and } [GSH] = 2(1-x)[GSSG]_0$$

Formal potential E'^{0} measurements were based on the Nernst equation at T=298.15 K. The prime is used to indicate pH dependent quantities:

$$E' = E^{0\prime} - (RT/2F)\ln Q' \quad (3)$$

where E' -applied potential and Q' —reaction (2) quotient:

$$Q' = \frac{[GSH]^2}{[GSSG]} = \frac{4(1-x)^2[GSSG]_0}{x} \quad (4)$$

Note that Q' is dependent on the glutathione concentration.

Absorption spectra were stable for several days at 298.15 K and could be cycled multiple times between fully reduced and fully oxidized states with small hysteresis at 0.1 mV/s. Solution absorbance was recorded periodically every 3 mV during the potential scan, thus obtaining acceptable S/N rate.

$E^{0\prime}$ values were obtained from the log Q' vs. E plot intersection with the abscissa drawn at log Q' =0 (Fig. 2). Plot linearity demonstrates nearly ideal Nernstian behavior for a two electron transfer with a 29 mV/dec slope. The reported formal potential value is valid for solution conditions T=298.15 K, pH=7.34 and I=0.1 mol/l. Further study is needed to explore formal potential dependence on the complex glutathione protonation equilibria.

Although our glutathione $E^{0\prime}$ value is on average 0.3 V more negative compared to potentiometric titration data, it is quite consistent with the results, obtained from the equilibrium measurements (Table 1). Freedman et al. [4] critically evaluated potentiometric data and concluded that reversibility conditions were not satisfied. The calculation of $E^{0\prime}$ from the equilibrium results for reaction (1) relies on a value of E° for the NADP/NADPH couple, the value of which also has some uncertainty in it. Given the fundamental importance of NADPH/NADH for bioprocesses, it would be justified to revisit the redox thermodynamics of NADP, NAD reactions.

4. Conclusion

In this study, using spectroelectrochemistry, we have shown that glutathione can be directly reduced on metal oxide electrodes. Reversible glutathione titration has demonstrated almost ideal Nernst behavior for a two-electron transfer reaction. At conditions close to physiological ([GSH]=0.001–0.005 mol/l, pH=7.34; I=0.1 mol/l; T=298.15 K), we have measured glutathione $E^{0\prime}$ =−0.22± 0.02 V (NHE), corroborating the results of equilibrium measurements [7].

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References

[1] F.Q. Schafer, G.R. Buettner, Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, Free Radic. Biol. Med. 30 (2001) 1191–1212.
[2] J.C. Ghosh, S.C. Ganguli, Das Redoxpotential des Glutathions, Biochem. Z. 279 (1935) 296–299.
[3] R.L. Ryklan, C.L. Schmidt, The oxidation potentials of cystine–cysteine and related systems, Univ. Calif. Pub. Physiol. 8 (1944) 257–276.
[4] L.D. Freedman, A.H. Corwin, Oxidation–reduction potentials of thiol–desulfide systems, J. Biol. Chem. 181 (1949) 601–621.
[5] R.P. Szajewski, G.M. Whitesides, Rate constants and equilibrium constants for thiol–disulfide interchange reactions involving oxidized glutathione, J. Am. Chem. Soc. 102 (6) (1980) 2011–2026.
[6] J. Rost, S. Rapoport, Reduction-potential of glutathione, Nature 201 (1964) 185.
[7] Y. Tewari, R. Goldberg, Thermodynamics of the oxidation–reduction reaction {2 glutathione$_{red}$(aq)+NADP$_{ox}$(aq)=glutathione$_{ox}$(aq)+ NADP$_{red}$(aq)}, J. Chem. Thermodyn. 35 (2003) 1361–1381.
[8] G. Gorin, A. Esfandi, G.B. Guthrie Jr., Glutathione: its reaction with NADP and its oxidation–reduction potential, Arch. Biochem. Biophys. 168 (1975) 450–454.
[9] V. Reipa, M.J. Holden, M.P. Mayhew, V.L. Vilker, Temperature dependence of the formal reduction potential of putidaredoxin, Biochim. Biophys. Acta 1459 (2000) 1–9.
[10] V. Reipa, M.P. Mayhew, M.J. Holden, V.L. Vilker, Redox control of the P450cam catalytic cycle: effects of the Y96F active site mutation and binding of a non-natural substrate, Chem. Commun. 4 (2002) 318–319.
[11] W. Boschloo, D. Fitzmaurice, Spectroelectrochemistry of highly doped nanostructured tin dioxide electrodes, J. Phys. Chem. 103 (1999) 3093–3098.
[12] M. Clark, Oxidation–Reduction Potentials of Organic Systems, Williams & Wilkins, Baltimore, MD, 1960, p. 131.
[13] J.P. Greenstein, M. Winitz, Chemistry of the Amino Acids, Wiley, New York, NY, 1961, pp. 1879–1928.