Thiol/Disulfide Exchange Reactions of Ovothiol A with Glutathione

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Introduction

Biological systems use enzymatic and nonenzymatic antioxidant systems to deal with oxidative stress, including the ubiquitous cysteine-containing tripeptide glutathione.[1,2] Recently a new family of antioxidant thiol compounds, the 1-methyl-4-mercaptohistidines (ovothiols) in Figure 1, has been discovered in marine invertebrate eggs.[3,4] The function of these compounds, which are present at millimolar concentrations, seems to be to protect the egg from oxidative damage during the respiratory burst which takes place immediately after fertilization.[3] For example, during the respiratory burst, sea urchin eggs produce extracellular H_2O_2 which is used in the construction of an envelope to protect the early embryo by cross-linking tyrosyl residues.[3d,e] Any H_2O_2 which crosses the plasma membrane is scavenged by nonenzymatic reaction with ovothiol (OvSH), with the formation of oxidized ovothiol (OvSSOv). The OvSSOv is then reduced by glutathione, which is present at millimolar concentrations in the egg in the reduced (GSH) state. The redox potential of ovothiol C (Figure 1) is reported to be 84 mV positive that of GSH.[5]

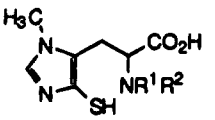
In view of the potential importance of aromatic thiols of the 1-methyl-4-mercaptohistidine type as biological antioxidants, it is of interest to characterize their redox properties and thiol/disulfide exchange reactions. In this paper, we report equilibrium constants for thiol/disulfide exchange reactions of ovothiol A (Figure 1) with glutathione (eqs 1–3) and redox potentials for ovothiol A over the pD range 3.4–10.4.

$$\mathrm{GSH} + \mathrm{OvSSOv} \rightleftharpoons \mathrm{OvSH} + \mathrm{OvSSG} \quad (1)$$

$$\mathrm{GSH} + \mathrm{OvSSG} \rightleftharpoons \mathrm{OvSH} + \mathrm{GSSG} \quad (2)$$

$$2\mathrm{GSH} + \mathrm{OvSSOv} \rightleftharpoons 2\mathrm{OvSH} + \mathrm{GSSG} \quad (3)$$

To account for the dependence of the thiol/disulfide exchange equilibrium constants and redox potentials on pD, we have also characterized in detail the acid/base chemistry of ovothiol A and ovothiol A disulfide.



Ovothiol	R^1	R^2
A	H	H
B	CH_3	H
C	CH_3	CH_3

Figure 1. Structures of ovothiols A, B, and C.

Results and Discussion

Equilibrium constants for thiol/disulfide exchange of ovothiol A with GSH were measured by ^{1}H NMR. The procedure involved determination of the concentration of reactants and products from resonance intensities in spectra measured as increasing amounts of GSH were added to solutions of OvSSOv. To illustrate, selected portions of ^{1}H NMR spectra from a typical experiment at pD 7.4 are presented in Figure 2. The resonances at 8.23, 7.76, and 7.69 ppm are for the imidazole C2–H protons of OvSH, OvSSOv, and OvSSG, respectively. The four-line resonance pattern centered at 2.96 ppm is the upfield half of the multiplet pattern for the $C_\beta H_2$ protons of the cysteinyl residue of GSSG. The four-line resonance pattern centered at 2.15 ppm is for the $C_\beta H_2$ protons of the glutamyl residues of GSH and GSSG, while the pattern centered at 2.10 ppm is for the same protons of OvSSG. Spectrum A in Figure 2 is for a solution which contained 12.1 mM OvSSOv and 2.23 mM OvSH. Addition of GSH to the solution (spectra B–D) causes the intensity of the OvSH resonance at 8.23 ppm to increase, the intensity of the OvSSOv resonance at 7.76 ppm to decrease, and resonances to appear at 7.69 and 2.10 ppm for OvSSG.[6]

Because the equilibrium constant for eq 1 lies far to the right, the concentration of GSH is small when OvSSOv is present, and thus precise values could not be determined for K_1 directly from resonance intensities. K_1 was determined indirectly by first determining the equilibrium constant K^* for eq 4 using

$$\mathrm{OvSSOv} + \mathrm{GSSG} \rightleftharpoons 2\mathrm{OvSSG} \quad (4)$$

concentrations obtained directly for OvSSOv, GSSG, and OvSSG from ^{1}H NMR spectra under conditions where the total concentration of GSH added to the OvSSOv solution was less than the initial OvSSOv concentration. K_2 was then determined under conditions where the total added GSH was greater than the initial OvSSOv concentration. The concentrations of OvSH and OvSSG were obtained directly from the intensities of their imidazolium resonances (Figure 2), and the concentration of GSH was then calculated by difference using these concentrations, the total added GSH and the stoichiometry of the reaction. The concentration of GSSG was determined either from

(1) Sies, H. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1058–1071.
(2) Sies, H. In *Oxidative Stress*; Sies, H., Ed.; Academic Press: New York, 1985; pp 1–8.
(3) (a) Turner, E.; Klevit, R.; Hopkins, P.; Shapiro, B. M. *J. Biol. Chem.* **1986**, *261*, 13056–13063. (b) Turner, E.; Klevit, R.; Hager, L. J.; Shapiro, B. M. *Biochemistry* **1987**, *26*, 4028–4036. (c) Shapiro, B. M.; Turner, E. *Biofactors* **1988**, *1*, 85–88. (d) Turner, E.; Hager, L. J.; Shapiro, B. M. *Science* **1988**, *242*, 939–941. (e) Shapiro, B. M. *Science* **1991**, *252*, 533–536.
(4) (a) Palumbo, A.; Misuraca, G.; d'Ischia, M.; Donandy, F.; Prota, G. *Comp. Biochem. Physiol.* **1984**, *78B*, 81–83. (b) Palumbo, A.; d'Ischia, M.; Misuraca, G.; Prota, G. *Tetrahedron Lett.* **1982**, *23*, 3207–3208. The structure of ovothiol was incorrectly identified in these references as having the methyl substituent on N-3 of the imidazole ring. (Holler, T. P.; Spaltenstein, A.; Turner, E.; Klevit, R. E.; Shapiro, B. M.; Hopkins, P. B. *J. Org. Chem.* **1987**, *52*, 4421–4423.)
(5) Heinecke, J. W.; Shapiro, B. M. *J. Biol. Chem.* **1992**, *267*, 7959–7962. The redox potential was originally reported to be 44 mV positive of that for glutathione.[3c]
(6) Thiol/disulfide exchange equilibrium was reached within the time required to obtain the first NMR spectrum (<6 min) over the pD range 3.4–10.4.

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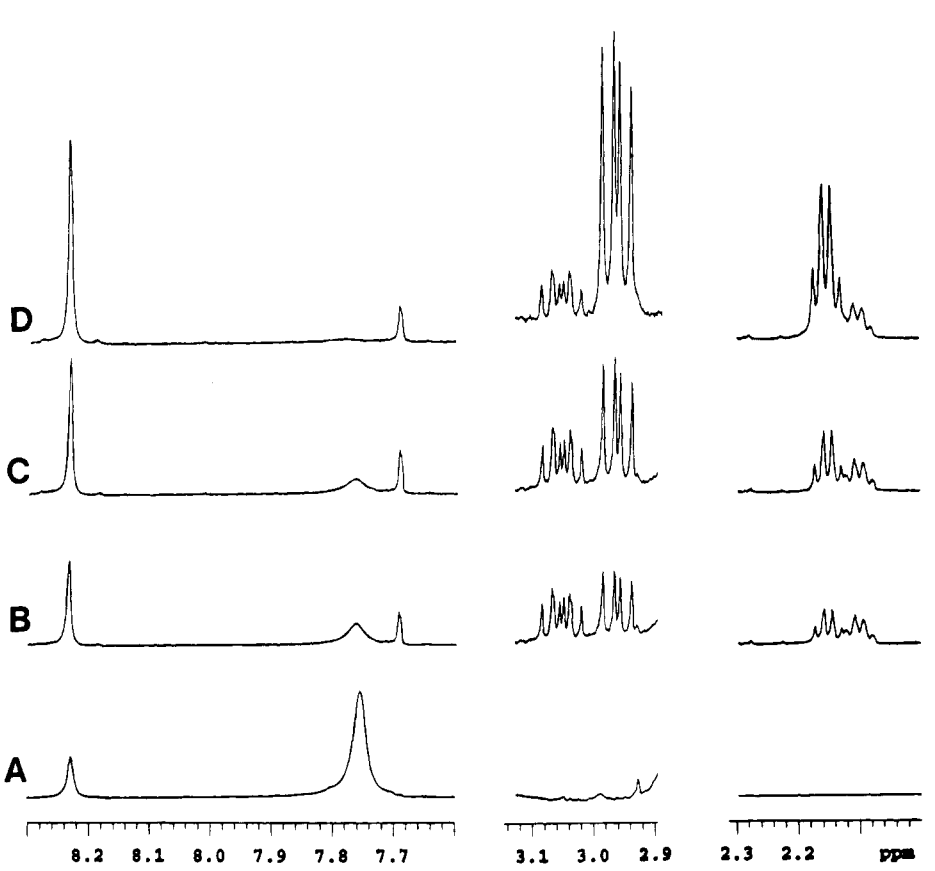

Figure 2. Portions of 500 MHz ^{1}H NMR spectra of 12.1 mM OvSSOv and 2.23 mM OvSH in D_2O at pD 7.4 to which (A) 0.00, (B) 4.43, (C) 6.45, and (D) 10.3 mM GSH were added. Resonance assignments are given in the text. The 2.9–3.14 ppm region is plotted with a vertical scale 4× that is used for the other regions.

Table 1. Thiol/Disulfide Exchange Equilibrium Constants and Redox Potential of Ovothiol [a]

pD	K^*	n[b]	K_1[c]	K_2	n[d]	K_3	$E^{o'}_{OvSSOv/OvSH}$[e]
3.4	3.0 ± 0.6	6	510	170 ± 40	3	8.7×10^4	0.146
7.0	1.1 ± 0.2	7	780	710 ± 90	7	5.5×10^5	0.170
7.4	1.0 ± 0.3	6	850	850 ± 180	13	7.2×10^5	0.173
9.4	0.91 ± 0.06	12	420	460 ± 110	6	1.9×10^5	0.156
9.9	1.2 ± 0.2	10	140	120 ± 16	4	1.7×10^4	0.125
10.4	1.2 ± 0.2	19	53	44 ± 7	8	2.3×10^3	0.099

[a] Conditions: 25 °C, D_2O solution, 0.15 **M** phosphate buffer at pD < 9, 0.15 **M** ammonia buffer at pD > 9. [b] Number of determinations of K^*. [c] Calculated using $K_1 = K_2K^*$. [d] Number of determinations of K_2. [e] Volts relative to $E^{o'}_{GSSG/GSH}$. Estimated uncertainty ±0.003 V.

the intensity of the resonance pattern centered at 2.96 ppm or from the total intensity of the resonances in the 2.05–2.20 ppm region, corrected for the concentrations of GSH and OvSSG. K_1 was then calculated from K^* and K_2 using the relationship $K_1 = K_2K^*$, and the equilibrium constant K_3 for the overall exchange reaction (eq 3) was calculated using the equation $K_3 = K_1K_2$.

Values determined for K^*, K_1, K_2, and K_3 over the pD range 3.4–10.4 are presented in Table 1. Also listed in Table 1 are redox potentials calculated for ovothiol A relative to the redox potential for GSH using the values for K_3 and eq 5.

$$E^{o'}_{OvSSOv/OvSH} = E^{o'}_{GSSG/GSH} + \frac{RT}{nF} \ln K_3 \qquad (5)$$

The relative redox potentials determined for ovothiol A indicate that GSH is more reducing than the ovothiols, in agreement with previous reports.[3c,5] However we find the difference in redox potential at pD 7.0 to be even larger than reported previously: 170 mV more positive than $E^{o'}_{GSSG/GSH}$ as compared to 84 mV reported for ovothiol C.[5] The difference might reflect differences in the redox properties of ovothiol A and C, but more likely is due to the different methods used to determine the thiol/disulfide exchange equilibrium constants from which the redox potentials were calculated.

The results in Table 1 indicate that the thiol/disulfide exchange equilibrium constants and $E^{o'}_{OvSSOv/OvSH}$ are strongly pD dependent. Since the thiolate anion is the reactive species in thiol/disulfide exchange reactions, thiol/disulfide exchange equilibrium constants will be pD dependent if the acid dissociation constants of the reactant and product thiol groups are different.[7] pK_A values have been reported for GSH but not for the ovothiols.[8] The ovothiols have four acidic groups. Chemical shift data measured as a function of pD for the C_2H, CH_3N, and $C_\alpha H$ protons for ovothiol A indicate that the thiol and carboxylic acid groups are titrated in a stepwise fashion (Figure 3), but that the imidazolium and ammonium groups titrate simultaneously. Thus macroscopic constants K_1 and K_2 are for the thiol and carboxylic acid groups, respectively, while K_3 and K_4 are each a function of the microconstants for both the imidazolium and ammonium groups. Acid dissociation constants determined for OvSH from chemical shift vs pD data for the C_2H proton of the imidazole ring and the $C_\alpha H$ proton

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(8) pK_A values of 2.3 and 6.7 have been reported for the thiol group of the imidazolium and imidazole forms, respectively, of 1,5-dimethyl-4-mercaptoimidazole. Holler, T. P.; Hopkins, P. B. *J. Am. Chem. Soc.* **1988**, *110*, 4837–4838.

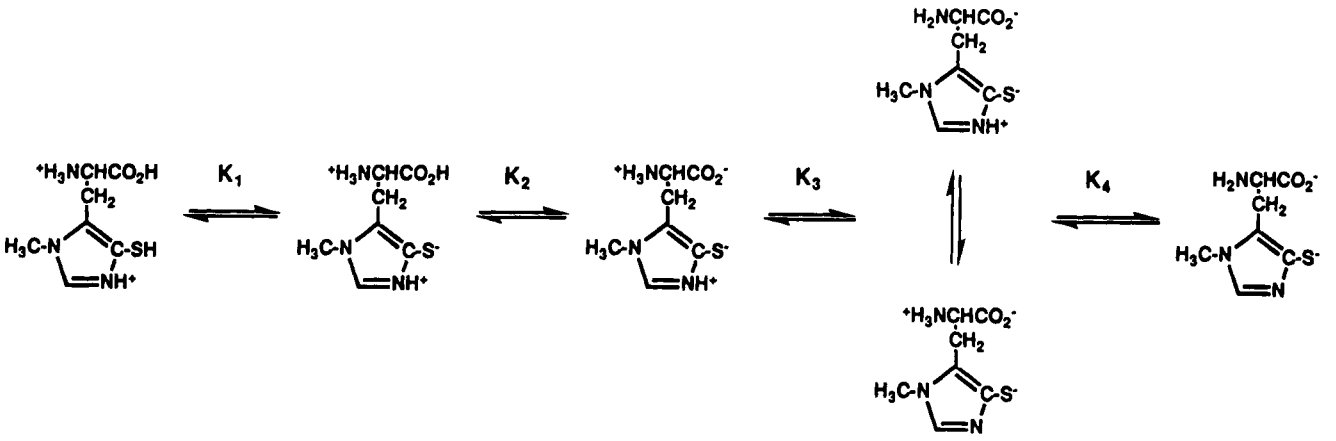

Figure 3. Deprotonation scheme for OvSH.

Table 2. Acid Dissociation Constants for Ovothiol and Ovothiol Disulfide[a]

	OvSH	OvSSOv
pK_1	1.42	1.60
pK_2	2.62	2.20
pK_3	8.83	3.38
pK_4	10.46	4.91
pK_5		8.72
pK_6		9.32
pk_1, pk_2		1.90
pk_{12}, pk_{21}		1.90
pk_{123}, pk_{124}		3.68
pk_{1234}, pk_{1243}		4.61
pk_{12345}, pk_{12346}		9.02
pk_{123456}, pk_{123465}		9.02

[a] Conditions: 25 °C, 0.15 M NaCl in D_2O solution.

using standard methods[9] are presented in Table 2. Titration of the six acid groups of OvSSOv can be described in terms of the macroscopic constants K_1–K_6 and the microscopic scheme defined in Figure 4. Chemical shift vs pD data indicate that the two CO_2H groups are titrated first, followed by the two imidazolium groups and then the two ammonium groups. Microscopic and macroscopic acid dissociation constants determined from chemical shift vs pD data for OvSSOv are listed in Table 2. At the microscopic level, the acidity of one carboxylic acid group of OvSSOv is independent of the protonation state of the other carboxylic acid group. The same is true for the two ammonium groups, but not for the imidazolium groups, which are separated by fewer bonds. The acid dissociation constant of the imidazolium group of OvSSG was determined from chemical shift vs pD data for the imidazole C_2H proton to be 4.64, i.e. essentially identical to pk_{1234}, the acid dissociation constant for one imidazolium group of OvSSOv when the other is deprotonated.

With a pK_A of 1.42, the thiol group of OvSH is in the thiolate form over the pD range 3.4–10.4, whereas the thiol group of GSH titrates with a pK_A of 9.33.[7] Thus, K_3 is predicted to increase by a factor of 100 per unit increase in pD as the pD is increased from 3.4 to 8.4 and then increase more slowly to a maximum when GSH is all in the thiolate form. Experimentally, K_3 increases as the pD is increased from 3.4–7.4 (Table 1), but much less than predicted, and then decreases above pD 7.4. The less than predicted increase in K_3 over the pD range 3.4–7.4 is apparently due to titration of the imidazolium groups of OvSSOv and OvSSG. Above pD 7.4, the difference between the predicted and observed dependence on pD is due to a change in the Brønsted basicity of the ovothiol thiolate anion when the imidazolium group is deprotonated. Thiol/disulfide equilibrium constants increase with an increase in the Brønsted basicity of the reactant thiolate anion and decrease with an increase in the Brønsted basicity of the product thiolate anion.[7] On the basis of pK_A values reported for 1,5-dimethyl-4-mercaptoimidazole by Holler and Hopkins,[8] the Brønsted basicity of the thiolate group of OvSH is predicted to increase by ~4.4 pK units when the imidazolium group is deprotonated. Thus, the predicted increase in K_3 due to deprotonation of GSH is more than offset by the increased basicity of the thiolate group of OvSH as the imidazolium group is titrated, with the result that K_3 actually decreases as the pD is increased from 7.4 to 10.4.

By using a value of −0.262 V vs the standard hydrogen electrode for $E^{o'}$ for GSH at pD 7.0[10] and the relative redox potential for OvSH in Table 1, $E^{o'}$ for the imidazolium form of OvSH is calculated to be −0.092V vs the standard hydrogen electrode, more positive than has been reported for any other organic thiol.[10–12] $E^{o'}$ cannot be calculated for the imidazole form of OvSH from K_3 at pD 10.4 because $E^{o'}$ for GSH is not known at this pD. However, $E^{o'}$ for the imidazole form of OvSH is estimated to be >0.2 V more negative than $E^{o'}$ for the imidazolium form from equilibrium constants for the reaction of the thiolate form of GSH (GS^-) with the imidazolium and imidazole forms of OvSSOv to yield GSSG and the imidazolium and imidazole forms of OvS^-.[13] Using the equation $\Delta E^{o'} = \Delta pK_A - 7.7$, which was obtained from redox potentials for alkyl thiols,[7] the imidazole form is predicted to be 0.27 V more reducing than the imidazolium form.

In summary, the redox potential for ovothiol A is considerably more positive than the redox potential for GSH and is strongly dependent on the protonation state of the imidazole ring. The large difference between the redox potentials of the imidazolium and imidazole forms

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(13) At pD 10.4, GSH is predominantly in the GS^- form, and OvS^- and OvSSOv are in the imidazole form. Thus, K_3 at pD 10.4 can be used directly. The equilibrium constant for the reaction involving GS^- and the imidazolium forms of OvSSOv and OvS^- was calculated using K_3 at pD 7.0, pK_A = 9.33 for the thiol group of GSH and pK_3 and pK_4 for OvSSOv in Table 2.

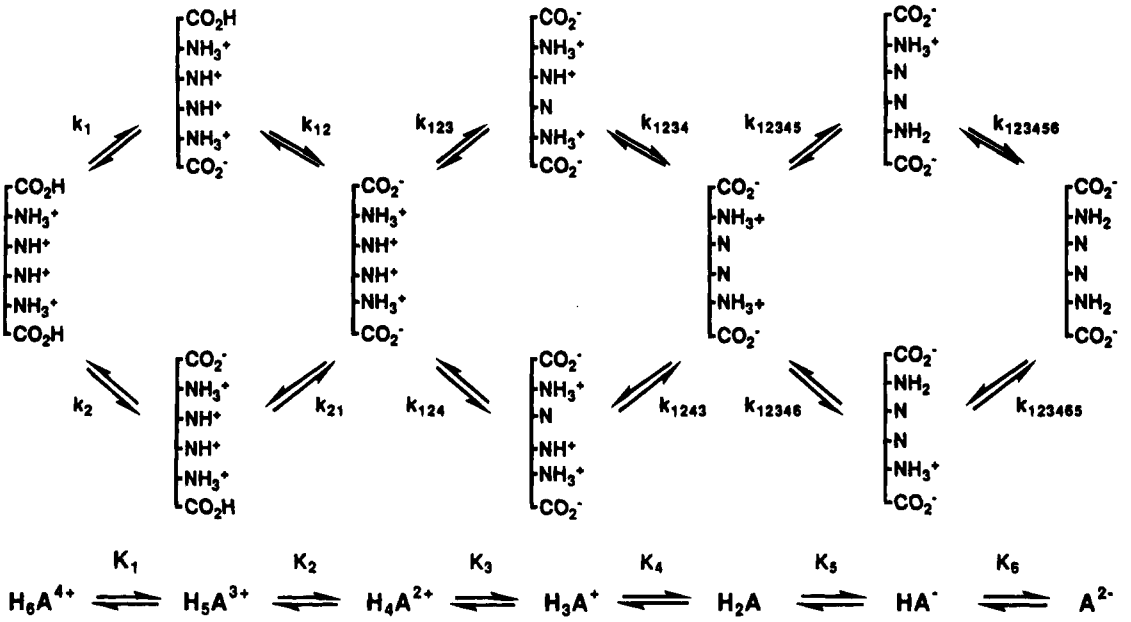

Figure 4. Macroscopic and microscopic deprotonation schemes for OvSSOv.

of OvSH is unprecedented in the dependence of the redox properties of an organic thiol on the protonation state of a neighboring group. The redox potential of the imidazolium form is 170 mV more positive than that of GSH at physiological pH, and thus ovothiol A in marine invertebrate eggs is predicted to be maintained completely in the reduced form by intracellular glutathione.

Experimental Section

Ovothiol A was prepared by deprotection of *S*-(4-methylphenyl)methyl-DL-ovothiol A by a literature procedure.[14] The *S*-(4-methylphenyl)methyl-DL-ovothiol A was a gift from Professor Paul Hopkins. Ovothiol A disulfide was prepared by bubbling an aqueous solution of ovothiol A with oxygen.[14] Oxidized and reduced glutathione and alanine were obtained from Sigma. ^{1}H NMR spectra were measured at 500 MHz and 25 °C. Solutions for pK_a determinations were prepared in D_2O and contained 0.15 M NaCl. The solutions used to determine the pK_as of OvSH also contained deuterated dithiothreitol (Cambridge Isotopes) to keep the OvSH in the reduced form. Solutions for determination of equilibrium constants were prepared in 0.15 M phosphate buffer or 0.15 M ammonia buffer. Alanine was added as an internal intensity reference. Resonance areas were determined by integration with the NMR software or by the cut and weigh method when resonances overlapped. All solutions were prepared under a nitrogen atmosphere in a glove bag and were deoxygenated by bubbling with oxygen-scrubbed argon.

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