# Kinetics and Equilibria of the Formation and Reduction of the Disulfide Bonds in Arginine-Vasopressin and Oxytocin by Thiol/Disulfide Interchange with Glutathione and Cysteine

Dallas L. Rabenstein* and Pauline L. Yeo

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

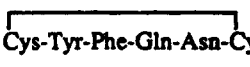
*Received March 4, 1994*®

Rate and equilibrium constants are reported for reduction of the disulfide bonds in the neurohypophyseal peptide hormones oxytocin (OT) and arginine-vasopressin (AVP) by thiol/disulfide interchange with glutathione (GSH) and cysteine (CySH) and for formation of the disulfide bonds by thiol/disulfide interchange with oxidized glutathione (GSSG) and cystine (CySSCy). The reactions take place in two steps. In the first step of the reduction reactions, AVP and OT react with GSH and CySH to form peptide-GSH and peptide-CySH mixed disulfides, which in turn react with another molecule of GSH or CySH to give the reduced dithiol form of the peptide and GSSG or CySSCy. Analysis of the forward and reverse rate constants indicates that which step is rate determining depends on the concentration of GSH or CySH. At physiological concentrations of GSH and CySH, intramolecular thiol/disulfide interchange in the mixed disulfides to reform the native disulfide bonds is faster than reaction with another molecule of GSH or CySH, even though intramolecular thiol/disulfide interchange involves closure of 20-membered rings. Rate constants for reaction of GSH and CySH with the disulfide bonds of AVP and OT are 1–2 orders of magnitude larger than for reaction with disulfide bonds formed by two cysteine-containing peptides, which suggests that the disulfide bonds in the neurohypophyseal peptide hormones are strained. Equilibrium constants are also reported for reaction of GSH with the hexapeptide analogs of AVP and OT, pressinoic acid (PA), and tocinoic acid (TA). A reduction potential of −0.216 V was calculated for the disulfide bonds of OT and TA from the thiol/disulfide interchange equilibrium constants. Reduction potentials of −0.229 V and −0.227 V were calculated for the disulfide bonds in AVP and PA, respectively. The similarity of the reduction potentials for OT and TA and for AVP and PA indicates that the acyclic tripeptide tails of OT and AVP have little affect on the redox properties of their disulfide bonds.

Thiol/disulfide interchange reactions provide a mechanism for the reversible formation of disulfide bonds in biological systems.[1] Such reactions are involved in maintaining the intracellular distribution of glutathione, coenzyme A, cysteine, and other thiols among their oxidized and reduced forms. The reversible formation of disulfide bonds is also utilized in biology as a cellular defense system, to regulate metabolism, and to transport reducing equivalents.[1b]

In previous studies, we have characterized the kinetics and equilibria of thiol/disulfide interchange reactions of biological monothiols.[2] These and other[3] studies have elucidated the molecular aspects of thiol/disulfide interchange reactions, including the effect of pH, thiol $pK_A$, and charge and steric factors. Mechanistically, thiol/disulfide interchange takes place via a simple nucleophilic displacement of a thiolate anion from the disulfide by another thiolate anion.[1b,2b,3a,d] No intermediates have been observed, which suggests a single transition state with a significant negative charge on the attacking and leaving thiolates and on the central sulfur atom.

In the next phase of this research program, we are studying the thiol/disulfide chemistry of peptides which have an intrapeptide disulfide bond. We report here the results of a study of the kinetics and equilibria of thiol/disulfide interchange reactions of the neurohypophyseal peptide hormones arginine vasopressin (AVP) and oxytocin (OT) with the tripeptide glutathione (γ-L-glutamyl-cysteinyl-glycine, GSH) and cysteine (CySH).




AVP


Oxytocin

AVP and OT have in common seven of their nine amino acids, a hexapeptide ring formed by residues 1–6 and an acyclic tripeptide tail. The hexapeptide ring in both AVP and OT is closed by a disulfide bond between cysteine residues at positions 1 and 6.

® Abstract published in *Advance ACS Abstracts,* June 15, 1994.

(1) (a) Kosower, E. M. in *Glutathione: Chemical, Biochemical and Medical Aspects;* Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1989; Part A, pp 103–146. (b) Gilbert, H. F. *Adv. Enzymol.* **1990**, *63*, 69–172.

(2) (a) Keire, D. A.; Strauss, E.; Guo, W.; Noszál, B.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 123–127. (b) Rabenstein, D. L., Theriault, Y. *Can. J. Chem.* **1984**, *62*, 1672–1680. (c) Rabenstein, D. L.; Theriault, Y. *Can. J. Chem.* **1985**, *63*, 33–39. (d) Theriault, Y.; Rabenstein, D. L. *Can. J. Chem.* **1985**, *63*, 2225–2231. (e) Keire, D. A.; Rabenstein, D. L. *Bioorg. Chem.* **1989**, *17*, 257–267. (f) Pleasants, J. C.; Guo, W.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1989**, *111*, 6553–6558. (g) Guo, W.; Pleasants, J. C.; Rabenstein, D. L. *J. Org. Chem.* **1990**, *55*, 373–376.

(3) (a) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011–2026. (b) Whitesides, G. M.; Lilburn, J. E.; Szajewski, R. P. *J. Org. Chem.* **1977**, 332–338. (c) Whitesides, G. M.; Houk, J.; Patterson, M. A. K. *J. Org. Chem.* **1983**, *48*, 112–115. (d) Wilson, J. M.; Bayer, R. J.; Hupe, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7922–7926. (e) Freter, R.; Pohl, E. R.; Wilson, J. M.; Hupe, D. J. *J. Org. Chem.* **1979**, *44*, 1771–1774. (f) Hupe, D. M.; Wu, D. *J. Org. Chem.* **1980**, *45*, 3100–3103.

0022-3263/94/1959-4223$04.50/0 

The disulfide bond is one of the most important structural features of AVP and OT.[4] In early reports, their biological activity[5] was accounted for in terms of a mechanism which assumed that interaction with their receptors involved covalent bond formation via thiol/disulfide interchange reactions.[6] However, structure–activity studies with analogs have shown the disulfide bond is not involved in the mechanism of action, but rather it is the cyclic arrangement of amino acids 1–6 that is essential for high biological activity.[4] The cyclic arrangement need not involve a disulfide bond; for example, analogs of deaminooxytocin in which the disulfide group is replaced by $CH_2S$ are biologically active. Thus, the main purpose of the disulfide bond in AVP and OT is to form and keep a molecular conformation suitable for noncovalent interaction with AVP and OT receptors.[7]

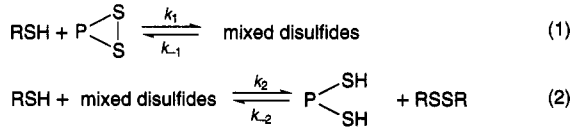
GSH and CySH were chosen for this study of the thiol/disulfide chemistry of AVP and OT because rate and equilibrium constants for their thiol/disulfide interchange reactions with a variety of other thiols and disulfides are available for comparison.[2a,3a] Also, GSH is the standard to which other thiols and disulfides are compared thermodynamically.[1b] Equilibrium constants and forward and reverse rate constants were determined for each step in the two-step reduction of AVP and OT by GSH and CySH, and redox potentials were calculated from the equilibrium constants for the overall reduction reactions.[8] Equilibrium constants were also determined for the reaction of GSH with the disulfide groups of the hexapeptide analogs of AVP and OT, pressinoic acid (PA), and tocinoic acid (TA). Major objectives of this study were to determine the tendency of the disulfide bonds in AVP and OT to undergo reduction by thiol/disulfide interchange, to characterize the kinetics of formation of the disulfide-containing 20-membered rings of AVP and OT by thiol/disulfide interchange, to determine redox potentials for the disulfide bonds in AVP and OT, and to determine the effect of the acyclic tripeptide tails on the thiol/disulfide interchange reactions and redox properties


Pressinoic Acid Tocinoic Acid

of AVP and OT. Such information is of interest not only with respect to the stability of the disulfide bonds of AVP and OT in biological systems, but also for understanding factors which influence the formation and stability of disulfide-containing loops in engineered peptides and proteins.

## The Model

Reduction and formation of the disulfide bonds in AVP, OT, PA, and TA by thiol/disulfide interchange is summarized by the reaction scheme:

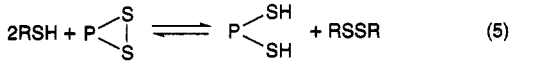



where RSH represents GSH and CySH. RSH reacts with the disulfide bond to form one of two possible mixed disulfides, which in turn react with RSH to give the reduced dithiol form of the peptide and RSSR.[9] Rate and equilibrium constants for reduction of the peptides by GSH and CySH and for formation of the peptide disulfide bonds by reaction of the reduced peptides with GSSG and CySSCy were determined by separation and analysis of reaction mixtures by high performance liquid chromatography (HPLC) using chromatographic conditions described previously.[10] To illustrate, the chromatogram for an AVP–GSH reaction mixture is shown in Figure 1. The kinetics and equilibria of the thiol/disulfide interchange reactions were characterized in terms of the total concentration of mixed disulfide, as summarized by eqs 1–4:

$$K_1 = \frac{[P\langle^{SSR}_{SH} + P\langle^{SH}_{SSR}]}{[RSH][P\langle^{S}_{S}]} \quad (3)$$

$$K_2 = \frac{[P\langle^{SH}_{SH}][RSSR]}{[RSH][P\langle^{SSR}_{SH} + P\langle^{SH}_{SSR}]} \quad (4)$$

The result of the overall reaction is reduction of the disulfide bond and formation of RSSR:




$$K_{OV} = \frac{[P\langle^{SH}_{SH}][RSSR]}{[RSH]^2[P\langle^{S}_{S}]} \quad (6)$$

Thiol/disulfide interchange occurs by a simple $S_N2$ nucleophilic displacement, with the thiolate anion being the reactive form of RSH.[1b,2b,3a,d] Thus, the rates of thiol/disulfide interchange reactions are pH dependent, as are the equilibrium constants if the reactant and product thiols have different $pK_A$ values. Conditional rate and equilibrium constants were measured at pH 7.00 and are expressed in terms of the total concentration of protonated and thiolate forms of the reactant and product thiols. Intrinsic rate and equilibrium constants were calculated in terms of the concentrations of the reactive

(4) Jošt, K. in *Handbook of Neurohypophyseal Peptide Hormones*; Jošt, K., Lebl, M., Brtnik, F., Eds.; CRC Press, Inc.: Boca Raton, FL, 1987; Vol. 1, part 2, 144–155.

(5) The major physiological activities of OT in mammals are milk ejection and uterine contraction; the major activities of AVP are vasoconstriction and antidiuretic functions.

(6) (a) Fong, C. T. O.; Schwartz, I. L.; Popenoe, E. A.; Silver, L.; Schoessler, M. A. *J. Am. Chem. Soc.* **1959**, *81*, 2592–2593. (b) Fong, C. T. O.; Silver, L.; Christman, D. R.; Schwartz, I. L. *Proc. Natl. Acad. Sci. U.S.A.* **1960**, *46*, 1273–1277. (c) Rasmussen, H.; Schwartz, I. L.; Schoesler, M. A.; Hochster, G. *Proc. Natl. Acad. Sci. U.S.A.* **1960**, *46*, 1278–1287. (d) Schwartz, I. L.; Fong, C.T.O.; Popenoe, E. A.; Silver, L.; Schoesler, M. A. *J. Clin. Invest.* **1959**, *38*, 1041. (e) Schwartz, I. L.; Rasmussen, H.; Schoesler, M. A.; Silver, L.; Fong, C.T.O. *Proc. Natl. Acad. Sci. U.S.A.* **1960**, *46*, 1288–1298.

(7) Hormone–receptor thiol/disulfide interchange reactions take place; however, they apparently involve thiol groups on the receptor that are not concerned with hormonal activity.[4]

(8) Redox potentials determined for thiol/disulfide systems by direct electrochemical measurement are not reliable because typically the reactions are thermodynamically irreversible due to reaction with the electrode surface. Jocelyn, P. C. *Biochemistry of the Sulfhydryl Group*; Academic Press: New York, 1972.

(9) Formation of double mixed disulfides by reaction of the single mixed disulfides with RSSR and of dimers by reaction of single mixed disulfides with AVP or OT is also possible. Low peptide concentrations were used to minimize formation of dimers and there was no evidence for their formation in the experiments reported here.

(10) Yeo, P. L.; Rabenstein, D. L. *Anal. Chem.* **1993**, *65*, 3061–3066.

**Table 1. Equilibrium Constants for Thiol/Disulfide Interchange Reactions[a]**

| | OT/GSH | AVP/GSH | OT/CySH | AVP/CySH | TA/GSH | PA/GSH |
|---|---|---|---|---|---|---|
| $K_1$, $M^{-1}$ | 140 ± 4 | 60 ± 2 | 67 ± 3 | 25.9 ± 0.3 | 130 ± 2 | 42 ± 1 |
| $K_2$ | 0.26 ± 0.01 | 0.24 ± 0.03 | 0.15 ± 0.02 | 0.124 ± 0.007 | 0.28 ± 0.01 | 0.36 ± 0.01 |
| $K_{OV}$, $M^{-1}$ | 36 ± 2 | 14.5 ± 1.8 | 10 ± 1 | 3.2 ± 0.2 | 35.8 ± 0.7 | 15.1 ± 0.5 |
| $f_1$[b] | 0.62 ± 0.01 | 0.69 ± 0.02 | 0.64 ± 0.02 | 0.65 ± 0.01 | 0.59 ± 0.01 | 0.73 ± 0.01 |
| $f_2$[c] | 0.38 ± 0.01 | 0.31 ± 0.02 | 0.36 ± 0.02 | 0.35 ± 0.01 | 0.41 ± 0.01 | 0.27 ± 0.01 |

[a] 25 °C and pH = 7.00; 0.15 M KCl. [b] Fractional concentration of the mixed disulfide which elutes first. [c] Fractional concentration of the mixed disulfide which elutes second.

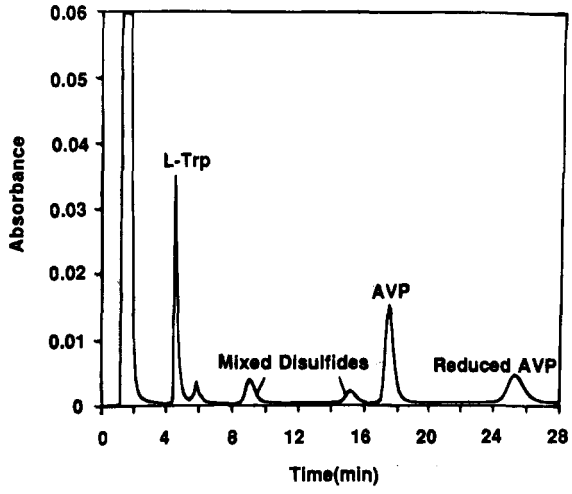



**Figure 1.** Chromatogram of an AVP/GSH reaction mixture at equilibrium. The initial concentrations were 32.6 μM AVP, 7.11 mM GSH and 1.23 mM GSSG at pH 7.0 and 25 °C. The peaks at 2 min. are for GSH, GSSG and the solvent front. The peak at ~6 min. is from the double mixed disulfide. The mobile phase contained 12% acetonitrile, 0.1 *M* $NaH_2PO_4$ and sufficient $H_3PO_4$ to adjust the pH to 2.5.

thiolate species from the conditional constants measured at pH 7.00.

## Results

**Thiol/Disulfide Interchange Equilibrium Constants.** The equilibrium constants defined by eqs 1–6 were determined for reaction of GSH and CySH with AVP and OT and for reaction of GSH with PA and TA. Determination of equilibrium constants for the GSH/AVP system will be described to illustrate the procedures used. Peaks are observed for the native disulfide, reduced dithiol, and two mixed disulfide forms of AVP, and for the internal intensity standard L-tryptophan in the chromatogram for an equilibrium reaction mixture (Figure 1). The peaks for AVP and reduced AVP were assigned using authentic samples of the two compounds. Evidence for assignment of the two peaks at 9 and 15 min to single mixed disulfides is that they are observed only in the presence of GSH and constant values are obtained for $K_1$ and $K_2$ over a range of [GSH]/[GSSG] ratios.[11-13] $K_1$ depends on 1/[GSH], while $K_2$ depends on [GSSG]/[GSH]. GSH and GSSG are essentially unretained with the mobile phase used, and thus their peaks are in the peak for the solvent front. The reaction solution contained known, excess concentrations of GSH and GSSG, and thus it was only necessary to determine the concentrations of the various forms of AVP by HPLC analysis.[10,14] Concentrations of the disulfide and reduced forms of AVP were determined from the areas of their peaks and the internal intensity standard by using the appropriate calibration factors.[10] Since pure samples of the mixed disulfides were not available for calibration of the detector response, the total concentration of mixed disulfide was calculated to be the difference between the initial AVP concentration and the sum of the concentrations determined for AVP and reduced AVP.

To establish that equilibrium is reached, a procedure was used in which equilibrium was approached from both directions.[10] AVP was reacted with a GSH/GSSG redox buffer. Aliquots were removed as a function of time, quenched, and analyzed by HPLC until equilibrium was reached, as indicated by no further change in the concentrations of the disulfide, dithiol, and mixed disulfide forms of AVP. The equilibrium was then perturbed by addition of a known amount of GSSG, and aliquots were removed and analyzed as the system approached equilibrium from the opposite direction. Equilibrium constants were calculated from the concentrations determined for the disulfide, dithiol, and mixed disulfide forms of AVP and the known concentrations of GSH and GSSG, corrected to account for changes in concentration due to the reactions in eqs 1 and 2. To illustrate, the values obtained for $K_1$, $K_2$, and $K_{overall}$ from the first equilibrium in a typical experiment are 62.2 $M^{-1}$, 0.223, and 13.9 $M^{-1}$, respectively, while those obtained from the second equilibrium are 61.6 $M^{-1}$, 0.216, and 13.3 $M^{-1}$. The average of 12 values for $K_1$, $K_2$, and $K_{OV}$ from six experiments of this type for the AVP/GSH system are reported in Table 1. Typically the AVP concentration was in the 50 μM range, while GSH and GSSG concentrations were in the 7–20 mM and 1–4 mM range, respectively.

Equilibrium constants determined by the above procedure are reported in Table 1 for all the systems studied.

Microscopic equilibrium constants in terms of the individual mixed disulfides (Figure 1) can be calculated from the values for $K_1$ and $K_2$ in Table 1 and the fractional concentrations reported in Table 1 for each of the mixed disulfides. For example, using the results reported in Table 1, microscopic equilibrium constants of $K_{1m}$ = 41 $M^{-1}$ and $K_{2m}$ = 0.35 are calculated for the first AVP–GSH mixed disulfide to elute, where $K_{1m}$ is for the reaction of AVP with GSH to form the mixed disulfide and $K_{2m}$ is for reaction of GSH with the mixed disulfide. For the second mixed disulfide to elute, $K_{1m}$ = 19 $M^{-1}$ and $K_{2m}$ = 0.77. The fractional concentrations of the individual mixed disulfides were determined from the ratios of the peak areas for the mixed disulfides.

**Rate Constants for Thiol/Disulfide Interchange.** Rate constants for the forward and reverse reactions in eqs 1 and 2 were determined for the OT/GSH, AVP/GSH, OT/CySH, and AVP/CySH systems. Rate constant $k_1$ was determined by measuring the concentration of OT or AVP

(11) Zhang, R.; Snyder, G. H. *J. Biol. Chem.* **1989**, *263*, 18472–18479.
(12) Huyghnes-Despointes, B. M. P.; Nelson, J. W. *Biochemistry* **1992**, *31*, 1476–1483.
(13) Zhang, R.; Snyder, G. H. *Biochemistry* **1988**, *27*, 3785–3794.
(14) Lin, T.-Y.; Kim, P. S. *Biochemistry* **1989**, *28*, 5282–5287.

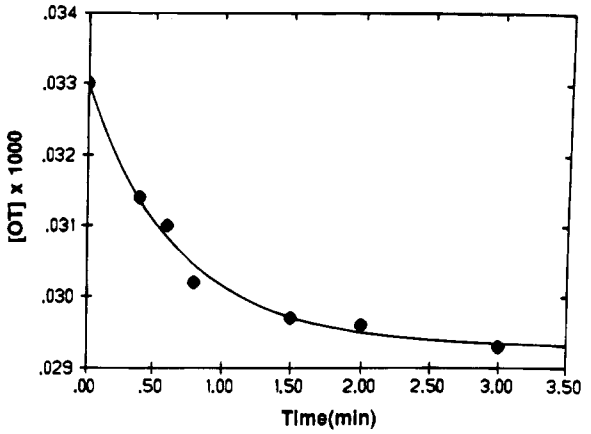



**Figure 2.** The concentration of OT as a function of time for the reaction of 33.0 $\mu$M OT with 0.849 mM GSH in 0.15 *M* KCl at pH 5.5 and 25 °C.

as a function of time after GSH or CySH was added to a solution of the peptide. The reactions were run under pseudo-first-order conditions by using an excess concentration of GSH or CySH. Typical time course data for the reaction of OT with GSH at pH 5.5 are presented in Figure 2. Peaks were observed for OT and the OT–GSH mixed disulfides in chromatograms for samples taken over the time period in Figure 2. However, no peaks were observed for the reduced, dithiol form of OT. Thus, the results in Figure 2 indicate that the reaction mixture rapidly comes to a pseudoequilibrium between OT and its mixed disulfides.[15] Pseudo-first-order rate constant $k'_1$ was obtained by fitting the time course data to the equation for a first order, reversible reaction:[16]

$$A_t = \frac{A_o(K'_1 + \exp(k'_1(1 + 1/K'_1)t))}{(K'_1 + 1)\exp(k'_1(1 + 1/K'_1)t)} \quad (7)$$

where $A_t$ is the concentration of OT at time $t$, $A_o$ the initial concentration of OT, $k'_1$ the pseudo-first-order rate constant for reaction of OT with GSH, and $K'_1$ a pseudoequilibrium constant ($K'_1$ = [mixed disulfides]/[OT] = $k'_1/k_{-1}$). The second-order rate constant $k_1$ at pH 5.5 was then calculated from $k'_1$ using the relationship $k'_1 = k_1$[GSH]. Rate constant $k_1$ at pH 7.0 was calculated from the rate constant at pH 5.5 using the relationship $k_1(7.0) = k_1(5.5)(\alpha_{7.0}/\alpha_{5.5})$ where $\alpha_{7.0}$ and $\alpha_{5.5}$ ($\alpha = K_A/(K_A + [H^+])$) are the fractional concentrations of GSH in the thiolate form at pH 7.0 and 5.5.[17]

This treatment assumes that the reaction of GSH with OT to form the mixed disulfides is second order.[18] To verify that this is the case, time course data were measured for the following reactant concentrations: 18.4 $\mu$M OT and 0.971 mM GSH, 36.9 $\mu$M OT and 0.971 mM GSH, and 36.9 $\mu$M OT and 0.466 mM GSH, all at pH 5.5. Values of 3.66, 3.43, and 3.76 $M^{-1}$ $s^{-1}$ were obtained for $k_1$ from the time course data.

Values determined for rate constant $k_1$ by the above procedure for all the systems studied are listed in Table 2. Also listed are values calculated for $k_{-1}$ using the relation $k_{-1} = k_1/K_1$.

Rate constant $k_{-2}$ for reaction of the reduced dithiol form of the peptides with GSSG or CySSCy was determined by measuring the concentration of reduced peptide as a function of reaction time. The reactions were run at pH 7.00 under pseudo-first-order conditions with respect to reduced peptide. Rate constant $k_{-2}$ was obtained from the initial slope of the time course data, and $k_2$ was then calculated using the relation $k_2 = K_2k_{-2}$. The results obtained for the OT/GSH, AVP/GSH, OT/CySH, and AVP/CySH systems are reported in Table 2.

**Table 2. Rate Constants for Thiol/Disulfide Interchange Reactions[a]**

| | OT/GSH | AVP/GSH | OT/CySH | AVP/CySH |
|---|---|---|---|---|
| $k_1$, $M^{-1}$ $s^{-1}$ | 110 ± 6 | 38 ± 6 | 41 ± 3 | 36 ± 6 |
| $k_{-1}$, $s^{-1}$ | 0.76 ± 0.04 | 0.63 ± 0.10 | 0.61 ± 0.04 | 1.4 ± 0.2 |
| $k_2$, $M^{-1}$ $s^{-1}$ | 0.84 ± 0.13 | 0.74 ± 0.09 | 1.3 ± 0.2 | 1.2 ± 0.2 |
| $k_{-2}$, $M^{-1}$ $s^{-1}$ | 3.2 ± 0.5 | 3.1 ± 0.1 | 8.8 ± 0.1 | 10.0 ± 1.4 |

[a] 25 °C and pH = 7.00; 0.15 M KCl.

**Table 3. Intrinsic Rate and Equilibrium Constants for Thiol/Disulfide Interchange[a]**

| | OT/GSH | AVP/GSH | OT/CySH | AVP/CySH |
|---|---|---|---|---|
| $K^i_{OV}$, $M^{-1}$ | 1 190 | 539 | 23 | 8.3 |
| $k^i_1$, $M^{-1}$ $s^{-1}$ | 10 400 | 3 600 | 1 020 | 900 |
| $k^i_{-1}$, $s^{-1}$ | 9 | 6 | 7 | 13 |
| $k^i_2$, $M^{-1}$ $s^{-1}$ | 79 | 70 | 32 | 30 |
| $k^i_{-2}$, $M^{-1}$ $s^{-1}$ | 37 | 29 | 100 | 94 |

[a] 25 °C and 0.15 M KCl.

## Discussion

The fraction of thiol in the reactive thiolate form, and thus rate and equilibrium constants for thiol/disulfide interchange reactions, are pH dependent. The equilibrium constants reported in Table 1 and the rate constants in Table 2 are conditional constants for pH 7.00. pH independent, intrinsic rate constants and overall equilibrium constants are reported in Table 3. The intrinsic equilibrium constants $K^i_{OV}$ were calculated using the relation $K^i_{OV} = (\alpha_2/\alpha^2_{RSH})K_{OV}$ where $\alpha_2$ is the fractional concentration of reduced peptide in the dithiolate form and $\alpha_{RSH}$ the fractional concentration of GSH or CySH in the thiolate form at pH 7.00. $\alpha_2$ was calculated to be $3.7 \times 10^{-3}$, $4.2 \times 10^{-3}$, $6.2 \times 10^{-5}$, and $9.3 \times 10^{-5}$ using literature p$K_A$ values for the two thiol groups of reduced OT, AVP, TA, and PA, respectively.[19] Intrinsic rate constants were calculated from the conditional rate constants in Table 2 using relationships of the type $k_1 = \alpha_{RS^-}k^i_1$, where $k^i_1$ is the intrinsic rate constant and $\alpha_{RS^-}$ the fractional concentration of RSH in the thiolate form. $k^i_1$ and $k^i_2$ were calculated using p$K_A$ values of 8.97 and 8.38 for GSH and CySH, respectively.[17] $k^i_{-2}$ and $k^i_{-1}$ were estimated using an average of $\alpha$ values calculated for the two thiol groups of the reduced peptides.[19]

(15) Reduction of the intramolecular disulfide bond in other peptides also takes place in distinct kinetic phases, with the first step fast relative to the second step.[13]

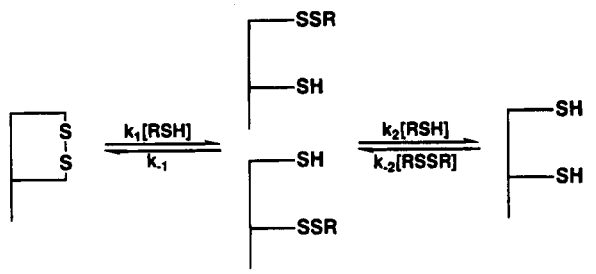
(16) Amdur, I.; Hammes, G. G. *Chemical Kinetics: Principles and Selected Topics*; McGraw Hill: New York, 1966. Note: eq 8 in ref 10 is incorrect; however, the correct equation was used to fit the time course data in ref 10.

(17) The rates of the reaction of GSH and CySH with AVP and OT are too fast at pH 7.00 and the concentrations used in this study to characterize by removal of aliquots followed by analysis by HPLC. The rates of the reactions with GSH and CySH are slower by factors of 0.032 and 0.033, respectively, at pH 5.5, because smaller fractions of GSH and CySH are present in the reactive thiolate forms. These factors were calculated using p$K_A$ values of 8.97 and 8.38 for the thiol groups of GSH and CySH, respectively (Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797–2803 and Backs, S. J.; Rabenstein, D. L. *Inorg. Chem.* **1981**, *20*, 410–415).

(18) It is well established that thiol/disulfide interchange reactions are mechanistically simple second-order $S_N2$ displacement reactions.[2b,3a]

(19) Noszál, B.; Guo, W.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 2327–2334.

**Kinetics and Equilibria at pH 7.00.** Since the cyclic arrangement of amino acids 1–6 is essential for high biological activity,[4] rate and equilibrium constants of thiol/disulfide interchange reactions of OT and AVP under near physiological conditions are of interest. It is convenient to discuss the stepwise kinetics for reduction and formation of the peptide disulfide bonds by thiol/disulfide interchange using the reaction scheme:




where $k_1$[RSH], $k_2$[RSH], and $k_{-2}$[RSSR] are pseudo-first-order rate constants.

The results reported in Table 2 for the reaction of AVP and OT with GSH and CySH indicate that the second-order rate constant, $k_1$, for the first step in the overall reduction of the disulfide bond in AVP and OT by thiol/disulfide interchange is much larger than $k_2$, i.e. the second step in the overall reduction of peptide by RSH is rate determining. For example, $k_1$ for reduction of OT by GSH is 130 times $k_2$. For comparison, rate constants for the second-order reaction of GSH and CySH with GSSG at pH 7.00 are 0.41 and 0.33 $M^{-1}$ $s^{-1}$, respectively,[2a,g], i.e. similar in magnitude to $k_2$. Using rate constants $k_2$ and $k_{-2}$ for the OT/GSH and AVP/GSH systems as characteristic thiol/disulfide interchange rate constants ($k_{ex}$) for these peptides in the absence of ring strain, a value of 1.5 $M^{-1}$ $s^{-1}$ is obtained for $k_{ex}$ after correcting for statistical factors. Rate constant $k_1$ for the OT/GSH and AVP/GSH systems is 73 and 25 times larger than $k_{ex}$. Thus, it is evident that the second step is rate determining not because it is slower than normal for thiol/disulfide interchange reactions, but rather because the first step is 1–2 orders of magnitude faster than normal. This suggests that the disulfide bonds in OT and AVP are somewhat strained.[20]

Even though $k_1 \gg k_2$, the peptides are not all rapidly converted to mixed disulfide by reaction with GSH or CySH. Rather, the initial reaction of peptide with RSH is rapidly reversed and a pseudoequilibrium is established between peptide and the peptide-RSH mixed disulfides, as shown by the kinetic data in Figure 2 for reaction of OT with GSH.[15] For example, taking the OT/GSH system as an example, $k_{-1} > k_1$[GSH], when [GSH] < 0.007 M. Thus, under the conditions used in this study, the mixed disulfides are relatively rapidly converted back to peptide by intramolecular thiol/disulfide interchange and a pseudoequilibrium is established. At the same time, some mixed disulfide reacts further with RSH. The relative rates of the competing intra- and intermolecular processes depend on the concentration of RSH. For example, at [GSH] < 0.9 M, $k_{-1} > k_2$[GSH] and intramolecular disulfide bond formation is faster than reaction with another molecule of GSH to form reduced OT and GSSG. This suggests that, in human blood plasma where the concentration of nonprotein thiol is $\ll$0.9 M,[21] mixed disulfides formed by reaction of AVP and OT with nonprotein thiols will undergo intramolecular thiol/disulfide interchange to reform biologically active AVP and OT rather than react with another molecule of nonprotein thiol to give the reduced dithiol forms of AVP and OT. At the plasma concentration of GSH,[21] the equilibrium concentration of mixed disulfides is also predicted to be small.[22]

The reaction of reduced OT and AVP with GSSG or CySSCy to form the native disulfide forms of the peptides is of interest since it involves closing a 20-membered hexapeptide ring by formation of an intrapeptide disulfide bond. The overall reaction involves two steps. In the first step, reduced peptide reacts with GSSG or CySSCy to form the mixed disulfide, which in turn reacts by intramolecular thiol/disulfide interchange to form the peptide disulfide bond. Which of the two steps is rate determining depends on whether $k_{-1}$ or the pseudo-first-order rate constant $k_{-2}$[RSSR] is larger. Taking the reaction of reduced OT with GSSG as an example, the first step will be rate limiting when [GSSG] < 0.24 M, i.e. closure of the 20-membered ring by intramolecular thiol/disulfide interchange is fast relative to the reaction of reduced OT with GSSG.

The formation of peptide rings by intramolecular thiol/disulfide interchange has been analyzed previously for several peptides and proteins in terms of an effective concentration, $C_{eff}$.[12,14,20,23] $C_{eff}$ represents a ratio of equilibrium constants for otherwise identical intra- and intermolecular processes. In such studies, glutathione is generally used as the reference thiol, and it can be shown that:

$$C_{eff} = \frac{K_{intra}}{K_{inter}} = \frac{[P\!<^{S}_{S}][GSH]^2}{[P\!<^{SH}_{SH}][GSSG]}$$

In terms of the equilibrium constants in Table 1, $C_{eff} = 1/K_{OV}$. The magnitude of $C_{eff}$ has been interpreted to indicate the tendency of the peptide or protein to keep the cysteine thiols in proximity even in the absence of a disulfide bond.[20] For example, $C_{eff}$ for disulfide bond formation at the active site of thioredoxin at pH 8.7 is 10 M.[14] In the presence of urea, $C_{eff}$ for thioredoxin decreases, reaching a minimum value of 0.026 M in 7.7 to 9 M urea. For comparison, $C_{eff}$ for the random coil peptide [Ac-Cys-(Gly)$_6$-Cys-NH$_2$] is constant at ~0.060 M at pH 8.7 and urea concentrations ranging from 0 to 7 M.[14] The values calculated for $C_{eff}$ from $K_{OV}$ in Table 1 are 0.07 M for the AVP/GSH system and 0.03 M for the OT/GSH system. By comparison to the value of 0.060 M for the [Ac-Cys-(Gly)$_6$-Cys-NH$_2$]/GSH system[14] and the values reported for random polymers,[13,14] we can conclude that the two cysteines of reduced AVP and OT are not constrained to be closer together than would be the case for an unstructured peptide. It also is of interest that $C_{eff}$ = 0.07 M for the PA/GSH system and 0.03 M for the TA/GSH system, i.e. the acyclic tripeptide tails of AVP and OT seem to have no effect on the proximity of the two thiol groups of reduced AVP and OT.

(20) Creighton, T. E. *Methods Enzymol.* **1986**, *131*, 83–106.

(21) The concentration of GSH in human plasma is in the range of 2 μm: Henning, S. M.; Zhang, J. Z.; McKee, R. W.; Swendseid, M. E.; Jacob, R. A. *J. Nutr.* **1991**, *121*, 1969–1975.

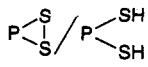
(22) Using the equilibrium constants in Table 1 and a plasma GSH concentration of 2 μM, the ratio of mixed disulfides to disulfide is predicted to be approximately 3 × $10^{-4}$ for AVP and 1 × $10^{-3}$ for OT at equilibrium.

(23) Creighton, T. E. *Biopolymers* **1983**, *22*, 49–58.

**Intrinsic Rate and Equilibrium Constants.** The intrinsic rate constants in Table 3 are all larger than the corresponding values in Tables 1 and 2 since the thiols are only fractionally deprotonated at pH 7.00. The differences in $k_1^i$ and $k_2^i$ are larger for the peptide/GSH systems than for the peptide/CySH systems because the $pK_A$ is larger for GSH than for CySH. Thus, for the peptide/GSH systems, $k_1^i \gg k_1$ and $k_2^i \gg k_2$ and, as a consequence, $K_{ov}^i \gg K_{OV}$. The inherent nucleophilicity is less for CySH, and thus $k_1^i$, $k_2^i$, and $K_{ov}^i$ are similar for the peptide/CySH systems. However, the values for the peptide/GSH and peptide/CySH systems are similar at pH 7 because the greater fraction of CySH in the reactive thiolate form compensates for its lower nucleophilicity.

The intrinsic rate and equilibrium constants in Table 3 can be compared with values reported for thiol/disulfide interchange reactions of other peptides which contain two cysteines.[11,13] For example, rate constants $k_1$ and $k_{-1}$ and equilibrium constant $K_1$ were reported for reaction of 12 peptides of the type Cys-Xaa$_m$-Cys where $m$ is the number of amino acids between the cysteines, with GSH in 3 M guanidine HCl at pH 6.9. Intrinsic constants were calculated from the pH 6.9 values by assuming the thiol $pK_A$ values to all be 8.9.[11] Of particular interest with respect to the results in Table 3 are rate constants for the series Cys-Ala$_3$-Cys, Cys-Ala$_4$-Cys, and Cys-Ala$_5$-Cys. The values reported for $k_1^i$ are 50, 16, and 18 $M^{-1}$ $s^{-1}$, respectively, values reported for $k_{-1}^i$ are 0.90, 0.90, and 0.55 $s^{-1}$, and values reported for $K_1^i$ are 56, 18, and 33 $M^{-1}$. Rate constant $k_{-1}^i$ is of particular interest for it involves peptide conformational transitions that bring a cysteine residue and a mixed disulfide bond into proximity for formation of an intramolecular disulfide bond.[20] Assuming that the $k_{-1}^i$ value for Cys-Ala$_4$-Cys is a good reference value for a random coil peptide with two cysteines separated by four amino acids, the somewhat larger $k_{-1}^i$ values in Table 3 suggest that for OT and AVP conformations in which the thiol and mixed disulfide groups are in close proximity are more populated than for completely random coil peptides. This conclusion must be considered somewhat tentative, however, since the values calculated for the intrinsic constants for the Cys-Ala$_m$-Cys series are dependent on the assumption that the $pK_A$s for all the thiol groups are 8.9.[11] It also is of interest to note that $k_1^i$ for OT and AVP is much larger than for Cys-Ala$_m$-Cys with $m$ = 3, 4, and 5, which is further evidence that the disulfide bonds in OT and AVP are strained.

**Redox Potentials of the Peptide Disulfide Bonds.** A major objective of this research has been to quantitatively characterize the oxidation–reduction chemistry of the disulfide group in AVP and OT. Equilibrium constants for the overall reduction of the peptide disulfide bonds by GSH and CySH are related to half-cell potentials for the



and RSSR/RSH redox couples:

The redox potentials listed in Table 4 were calculated using the values for $K_{OV}$ in Table 1 and reference values of $E^{o'}_{GSSG/GSH} = -0.262$ V and $E^{o'}_{CySSCy/CySH} = -0.245$ V.[24]

**Table 4. Redox Potentials for Oxytocin, Arginine Vasopressin, Tocinoic Acid, and Pressinoic Acid[a,b]**

| peptide | thiol/disulfide system | $E^{o'}$ (V) |
|---|---|---|
| OT | OT/GSH | −0.216 |
| OT | OT/CySH | −0.216 |
| TA | TA/GSH | −0.216 |
| AVP | AVP/GSH | −0.228 |
| AVP | AVP/CySH | −0.230 |
| PA | PA/GSH | −0.227 |

[a] pH 7.0 and 25 °C. 0.15 M KCl. [b] The uncertainty estimated for each $E^{o'}$ value on the basis of the uncertainty in the $K_{OV}$ values is ±0.001 − 0.002 V; however, the actual uncertainty is probably larger due to uncertainty in $E^{o'}_{GSSG/GSH}$ and $E^{o'}_{CySSCy/CySH}$.[24]

There is excellent agreement between the two redox potentials calculated for OT from $K_{OV}$ for the OT/GSH and OT/CySH systems. The same is true for the redox potentials for AVP. This is significant since the redox potentials serve as a check on the validity of the equilibrium constants. It also is of interest to note that $E^{o'}$ is essentially identical for OT and TA and for AVP and PA, which suggests that the acyclic tripeptide tails of OT and AVP apparently have no affect on the redox properties of their disulfide bonds.

It is of interest to compare the results in Table 4 to $E^{o'}$ values for other intramolecular disulfide bonds. $E^{o'}$ values reported recently for molecules which form 5–11-membered disulfide-containing rings range from −0.354 to −0.240 V.[25] For example, the $E^{o'}$ values for dithiothreitol (DTT), lipoic acid, and 6,6′-sucrosedithiol, which form 6-, 5- and 11-membered rings, are −0.327, −0.288, and −0.245 V, respectively. The $E^{o'}$ values for OT and AVP are less reducing; however, they are larger than might be expected, considering that formation of their disulfide bonds involves closure of 20-membered rings.

## Experimental Section

**Chemicals.** AVP, OT, PA, and TA were obtained from Bachem Inc., Torrance, CA. HPLC traces provided by the supplier showed that >98% of the peptide present was AVP, OT, PA, or TA. Peptide content was determined by 500-MHz $^1$H NMR to be in the range 70–90%, the remainder being water, in good agreement with the certificate of analysis provided by the supplier. Dithiothreitol (DTT) was obtained from Aldrich Chemical Co. Oxidized DTT, GSH, the sodium salt of GSSG, the hydrochloride forms of CySH and CySSCy, and *N*-acetyl-L-leucine (Ac-Leu) and *N*-acetyl-L-phenylalanine (Ac-Phe) were obtained from Sigma Chemical Co. Mobile phases used in the HPLC experiments and buffer solutions were prepared using sodium dihydrogen phosphate, sodium acetate, potassium chloride, phosphoric acid (85%), and acetonitrile (Optima) from Fisher Scientific Co.

**HPLC Apparatus.** Reverse-phase HPLC separations were performed with a liquid chromatograph equipped with a 20 μL sample loop, a 100 × 3.2 mm ODS ($C_{18}$) column (particle size 3 μm, Bioanalytical Systems MF-6213), and a dual channel UV detector. The detector was set at 215 nm. Mobile phases were prepared by addition of $NaH_2PO_4$ (0.1 M final concentration) and acetonitrile to water which had been purified with a Millipore water purification system, and then the pH was adjusted to 2.5 with 85% $H_3PO_4$. On the basis of results of a previous study of the chromatography of the native disulfide and reduced dithiol forms of AVP, OT, PA, and TA,[10] a mobile phase containing 12% acetonitrile was used for the AVP/GSH, AVP/CySH, and TA/GSH systems, 14% acetonitrile for the PA/

(24) Millis, K. K.; Weaver, K. H.; Rabenstein, D. L. *J. Org. Chem.* **1993**, *58*, 4144–4146.
(25) Lees, W. J.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 642–647.

GSH system, and 18% acetonitrile for the OT/GSH and OT/CySH systems. Mobile phases were filtered through a 0.45 μm cellulose nitrate filter membrane (Whatman 7184 004) and sparged with helium gas for at least 15 min before use.

Concentrations were obtained from chromatographic peak areas by using L-tryptophan, Ac-Leu, or Ac-Phe as an internal intensity standard.[10] Calibration solutions of reduced AVP, OT, PA, and TA were prepared by reduction of stock solutions of the peptides with an excess of DTT.

**Kinetic and Equilibrium Studies.** Stock solutions of AVP, OT, PA, and TA were prepared by weighing 1–2 mg of peptide on a Mettler M5SA microbalance, transferring the peptide to a 5 or 10 mL volumetric flask, and then adding 0.05 M sodium acetate–0.05 M $NaH_2PO_4$ solution (pH 5.6) which had been deoxygenated by bubbling with either argon or nitrogen. In the procedure used to study the kinetics of the reaction of GSH and CySH with AVP and OT, aliquots of peptide and internal standard stock solutions were combined and the pH adjusted to 5.5.[17] The solution was deoxygenated and placed in a water bath (25 °C) in a nitrogen-filled glove bag. Reaction was initiated by addition of GSH or CySH stock solution (pH 5.5). Aliquots of the reaction mixture were removed at time intervals of 12 s or more and quenched by lowering the pH to ~3 with HCl. The quenched solutions were then analyzed by HPLC. The quenching procedure was verified previously by analyzing a quenched reaction mixture as a function of time.[10]

Rate constants were determined for the reaction of reduced AVP and OT with GSSG and CySSCy at pH 7.00 by a similar procedure. Reduced AVP and OT were prepared by electrochemical reduction at a mercury pool electrode for ~3 h.[26]

Equilibrium constants for the thiol/disulfide exchange reactions were determined at pH 7.00 by measuring the concentrations of the native disulfide, reduced dithiol and mixed disulfide forms of the peptides in solutions containing known, excess concentrations of CySH and CySSCy or GSH and GSSG, i.e. in solutions containing an RSH/RSSR redox buffer.[27] As described above, a procedure was used in which equilibrium was approached from both directions to ensure that equilibrium was achieved.

**Acknowledgment.** This research was supported in part by National Institutes of Health Grant GM 37000. A Dissertation Fellowship from UC Riverside (P.L.Y.) is gratefully acknowledged.

(26) Saetre, R.; Rabenstein, D. L. *Anal. Chem.* **1978**, *50*, 276–280.
(27) Snyder, G. H. *Biochemistry* **1987**, *26*, 688–694.