This Research Contribution is in Commemoration of the Life and Science of I. M. Kolthoff (1894-1993).

Characterization of the Thiol/Disulfide Chemistry of Neurohypophyseal Peptide Hormones by High-Performance Liquid Chromatography

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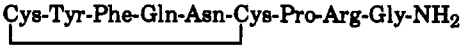
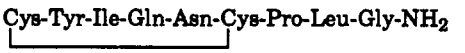
Methodology is described for characterization of the kinetics and equilibria of thiol/disulfide interchange reactions of the disulfide bonds in the neurohypophyseal peptide hormones arginine vasopressin and oxytocin and the related peptides pressinoic acid and tocinoic acid. Thiol/disulfide interchange reaction mixtures are analyzed by reversed-phase high-performance liquid chromatography. The effect of mobile-phase composition and pH on the HPLC capacity factors for the native disulfide and reduced dithiol forms of each peptide was examined. In each case, the capacity factor decreases as the acetonitrile content of the mobile phase increases. For each disulfide/dithiol peptide pair, the capacity factor is larger for the dithiol form of the peptide, indicating that the hydrophobic side chains of the linear peptide are more accessible for interaction with the hydrophobic stationary phase. To illustrate application of the methodology, rate and equilibrium constants are reported for the thiol/disulfide interchange reactions of cysteine with arginine vasopressin at pH 7.0. Cysteine reacts with arginine vasopressin to form two mixed disulfides, which in turn react with another molecule of cysteine to give the dithiol form of arginine vasopressin and cystine. Rate and equilibrium constants were determined for each step by analysis of reaction mixtures by HPLC. The results are compared to rate and equilibrium constants for reaction of cysteine with oxidized glutathione.

INTRODUCTION

The neurohypophyseal peptide hormones arginine vasopressin (AVP) and oxytocin have in common seven of their nine amino acids, a disulfide-containing hexapeptide ring formed from the six N-terminal amino acids, and a C-terminal acyclic tripeptide tail. Since their isolation and synthesis in the early 1950s,[1-8] they have been the subject of many studies to characterize their biological activity and chemical properties. However, the chemistry of their Cys^1–Cys^6 disulfide bonds has not been characterized, even though reduction of



arginine vasopressin

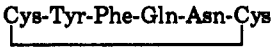


oxytocin

the disulfide bond might be a key step in the inactivation of the hormones.[9,10]

We have initiated studies to characterize the chemistry of the disulfide groups of AVP and oxytocin, focusing on their thiol/disulfide interchange reactions and their oxidation/reduction properties. The disulfide bonds of AVP and oxytocin can be reduced by thiol/disulfide interchange reactions, as described by the reaction sequence in Figure 1. In this sequence, a monothiol RSH reacts with the disulfide bond by a thiol/disulfide interchange reaction to form one of two possible peptide-RSH mixed disulfides. The mixed disulfides then react with another molecule of RSH to give the dithiol form of AVP or oxytocin and RSSR.

In this paper, we report conditions for the separation and quantitation by high-performance liquid chromatography (HPLC) of the native disulfide, reduced dithiol, and mixed disulfide forms of AVP and oxytocin, and of the related compounds pressinoic acid and tocinoic acid, and we present methodology for characterization of the kinetics and equilibria of thiol/disulfide interchange reactions of the peptide hormones. Application of the methodology is illustrated by the results of a study of the kinetics and equilibria of the thiol/disulfide interchange reactions of cysteine with AVP.



pressinoic acid

tocinoic acid

EXPERIMENTAL SECTION

Apparatus. Reversed-phase HPLC separations were performed with a Bioanalytical Systems BAS 200 liquid chromatograph equipped with a Rheodyne Model 7125 sample injector, a 100 × 3.2 mm ODS (C_{18}) Phase II column (particle size 3 μm)

(1) du Vigneaud, V.; Ressler, C.; Trippett, S. *J. Biol. Chem.* **1953**, *205*, 949–957.
(2) du Vigneaud, V.; Ressler, C.; Swan, J. M.; Roberts, C. W.; Katsoyannis, P. G.; Gordon, S. *J. Am. Chem. Soc.* **1953**, *75*, 4879–4880.
(3) Tuppy, H.; Michl, H. *Monatsch. Chem.* **1953**, *84*, 1011–1020.
(4) Maier-Huser, H.; Clauser, H.; Fromageot, P.; Plongeron, P. *Biochim. Biophys. Acta* **1953**, *11*, 252–257.
(5) du Vigneaud, V.; Lawler, H. C.; Popenoe, E. A. *J. Am. Chem. Soc.* **1953**, *75*, 4880–4881.
(6) Acher, R.; Chauvet, J. *Biochim. Biophys. Acta* **1953**, *12*, 487–488.
(7) Acher, R.; Chauvet, J. *Biochim. Biophys. Acta* **1954**, *14*, 421–429.
(8) Fromageot, P.; Acher, R.; Clauser, H.; Maier-Huser, H. *Biochim. Biophys. Acta* **1953**, *12*, 424–431.
(9) Jošt, K. In *Handbook of the Neurohypophyseal Hormone Analogs*; Jošt, K., Lebl, M., Brtnik, F., Eds.; CRC Press, Inc.: Boca Raton, FL, 1987; Vol. 2, pp 15–30.
(10) Branda, L. A.; Ferrier, B. M.; Celhoffer, L. *Can. J. Biochem.* **1972**, *50*, 507–509.

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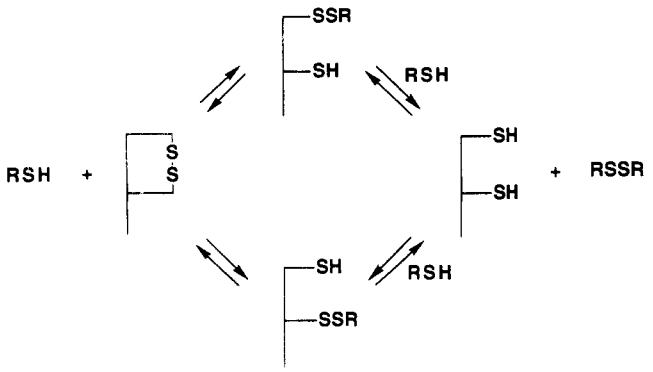


Figure 1. Microscopic scheme for thiol/disulfide interchange reactions of monothiol RSH with neurohypophyseal peptide hormones.

and a Linear Instruments Model 204 dual-channel UV detector. A 20-μL sample loop was used, and injections were made by overloading the injector loop with 70 μL of sample. The detector was set to a wavelength of 215 nm. All connecting tubing was stainless steel to exclude oxygen.

Mobile Phase. Mobile phases were prepared by addition of NaH_2PO_4 (0.1 M final concentration) and acetonitrile (Optima grade, Fisher Scientific Co.) to water purified with a Millipore water purification system, and then the pH was adjusted with 85% H_3PO_4 (Fisher Scientific Co.). 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. Mobile phase was pumped at a flow rate of 0.5 mL/min. The acetonitrile content of the mobile phase was varied from 11% to 19% and the pH was varied from 2 to 4. The mobile phase used in the study of the cysteine/AVP thiol/disulfide interchange reaction contained 11.8% acetonitrile and the pH was 2.5.

Chemicals. Arginine vasopressin, oxytocin, pressinoic acid, and tocinoic acid were obtained from Bachem California. Dithiothreitol (DTT), *N*-acetyl-L-phenylalanine (Ac-Phe), *N*-acetyl-L-leucine (Ac-Leu), L-cysteine (CySH), and cystine (CySSCy) were obtained from Sigma Chemical Co.

Detector Calibration. Concentrations were obtained from peak areas by using an internal intensity standard. Detector response was calibrated by plotting the peptide to internal reference peak area ratio vs the peptide to internal reference concentration ratio. Calibration plots were linear from the minimum detectable concentrations up to the highest concentrations used in this work ($\sim 1 \times 10^{-4}$ M). Calibration solutions of the reduced forms of oxytocin, AVP, tocinoic acid, and pressinoic acid were prepared by reduction of stock solutions of their native disulfide forms with an excess of DTT for at least 15 min.

Kinetic and Equilibrium Studies. Stock solutions were prepared in degassed 0.05 M sodium acetate–0.05 M NaH_2PO_4 solution (pH 5.6). In the study of the kinetics of the reaction of AVP with cysteine, aliquots of the AVP and internal standard (Ac-Leu) stock solutions were combined together with KCl (final concentration 0.15 M) in a volumetric flask, the pH was adjusted to the appropriate value, and the solution was brought to volume. The solution was then degassed and placed in a water bath (25 °C) in a nitrogen-filled glovebag. Reaction was initiated by addition of CySH stock solution, which had been degassed and adjusted to the appropriate pH. Aliquots of the reaction mixture were quenched at time intervals of 12 s or more by addition of HCl to lower the pH to ~3. The quenched solutions were then analyzed by HPLC to determine the concentrations of the disulfide and dithiol forms of AVP. To establish the validity of the quenching procedure, a reaction mixture which had not reached equilibrium was quenched and then analyzed repeatedly at 20-min intervals for a period of 2 h. No further reaction was detected in the quenched solution. CySH:AVP concentration ratios of 25–50:1 were used in the kinetic studies.

The rate constant for the reaction of reduced AVP with cystine was determined by a similar procedure. Reduced AVP solutions were prepared by electrochemical reduction of a solution of AVP at a mercury pool electrode[11] for ~3 h. The cystine stock solution was prepared in degassed Millipore water at pH 11 to dissolve the cystine.

Equilibrium constants for the AVP/CySH thiol/disulfide interchange reaction were determined by measuring the concentrations of the dithiol, disulfide, and mixed disulfide forms of AVP in solutions containing known, excess concentrations of CySH and CySSCy. Solutions were analyzed as a function of time to ensure that equilibrium was achieved. To verify that equilibrium is reached from both directions, solutions of AVP and CySH were reacted until there was no further change in concentration. The concentration of CySSCy was then increased by addition of CySSCy stock solution to shift the equilibrium back toward reactants, and the solution was analyzed as a function of time until the concentrations reached new constant values. The concentration of CySH was then increased by addition of CySH stock solution to shift the equilibrium toward products, and the solution was analyzed until the concentrations again reached new constant values. Equilibrium constants were calculated using the concentrations of the dithiol, disulfide, and mixed disulfide forms of AVP and the known excess concentrations of CySH and CySSCy for each of the equilibrium solutions.

RESULTS AND DISCUSSION

The thiol RSH reacts with the disulfide bonds of AVP, oxytocin (OT), pressionic acid (PA), and tocinoic acid (TA) to form mixed disulfides (Figure 1), which in turn react with another molecule of RSH to give the reduced dithiol form of the peptide and RSSR. In addition, the free thiol groups of the mixed disulfides in Figure 1 can react with RSSR to form the double mixed disulfide. As described below, rate constants can be measured under conditions of an excess of RSH or RSSR, i.e., under pseudo-first-order reaction conditions, while the equilibrium constants can be measured under conditions where the concentrations of both RSH and RSSR are known and in excess of the concentration of peptide. Thus it is only necessary to quantitate by HPLC the various forms of the peptide hormones in the reaction mixtures to characterize the kinetics and equilibria of the thiol/disulfide interchange reactions.

Chromatography of Peptide Hormones. The neurohypophyseal peptide hormones can be separated from each other and from diastereoisomeric forms by reversed-phase HPLC using aqueous–organic mobile phases.[12-19] On the basis of previous reports, we are using a water–acetonitrile mobile phase, with added phosphoric acid as a hydrophilic ion pairing agent.[20]

The capacity factor, k', is plotted in Figure 2 as a function of the acetonitrile concentration for the oxidized and reduced forms of AVP, PA, OT, and TA. The mobile phase contained 0.10 M NaH_2PO_4, adjusted to pH 2.5 with phosphoric acid. The results in Figure 2 show that as the acetonitrile concentration is increased from 11% to 19%, the capacity factors decrease rapidly. Plots of ln k' vs percent acetonitrile are linear, indicating that k' decreases exponentially with increasing acetonitrile concentration over this concentration range. Similar results have been reported for other peptides

(11) Saetre, R.; Rabenstein, D. L. *Anal. Chem.* **1978**, *50*, 276–280.
(12) Frei, R. W.; Michel, L.; Santi, W. *J. Chromatogr.* **1976**, *126*, 665–677.
(13) O'Hare, M. J.; Nice, E. C. *J. Chromatogr.* **1979**, *171*, 209–226.
(14) Larsen, B.; Fox, B. L.; Burke, M. F.; Hruby, V. J. *Int. J. Pept. Protein Res.* **1979**, *13*, 12–21.
(15) Blevins, D. D.; Burke, M. F.; Hruby, V. J.; Larsen, B. R. *J. Liq. Chromatogr.* **1980**, *3*, 1299–1318.
(16) Blevins, D. D.; Burke, M. F.; Hruby, V. J. *Anal. Chem.* **1980**, *52*, 420–424.
(17) Lindeberg, G. *J. Chromatogr.* **1980**, *193*, 427–431.
(18) Rhodes, G. R.; Boppana, V. K. *J. Chromatogr.* **1988**, *444*, 123–131.
(19) Sun, Y.; Andrews, P. C.; Smith, D. L. *J. Protein Chem.* **1990**, *9*, 151–157.
(20) Hearn, M. T. W. *Adv. Chromatogr.* **1982**, *20*, 1–82.

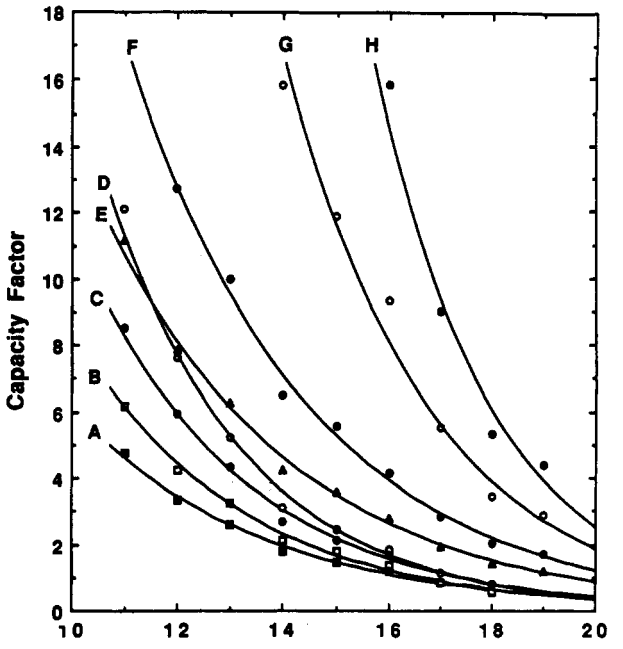


Figure 2. Capacity factor vs percent acetonitrile for the disulfide and dithiol forms of AVP (curves C and D, respectively), PA (E and F), OT (G and H), and TA (A and B). The mobile phase contained 0.1 M NaH_2PO_4 and sufficient H_3PO_4 to adjust the pH to 2.5.

when $0 < \ln k' < 2$.[20] The capacity factors for the internal intensity standards Ac-Leu and Ac-Phe show similar behavior.

The capacity factors for AVP, OT, PA, and TA cover a wide range and are different for the oxidized and reduced forms of each peptide. For example, at 14% acetonitrile, the capacity factors decrease in the order reduced OT > OT > reduced PA > PA > reduced AVP > AVP > reduced TA > TA. This order can be accounted for in terms of the amino acid composition of the peptides. For example, the capacity factor for PA is larger than that for TA, whereas the reverse is true for the parent peptides AVP and OT. The side chain of Phe of PA is more lipophilic than that of Ile of TA[16] resulting in a larger capacity factor for PA. In AVP, however, the greater lipophilicity of the side chain of Phe is more than offset by the effect of the positively-charged side chain of Arg, with the result that the capacity factor of AVP is less than that of PA and OT.

For each reduced/oxidized peptide pair, the capacity factor is larger for the reduced form of the peptide. The oxidized form exists as a compact cyclic peptide. Reduction of the disulfide bond can be expected to result in a rather drastic change in the structure of the peptide, with the result that additional nonpolar side chains are accessible for interaction with the hydrophobic stationary phase.

The effect of pH on capacity factors for the reduced and oxidized forms of the peptides was studied using a mobile phase which contained 12% acetonitrile and NaH_2PO_4/phosphoric acid buffer. For both the reduced and oxidized forms of PA and TA, the capacity factor remained constant as the pH was increased from 2 to 2.5 and then decreased as the pH was increased to 4, i.e., as their C-terminal carboxylic acid groups are titrated.[21] The capacity factors for the oxidized and reduced forms of OT are essentially independent of pH over the pH range 2–4, as expected since there is no change in the protonation state of OT. For AVP, the capacity factors are essentially constant for both the oxidized and reduced forms from pH 2 to 3, and then they increase rapidly

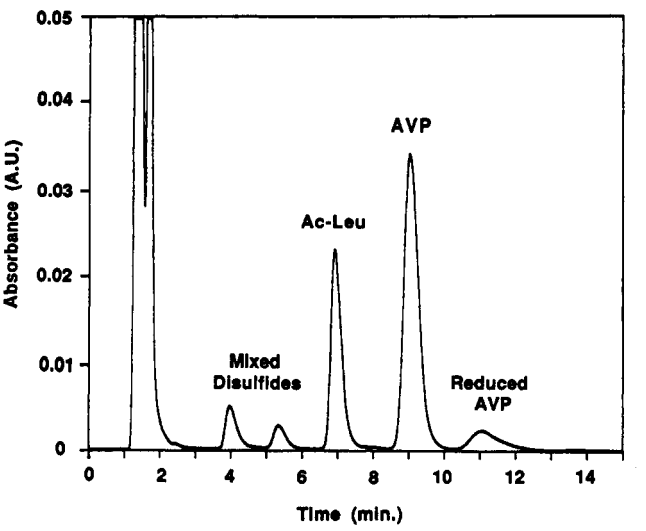
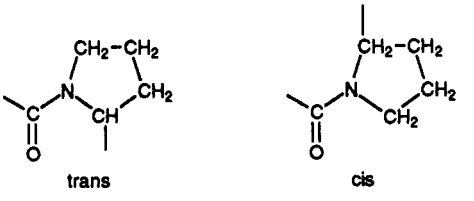


Figure 3. Chromatogram for a sample from a mixture which contained initially 3.55×10^{-5} M AVP and 7.17×10^{-3} M CySH at pH 7.0. *N*-Acetylleucine (Ac-Leu) was added as an internal intensity standard. The peaks at 1–2.5 min are from the solvent front, CySH, and CySSCy. The pH 2.5 mobile phase contained 11.8% acetonitrile.

as the pH is increased from 3 to 4. For example, the capacity factor for reduced AVP increases from 9.6 at pH 3 to 15.3 at pH 4. Since there is no change in protonation state of AVP over this pH range, the increase in capacity factor apparently reflects a decrease in the total phosphate/phosphoric acid concentration, and thus a decrease in the extent to which the arginine side chain forms hydrophilic ion pairs at the higher pH values.

The oxidized and reduced forms of AVP and oxytocin exist in solution as a mixture of the trans and cis conformations with respect to the conformation across the Cys[6]–Pro peptide bond.[22] For example, AVP is 94% trans/6% cis in aqueous



solution at pH 3 and 25 °C, while OT is 92% trans/8% cis.[22] However, separate peaks were not observed for the trans and cis isomers in chromatograms of the oxidized or reduced forms of AVP or oxytocin. This is consistent with the results of a recent study of the kinetics of cis/trans isomerization by rotation around the Cys[6]–Pro peptide bond of AVP and oxytocin.[23] The half-lives calculated for the cis isomers of AVP and oxytocin from the reported rate constants are 10.3 and 16.5 s, respectively, at pH 3 and 25 °C. Thus, interchange is sufficiently fast that the peptides interconvert between their cis and trans conformations during the separation. However, it is of interest to note that the chromatographic peaks for the various forms of the peptide are slightly broader than expected, possibly because, even though cis/trans interconversion is relatively fast, it is not instantaneous on the chromatography time scale and the retention properties are different for the cis and trans isomers. For example, the chromatogram for a mixture obtained by reaction of 3.55×10^{-5} M AVP with 7.17×10^{-3} M cysteine at pH 7.0 is shown in Figure 3. Peaks are observed for the reduced dithiol, native

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

(22) Larive, C. K.; Guerra, L.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1992**, *114*, 7331–7337.

(23) Larive, C. K.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1993**, *115*, 2833–2836.

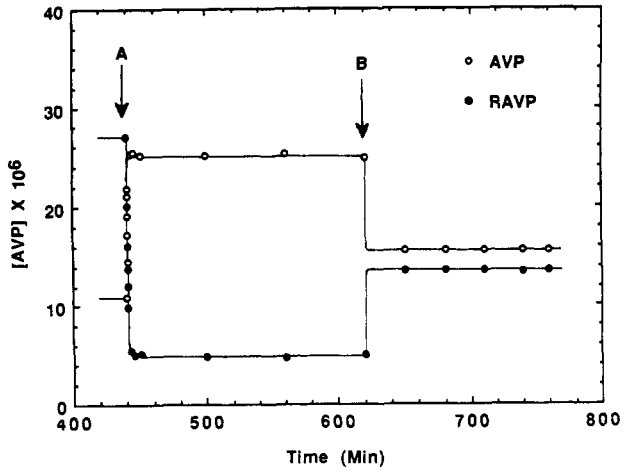


Figure 4. Concentration of AVP and reduced AVP as a function of time. The solution initially contained 4.02×10^{-5} M AVP and 9.71×10^{-3} M CySH. At 440 min (A), the concentration of CySSCy was increased to 1.46×10^{-3} M; at 620 min (B), the concentration of CySH was increased to 1.92×10^{-2} M. 25 °C and pH 7.0.

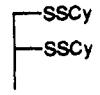
disulfide, and mixed disulfide forms of AVP and for Ac-Leu, which was added as an internal intensity standard. Even though the retention time is longer for Ac-Leu (6.8 min), the widths at half-height for the mixed disulfide peaks are larger (0.35 and 0.39 min for the peaks at 4.0 and 5.3 min, respectively) as compared to that for the Ac-Leu peak (0.33 min).

The peaks for AVP and reduced AVP were assigned by comparison with chromatograms for the native disulfide and reduced dithiol forms of AVP, respectively. The reduced AVP was prepared by reduction with excess DTT. Pure samples of the mixed disulfides were not available. Evidence for assignment of the peaks at 4.0 and 5.3 min to mixed disulfides is that two mixed disulfide peaks are expected (Figure 1), the two peaks are observed only in the presence of CySH, and, as discussed below, constant values are obtained for equilibrium constants K_1 and K_2 from the peak areas for a range of CySH:CySSCy ratios. K_1 depends on 1/[CySH], while K_2 depends on [CySSCy]/[CySH]. It is also of interest to note in Figure 3 that the retention times for the mixed disulfides are shorter than for the native disulfide and reduced dithiol forms of AVP, apparently due to the effect of the additional charges on the cysteine moiety.

The minimum detectable concentrations at a signal-to-noise ratio of 2 were determined to be 15 nM for AVP and 22 nM for oxytocin with the UV detector set at its most sensitive range (0.001 absorbance unit full scale). This corresponds to 0.30 and 0.44 pmol, respectively, on the column. Calibration graphs were linear up to concentrations of 1×10^{-4} M (the highest concentrations used) for both the oxidized and reduced forms of AVP and OT.

Kinetics and Equilibria of Cysteine/AVP Interchange Reactions. Equilibrium constants were determined for the cysteine/AVP thiol/disulfide interchange reactions at pH 7.0. The procedure involved determination by HPLC of the concentrations of the native disulfide and reduced dithiol forms of AVP in the presence of a large excess of CySH and CySSCy, i.e., in a CySH/CySSCy redox buffer. Since pure samples were not available for calibration of the detector response for the mixed disulfides it was not possible to obtain their concentrations directly from their peak areas. The sum of the concentrations of the mixed disulfides was calculated as the difference between the initial AVP concentration and the sum of the concentrations determined for the disulfide and dithiol forms.

In the presence of an excess of CySSCy, an additional peak is observed in the chromatogram for the double mixed disulfide



The concentrations of the single (Figure 1) and double mixed disulfides were calculated from the total concentration of mixed disulfide by assuming detector response factors to be the same for the three species. Equilibrium constants were calculated for the thiol/disulfide interchange reactions in terms of the total single mixed disulfide (Figure 1) concentration, as defined by the following modified equilibrium scheme:

$$\text{CySH} + \text{AVP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{mixed disulfides} \quad (1)$$

$$\text{CySH} + \text{mixed disulfides} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{CySSCy} + \text{reduced AVP} \quad (2)$$

$$K_1 = \frac{[\text{mixed disulfides}]}{[\text{CySH}][\text{AVP}]} \quad (3)$$

$$K_2 = \frac{[\text{reduced AVP}][\text{CySSCy}]}{[\text{CySH}][\text{mixed disulfides}]} \quad (4)$$

$$K_{\text{overall}} = \frac{[\text{reduced AVP}][\text{CySSCy}]}{[\text{CySH}]^2[\text{AVP}]} \quad (5)$$

The following procedure was used to establish that the same results are obtained when equilibrium is approached from either direction. First, a reaction mixture was analyzed until equilibrium was reached, as indicated by no further change in the concentrations of the native disulfide and reduced dithiol forms of AVP. Then the concentration of either CySH or CySSCy was increased to shift the equilibrium to the right or left, respectively, and the new equilibrium concentrations were determined. Data from a typical experiment are plotted in Figure 4. In this experiment, 9.71×10^{-3} M CySH was reacted with 4.02×10^{-5} M AVP for 440 min. The concentration of CySSCy was then increased to 1.46×10^{-3} M to shift the equilibrium in eqs 1 and 2 to the left and the concentrations of the disulfide and dithiol forms were determined as a function of time. As indicated by the results in Figure 4, the concentrations rapidly reach new, constant values. After ~3 h, the concentration of CySH was increased to 1.94×10^{-2} M and the concentrations of AVP and reduced AVP were determined as a function of time. The values obtained for K_1, K_2, and K_{overall} from the concentrations determined for AVP and reduced AVP, the total concentration of the single mixed disulfides, and the known, excess concentrations of CySH and CySSCy at the second and third equilibria are 25.5 M^{-1}, 0.125, and 3.2 M^{-1}, and 25.8 M^{-1}, 0.128, and 3.3 M^- respectively. Average values obtained from experiments of this type are listed in Table I. These constants are conditional equilibrium constants for pH 7.0 and 25 °C.

At the second and third equilibria a peak is observed for the double mixed disulfide. The concentration of the double mixed disulfide was determined as described above, from which an average value of 14.4 ± 0.5 was calculated for the equilibrium constant for the double mixed disulfide, K_{DD}.

$$2\text{CySSCy} + \text{reduced AVP} \rightleftharpoons \text{double disulfide} + 2\text{CySH} \quad (6)$$

$$K_{\text{DD}} = \frac{[\text{double disulfide}][\text{CySH}]^2}{[\text{reduced AVP}][\text{CySSCy}]^2} \quad (7)$$

With the assumption that the detector calibration factors are the same for the two mixed disulfides in Figure 4, a 65:35

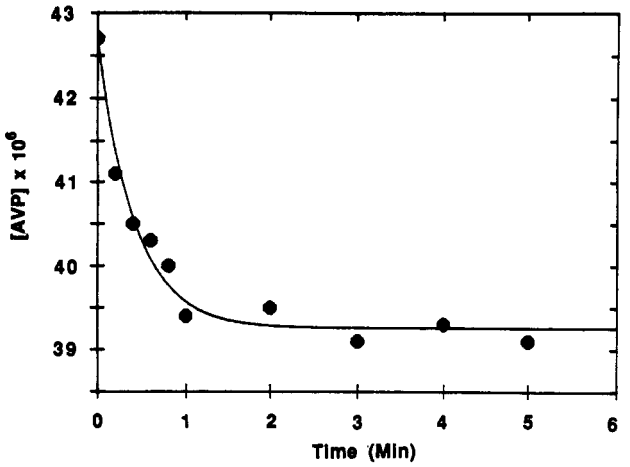


Figure 5. Concentration of AVP as a function of time after the addition of CySH to AVP (final concentrations, 2.34×10^{-3} M and 4.27×10^{-5} M, respectively). pH 5.5, 25 °C. The solid curve is the nonlinear least squares best fit of the data to eq 8.

Table I. Equilibrium and Rate Constants for the Arginine-Vasopressin/Cysteine Thiol/Disulfide Interchange Reactions[a]

K_1 (M^{-1})	25.9 ± 0.3	k_1 (M^{-1} s^{-1})	36 ● 6
K_2	0.124 ± 0.007	k_{-1} (s^{-1})	1.4 ± 0.2
$K_{overall}$ (M^{-1})	3.2 ± 0.2	k_2 (M^{-1} s^{-1})	1.2 ± 0.2
K_{DD}	14.4 ± 0.5	k_{-2} (M^{-1} s^{-1})	10.0 ± 1.4

[a] At 25 °C and pH 7.00.

ratio is obtained for the distribution between the two mixed disulfides. Using these relative concentrations, microscopic equilibrium constants of K_1^{MD1} = 16.8 M^{-1}, K_2^{MD1} = 0.191, K_1^{MD2} = 9.1 M^{-1}, and K_2^{MD2} = 0.354 are calculated from the values for K_1 and K_2 in Table I. MD1 represents the mixed disulfide peak with the shortest retention time.

The rate constant for reaction of CySH with AVP was determined by measuring the concentration of AVP as a function of time after the addition of a known, excess concentration of CySH. At pH 7.0 and 25 °C, the first step of the overall reaction (eqs 1 and 2) is too fast to characterize by quenching aliquots of the reaction mixture as a function of time and then analyzing the aliquots by HPLC. To decrease the reaction rate, the reaction was run at pH 5.5. Since the thiolate anion is the reactive species in thiol/disulfide interchange reactions,[24-28] the rate of reaction of CySH with disulfide bonds is reduced by a factor of ~300 by decreasing the pH to 5.5.

As shown by the time course data in Figure 5, the concentration of AVP decreases rapidly following the addition of excess CySH to AVP at pH 5.5. Peaks were detected for the mixed disulfides; however, no reduced AVP was detected during the first 5 min of reaction, which indicates that the second step in the overall reaction (eq 2) is slow at these concentrations. Under the conditions used (CySH to AVP concentration ratios in the range 27:1–55:1) the reactions are pseudo first order, and values were obtained for k'_1 and K'_1 by fitting the time course data obtained during the first 5 min to the equation for a first-order, reversible reaction:[29]

$$A_t = \frac{A_0(K'_1 + e^{k'_1(1+1/K'_1)t}}{(K'_1 + 1)e^{k'_1(1+1/K'_1 t}} \quad (8)$$

where k'_1 is the pseudo-first-order rate constant for reaction of AVP with CySH and $K'_1 = k'_1/k_{-1}$. k_{-1} is defined in eq 1. The smooth curve through the points in Figure 5 is the nonlinear least squares fit to the time course data. The values obtained for k'_1 and K'_1 from the nonlinear least squares fit are 0.193 min^{-1} and 0.0877, respectively, from which values of 1.37 M^{-1} s^{-1} and 37.5 M^{-1} were calculated for k_1 and K_1. Average values of 1.19 ± 0.19 M^{-1} s^{-1} and 37.4 ± 0.6 were obtained for k_1 and K_1, respectively, at pH 5.5 by this method. The value reported in Table I for k_1 at pH 7.0 was calculated from the average value 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 $\alpha_{5.5}$ are the fractional concentrations of CySH in the reactive thiolate form at pH 7.0 and 5.5, respectively. The α values were calculated using the equation $\alpha = K_A/(K_A + [H^+])$ and a pK_A = 8.38 for the thiol group of CySH.[30] k_{-1} was calculated using the relationship $k_{-1} = k_1/K_1$ and the value listed in Table I for K_1. Because the values determined for K_1 at pH 5.5 and 7.0 are conditional constants, they depend on the pK_A values for the thiol groups of CySH and the mixed disulfides and thus the pH.

The rate constant for reaction of reduced AVP with cystine (k_{-2} in eq 2) was determined from the initial rate of decrease in the concentration of reduced AVP at pH 7.0 under pseudo-first-order conditions with respect to reduced AVP. The result is reported in Table I. The value listed for k_2 was calculated from k_{-2} and K_2 using the relation $k_2 = k_{-2}K_2$.

Although thiol/disulfide interchange reactions of simple thiols and dithiols[26,31-33] and dithiol-containing peptides[34-39] have been the subject of previous studies, there are no previous reports on the kinetics and equilibria of thiol/disulfide interchange reactions of the neurohypophyseal peptide hormones for comparison with the results in Table I. It is of interest, however, to compare the results to results for other thiol/disulfide interchange reactions. At pH 7.0, the rate constant for reaction of CySH with glutathione disulfide, GSSG, to form CySSG and CySH is 0.33 M^{-1} s^{-1},[32] as compared to 36 M^{-1} s^{-1} for the reaction of CySH with AVP. These are conditional rate constants, however, the fraction of CySH in the reactive thiolate form is the same in both cases and thus they can be compared directly. The difference of a factor of ~100 in rate constants is probably due to several factors. The net charges on the reactants is predicted to favor the reaction of CySH with AVP since the reactive thiolate form of CySH has a net charge of −1, while the most abundant forms of GSSG and AVP at pH 7 have net charges of −2 and +2, respectively. The disulfide bond of AVP is also predicted

(24) Kolthoff, I. M.; Stricks, W.; Kapoor, R. C. *J. Am. Chem. Soc.* **1955**, *77*, 4733–4739.
(25) Eldjarn, L.; Pihl, A. *J. Am. Chem. Soc.* **1957**, *79*, 4589–4593.
(26) Szajewski, R. P.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 2011–2026.
(27) Rabenstein, D. L.; Theriault, Y. *Can. J. Chem.* **1984**, *62*, 1672–1680.
(28) Wilson, J. M.; Bayer, R. J.; Hupe, D. J. *J. Am. Chem. Soc.* **1977**, *99*, 7922–7926.
(29) Amdur, I.; Hammes, G. G. *Chemical Kinetics: Principles and Selected Topics*; McGraw-Hill: New York, 1966.
(30) Backs, S. J.; Rabenstein, D. L. *Inorg. Chem.* **1981**, *20*, 410–415.
(31) Houk, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 6825–6836.
(32) Keire, D. A.; Strauss, E.; Guo, W.; Noszál, B.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 123–127.
(33) Gilbert, H. F. *Adv. Enzymol.* **1990**, *63*, 69–172.
(34) Snyder, G. H. *Biochemistry* **1987**, *26*, 688–694.
(35) Zhang, R.; Snyder, G. H. *Biochemistry* **1988**, *27*, 3785–3794.
(36) Milburn, P. J.; Konishi, Y.; Meinwald, Y. C.; Scheraga, H. A. *J. Am. Chem. Soc.* **1987**, *109*, 4486–4496.
(37) Milburn, P. J.; Meinwald, Y. C.; Takahashi, S.; Ooi, T.; Scheraga, H. A. *J. Pept. Protein Res.* **1988**, *31*, 311–321.
(38) Falcomer, C. M.; Meinwald, Y. C.; Choudhary, I.; Talluri, S.; Milburn, P. J.; Clardy, J.; Scheraga, H. A. *J. Am. Chem. Soc.* **1992**, *114*, 4036–4042.
(39) Huyghnes-Despointes, B. M. P.; Nelson, J. W. In *Current Research in Protein Chemistry*; Villfanca, J. J., Ed.; Academic Press: New York, 1990; pp 457–466.

to be more accessible to nucleophilic attack since one of the two cysteine residues is a terminal amino acid. Ring strain might also be a factor in the reactivity of the disulfide bond of AVP, although there is not expected to be much ring strain since the disulfide bond is part of a 20-membered ring.

The reverse reaction, i.e., the reaction of reduced AVP with CySSCy, results in formation of the 20-membered ring. For the conditions used here, e.g., 10^{-3} M CySSCy and 10^{-5} M reduced AVP, the slow step is the first step, i.e., reaction of reduced AVP with CySSCy to form the mixed disulfides. The results indicate that, once formed, the mixed disulfides have a much greater tendency to undergo intramolecular thiol/disulfide interchange with closure of the 20-membered ring than reaction with CySH to reform reduced AVP and CySSCy.

CONCLUSIONS

The results presented for the cysteine/AVP system demonstrate that the kinetics and equilibria of thiol/disulfide interchange reactions involving the neurohypophyseal peptide hormones can be characterized by methodology based on analysis of reaction mixtures by reversed-phase HPLC. By use of the capacity factor information in Figure 2, appropriate conditions can be identified for separation of the native disulfide and reduced dithiol forms of AVP and OT and of their analogs PA and TA in thiol/disulfide reaction mixtures. Under these conditions, the majority of small thiol-containing biological molecules, and their symmetrical and mixed disulfides, were found to elute before the disulfide and dithiol forms of the peptides and thus do not interfere in the analysis. This methodology should be widely applicable to the study of the thiol/disulfide and redox chemistry of the neurohypophyseal peptide hormones.

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