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Thiol/Disulfide Exchange Reactions of Captopril and Penicillamine with Arginine Vasopressin and Oxytocin

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The kinetics and equilibria of the reaction of the thiol-containing drugs captopril (D-3-mercapto-2-methylpropanoyl-L-proline, CpSH) and penicillamine (β, β-dimethylcysteine, PSH) with the disulfide bonds of the neurohypophyseal peptide hormones arginine vasopressin (AVP) and oxytocin (OT) have been characterized. CpSH reacts with AVP and OT by thiol/disulfide interchange to form two peptide–CpSH mixed disulfides, which in turn react with another molecule of CpSH to form the reduced peptide and CpSSCp. Forward and reverse rate constants and the equilibrium constant are reported for both steps in the reaction of CpSH with AVP and OT at pH 7.00. The rate constant for the first step (k_1) is much larger than that for the second step (k_2). Also, once formed, the peptide–CpSH mixed disulfides rapidly undergo intramolecular thiol/disulfide interchange with reformation of the cyclic peptide and CpSH. PSH reacts with AVP and OT by the same two-step reaction sequence; however, the rate of the second step is very slow due to steric hindrance from the methyl groups of PSH and the PSH moiety of the peptide–PSH mixed disulfides. Using rate constants determined in this study and PSH levels in the plasma of patients on PSH therapy, it is predicted that *in vivo* reduction of the disulfide bonds of AVP and OT by PSH and CpSH has little effect on the plasma half-lives of AVP or OT.

INTRODUCTION

The neurohypophyseal peptide hormones arginine vasopressin (AVP) and oxytocin (OT) (see Scheme 1) both contain a hexapeptide ring formed by a disulfide bond between cysteine residues at positions 1 and 6. Structure–activity studies with analogs have shown that the disulfide bonds are 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 (*1*). Thus, the main purpose of the disulfide bonds in AVP and OT is to form and keep a molecular conformation suitable for noncovalent interaction with their respective receptors.

Reduction of the disulfide bonds of AVP and OT by thiol/disulfide interchange reactions provides a mechanism for opening the hexapeptide rings, and such reactions with the tripeptide glutathione and other endogenous thiols may be involved in inactivation and removal of AVP and OT from the circulatory system (*2*). In a recent study, it was found that rate constants for reduction of the disulfide bonds of AVP and OT by glutathione (GSH) and cysteine (CySH) are 1–2 orders

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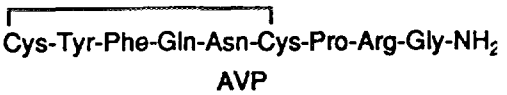
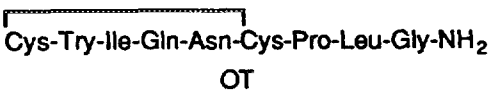


AVP



OT

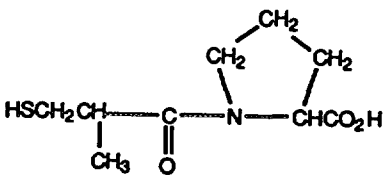
SCHEME 1

of magnitude larger than for reduction of intermolecular disulfide bonds formed by two cysteine-containing peptides (*3*). This finding raises questions about the reaction of AVP and OT with thiol-containing drugs. Such reactions are potentially important not only with respect to the metabolism of the drug but also for their effect on the biological activity of the peptide hormones. For example, previous studies have shown that preincubation of AVP with the thiol-containing drug molecule captopril reduces the activity of AVP administered intravenously to rats (*4*).

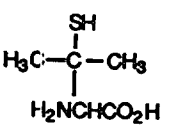
In this paper, we report the results of a study of the kinetics and equilibria of the reaction of captopril (CpSH) and penicillamine (PSH) with AVP and OT (see Scheme 2). Captopril (D-3-mercapto-2-methylpropanoyl-L-proline) is effective as an orally active inhibitor of angiotensin I-converting enzyme (*5*) in reducing arterial blood pressure in most forms of hypertension (*6*). Penicillamine (β,β-dimethylcysteine) is used in the treatment of Wilson's disease, cystinuria, metal intoxication, and rheumatoid arthritis (*7*).

EXPERIMENTAL

Chemicals. AVP and OT were obtained from Bachem Inc. (Torrance, CA). Peptide purity was determined by 500-MHz ^{1}H NMR and was found to be in good agreement with the certificate of analysis provided by the supplier. Dithiothreitol (DTT) was obtained from Aldrich Chemical Co. CpSH and CpSSCp were gifts from



CpSH



PSH

SCHEME 2

the Squibb Institute for Medical Research (Princeton, NJ). PSH, L-tryptophan, and *N*-acetyl-L-phenylalanine 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 Bioanalytical Systems BAS 200 liquid chromatograph equipped with a Rheodyne Model 7125 sample injector with a 20-μl loop, a 100 × 3.2-mm ODS (C_{18}) Phase II column (particle size 3 μm), and a Linear Instruments Model 204 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. A mobile phase containing 11.5% acetonitrile was used for the AVP/PSH and AVP/CpSH systems and 15.5% acetonitrile for the OT/PSH and OT/CpSH systems (*8*). 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.

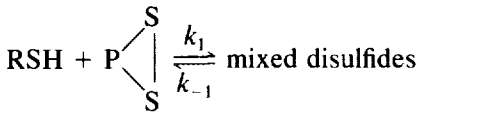
Concentrations were obtained from chromatographic peak areas by using L-tryptophan and *N*-acetyl-L-phenylalanine as internal intensity standards (*8*). Calibration solutions of reduced AVP and OT were prepared by reduction of stock solutions of the peptides with an excess of DTT.

Kinetic and equilibrium studies. Stock solutions of AVP and OT 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 pH 7.0 phosphate buffer which had been deoxygenated by bubbling with either argon or nitrogen. In the procedure used to study the kinetics of the reaction of PSH and CpSH with AVP and OT, aliquots of the peptide and internal standard stock solutions were combined, and the solution was deoxygenated and placed in a water bath (25°C) in a nitrogen-filled glove bag. The reaction was initiated by addition of pH 7.0 PSH or CpSH stock solution. Aliquots of the reaction mixture were removed as a function of time, quenched by lowering the pH to ~3 with HCl, and then analyzed by HPLC. The quenching procedure was verified previously (*8*).

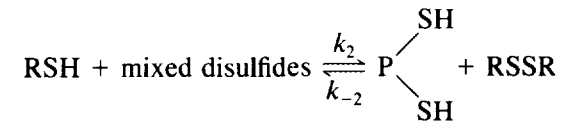
Rate constants were determined for reaction of reduced AVP and OT with CpSSCp at pH 7.0 by a similar procedure. Reduced AVP and OT were prepared by electrochemical reduction at a mercury pool electrode for ~3 h (*9*). Equilibrium constants were determined at pH 7.00 for the AVP/CpSH and OT/CpSH systems by measuring the concentrations of the disulfide and reduced dithiol forms of the peptides in solutions containing a CpSH/CpSSCp redox buffer (*3*, *8*).

RESULTS

PSH and CpSH react with the disulfide bonds of AVP and OT by the two-step reaction sequence, where RSH represents PSH and CpSH:

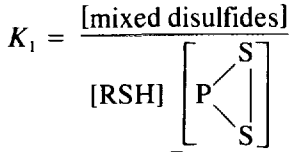


$$\text{RSH} + \text{P}(\text{S})(\text{S}) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{mixed disulfides} \qquad [1]$$

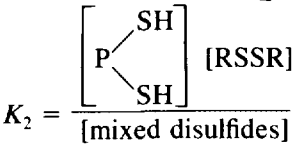


$$\text{RSH} + \text{mixed disulfides} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{P}(\text{SH})(\text{SH}) + \text{RSSR} \qquad [2]$$

In the first step, RSH reacts with the disulfide bond to form one of two possible mixed disulfide conjugates, which in turn react with another molecule of RSH to give the reduced dithiol form of the peptide and RSSR. The reactions are equilibrium reactions:



$$K_1 = \frac{[\text{mixed disulfides}]}{[\text{RSH}]\left[\text{P}(\text{S})(\text{S})\right]} \qquad [3]$$



$$K_2 = \frac{\left[\text{P}(\text{SH})(\text{SH})\right][\text{RSSR}]}{[\text{mixed disulfides}]} \qquad [4]$$

Rate and equilibrium constants for the reaction of PSH and CpSH with AVP and OT were determined by separation and analysis of reaction mixtures by high-performance liquid chromatography (HPLC).

Reaction of PSH with AVP and OT. The chromatogram of an aliquot from a reaction mixture which initially contained 32.0 μM AVP and 7.15 mM PSH at pH 7.0 and 25°C is shown in Fig. 1. The aliquot was removed from the reaction mixture after 5 h of reaction. Well-resolved peaks are obtained for the native disulfide, reduced dithiol, and mixed disulfide forms of AVP and for the internal intensity standard. The peaks for AVP and reduced AVP were assigned using authentic samples of the two compounds. The peaks at 6.5 and 8.2 min were assigned to the two mixed disulfides on the basis of the constant values obtained for equilibrium constants over a range of AVP/PSH ratios and by comparison with retention times of mixed disulfides of AVP with GSH and CySH (*3*). PSH is essentially unretained with the mobile phase used, and thus the peak for PSH is close to the peak for the solvent front.

The low intensity of the peak for reduced AVP in the chromatogram in Fig. 1 indicates that the rate of the second step, i.e., the reaction of PSH with the two possible AVP–PSH mixed disulfides, is very slow relative to the rate of reaction of PSH with AVP. This is also the case for the reaction of PSH with oxidized glutathione and cystine, presumably due to steric effects from the two methyl groups adjacent to the sulfur atom of PSH (*10*, *11*). Thus, only the first step was characterized in the reaction of PSH with AVP (Eqs. [1] and [3]).

The kinetics and equilibria of the reaction of AVP with PSH were characterized at pH 7.0 by removing aliquots from reaction mixtures as a function of time,

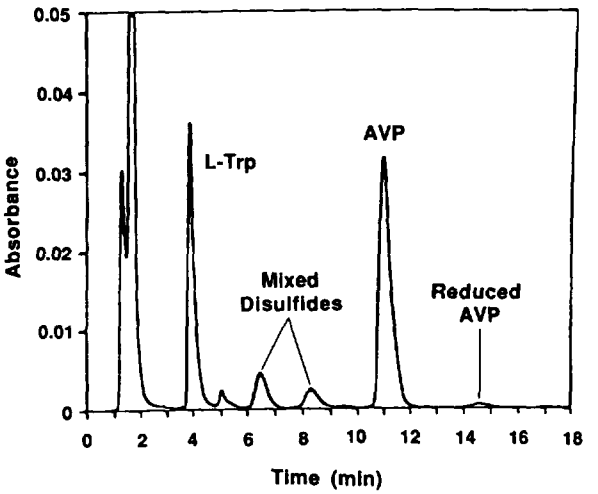


FIG. 1. Chromatogram of an AVP/PSH reaction mixture. The initial concentrations were 32.0 μM AVP and 7.15 mM PSH at pH 7.00 and 25°C. The concentration of the internal standard, L-Trp, was 13.2 μM. The chromatogram is for an aliquot removed after 5 h of reaction.

quenching the aliquots by lowering the pH to ~3, and then analyzing the reaction mixtures. The reactions were run under pseudo-first-order conditions by using an excess concentration of PSH. Typical time-course data are presented in Fig. 2. Over the time period in Fig. 2, peaks are observed for AVP and the two mixed disulfides, but not for reduced AVP. Thus, the results in Fig. 2 indicate that the reaction mixture rapidly comes to a pseudo equilibrium between AVP and its

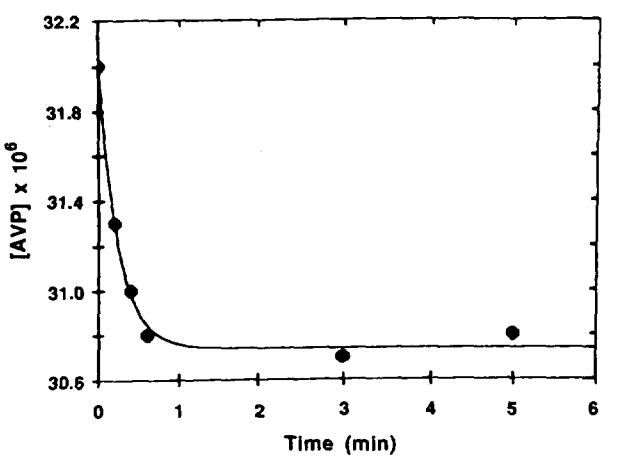


FIG. 2. The concentration of AVP as a function of time for the reaction of 32.0 μM AVP with 0.905 mM PSH in 0.15 M KCl at pH 7.00 and 25°C.

TABLE 1

Rate and Equilibrium Constants for the Reaction of Thiol-Containing Compounds with AVP and OT[a]

	PSH/AVP	CpSH/AVP	GSH/AVP[b]	PSH/OT	CpSH/OT	GSH/OT[b]
$k_1(M^{-1} s^{-1})$	2.9 ± 0.6	3.6 ± 0.5	38 ± 6	17.9 ± 1.6	4.2 ±0.6	110 ± 6
$k_{-1}(s^{-1})$	0.076 ± 0.008	0.023 ± 0.003	0.63 ± 0.10	0.132 ± 0.017	0.013 ± 0.003	0.76 ± 0.04
$k_2(M^{-1} s^{-1})$	[c]	0.37 ± 0.07	0.74 ± 0.09	[c]	0.16 ± 0.02	0.84 ± 0.13
$k_2(M^{-1} s^{-1})$	[c]	2.5 ± 0.5	3.1 ± 0.1	[c]	0.65 ± 0.09	3.2 ± 0.5
$K_1(M^{-1})$	39 ± 11	154 ± 28	60 ± 2	137 ± 14	320 ± 70	140 ± 4
K_2	[c]	0.15 ± 0.03	0.24 ± 0.03	[c]	0.24 ± 0.02	0.26 ± 0.01
$K_{OV}(M^{-1})$	[c]	24.8 ± 2.3	14.5 ± 1.8	[c]	79 ± 14	36 ± 2

[a] 25°C and pH 7.00, 0.15 M KCl.
[b] Ref. *3*.
[c] Not determined.

mixed disulfides. Pseudo-first-order rate constant k_1' was obtained by fitting the time-course data to the equation for a first-order, reversible reaction (*12*)

$$A_t = \frac{A_0(K_1' + \exp(k_1'(1 + 1/K_1')t)}{(K_1' + 1)\exp(k_1'(1 + 1/K_1')t)}, \quad [5]$$

where A_t is the concentration of AVP at time t, A_0 the initial concentration of AVP, k_1' the pseudo-first-order rate constant for reaction of PSH with AVP, and K_1' a pseudo equilibrium constant (K_1' = [mixed disulfides]/[AVP] = k_1'/k_{-1}). The second-order rate constant k_1 was calculated from k_1' using the relation k_1' = k_1[PSH]. This treatment assumes that reaction of PSH with AVP to form the mixed disulfides is second order. It is well established that thiol/disulfide exchange reactions are mechanistically simple, second-order S_N2 nucleophilic displacement reactions (*13–15*) and it has been shown that the reaction of GSH with neurohypophyseal peptide hormones and the reaction of PSH with oxidized glutathione are both second-order reactions (*10*, *16*).

The smooth curve through the points in Fig. 2 is the nonlinear least-squares fit of the time-course data to Eq. [5]. Average values obtained for k_1 and K_1 from experiments of this type are reported in Table 1. The value reported for rate constant k_{-1} was calculated using the relation k_{-1} = k_1/K_1. Rate and equilibrium constants determined for the reaction of PSH with OT by the procedures described above are also reported in Table 1. Also included for comparison are rate and equilibrium constants for the corresponding reactions of glutathione with AVP and OT (*3*).

Reaction of CpSH with AVP and OT. The reactions of CpSH with AVP and OT differ from those of PSH in that the second step (Eq. [2]) also takes place, although at a somewhat slower rate than the first step. Thus, the result of the overall reaction of CpSH with AVP and OT is reduction of the disulfide bond and formation of CpSSCp.

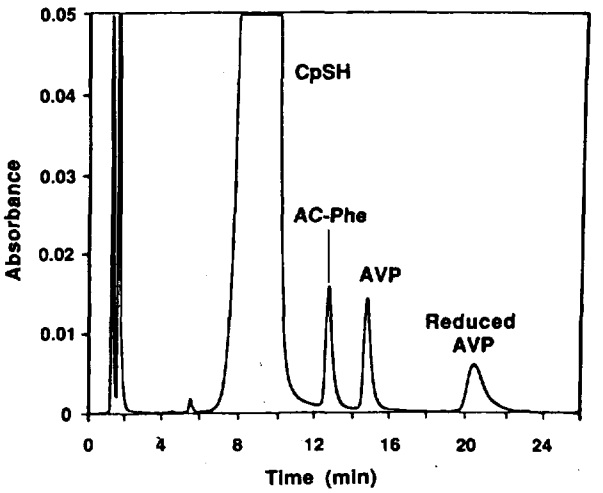


FIG. 3. Chromatogram of an AVP/CpSH reaction mixture at equilibrium. The initial concentrations were 32.7 μM AVP, 0.995 mM CpSSCp, and 6.68 mM CpSH in 0.15 M KCl at pH 7.00 and 25°C. The concentration of the internal standard, *N*-acetylphenylalanine, was 42.2 μM.

$$2\text{CpSH} + \text{Pr}\begin{matrix}\text{S}\\|\\\text{S}\end{matrix} \rightleftharpoons \text{Pr}\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix} + \text{CpSSCp} \qquad [6]$$

$$K_{OV} = \frac{\left[\text{Pr}\begin{matrix}\text{SH}\\\\\text{SH}\end{matrix}\right][\text{CpSSCp}]}{[\text{CpSH}]^2\left[\text{Pr}\begin{matrix}\text{S}\\|\\\text{S}\end{matrix}\right]} \qquad [7]$$

Equilibrium constants K_1, K_2, and K_{OV} were determined for the reaction of CpSH with AVP and OT by analysis of equilibrium mixtures by HPLC. A typical chromatogram for an equilibrium reaction mixture is shown in Fig. 3. The solution was prepared by reaction of 32.7 μM AVP with a CpSH/CpSSCp redox buffer, which contained 6.68 mM CpSH and 0.995 mM CpSSCp at pH 7.0 and 25°C. The solution also contained 42.2 μM *N*-acetyl-L-phenylalanine as an internal intensity standard. The peak for CpSH overlaps those for the mixed disulfides, while CpSSCp has a long retention time for the chromatographic conditions used and its peak is not shown in Fig. 3. The concentrations of AVP and reduced AVP were determined from their peak intensities. The total concentration of the mixed disulfides was then calculated using these concentrations and the known total concentration of AVP.

To establish that equilibrium was reached, a procedure was used in which equilibrium was approached from both directions (*3*, *8*). AVP was reacted with a CpSH/CpSSCp 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 AVP and reduced AVP. The equilibrium was then perturbed by addition of a known amount of CpSSCp, and aliquots were removed and analyzed as the system approached equilibrium from the opposite direction. Equilibrium constants were calculated from the concentrations determined at each equilibrium for the disulfide, dithiol, and mixed disulfide forms of AVP and the known excess concentrations of CpSH and CpSSCp, corrected to account for changes in their concentrations due to the reactions in Eqs. [1] and [2]. Average values determined for K_1, K_2, and K_{OV} from four experiments of this type are presented in Table 1. Also listed in Table 1 are values obtained by the same procedure for the reaction of CpSH with OT.

Rate constants for both the forward and reverse reactions in Eqs. [1] and [2] were determined for the CpSH/AVP and CpSH/OT systems. Rate constant k_1 was determined by measuring the concentration of AVP or OT as a function of time after CpSH was added to a solution of the peptide at pH 7.0. The reaction was run under pseudo-first-order conditions by using an excess concentration of CpSH. Time-course data similar to those shown in Fig. 2 were observed over reaction times of 6–8 min, i.e., the concentration of the disulfide form decreased and then leveled off as AVP or OT reached a pseudo equilibrium with its mixed disulfides. No peaks were detected for reduced AVP or OT over this time period. Time-course data were fitted to Eq. [5], as described above, and values for k_1 and k_{-1} were then calculated from the best fit values for k_1' and K_1'. The results are reported in Table 1.

Rate constant k_{-2} for the reaction of the reduced dithiol forms of AVP and OT with CpSSCp was determined by measuring the concentration of reduced peptide as a function of time after CpSSCp was added to a solution of the reduced peptide at pH 7.00. The reactions were run under pseudo-first-order conditions by using an excess concentration of CpSSCp. Rate constant k_{-2} was obtained from the initial slope of the time-course data, and k_2 was then calculated using the relation $k_2 = K_2 k_{-2}$. The results obtained for the AVP/CpSH and OT/CpSH systems are reported in Table 1.

DISCUSSION

The results in Table 1 indicate that both PSH and CpSH react rapidly with AVP and OT. In all cases, rate constant k_1 is much larger than k_2, and thus the second step is rate determining in the overall reduction of AVP and OT to their dithiol forms. However, even though $k_1 \gg k_2$, AVP and OT are not converted completely to their mixed disulfides by reaction with PSH and CpSH. Rather, the mixed disulfides are rapidly converted back to AVP and OT by intramolecular thiol/disulfide interchange and a pseudo equilibrium is established. For example, for the AVP/PSH system, $k_{-1} > k_1$[PSH] when [PSH] < 0.026 M. At the same time, some mixed disulfide reacts further with PSH or CpSH. The relative rates of the competing intramolecular thiol/disulfide reaction to reform the disulfide bond and the intermolecular reaction to give the dithiol form of the peptide depend on the rate constants (k_{-1} and k_2) and the concentration of PSH or CpSH. For example,

$k_{-1} > k_2$[CpSH] and thus intramolecular thiol/disulfide interchange to displace CpSH and reform the AVP disulfide bond is faster when [CpSH] < 0.062 M.

Rate constants for the reaction of PSH and CpSH with AVP and OT are consistently smaller than those for the corresponding reactions of GSH with AVP and OT (Table 1). Since the thiolate anion is the reactive species, the rates of thiol/disulfide exchange reactions are governed by solution pH and the pK_A of the thiol group (*1*, *14*, *15*). The pK_As of the thiol groups of PSH, CpSH, and GSH are 8.03, 9.71, and 8.97 (*17*, *18*), respectively, from which it can be calculated that the fractions of the thiol groups present in the reactive thiolate form at pH 7.00 are 8.7×10^{-2}, 2.0×10^{-3}, and 1.1×10^{-2}. Thus, k_1 is predicted to increase in the order $k_{1,\text{CpSH}} < k_{1,\text{GSH}} < k_{1,\text{PSH}}$. As predicted, $k_{1,\text{CpSH}}$ is less than $k_{1,\text{GSH}}$; however, $k_{1,\text{PSH}}$ is also significantly less than $k_{1,\text{GSH}}$. k_2 is predicted to increase in the same order as k_1; however, $k_{2,\text{PSH}} <<< k_{2,\text{CpSH}} < k_{2,\text{GSH}}$. Presumably $k_{1,\text{PSH}}$ and $k_{2,\text{PSH}}$ are less than predicted due to steric effects from the two methyl groups adjacent to the sulfur atom (*10*). This is particularly important for $k_{2,\text{PSH}}$, since the reaction involves nucleophilic attack by the sulfur of PS^- at the penicillamine sulfur of the AVP–PSH and OT–PSH mixed disulfides. Molecular models show steric interference between the methyl groups on the two PSH moieties.

A major objective of this study was to determine the extent to which PSH and CpSH might reduce the disulfide bonds of AVP and OT by thiol/disulfide exchange *in vivo*. The time-course data in Fig. 2 show that PSH reacts rapidly with AVP. Concentrations of PSH ranging from 3×10^{-7} to 2×10^{-5} M were found in plasma in a study of the pharmacokinetics of PSH in patients with rheumatoid arthritis (*19*). Using rate constants calculated for pH 7.4 from the rate constants in Table 1 and the pK_A of the thiol group of PSH, half-lives for the reaction of AVP and OT with PSH are predicted to be 90 and 14.5 min, respectively, at a PSH concentration of 2×10^{-5} M, while they are predicted to be 6000 and 962 min at a PSH concentration of 3.7×10^{-7} M PSH. For comparison, the plasma half-life of AVP is reported to be in the range of 5–15 min (*20*), while the plasma half-life of OT is 1.8–10.3 min (*21*). Also, once formed, the PSH and CpSH mixed disulfides of AVP and OT rapidly convert back to the native disulfide forms by intramolecular thiol/disulfide interchange. For example, using the values for k_{-1} in Table 1, half-lives of 9 and 30s are predicted for the mixed disulfides of AVP with PSH and CpSH, respectively, while those predicted for the mixed disulfides of OT with PSH and CpSH are 5 and 53 s. Thus, the results of this study indicate that, even though the disulfide bonds of AVP and OT are reactive to thiol/disulfide exchange with PSH and CpSH, *in vivo* reduction by PSH and CpSH is not likely to have a significant effect on the plasma half-lives of AVP and OT.

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