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Nuclear Magnetic Resonance Studies of Thiol/Disulfide Chemistry

I. Kinetics and Equilibria of the Reduction of Captopril Disulfide and Captopril–Glutathione Mixed Disulfide by Glutathione

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The kinetics and equilibria of the reduction of captopril disulfide (CpSSCp) and captopril–glutathione mixed disulfide (GSSCp) by glutathione (GSH) have been studied by ^1H and ^{13}C NMR. Reduction takes place by thiol/disulfide exchange:

$$GSH + CpSSCp \rightleftarrows GSSCp + CpSH$$

$$GSH + GSSCp \rightleftarrows GSSG + CpSH.$$

Equilibrium constants were determined as a function of pH for the two reactions; at physiological pH, the equilibrium constants for the first and second reactions are 1.46 and 0.300, respectively. The equilibrium constant for the overall reaction is 0.436, which corresponds to a difference in formal electrode potential for the two half reactions ($E^{\circ\prime}_{GSSG/GSH} - E^{\circ\prime}_{CpSSCp/CpSH}$) of 0.011 V; i.e., CpSH is more strongly reducing than GSH. Rate constants were determined as a function of pD for the forward and reverse reactions of both steps. The results indicate that, at physiological pH, the rate predicted for the *in vivo* reduction of CpSSCp by endogenous thiols is much slower than that oberved experimentally, which supports previous conclusions that reduction of CpSSCp to CpSH involves both enzymatic and nonenzymatic, i.e., thiol/disulfide exchange, processes.

INTRODUCTION

Captopril, D-3-mercapto-2-methylpropanoyl-L-proline (CpSH),[2] is an orally active drug used for the treatment of hypertension. Inhibition of angiotensin I converting enzyme is the molecular basis for the antihypertensive activity of captopril (*1–3*). Major metabolites include the mixed disulfides of captopril with cysteine and glutathione (GSH) (*4–6*), captopril disulfide, and the *S*-methyl derivative and the sulfoxide of captopril (*5–9*). Captopril also forms mixed disulfides with plasma proteins (*6, 10*).

Because CpSH is the active form of captopril, the ease of reduction of its various mixed and symmetrical disulfides back to CpSH determines to some

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[2] Abbreviations used: CpSH, captopril; GSH, glutathione; CpSSCp, captopril disulfide; GSSCp, glutathione–captopril mixed disulfide; TBA, *tert*-butyl alcohol; DSS, sodium-2,2-dimethyl-2-silapentene-5-sulfonate; GSSG, glutathione disulfide.

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extent its pharmacokinetics and duration of activity. (*11*). Previous metabolic studies have shown that captopril–plasma protein mixed disulfides dissociate rapidly *in vivo* by thiol/disulfide exchange reactions with endogenous thiols such as cysteine and GSH (*6, 12*). The reduction of captopril disulfide (CpSSCp) by GSH has also been qualitatively characterized *in vitro* (*13*), and a preliminary estimate of the equilibrium constant for the reaction of GSH with CpSSCp at pH 6.0 has been reported (*14*). Because knowledge of the rates and mechanism of the reduction of CpSSCp and of mixed disulfides of captopril with GSH, cysteine, and other endogenous thiols is so important to understanding the pharmacokinetics and duration of activity of captopril, we have studied in detail the kinetics and equilibria for the reduction of CpSSCp and the captopril–glutathione mixed disulfide (CpSSG) by GSH.

The reduction of CpSSCp by GSH takes place in two steps: in the first step, GSH reacts with CpSSCp to form GSSCp (Eq. [1]), which in turn reacts with another molecule of GSH in the second step (Eq. [2]). The overall result of the two reactions is the reduction of CpSSCp and the oxidation of GSH:

$$\text{GSH} + \text{CpSSCp} \rightleftarrows \text{GSSCp} + \text{CpSH} \quad [1]$$

$$\text{GSH} + \text{GSSCp} \rightleftarrows \text{GSSG} + \text{CpSH} \quad [2]$$

$$2\text{GSH} + \text{CpSSCp} \rightleftarrows \text{GSSG} + 2\text{CpSH}. \quad [3]$$

We have measured the rates and equilibrium constants for both steps in the overall reaction by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy.

EXPERIMENTAL

Chemicals. Captopril and captopril disulfide were gifts from the Squibb Institute for Medical Research (Princeton, NJ). Free acid and sodium salt forms of glutathione and glutathione disulfide were obtained from Sigma Chemical Co. The 99.7% D_2O was obtained from Merck Sharpe and Dohme Ltd. NaOD (30%) in D_2O and DCl (20%) in D_2O were obtained from Aldrich Chemical Co.

NMR measurements. ^{1}H and ^{13}C NMR measurements were performed on a Varian VXR-500S NMR spectrometer at 500 and 125 MHz, respectively. The probe temperature was maintained at 25°C for all experiments. ^{13}C spectra were acquired with WALTZ broadband proton decoupling gated on only during acquisition to minimize NOE effects. ^{1}H NMR chemical shifts were measured relative to internal *tert*-butyl alcohol (TBA), which has a chemical shift of 1.2365 ppm relative to the resonance for the methyl protons of sodium-2,2-dimethyl-2-silapentene-5-sulfonate (DSS). ^{13}C NMR chemical shifts were measured relative to dioxane, which has a chemical shift of 67.4 ppm relative to $(CH_3)_4Si$ (TMS). All ^{1}H spectra were acquired on D_2O solutions, while ^{13}C spectra were measured on H_2O solutions with sufficient D_2O added (5–10%) for a spectrometer lock signal.

Spin-lattice (T_1) relaxation times for the various species present in the exchange reactions were measured by the inversion-recovery method (*15*). To obtain quantitative resonance intensities in the kinetic and equilibrium constant measure-

ments, a repetition time of five times the longest T_1 of the resonances of interest was used. ^{13}C T_1's for CpSH, CpSSCp, CpSSG, GSH, and GSSG ranged from 0.2 to 1.6 s.

pH measurements were made with an Orion Research Model 701A pH meter equipped with Fisher Scientific Accu-Phast double junction combination or microcombination electrodes. Ingold Electrode combination ultramicroelectrodes were used for pH measurements made directly in 5-mm NMR tubes. The pH meters were calibrated with Fischer Scientific certified pH 4.00, 7.00, and 10.00 standard solutions. The exact pH values of the standard solutions were determined by comparison with N.B.S. primary standard buffers. pH measurements in D_2O solutions were corrected for the deuterium isotope effect with the equation pD = pH meter reading +0.40 (*16*).

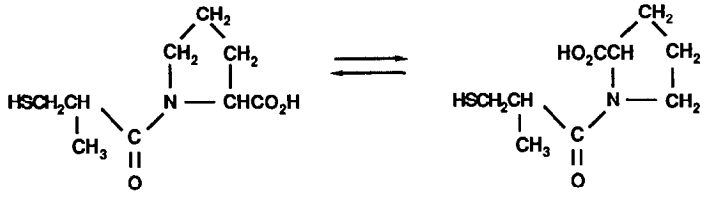
Sample preparation. The solutions used for the measurement of equilibrium constants by ^{13}C NMR were prepared in a solvent mixture of distilled deionized water, 5–10% D_2O for a lock signal, and approximately 0.1 M dioxane for a chemical shift reference. The CpSH and GSSG solutions were prepared separately, their pH values adjusted, and then mixed to give initial concentrations of 0.1 and 0.05 M, respectively. Care was taken to exclude oxygen; all solvents were degassed with nitrogen or argon prior to use, nitrogen or argon was bubbled into the solution during pH adjustment with concentrated HCl or NaOH, and NMR tubes were flushed with nitrogen or argon before and after sample addition and then were sealed with Parafilm. To ensure that the thiol/disulfide exchange reactions were at equilibrium, samples were allowed to equilibrate at least 24 h prior to measurement of ^{13}C NMR spectra, with longer equilibration time periods used for the lower pH samples.

Solutions for the kinetics experiments were prepared by mixing 750 μl of GSSG or CpSSCp and 50 μl of CpSH or GSH in a 5-mm NMR tube with the concentrations of the reagent solutions chosen so that the final concentrations were approximately 20 mM disulfide and 40 mM thiol, respectively. ^1H NMR spectra were then measured as a function of time at predetermined intervals of 10–30 min. After the NMR time course measurements were completed, the final pH of the mixture was measured directly in the tube with the ultramicroelectrode. At pH values greater than 7, the reactions are too fast to adequately characterize by the above method. To determine rate constants in this pH range, reactants were mixed in a constant temperature bath. Aliquots were then taken for analysis by ^1H NMR at approximately 1-min intervals and quenched to pH $\leq$2.0, by addition of 25 μl of 20% DCl. Spectra were measured for all aliquots once the reaction was completed. All kinetics experiments were carried out in degassed D_2O with 0.15 M NaCl added for ionic strength control and approximately 10 mM TBA for a chemical shift reference. pH adjustments were made with DCl or NaOD in D_2O.

Curve-fitting. The ^{13}C and ^1H NMR signal areas were obtained by integration using the software supplied with the Varian VXR-500S spectrometer when the resonances were sufficiently separated so that an integration range of 32 times the width at half height to either side of the resonance of interest could be used. When the resonances were more closely spaced, resonance areas were determined using a curvefitting program which fits Lorentzian lineshapes to the peaks. The curvefitting was performed on a Sun 3/160 work station running Varian software.

RESULTS

The reduction of CpSSCp by GSH takes place in two steps, as described by Eqs. [1] and [2]. However, the equilibria are somewhat more complicated than indicated by these reactions because CpSH and its symmetrical and mixed disulfides exist as mixtures of conformational isomers as a result of *cis*/*trans* isomerism across the captopril amide bond. For example, for CpSH:



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The complete thiol/disulfide exchange and conformational isomerization scheme is shown in Fig. 1. The rate of interchange between the *cis* and *trans* isomers is slow on the NMR time scale and thus NMR spectra of CpSH and GSSCp are each a composite of the spectra of two isomers, while that for CpSSCp is a composite of the spectra of three isomers.

In this study, conditional equilibrium and rate constants, i.e., constants which describe the equilibria and kinetics of the reactions at a specific pH, were determined for the thiol/disulfide exchange reactions. The conditional equilibrium (K_{1c} and K_{2c}) and rate (k_{1c}, k_{-1c}, k_{2c}, and k_{-2c}) constants are defined in Eqs. [4]–[8]:

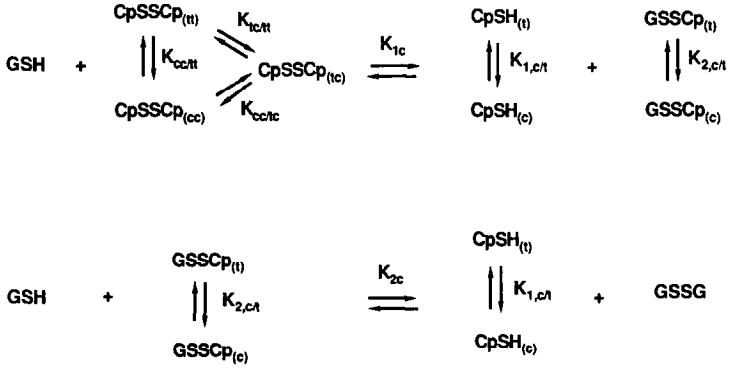

FIG. 1. The thiol/disulfide exchange equilibria and CpSH, GSSCp, and CpSSCp conformational equilibria occurring in solutions prepared by reacting GSH with CpSSCp or CpSH with GSSG. The subscripts c and t indicate *cis* and *trans* conformations across the amide bonds of CpSH and CpSSCp and the captopril part of GSSCp.

$$\text{GSH} + \text{CpSSCp} \underset{k_{-1c}}{\overset{k_{1c}}{\rightleftharpoons}} \text{GSSCp} + \text{CpSH} \quad [4]$$

$$\text{GSH} + \text{GSSCp} \underset{k_{-2c}}{\overset{k_{2c}}{\rightleftharpoons}} \text{GSSG} + \text{CpSH} \quad [5]$$

$$K_{1c} = \frac{[\text{GSSCp}]_{\text{total}}[\text{CpSH}]_{\text{total}}}{[\text{GSH}]_{\text{total}}[\text{CpSSCp}]_{\text{total}}} \quad [6]$$

$$K_{2c} = \frac{[\text{GSSG}]_{\text{total}}[\text{CpSH}]_{\text{total}}}{[\text{GSH}]_{\text{total}}[\text{GSSCp}]_{\text{total}}} \quad [7]$$

$$K_{3c} = K_{1c}K_{2c}. \quad [8]$$

The constants are defined in terms of the total concentrations which include all the various protonated species for each reactant and, as summarized in Fig. 1, two or more conformational isomers for each captopril species.

Equilibrium constants. Conditional equilibrium constants were determined over the pH range 5.0–10.5 by ^{13}C NMR. Measurements were made by ^{13}C NMR, rather than ^{1}H NMR, because it was not possible to obtain a sufficient number of resolved resonances in the ^{1}H NMR spectrum (Fig. 2). In contrast, resolution in the 125-MHz ^{13}C spectrum is sufficient to resolve resonances for four of the five species in the equilibria. To illustrate, the 39 to 43-ppm region of the ^{13}C spectrum of a reaction mixture at pH 7.04 is shown in Fig. 3. Resolved resonances are observed for the methine carbon of the propanoyl part of the *cis* and *trans* forms of CpSH at 42.89 and 42.37 ppm, respectively, and for the methylene carbons of the cysteine residues of GSSG and the *cis* and *trans* forms of GSSCp at 39.60, 39.20, and 39.40 ppm. Overlapping resonances are observed for the methine car-

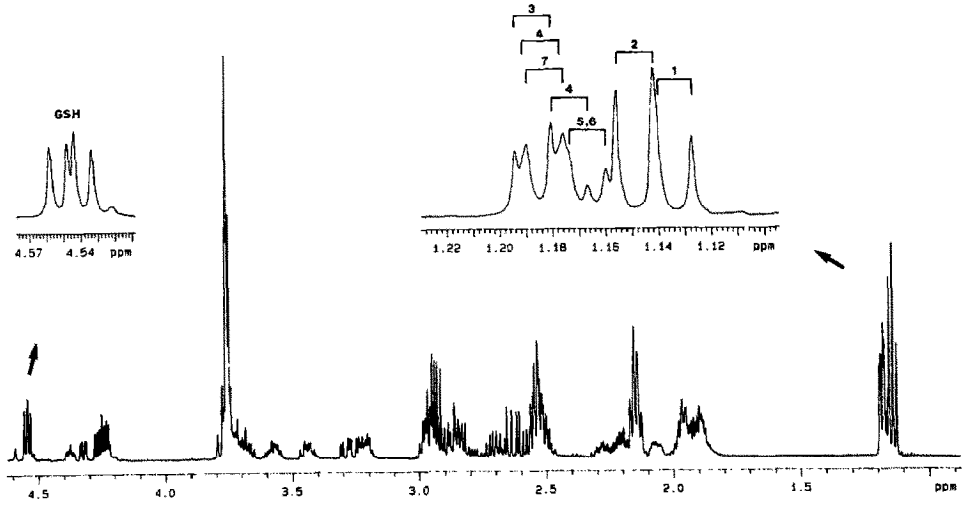

FIG. 2. A portion of the 500-MHz ^{1}H NMR spectrum of an equilibrium mixture prepared by reacting 40 mM CpSH with 20 mM GSSG at pD 7.80. The resonances centered at 4.56 ppm are for the C-α proton of the cysteine residue of glutathione. The resonances in the 1.1 to 1.2-ppm region are from the methyl groups of CpSH, GSSCp, and CpSSCp; the resonance assignments are 1, CpSH_{cis}; 2, CpSH_{trans}; 3, $\text{CpSSCp}_{trans,trans}$; 4, $\text{CpSSCp}_{trans,cis}$; 5, $\text{CpSSCp}_{cis,cis}$; 6, GSSCp_{cis}; 7, GSSCp_{trans}.

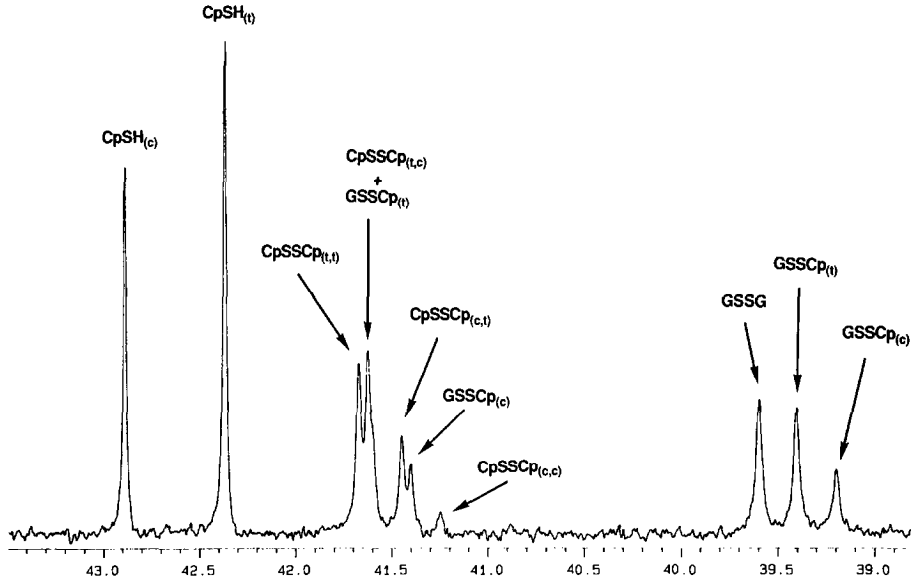

FIG. 3. A portion of the 125-MHz ^{13}C NMR spectrum of an equilibrium in mixture prepared by reacting 0.10 M CpSH with 0.05 M GSSG at pH 7.04. The specific carbons giving the assigned resonances are identified in the text.

bon of the propanoyl parts of the *cis* and *trans* forms of CpSSCp and GSSCp in the 41 to 42-ppm region. In addition, resolved resonances are observed for GSH at 26 and 56 ppm for the methylene and methine carbons, respectively, of the cysteine residue. The concentrations of all five species present at equilibrium were calculated from the integrated intensities and the initial concentrations of CpSH and GSSG.

Values were obtained for the conditional equilibrium constants K_{1c} and K_{2c} from 20 experiments covering the pH range 5–10.5. The conditional equilibrium constants were found to be invariant over the pH range 5–7; average values calculated from 12 experiments over this pH range are listed in Table 1. At pH >7, the conditional equilibrium constant decreases; average values at pH 8.02, 9.05, 10.00, and 10.50 are also listed in Table 1.

Thiol/disulfide exchange kinetics. Conditional rate constants for the reaction of GSH with CpSSCp and of CpSH with GSSG were determined from ^1H NMR spectra measured as a function of time. Thiol/disulfide exchange reactions are second-order reactions, first order in the concentrations of thiol and disulfide (*17–20*). Thus, the overall reaction sequence for the reaction of GSH with CpSSCp or of CpSH with GSSG (Eqs. [4] and [5]) consists of two consecutive, reversible second-order reactions. Because of the complexity of analyzing kinetic data for such a kinetic scheme, we have characterized the kinetics by measuring initial rates.

The areas of the resonances for the α protons of the cysteine residue of GSH (4.5–4.6 ppm in Fig. 2) and the methyl protons of CpSH (1.1–1.2 ppm) were used.

TABLE 1

Conditional Equilibrium Constants for the Reaction of Glutathione with Captopril Disulfide[a]

pH	K_{1c}	K_{2c}	K_{3c}
5–7	1.46 ± 0.13[b]	0.300 ± 0.022[b]	0.436 ± 0.046[b]
8.02	1.27	0.260	0.330
9.05	1.26	0.227	0.287
10.00	0.440	0.0412	0.0183
10.50	0.0952	0.0239	0.00228

[a] Unless noted otherwise, uncertainties are estimated to be ±7% for K_{1c} and K_{2c} and ±10% for K_{3c}.

[b] Average and standard deviation of values obtained from 12 experiments over pH range 5–7.

The areas of the resonances for the methyl protons of the CpSH were determined by curve fitting. Conditional rate constants were measured for the reaction of CpSH with GSSG over the pD range 4–9, and conditional rate constants for the reaction of GSH with CpSSCp were determined over the pD range 4.6–6.3. In the pD range 4–5.7, the rate of the reaction of CpSH with GSSG was determined from the decrease in the intensity of the CpSH methyl resonance, while at higher pH (7–9), where the reaction is more rapid, more reproducible results were obtained by following the increase in the intensity of the cysteine α proton resonance of GSH. The initial rate of the reaction of GSH with CpSSCp was determined from the decrease in the intensity of the cysteine α proton resonance of GSH with time.

The conditional rate constants k_{1c} and k_{-2c} were determined from the slope at time zero of plots of the concentration of GSH or CpSH vs time. The slopes were determined either by linear regression of the initial data points or from the derivative at $t = 0$ of a polynomial which was fitted to the data. For the reaction of GSH with CpSSCp, the initial rate equation is

$$\text{Rate} = k_{1c}[\text{GSH}]_0[\text{CpSSCp}]_0. \qquad [9]$$

Rate constant k_{1c} was determined from the slope at $t = 0$ and the initial concentrations. Rate constant k_{-2c} was obtained using the analogous initial rate equation for the reaction of CpSH with GSSG. The results are presented in Table 2. Also listed in Table 2 are values calculated for k_{-1c} and k_{2c} using the values for k_{1c} and k_{-2c} in Table 2 and K_{1c} and K_{2c} in Table 1 and the relationships $K_{1c} = k_{1c}/k_{-1c}$ and $K_{2c} = k_{2c}/k_{-2c}$.

pH independent rate and equilibrium constants. Thiol/disulfide exchange reactions proceed via the thiolate anion (*17–25*). For glutathione, the GSH^- (CO_2^-, ND_3^+, SD) form is the predominant species present over the pD range where the rate constant for the reaction of GSH with CpSSCp was measured, with the concentration of the GS^{2-}(CO_2^-, ND_3^+, S^-) form increasing as the pD increases (*26*). The increase in the fraction of glutathione in the GS^{2-} form with pD parallels the increase in k_{1c} with pD over the pD range in Table 2, confirming that GS^{2-} is the reactive glutathione species. Over this pD range, CpSSCp is present in the

TABLE 2

Conditional Rate Constants for Thiol/Disulfide Exchange

pD	k_{1c} (liters/mol-s)	k_{-1c}[a] (liters/mol-s)	k_{-2c} (liters/mol-s)	k_{2c}[b] (liters/mol-s)
4.38			$3.13 \pm 0.07 \times 10^{-4}$	
4.60	$1.34 \pm 0.01 \times 10^{-4}$			
4.80	$1.91 \pm 0.17 \times 10^{-4}$	$1.31 \pm 0.16 \times 10^{-4}$		
4.89			$5.89 \pm 0.20 \times 10^{-4}$	$1.77 \pm 0.14 \times 10^{-4}$
5.26	$3.35 \pm 0.23 \times 10^{-4}$	$2.30 \pm 0.26 \times 10^{-4}$		
5.43			$1.67 \pm 0.08 \times 10^{-3}$	$5.01 \pm 0.44 \times 10^{-4}$
5.70			$3.35 \pm 0.16 \times 10^{-3}$	$1.01 \pm 0.09 \times 10^{-3}$
6.25	$2.56 \pm 0.10 \times 10^{-3}$	$1.75 \pm 0.17 \times 10^{-3}$		
7.40[c]	8.9×10^{-3}	6.1×10^{-3}	1.5×10^{-2}	5.0×10^{-2}
7.76			$5.09 \pm 0.86 \times 10^{-2}$	
8.20			$8.1 \pm 1.4 \times 10^{-2}$	
9.10			0.162 ± 0.012	

[a] Calculated from k_{1c} and $K_{1c} = 1.46 \pm 0.13$ (Table 1).

[b] Calculated from k_{-2c} and $K_{2c} = 0.300 \pm 0.22$ (Table 1).

[c] Rate constants at pD = 7.40 were calculated from the pH independent rate constants as described in the text.

carboxylic acid deprotonated $CpSSCp^{2-}$ form. For captopril, the $CpSH^-(CO_2^-$, SD) form is the predominant species present over the pD range where the kinetics for the reaction of captopril with GSSG were studied (Table 2), with the concentration of the $CpS^{2-}(CO_2^-, S^-)$ form increasing as the pD is increased (*27*). A comparison of the pD dependence of the fractional concentration of CpS^{2-} and k_{-2c} confirms that CpS^{2-} is the reactive captopril species. Over this pD range, the $GSSG^{2-}(CO_2^-, ND_3^+)$ form is the predominant GSSG species present, with some protonation of carboxylate groups to form $GSSG^-$ and $GSSG^0$ at the low pD end and some deprotonation of ammonium groups to form $GSSG^{3-}$ and $GSSG^{4-}$ at the upper pD end. Thus, over the pD region studied, the thiol/disulfide exchange reactions proceed via the reaction sequence

$$GSH^- \rightleftharpoons GS^{2-} + H^+ \quad [10]$$

$$GS^{2-} + CpSSCp^{2-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} GSSCp^{2-} + CpS^{2-} \quad [11]$$

$$GS^{2-} + GSSCp^{2-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} GSSG^{2-} + CpS^{2-} \quad [12]$$

$$CpSH^- \rightleftharpoons CpS^{2-} + H^+, \quad [13]$$

where k_1, k_{-1}, k_2, and k_{-2} are pD independent rate constants.

The equilibrium constants for the reactions represented by Eqs. [11] and [12] are defined as

$$K_1 = \frac{[GSSCp^{2-}][CpS^{2-}]}{[GS^{2-}][CpSSCp^{2-}]} \quad [14]$$

$$K_2 = \frac{[GSSG^{2-}[CpS^{2-}]}{[GS^{2-}[GSSCp^{2-}]}. \quad [15]$$

K_1 and K_2 were calculated from K_{1c} and K_{2c} using the equations

$$K_1 = K_{1c}\frac{\alpha_1\alpha_2}{\alpha_3\alpha_4} \quad [16]$$

$$K_2 = K_{2c}\frac{\alpha_5\alpha_2}{\alpha_3\alpha_1}, \quad [17]$$

where α_1 is the fraction of $GSSCp_{(total)}$ which is in the form $GSSCp^{2-}$, α_2 the fraction of $CpSH_{(total)}$ in the CpS^{2-} form, α_3 the fraction of $GSH_{(total)}$ in the GS^{2-} form, α_4 the fraction of $CpSSCp_{(total)}$ in the $CpSSCp^{2-}$ form, and α_5 the fraction of $GSSG_{(total)}$ in the $GSSG^{2-}$ form. α_1, α_4, and α_5 are essentially 1 over the pD range 5–7, α_2 was calculated using $pK_{SH(cis)} = 9.79$ and $pK_{SH(trans)} = 10.10$ and α_3 was calculated using $pK_{SH} = 8.93$ (*26, 27*). The values obtained for K_1, K_2, and K_3 ($= K_1K_2$) using Eqs. [16] and [17] and the conditional equilibrium constants over the pD range 5–7 are 0.215 ± 0.019, 0.0445 ± 0.0036, and $9.57 \pm 0.97 \times 10^{-3}$, respectively. The values used for α_2 are the total fraction of captopril in the CpS^{2-} form, i.e., the total of the *cis* and *trans* isomers as CpS^{2-}, at a given pD and were calculated using the pK_a's for the two isomers and K_{eq} for the *cis/trans* isomerism (*27*).

Values were calculated for the pH independent rate constants k_1 and k_{-2} from the values for k_{1c} and k_{-2c} in Table 2 using the equations

$$k_1 = k_{1c}/\alpha_3 \quad [18]$$

$$k_{-2} = k_{-2c}/\alpha_2. \quad [19]$$

The value calculated for k_1 using the values determined for k_{1c} over the pD range 4.60–6.25 is 5.62 ± 0.82 liters/mol-s; the value calculated for k_{-2} using values determined for k_{-2c} over the pD range 4.5–5.3 is 106 ± 10 liters/mol-s. Using these values for k_1 and k_{-2} and the relations $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$, values of 26.1 and 4.72 liters/mol-s were calculated for k_{-1} and k_2, respectively.

DISCUSSION

It is well established that thiol/disulfide exchange reactions are mechanistically simple S_N2 displacement reactions (*17, 19*) and that they proceed via the thiolate anion (*17–25*). The pD dependence of the conditional rate constants indicates that GS^{2-} is the reactive species in the reduction of CpSSCp and GSSCp by glutathione and that CpS^{2-} is the reactive species in the reverse reactions (Eqs. [11] and [12]). Both involve the reaction of a thiolate with a disulfide in both directions, and, thus, if the position of equilibrium for the two stepwise reactions was governed by random distribution, K_1 and K_2 would be 2 and 0.5, respectively, and K_3

would equal 1. The experimental values for K_1–K_3 differ from these random distribution values by factors of 9.3, 11.2, and 104, respectively, indicating that CpS^{2-} is a better reducing agent than GS^{2-}. This difference in reducing ability can be quantitated in terms of the difference in their standard electrode potentials, which can be calculated from the overall equilibrium constant K_3:

$$\Delta E^{\circ\prime} = E^{\circ\prime}_{\text{GSSG/GSH}} - E^{\circ\prime}_{\text{CpSSCp/CpSH}} = \frac{RT}{nF} \ln \frac{1}{K_3}. \qquad [20]$$

$\Delta E^{\circ\prime}$ is calculated to be 0.0596 V; that is, CpS^{2-} is 0.0596 V more reducing than GS^{2-}.

K_3, and thus this difference in reducing potential, is given in terms of the species which react. However, at physiological pH, the species GS^{2-} and CpS^{2-} are a relatively small fraction of the total glutathione and captopril. Also, since the pK_a's of the thiol groups of GSH and CpSH are different, the fractions of GSH and CpSH present as GS^{2-} and CpS^{2-}, respectively, will be different. Thus, at physiological pH, the conditional equilibrium and rate constants differ from those calculated above for $K_1 - K_3$ and k_1, k_{-1}, k_2, and k_{-2}. The conditional constants for physiological pH are $K_{1c} = 1.46$, $K_{2c} = 0.300$ (Table 1), and $K_{3c} = 0.438$, and $k_{1c} = 8.9 \times 10^{-3}$, $k_{-1c} = 6.1 \times 10^{-3}$, $k_{2c} = 0.015$, and $k_{-2c} = 0.05$ liters/mol-s.

From the above value for K_{3c}, $\Delta E^{\circ\prime}$ is calculated to be 0.011 V at physiological pH. In contrast, $E^{\circ\prime}_{\text{GSSG/GSH}} - E^{\circ\prime}_{\text{CSSC/CSH}}$, where CSSC and CSH represent cystine and cysteine respectively, is reported to be in the range −0.013 to −0.018 V at pH 6.6–7.0 (*21, 23, 28*); i.e., GSH is more strongly reducing than CSH by −0.013 to −0.018 V at physiological pH. Thus, the equilibrium constants for thiol/disulfide exchange predict that CpSH has a stronger tendency to reduce disulfide bonds than do GSH and CSH. This prediction is in agreement with experiments: e.g., it has been found that CpSH is a more efficient activator of papain by disulfide reduction than is CSH (*29*). CpSH also has been shown to inactivate oxytocin and vasopressin and to cleave gamma-globulin to light and heavy chains by reduction of disulfide bonds (*29*).

It is of interest to consider the significance of these results with respect to the pharmacokinetics of captopril. The conditional equilibrium constants, and $\Delta E^{\circ\prime}$, indicate that the thiol group of captopril is more strongly reducing than the thiol group of GSH, which is the most abundant nonprotein thiol in biological systems (*30*). Thus, the relative reducing strengths will favor the formation of CpSSCp and its mixed disulfides, which have been detected as the major metabolites of captopril (*4–11*). The rate constants indicate that CpSSCp and GSSCp can be reduced to therapeutically active CpSH by thiol/disulfide exchange; however, they also indicate that such reactions will be slow. For example, at a GSH concentration of 2 mM, as found in erythrocytes (*31*), the conditional rate constants k_{1c} and k_{2c} predict the half-life of CpSSCp and GSSCp to be 10.8 and 6.4 h, respectively. These half-lives are somewhat longer than have been observed in studies of the reduction of CpSSCp in hemolysate (half-life ~1 h at 37°C). The rate constants in the present work were measured at 25°C; even if the rate constants are two to three times larger at 37°C, the predicted half-life for CpSSCp in erythrocytes is still longer than that observed, which supports the conclusion of Lan *et al.* (7) that

in vivo reduction of symmetrical and mixed disulfides of captopril involves both enzymatic and nonenzymatic, i.e., thiol/disulfide, processes.

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