A nuclear magnetic resonance study of the formation and conformational equilibria of symmetrical and mixed disulfides of captopril

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The oxidation of captopril (CpSH, 1-(D-3-mercapto-2-methylpropanoyl)-1-proline) by glutathione disulfide (GSSG) via thiol/disulfide exchange to form, in the first step, CpSSG and GSH and, in the second step, CpSSCp and GSH, has been studied in aqueous solution by ^{1}H nmr. Due to slow rotation around the amide bond(s) of CpSH and CpSSCp and of the captopril part of CpSSG, separate resonances are observed for the *cis* and *trans* conformations across these bonds. Conformational equilibrium constants were estimated as a function of pH for CpSH, CpSSCp, and CpSSG from the intensities of resonances for the *cis* and *trans* isomers. These equilibrium constants were used in the determination of equilibrium constants for the two steps in the oxidation of CpSH by GSSG. The results suggest that CpSH has a greater tendency to reduce disulfide bonds by thiol/disulfide exchange at physiological pH, and thus form mixed disulfides, than do the thiol groups in amino acids. Also, the conformational equilibrium constants indicate that, at physiological pH, approximately two thirds of the captopril, either free or in a disulfide form, has the *trans* conformation.

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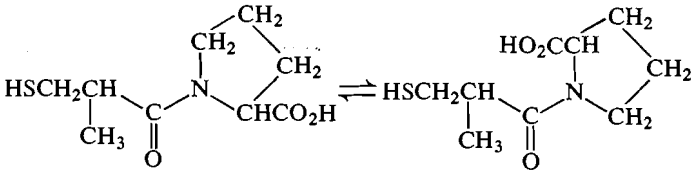
Faisant appel à la rmn du ^{1}H et opérant en solutions aqueuses, on a étudié l'oxydation du captoprile (CpSH, (D-mercapto-3 méthyl-2 propanoyl)-1 proline-1) par le disulfure de glutathion (GSSG) via un échange thio/disulfure qui permet de former le CpSSG et le GSH dans une première étape et le CpSSCp et le GSH dans une deuxième étape. A cause de la rotation lente autour des liaisons amide(s) du CpSH, du CpSSCp et de la partie captoprile du CpSSG, on a pu observer des résonances distinctes pour les conformations *cis* et *trans* par rapport à ces liaisons. Utilisant les intensités des résonances des isomères *cis* et *trans*, on a évalué les constantes d'équilibres conformationnels du CpSH, du CpSSCp et du CpSSG en fonction du pH. On a utilisé ces constantes d'équilibre pour déterminer les constantes d'équilibre des deux étapes de l'oxydation du CpSH par le GSSG. Les résultats suggèrent que, par comparaison avec les thiols des acides aminés, le CpSH a une plus grande tendance à réduire les liaisons disulfures, par un échange thiol/disulfure à pH physiologique, et ainsi à former des disulfures mixtes. Les constantes d'équilibres conformationnels indiquent également, à pH physiologique, qu'approximativement les deux tiers du captoprile, tant sous la forme libre que sous la forme de disulfure, existe sous une conformation *trans*.

[Traduit par le journal]

Introduction

Captopril, 1-(D-3-mercapto-2-methylpropanoyl)-1-proline, is used clinically for the treatment of hypertension (1–3). Antihypertensive activity is thought to result from inhibition of angiotensin I-converting enzyme, with the thiol group of captopril binding to the active site of the enzyme (1–3). The thiol group is also the key functional group in the metabolism of captopril, with major metabolites being captopril disulfide and mixed disulfides with thiol-containing amino acids, peptides, and proteins (4–7).

Captopril exists in solution as an equilibrium mixture of *trans* (**1**) and *cis* (**2**) isomers with respect to the conformation



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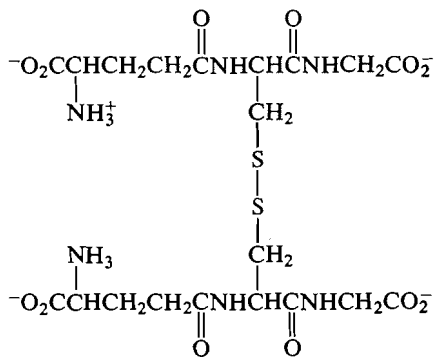
across the peptide bond (8). Although the conformational equilibrium of captopril has been characterized in detail (8), the chemistry of formation of disulfides from captopril and their conformational equilibria have not. This is of interest since it has been suggested that disulfide metabolites may play a role in determining the nature and time course of adverse reactions to captopril (9). In this paper, we report the results of an ^{1}H nmr study of the formation of symmetrical and mixed disulfides by



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oxidation by glutathione disulfide (**3**) via thiol/disulfide exchange as described by eqs. [1] and [2].

[1] CpSH + GSSG ⇄ CpSSG + GSH

[2] CpSH + CpSSG ⇄ CpSSCp + GSH

where CpSH, CpSSCp, GSH, GSSG, and CpSSG are captopril, captopril disulfide, glutathione, glutathione disulfide, and captopril–glutathione mixed disulfide, respectively. As part of this study, the *cis–trans* conformational equilibrium across the amide bond(s) of CpSH and CpSSCp and the captopril part of CpSSG have also been characterized by ^{1}H nmr.

Glutathione disulfide was chosen for this study because it occurs naturally (10), captopril–glutathione mixed disulfide (CpSSG) has been detected as a metabolite of captopril (4, 5), and, with the cystine between two amino acid residues, GSSG

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serves to a first approximation as a model for disulfide bonds in proteins.

Experimental

The captopril and captopril disulfide used in this study were gifts from The Squibb Institute for Medical Research, Princeton, N.J. Glutathione disulfide was obtained from Sigma Chemical Co. The 99.7% D_2O, 40% NaOD in D_2O, and 35% DCl in D_2O were obtained from Merck Sharp and Dohme, Ltd.

The ^1H nmr spectra were measured at 360 MHz on a Bruker WM-360 spectrometer operating in the pulse/Fourier transform mode. The probe temperature was 25 ± 1°C. Chemical shifts were measured relative to internal *tert*-butyl alcohol and are reported relative to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The resolution was increased in some spectra with the Gaussian resolution enhancement routine in the Aspect 2000 software.

The pH measurements were made at 25°C with a Fisher Accumet pH meter equipped with a standard glass electrode and a fiber-tipped, saturated calomel reference electrode, or a microcombination electrode. Fisher certified standard solutions having nominal pH values of 4.00, 7.00, and 10.00 were used for calibrating the pH meter. The exact pH of each standard solution was determined by comparison with freshly prepared N.B.S. pH standard solutions (11). pH values reported are the meter readings; to indicate that pH readings for D_2O solutions were not corrected for the deuterium isotope effect (12), they are reported as pH*.

Solutions were prepared in 99.7% D_2O containing 1 *M* KCl and approximately 0.0001 *M* *tert*-butyl alcohol. Na_2H_2EDTA (0.003 *M*) was also added to solutions containing thiols, e.g. the equilibrium mixture resulting from the reaction of CpSH with GSSG, to complex traces of heavy metals which would catalyze the oxidation of the thiols (13). The solvent mixture was deoxygenated by bubbling with argon or nitrogen before addition of thiol to minimize oxidation by dissolved oxygen. Inert gas was also blown over the solution during adjustment of pH, and nmr tubes were flushed out with the gas before transfer of an aliquot of solution to the tube. To ensure that no air oxidation of thiol occurred in the measurement of equilibrium constants for thiol/disulfide exchange reactions of CpSH with GSSG, the sample solutions were degassed further by taking them, in an nmr tube, through at least three freeze–pump–thaw cycles on a vacuum line.

Results

In aqueous solution, captopril and its disulfides exist in a conformational equilibrium due to *cis*/*trans* isomerization across the captopril amide bond. To quantitatively characterize the oxidation of CpSH by GSSG, it was necessary to first characterize these conformational equilibria for CpSH, CpSSCp, and CpSSG.

Conformational equilibria

The 360-MHz ^1H nmr spectra of captopril and captopril disulfide are shown in Fig. 1. Each spectrum consists of two highly coupled spin systems: the $—CH_2CHCH_3$ spin system from the 3-mercapto-2-methylpropanoyl part and the $—CH_2CH_2CH_2CH—$ spin system from the proline residue. The spectra are further complicated by the presence of *cis* and *trans* isomers with respect to the conformation across the amide bond. This results in a doubling of the resonances in the spectrum of captopril, as is clearly evident in the multiplet patterns at 3.4–3.9 and 4.26–4.39 ppm for the hydrogens on C_δ and C_α respectively of the proline residue and the methyl resonances at 1.12–1.17 ppm (Fig. 2). In the 3.4–3.9 ppm region, the resonances at 3.4–3.66 and 3.66–3.9 ppm are from the *cis* and *trans* isomers respectively (8). In the 4.26–4.39 and 1.12–1.17 ppm regions, the more intense multiplets are from the *trans* isomer. The assignment of the other resonances for captopril are: 1.8–2.4 ppm, the hydrogens on C_β and C_γ of the proline residue; 2.48–3.06 ppm, the two hydrogens on C3 and the one hydrogen on C2 of the 3-mercapto-2-methylpropanoyl part. The spectrum of captopril disulfide is composed of the spectrum for the captopril unit in four different environments due to the *cis–trans* isomerism: $CpSSCp_{(tt)}$ in which both amide bonds are *trans*, $CpSSCp_{(tc)}$ in which one amide bond is *cis* and the other is *trans*, and $CpSSCp_{(cc)}$ in which both amide bonds are *cis*. This results in an apparent lower resolution over the 1.9–2.4 and 3.2–3.8 ppm regions due to the overlap of resonances for the two *trans* environments ($CpSSCp_{(tt)}$ and $CpSSCp_{(tc)}$) and overlap of resonances for the two *cis* environments ($CpSSCp_{(tc)}$ and $CpSSCp_{(cc)}$). Four doublets from the four different environments are observed in the methyl region (Fig. 2). The assignments are given in Table 1; the basis of these assignments is discussed below.

TABLE 1. Identification of captopril-containing species

Species	Conformation of the captopril amide bond(s)	Species number
$CpSH_{(t)}$	*trans*	1
$CpSH_{(c)}$	*cis*	2
$CpSSCp_{(tt)}$	*trans*, *trans*	3
$CpSSCp_{(tc)}$	*trans*, *cis*	4
$CpSSCp_{(cc)}$	*cis*, *cis*	5
$CpSSG_{(t)}$	*trans*	6
$CpSSG_{(c)}$	*cis*	7

TABLE 2. *cis–trans* Conformational equilibrium constants for proline-containing molecules[a]

Compound	Low pH[b]	Neutral pH[c]	High pH[d]
Glycyl-L-proline[e]	0.19	0.54	0.72
Glycyl-L-hydroxyproline[e]	0.14	0.52	0.59
L-Alanyl-L-proline[e]	0.12	0.54	0.89
Captopril	0.15	0.59	0.41
Captopril disulfide	0.20[f]	0.39[f]	0.39[f]
Captopril–glutathione mixed disulfide	0.16	0.49	0.35

[a] Defined as $K_{c/t} = [cis]/[trans]$ where *cis* and *trans* refer to the conformation across the proline amide bond.
[b] Carboxyl, thiol, and amino groups, if present, are protonated.
[c] Carboxyl groups deprotonated; thiol and amino groups, if present, protonated.
[d] Carboxyl, thiol, and amino groups, if present, deprotonated.
[e] Reference 13.
[f] $K_{c/t} = [cis]_{total}/[trans]_{total}$.

Fractional concentrations of the two isomers of captopril were determined as a function of pH* from the relative intensities of the two methyl doublets and from the relative intensities of the resonances for the hydrogens on C_δ of the proline residue in the *cis* and *trans* isomers. The fractional concentrations of the *trans* isomer are 0.87 ± 0.01, 0.63 ± 0.01, and 0.71 ± 0.01 for the fully-protonated, monoprotonated, and deprotonated forms of captopril, which correspond to the equilibrium constants, $K_{1c/t} = [cis]/[trans]$, listed in Table 2.

The fractional concentrations of the three isomers of captopril disulfide were determined from the relative intensities of the methyl resonances (Fig. 2). Equations [3]–[6] describe the equilibria between the three forms of captopril disulfide.

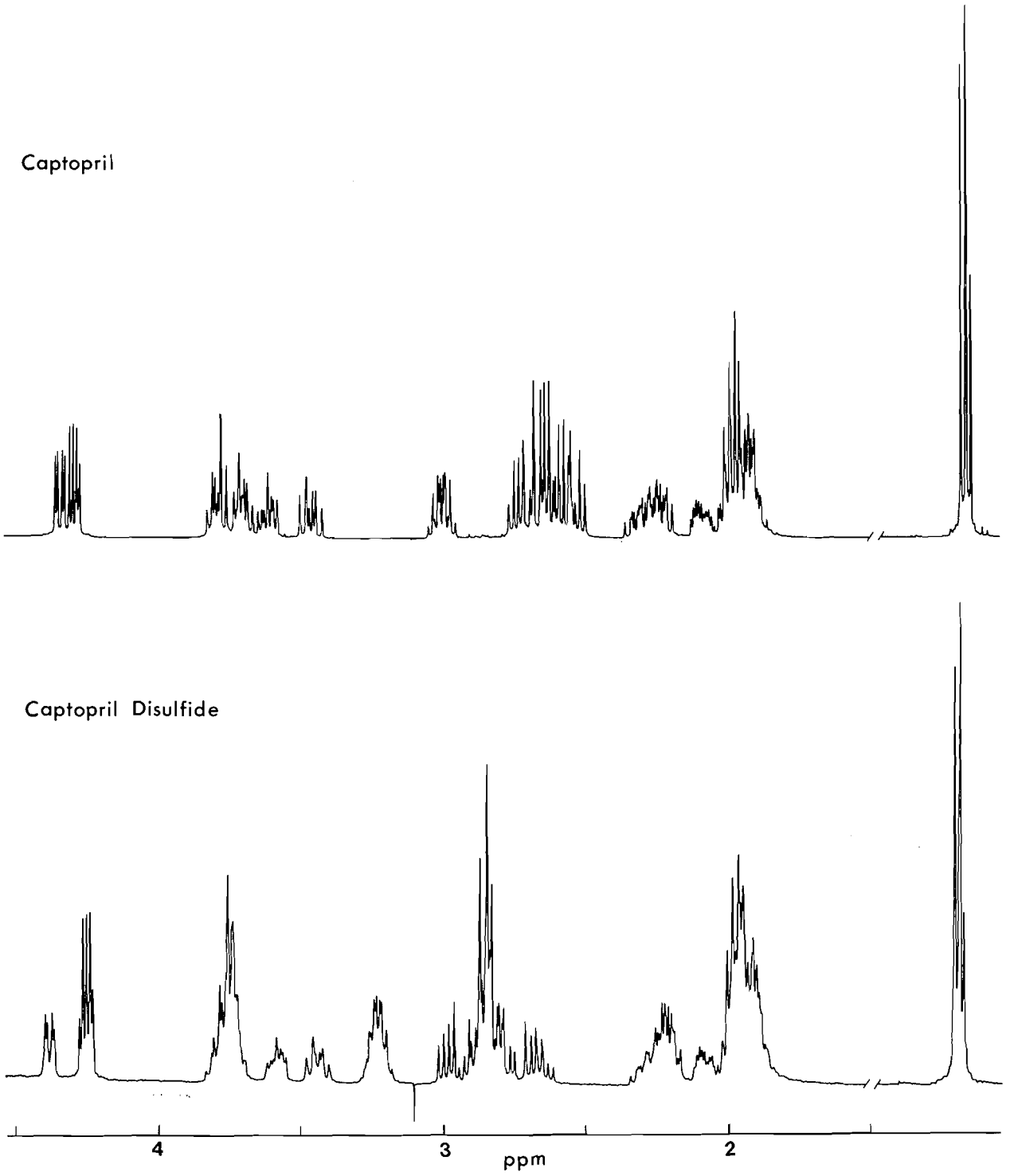


FIG. 1. The 360-MHz ^{1}H nmr spectra of 0.062 *M* captopril (pH* 6.0) and 0.042 *M* captopril disulfide (pH* 6.0). The gain was reduced by 2 in the 1–1.5-ppm region.

$$\begin{array}{ccc} & \mathrm{CpSSCp}_{(tt)} & \\ \swarrow & & \searrow \\ \mathrm{CpSSCp}_{(tc)} & \rightleftharpoons & \mathrm{CpSSCp}_{(cc)} \end{array} \quad [3]$$

$$K_{cc/tt} = \frac{[\mathrm{CpSSCp}_{(cc)}]}{[\mathrm{CpSSCp}_{(tt)}]} \quad [4]$$

$$K_{tc/tt} = \frac{[\mathrm{CpSSCp}_{(tc)}]}{[\mathrm{CpSSCp}_{(tt)}]} \quad [5]$$

$$K_{cc/tc} = \frac{[\mathrm{CpSSCp}_{(cc)}]}{[\mathrm{CpSSCp}_{(tc)}]} \quad [6]$$

Equilibrium constants were determined at pH* 5.90, 5.99, and 6.10 and the results are: $K_{cc/tt} = 0.144 \pm 0.005$, $K_{tc/tt} = 0.84 \pm 0.01$, and $K_{cc/tc} = 0.171 \pm 0.006$. Total fractional concentrations of the *cis* and *trans* environments[2] were also deter-

[2] Fractional concentration of *trans* = $(2[\mathrm{CpSSCp}_{(tt)}] + [\mathrm{CpssCp}_{(tc)}])/2[\mathrm{CpSSCp}]_{\mathrm{total}}$.

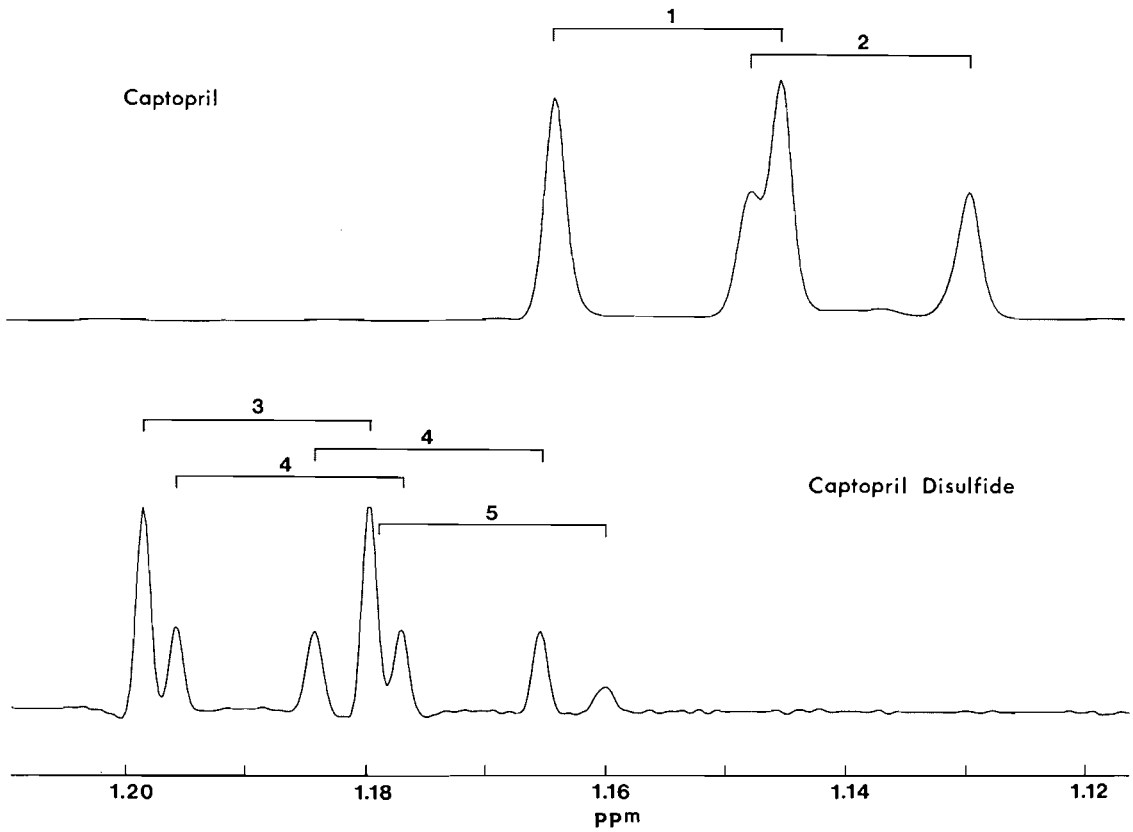
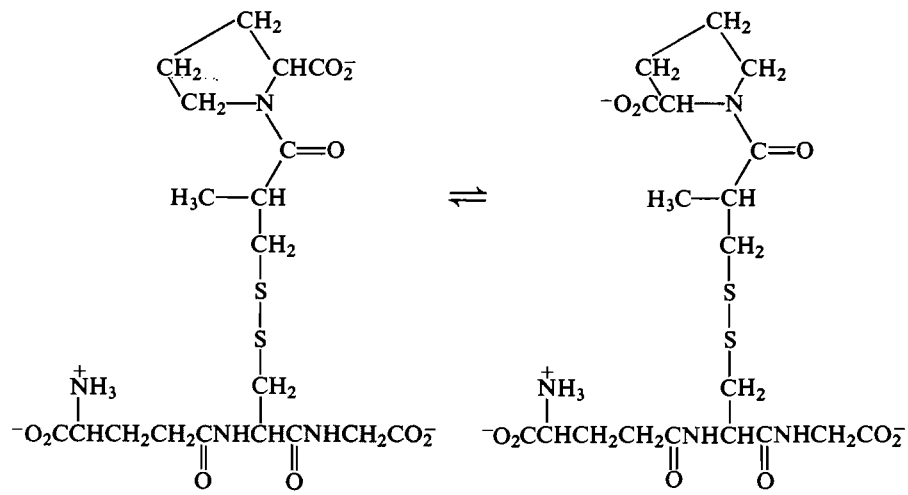


FIG. 2. The methyl resonances for the solutions described in the legend to Fig. 1. Resolution was enhanced by doing a Gaussian multiplication with the Bruker Aspect 2000 software. Resonance assignments are given in Table 1.

mined as a function of pH* directly from relative intensities of the *cis* and *trans* C_δ—H resonances. The fractional concentration of the *trans* conformation is 0.72 ± 0.03, independent of pH over the pH* range 3.75–11.02, corresponding to an equilibrium constant, $K_{c/t} = [cis]_{total}/[trans]_{total}$, of 0.39 ± 0.05. This is in excellent agreement with the value predicted by the above values for $K_{cc/tt}$, $K_{tc/tt}$, and $K_{cc/tc}$.[3] As the pH is decreased and the carboxylate groups are protonated, the fractional concentration of the *trans* isomer increases to 0.83 at pH* 0.75, corresponding to $K_{c/t} = 0.20$.

To obtain a solution containing CpSSG for the study of its conformational equilibrium (eq. [7]) 0.009 *M* CpSH was reacted with 0.18 *M* GSSG at pH* 7.1 in 1 *M* KCl solution. CpSSG, GSH, and CpSSCp were formed by thiol/disulfide exchange, as described by eqs. [1] and [2]. After reaction for 15 min, the solution was stirred in air for 18 days to oxidize CpSH to CpSSCp and GSH to GSSG to simplify the nmr spectrum. The methyl region of the ^{1}H nmr spectrum is shown in Fig. 3 and the assignments are given in Table 1. As indicated in Fig. 3, the dominant resonances are from CpSSG. Using the

[7]



[3] $K_{c/t} = (2K_{cc/tt} + K_{tc/tt})/(2 + K_{tc/tt})$.

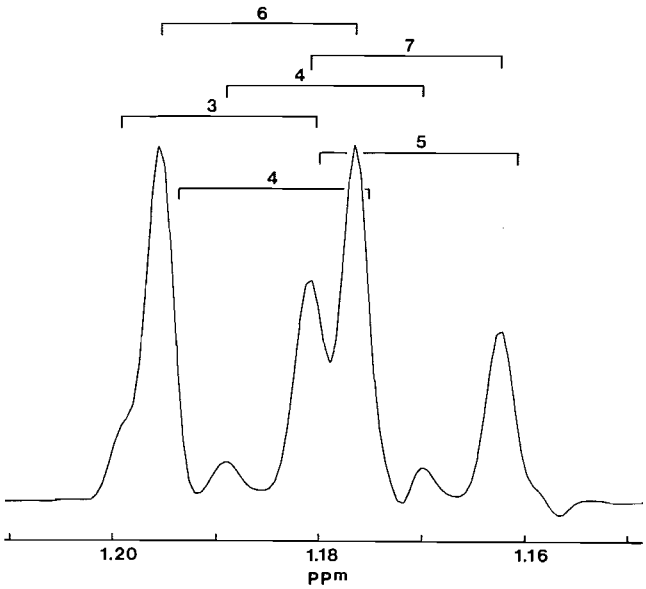


FIG. 3. The methyl region of the ^{1}H nmr spectrum of a pH* 7.0 solution contaning captopril–glutathione mixed disulfide and captopril disulfide. Details of the preparation of the mixed disulfide are given in the text. Resolution was enhanced by doing a Gaussian multiplication with the Bruker Aspect 2000 software.

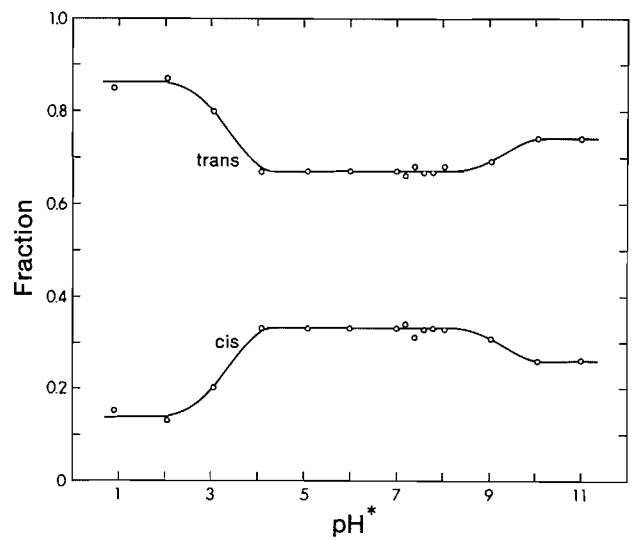


FIG. 4. The fractional concentrations of captopril–glutathione mixed disulfide in which the amide bond of the captopril part has the *trans* and *cis* conformations.

relative intensities of the methyl doublets for CpSSG, the fractional concentrations of the *trans* and *cis* forms of CpSSG were determined as a function of pH* over the pH* range 0.92–10.98 (Fig. 4). The equilibrium constants listed in Table 2 were obtained from these fractional concentrations.

Oxidation of CpSH by GSSG

To characterize the oxidation of CpSH by GSSG, equilibrium constants were determined for the reactions described by eqs. [1] and [2]. CpSH was reacted with GSSG in sealed nmr tubes, which had been degassed by the freeze–pump–thaw technique to minimize air oxidation, and then concentrations at equilibrium were determined by ^{1}H nmr. Figure 5 shows the methyl region of the nmr spectrum for a pH*

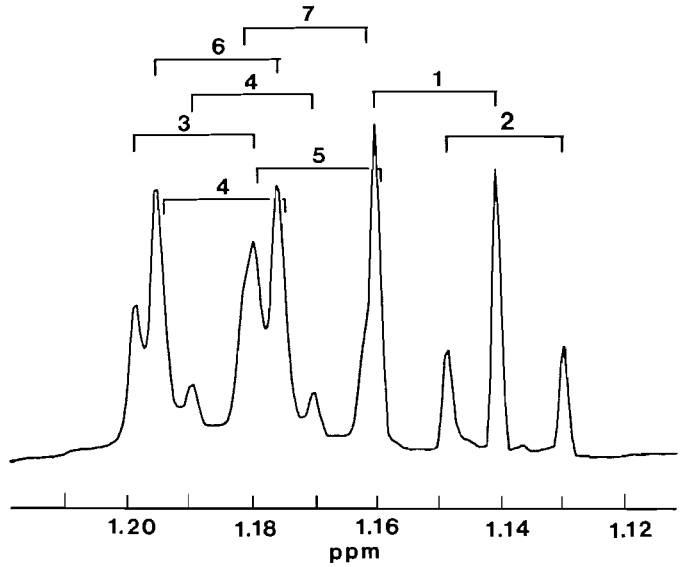


FIG. 5. The methyl region of the ^{1}H nmr spectrum of a solution prepared by reacting 0.045 *M* CpSH with 0.041 *M* GSSG at pH* 6.0 for 341 h in a degassed, sealed nmr tube. Resolution was enhanced by doing a Gaussian multiplication. Resonance assignments are given in Table 1.

6.0 solution at equilibrium; the spectrum was obtained 341 h after mixing the reactants to ensure that equilibrium had been achieved. As indicated in Fig. 5, the spectrum contains resonances for the *cis* and *trans* forms of CpSH, CpSSCp, and CpSSG. The concentrations of the various species were determined from the relative intensities of the resonances in Fig. 5 and the initial reactant concentrations. Using the determined concentrations, the conditional equilibrium constants, K_{1c} and K_{2c}, as defined by eqs. [8] and [9] and the equilibrium reactions in Fig. 6, were calculated.

$$[8] \quad K_{1c} = \frac{[\text{CpSSG}]_{\text{total}}[\text{GSH}]}{[\text{CpSH}]_{\text{total}}[\text{GSSG}]}$$

$$[9] \quad K_{2c} = \frac{[\text{CpSSCp}]_{\text{total}}[\text{GSH}]}{[\text{CpSH}]_{\text{total}}[\text{CpSSG}]_{\text{total}}}$$

where $[\text{CpSH}]_{\text{total}}$, $[\text{CpSSCp}]_{\text{total}}$, and $[\text{CpSSG}]_{\text{total}}$ are the total concentration (*cis* + *trans*) of CpSH, CpSSCp, and CpSSG. The procedure involved determining the fractions of the total area of the methyl resonances due to CpSH, CpSSCp, and CpSSG. The total area of the resonances for CpSH was obtained by doubling the sum of the areas of its resonances at 1.13 and 1.14 ppm. That due to CpSSCp was calculated from the area of its resonances at 1.17 and 1.19 ppm and the values determined above for $K_{tc/tt}$, $K_{cc/tt}$, and $K_{cc/tc}$, and that for CpSSG was calculated as the difference between the total methyl resonance area and the areas for the CpSH and CpSSCp resonances. Using these fractions of the total methyl resonance area and the initial concentration of CpSH, the concentrations of CpSH, CpSSCp, and CpSSG were obtained. From these concentrations and the initial GSSG concentration, the concentrations of GSH and GSSG were calculated. The values calculated for K_{1c} and K_{2c} from three separate experiments in the pH* range 5.9–6.1 are 2.1 ± 0.1 and 1.5 ± 0.6 respectively. The rather large uncertainties reflect the accumulation of errors that results from obtaining concentrations by difference.

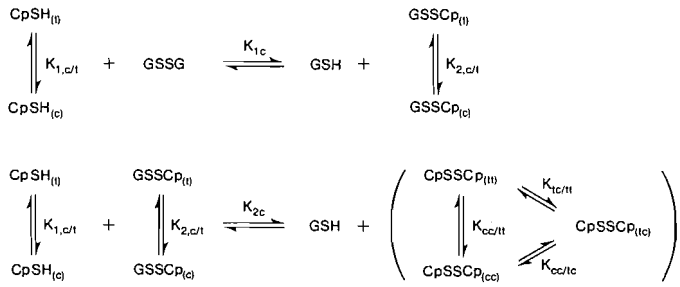


FIG. 6. The thiol/disulfide exchange equilibria and CpSH, CpSSCp, and CpSSG conformational equilibria occurring in a solution prepared by reacting CpSH and GSSG. The subscripts *t* and *c* indicate *trans* and *cis* conformations across the amide bond(s) of captopril in CpSH and CpSSCp or the captopril part of CpSSG.

Discussion

Conformational equilibria

Assignment of the *trans* conformation to the more abundant isomer(s) of CpSSCp and CpSSG is based on previous findings that the *trans* conformation is the more abundant for proline-containing dipeptides and related molecules, e.g. *tert*-butyloxycarbonyl-glycyl-L-proline-benzylester (14), glycyl-L-proline, glycyl-L-hydroxyproline, L-alanyl-L-proline, *N*-acetylsarcosine, glycylsarcosine (15), and captopril (8), and that the fractional concentration of the *trans* conformation increases when the carboxylate group is protonated (15). For example, the fractional concentration of the *trans* conformation of glycyl-L-proline increases from 0.65 to 0.84 when the carboxylate group is protonated (15), which is very similar to the increases found in this study for the more abundant isomers of CpSSCp and CpSSG.

The *cis/trans* equilibrium constants for the carboxyl-protonated (low pH) forms of CpSH, CpSSCp, and CpSSG are quite similar to each other and to those for dipeptides in which proline is the C-terminal residue, as indicated by the results in Table 2. The enhanced stability of the *trans* isomer at low pH probably results from intramolecular hydrogen bonding between the carboxylic acid hydrogen and the captopril amide carbonyl oxygen, which is not possible in the *cis* isomer. Evidence has been presented previously which indicates this to be the origin of the enhanced stability of the *trans* isomer of peptides in which protonated proline or sarcosine is the C-terminal residue (15–17). It is interesting to note that $K_{c/t}$ is approximately the same for the high pH forms of CpSH, CpSSCp, and CpSSC but is somewhat less than for the high pH forms of the proline-containing dipeptides. The origin of the enhanced stability of the *trans* conformation in the captopril unit at high pH is not obvious.

The magnitude of the *cis/trans* equilibrium constants for the neutral pH forms of CpSH, CpSSCp, and CpSSG suggests that, at physiological pH, approximately two thirds of the captopril has the *trans* conformation, regardless of whether it is free or in a disulfide form.

Oxidation of CpSH by GSSG

The reactive species in the oxidation of CpSH by GSSG via thiol/disulfide exchange (eqs. [1] and [2]) is presumably CpS^-, since it is well established that thiol/disulfide exchange proceeds via the thiolate anion (18–27). At pH 6, only a small fraction of CpSH is in the form of CpS^-, and thus the constants obtained in this work for eqs. [1] and [2] are conditional equilibrium constants. Conditional equilibrium constants for thiol/disulfide exchange in related systems are essentially pH independent up to pH values at which deprotonation of the thiol groups begins to occur (18), suggesting that the conditional equilibrium constants obtained in this work describe the reaction of CpSH with GSSG up to at least pH ~8.

The conditional equilibrium constant for the overall reaction (eq. [10])

[10] $2CpSH + GSSG \rightleftarrows CPSSCp + 2GSH$

K_{3c}, is calculated to be 3.2 ± 1.2 using the relation $K_{3c} = K_{1c}K_{2c}$. If the equilibrium position were governed by random distribution, K_{1c} and K_{2c} would be 2 and 0.5 respectively and K_{3c} would be 1. For comparison with the values for the reaction of CpSH with GSSG, $K_1 = 1.27$, $K_2 = 0.27$, and $K_3 = 0.34$ for the reaction of cysteine (CSH) with GSSG at pH 6.6 and 25°C (27) and $K_{1c} = 1.36$, $K_{2c} = 0.039$, and $K_{3c} = 0.053$ for the reaction of penicillamine (PSH) with GSSG at pH 7.4 and 25°C (18). The values for K_3 for the reactions involving CpSH and CSH are within a factor of 3 of the random distribution value, whereas that for PSH is somewhat smaller. PSH differs from CpSH and CSH in that it has two bulky methyl groups next to its thiol group, which hinders its reaction with penicillamine–glutathione mixed disulfide in the second step (18).

The overall equilibrium constant K_{3c} is related to the difference between the formal electrode potentials, $E^{0'}$, of the GSSG/GSH and CpSSCp/CpSH couples according to eq. [11].

$$[11] \quad \Delta E^{0'} = E^{0'}_{GSSG/GSH} - E^{0'}_{CpSSCp/CpSH} = \frac{RT}{nF} \ln K_{3c}$$

With the value of 3.2 for K_{3c}, $\Delta E^{0'}$ is calculated to be 0.015 V. For comparison, $E^{0'}_{GSSG/GSH} - E^{0'}_{CSSC/CSH}$, where CSSC is cystine, is reported to be in the range −0.013 to −0.018 V at pH 6.6–7.0 (21, 23, 27) and $E^{0'}_{GSSG/GSH} - E^{0'}_{PSSP/PSH}$, where PSSP is penicillamine disulfide, is −0.0378 V at pH 7.4 (18). Values reported for $E^{0'}_{GSSG/GSH}$ are −0.205 V vs. the standard hydrogen electrode at 30°C and pH 7 (19) and −0.24 V at 40°C and pH 7 (28). These results indicate that CpSH has a greater tendency to reduce disulfide bonds by thiol/disulfide exchange than do the thiol groups in amino acids and that, if the disulfide bond in GSSG is similar to those in proteins, CpSH is capable of reducing protein disulfide bonds. These conclusions are supported by the finding that CpSH can activate papain by reduction of a disulfide bond at the active site and that CpSH is a more efficient activator of papain than is cysteine (29).

Acknowledgements

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