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# Free Radical Scavenging and Copper Chelation: A Potentially Beneficial Action of Captopril

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Captopril (CpSH), an angiotensin converting enzyme (ACE) inhibitor, is reported to provide protection against free-radical mediated damage. The purpose of this study was to investigate, by means of pulse radiolysis technique, the behaviour of CpSH towards radiation-induced radicals in the absence and in the presence of copper(II) ions, which can play a relevant role in the metal catalysed generation of reactive oxygen species. The results indicate that the –SH group is crucial in determining the radical scavenging action of CpSH and the nature of the resulting CpSH transient products in the absence or in the presence of oxygen.

In the presence of Cu(II), the –SH group is still involved in the biological action of the molecule participating both in the one-electron reduction of Cu(II) with formation of CpSSCp, and in Cu(I) chelation. This conclusion is supported by the Raman spectroscopic data which allow to identify the CpSH sites involved in the copper complex at different pH.

These results suggest that CpSH may potentially inhibit oxidative damage both through free radical scavenging and metal chelation. Considering the low CpSH concentration *in vivo*, the metal chelation mechanism, more than the direct radical scavenging, could play the major role in moderating the toxicological effects of free radicals.

*Keywords:* Captopril, captopril/copper complexes, hydroxyl radicals, peroxyl radicals, Raman spectroscopy, pulse radiolysis

## INTRODUCTION

Captopril (CpSH), a synthetic 3-mercapto-2-methylpropionyl derivative of L-proline, has been specifically designed to bind to the active site of the angiotensin converting enzyme (ACE), which catalyses the conversion of angiotensin I to angiotensin II, a potent vaso-constrictor.[1,2] The peculiarity of CpSH, responsible for its specific affinity toward ACE, is the presence of a sulfhydryl group which complexes the zinc ion of the enzyme. The competitive inhibition of ACE by CpSH results in a reduction of hypertension.[3]

Thiol-containing ACE inhibitors, including CpSH, are reported to possess anti-inflammatory activity and are widely used in the treatment of congestive heart failure, chronic renal failure and diabetic nephropathy.[4–6] More recently, CpSH was found to be capable of providing protection

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against arrhythmias caused by ischaemia/reperfusion.[7] Since post-ischaemic myocardial dysfunction is mediated, at least in part, by the generation of free radicals, the beneficial effect of CpSH could be related to its ability to scavenge free radicals.[8] In this regard, CpSH has been found to react fast *in vitro* with $^{\bullet}OH$ and other reactive oxygen species (ROS), and to be a powerful scavenger of hypohalite species ($OCl^-$) and hypochlorous acid (HOCl), another ROS that can damage the myocardium.[9] Conversely, CpSH reacts very slowly with other ROS, like $O_2^{-\bullet}$ and $H_2O_2$, which are involved in post-ischaemic myocardial injury, too. However, it is unlikely that CpSH can act as an antioxidant *in vivo* by scavenging free radicals. In fact, its therapeutically achievable concentration in body fluids is in the micromolar range.[10]

It is to be considered that $O_2^{-\bullet}$ and $H_2O_2$ in the presence of transition metal ions as iron and copper, may be converted into highly reactive $^{\bullet}OH$.[11] Since Fe and Cu ions are mobilised following myocardial ischaemia, they could play a relevant role in the oxidative stress-induced damage. Thus, taking into account the foregoing information, it is of interest to investigate the interaction of CpSH with Cu(II) ions. In fact, one of the mechanisms by which an antioxidant can protect the potential biological targets from oxidative stress is the chelation of transition metal ions, so preventing the metal-catalysed generation of ROS.[12]

CpSH contains three possible sites capable of metal binding: the carboxylic, amide and mercapto groups (Figure 1). Therefore, its antioxidant action *in vivo* could be also related to the interaction of the drug with metals, interfering with the ROS-mediated processes.

In order to gain further insight into the molecular mechanism involved in the antioxidant action of CpSH, a comparative study on the behaviour of the molecule towards radiation-induced radicals was undertaken both in the absence and in the presence of copper(II) ions. Raman spectroscopic analysis was also performed to identify the functional groups of the molecule involved in the potential coordination of copper ions.

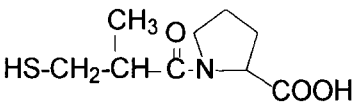


FIGURE 1 Chemical structure of captopril (CpSH).

## MATERIALS AND METHODS

Captopril (1-[2(S)-3-mercapto-2-methylpropionyl]-L-proline) by Sigma, $CuCl_2 \cdot 2H_2O$, by Fluka, p-nitroacetophenone (PNAP) by Merck and all other chemicals of Analar grade were used as supplied. Water was obtained from Millipore (Milli-Q) water purification systems. The complexes were obtained by mixing freshly prepared solutions of $CuCl_2$ and CpSH in the 1 : 4 ratio just before experiment. Solutions were saturated immediately prior to irradiation, with $N_2O$ or varying percentages in $N_2O/O_2$ by flushing gas mixtures. The pH of the solutions was adjusted by means of $HClO_4$ or NaOH.

Pulse radiolysis experiments were performed with a 12 MeV electron linear accelerator (LINAC). Spectral cells of 0.5 dm optical pathlength were used throughout. Optical filters were employed to minimise photochemical effects. Dosimetry was based on the initial yield of dithiocyanate radical anion, $(SCN)_2^{\bullet -}$ ($G = 0.3\,\text{mmol J}^{-1}$ and $\varepsilon = 710\,\text{m}^2\,\text{mol}^{-1}$ at 480 nm) obtained in irradiated aerated–saturated solution of $10^{-1}$ M KCNS. The signals from the photomultiplier were recorded on Tektronix AD7912 and analysed by a personal computer.

The Raman spectra were obtained by a Bruker IFS 66 spectrometer equipped with a FRA-106 Raman module and a cooled Ge-diode detector. The excitation source was a $Nd^{3+}$-YAG laser (1064 nm) in the backscattering (180°) configuration. The focused laser beam diameter was $\sim 100\,\mu\text{m}$, the spectral resolution $4\,\text{cm}^{-1}$, the encoding interval 1 point/$\text{cm}^{-1}$, and the apodization

function Blackman Harris, 4-term. On the sample, the laser power was 200 mW. The total number of scans for each spectrum was 10 000.

Because of the strong water absorption in the NIR range, the Raman spectra of solid samples, obtained by dehydration of CpSH–Cu(II) solutions at different pH, were recorded.

A linear correction brought the base line of the Raman spectra to approximately zero intensity, removing the curvature of the base line due to the sensitivity of the spectrometer.

## RESULTS AND DISCUSSION

### 1 Reaction with •OH Radicals

#### *1.1 Captopril*

Figure 2 shows the transient optical absorption spectra obtained on pulse irradiation of aqueous $N_2O$-saturated solutions containing $1 \times 10^{-3}$ mol dm$^{-3}$ CpSH at two pH values, 7 and 9.8. Under these experimental conditions, essentially all the $e^-_{aq}$ ($\geq 98\%$) are converted to •OH radicals according to the reaction:

$$e^-_{aq} + N_2O \xrightarrow{H_2O} N_2 + {}^{\bullet}OH + OH^- \qquad (1)$$

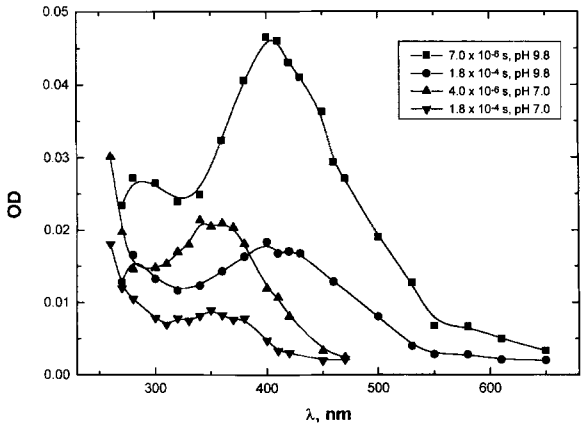


FIGURE 2 Time-resolved absorption spectra in the pulse radiolysis of $N_2O$-saturated CpSH solutions (1 mM) at different pH values. Path length = 0.5 dm; dose ≈ 13 Gy.

At pH 7 the transient spectrum exhibits a maximum of low intensity at 340–360 nm which, according to the literature on thiol compounds,[13] may be ascribed to the CpS• radicals, formed by the following reaction:

$$CpSH + {}^{\bullet}OH \longrightarrow CpS^{\bullet} + H_2O \qquad (2)$$

Further proof on the formation of thiyl free radicals from reaction (2) was obtained at pH 9.8, where the transient spectrum shows a more intense absorption maximum shifted towards $\lambda \approx 400$–$410$ nm (Figure 2). At pH 9.8. CpSH ($pK_{SH} = 9.7$)[14] exists partially in the anionic form, $CpS^-$ ($\approx 50\%$), and the band at 400–410 nm is attributable to the $2\sigma/1\sigma^*$ three-electron bonded disulfide radical anion, $CpSSCp^{-\bullet}$, formed via the following reactions:

$$CpSH \rightleftharpoons CpS^- + H^+ \qquad (3)$$

$$CpS^- + {}^{\bullet}OH \longrightarrow CpS^{\bullet} + OH^- \qquad (4)$$

$$CpS^{\bullet} + CpS^- \rightleftharpoons [CpS \therefore SCp]^- \qquad (5)$$

Therefore, the transient spectra of the radicals produced from the reaction of •OH with CpSH at both pH values resemble those observed for other thiols,[13,15] indicating that the –SH group of the molecule is the preferential site for the •OH attack.

To further support the formation of $CpSSCp^{-\bullet}$, a radical which should exhibit reducing properties, $N_2O$-saturated solutions of CpSH ($10^{-3}$ M) were irradiated in the presence of PNAP (50–100 μM) at different pH's.

PNAP is a powerful electron-affinic molecule ($E^0 = -0.36$ V)[16] and its electron adduct, $PNAP^{-\bullet}$, is characterised by two intense absorption peaks at 350 and 550 nm.[17]

The formation of $PNAP^{-\bullet}$ in the presence of $CpSSCp^{-\bullet}$ will indicate the occurrence of the following one-electron transfer reaction:

$$CpSSCp^{-\bullet} + PNAP \longrightarrow CpSSCp + PNAP^{-\bullet} \qquad (6)$$

The reference yield of $PNAP^{-\bullet}$ (100%) was estimated by the comparison with the absorption at 550 nm obtained with $N_2O$-saturated solutions containing Na formate ($5 \times 10^{-2}$ M) and PNAP (50–100 μM). Under these conditions practically all the $^{\bullet}OH$ radicals primarily formed are finally converted to $PNAP^{-\bullet}$:

$$HCOO^- + {}^{\bullet}OH \longrightarrow HCOO^{\bullet} + OH^- \quad (7)$$

$$HCOO^{\bullet} \rightleftharpoons CO_2^{-\bullet} + H^+ \quad (8)$$

$$CO_2^{-\bullet} + PNAP \longrightarrow CO_2 + \underset{(\lambda=550\,nm)}{PNAP^{-\bullet}} \quad (9)$$

In the presence of CpSH, the extent of the $PNAP^{-\bullet}$ formation (not observed at neutral pH) increases with increasing pH and reaches a yield of about 20% at pH 10.8. This result is in line with the equilibrium reaction (5) being more shifted towards $CpSSCp^{-\bullet}$ when the $CpS^-$ concentration increases. In spite of the fact that $^{\bullet}OH$ radical attack on CpSH should give rise mainly to $CpSSCp^{-\bullet}$ radicals (at $pH \geq pK_{SH}$), the observed $PNAP^{-\bullet}$ yields are lower than expected. One reason could be a formation of a transient radical adduct between $CpSSCp^{-\bullet}$ and PNAP itself which is only partially converted to $PNAP^{-\bullet}$. In fact, the absorption development at 550 nm is observed to take place in two steps with different rates.

The rate constant of the reaction of CpSH with $^{\bullet}OH$ radicals was evaluated by an indirect method based on the competition between the $^{\bullet}OH$ attack to KCNS and CpSH. $N_2O$-saturated solutions of KCNS ($10^{-3}$ M) were pulse irradiated in the presence of different concentrations of CpSH (from $10^{-4}$ to $5 \times 10^{-4}$ M) at pH 7 and 10. In the presence of $CNS^-$ ions, the following reactions take place:

$$^{\bullet}OH + CNS^- \longrightarrow CNS^{\bullet} + OH^- \quad (10)$$

$$CNS^{\bullet} + CNS^- \rightleftharpoons (CNS)_2^{\bullet-} \quad (11)$$

where the rate constant of the reaction of $CNS^-$ with $^{\bullet}OH$ ($k_{10}$) has been reported to be $1.1 \times 10^{10}\,M^{-1}\,s^{-1}$.[18]

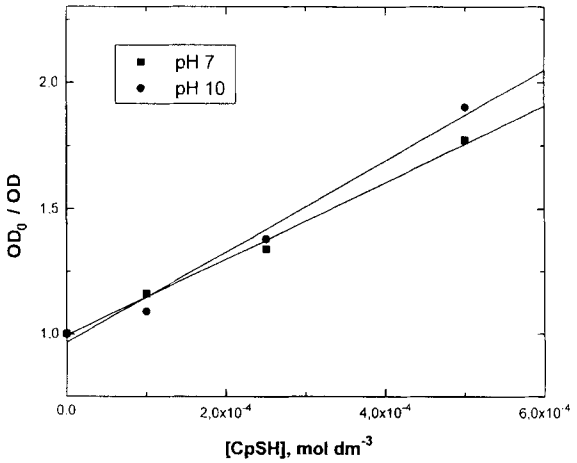


FIGURE 3 Indirect method for the determination of $k$(CpSH + $^{\bullet}OH$). Plot of $OD_0/OD$ (from $(CNS)_2^{-\bullet}$ measured at 500 nm) against [CpSH] at different pH. Dose ≈ 8.3 Gy.

The rate constants of the reaction between CpSH and $^{\bullet}OH$ radicals ($k_{12}$) were determined following the decrease of the absorption at 500 nm due to $(CNS)_2^{\bullet-}$ at different [CpSH]/[$CNS^-$] ratios:

$$^{\bullet}OH + CpS^-(H) \longrightarrow CpS^{\bullet} + OH^-(H_2O) \quad (12)$$

Under these conditions, the following competition equation was applied:

$$\frac{OD_0}{OD} = 1 + \frac{k_{12}[CpSH]}{k_{10}[CNS^-]}. \quad (I)$$

From the slope of the lines (Figure 3) the values of $1.7 \times 10^{10}$ and $2.0 \times 10^{10}\,M^{-1}\,s^{-1}$ for $k_{12}$ at pH 7 and 10, respectively, were calculated. These relatively high rate constant values, which agree with those reported for other thiols of biological importance,[13] evidence that CpSH is a powerful scavenger of $^{\bullet}OH$. The slight enhancement of the rate constant with pH may be correlated to the ionisation of the SH group at pH 10 which makes easier the $^{\bullet}OH$ electrophilic attack.

### 1.2 *Captopril/Cu(II) System*

When $N_2O$-saturated aqueous solutions containing $1 \times 10^{-3}$ M CpSH and $0.25 \times 10^{-3}$ M $Cu^{2+}$ were pulse irradiated at pH 7 and 9.8, the $^{\bullet}OH$

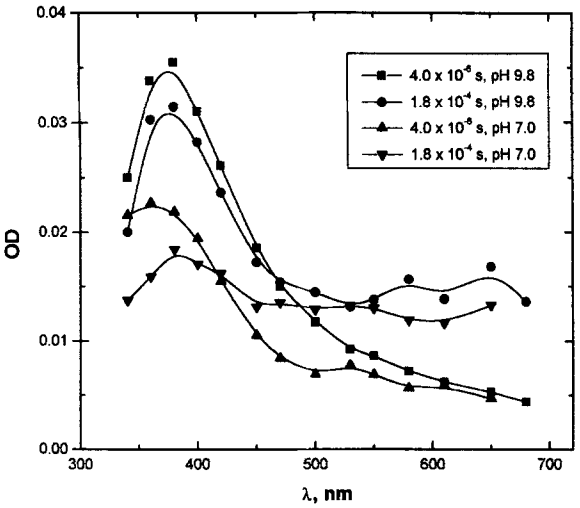


FIGURE 4 Time-resolved absorption spectra in the pulse radiolysis of $N_2O$-saturated solutions at different pH: Cu(II)/CpSH (1 : 4), [Cu(II)] = 0.25 mM, [CpSH] = 1 mM. Path length = 0.5 dm. Dose ≈ 13 Gy.

radicals reacted rapidly with the complex giving transient optical absorption spectra characterised by an absorption maximum at about 370 nm (Figure 4), with stronger intensity at basic pH. The absorption decay at 370 nm gives rise to a broad absorption at $\lambda > 470$ nm via a first order kinetic ($k_f \approx 1.4 \times 10^4\ s^{-1}$ at pH 9.8).

As far as the nature of the radical(s) responsible for the above absorption bands is concerned, it is worthnoting that CpSH, like a few other thiols (i.e. glutathione, penicillamine, etc.), forms a Cu(I)-type complex ($(CpS)_n$–Cu(I)) when reacting with Cu(II).[19–21] In fact, copper seems to be reductively inactivated by CpSH in a reaction from which no reactive thiyl radicals are generated:[19,22]

$$2CpSH + 2Cu(II) \rightleftharpoons CpSSCp + 2Cu(I) + 2H^+ \quad (13)$$

$$nCpSH + Cu(I) \rightleftharpoons (CpS)_n\text{–}Cu(I) + nH^+ \quad (14)$$

Whereas a 1:1 stoichiometry for the reaction (13) has been demonstrated,[19] different metal complexes can be obtained depending on pH or ligand/metal ratio. For example, in the CpSH–zinc system the most stable complex was the $ML_2$ species which binds > 50% of zinc at ligand/metal ratios greater than 3 : 1.[23] Since glutathione, which is characterised by a copper(II)-reducing capacity similar to CpSH, is markedly inhibitory on copper-dependent oxidative injury beginning at a 4 : 1 molar ratio with respect to the metal,[24] the same initial ratio was chosen. Moreover, as suggested by an EPR study, at this molar ratio the percentage of the copper present in the Cu(II) form is less than that at both 2 : 1 and 3 : 1 ratios.[25]

At pH 9.8 the band at 370 nm of the transient absorption spectra does not resemble that of a $CpSSCp^{-\bullet}$ radical species (see Figure 2), thus suggesting the involvement of the –SH group of CpSH in the metal coordination.

Similar results were obtained for solutions containing Cu(II)/CpSH in the ratio of 1 : 2, indicating that the eventual presence of free –SH groups is not able to induce significant modifications in the related transient spectra.

In order to verify the lack of reducing transient species, such as $CpSSCp^{-\bullet}$ type radicals, experiments with PNAP (100 μM) similar to those previously reported for CpSH alone were carried out. Solutions containing Cu(II)/CpSH in the ratio of 1 : 4 were investigated at different pH values, but in all cases no absorption due to $PNAP^{-\bullet}$ at 550 nm was evidenced.

Therefore, the band at 370 nm could be ascribed to the formation of an $^\bullet OH$-radical adduct of the $(CpS)_n$–Cu(I) complex:

$$(Cp\text{–}S)_n\text{–}Cu(I) + {}^\bullet OH \longrightarrow Cp\text{–}\underset{\displaystyle OH}{\overset{\displaystyle \bullet}{S}}\text{–}Cu(I)\text{–}(S\text{–}Cp)_{n-1} \quad (15)$$

The slow increase of the absorption at $\lambda > 470$ nm, after the 370 nm peak has partially decayed, might be due to an inter-molecular rearrangement of the above adduct leading, in example, to the

formation of a sulphur radical cation:

$$\mathrm{Cp{-}\overset{\bullet}{S}{-}Cu(I){-}(S{-}Cp)_{n-1}} \quad (\mathrm{OH}) \longrightarrow \mathrm{Cp{-}\overset{+\bullet}{S}{-}Cu(I){-}(S{-}Cp)_{n-1}} + \mathrm{OH^-} \qquad (16)$$

Sulphur radical cations with the unpaired electron located in the sulphur p-orbital are reported to generally yield transient absorptions in the visible region considered above.[15]

## 2 Effect of Oxygen

### 2.1 *Captopril*

The reaction of oxygen with the OH-induced captopril radicals was investigated in solutions containing $N_2O/O_2$ gas mixtures: under the experimental conditions used (60% $N_2O$ and 40% $O_2$) essentially every $e^-_{aq}$ is converted to $^\bullet$OH (reaction (1)).

The time-resolved optical absorption spectrum at pH 7 (Figure 5(a)) is characterised, immediately after the pulse, by a weak band at $\lambda \approx 350$ nm and a maximum at $\lambda \approx 550$ nm which further increases after the absorption at 350 nm has decayed away. The transient absorption at 350 nm represents the contribution of thiyl radicals (CpS$^\bullet$) (reaction (2)) that are known to absorb in the above spectral region,[13,26] whereas the 550 nm band can be attributed to thiyl peroxyl radicals (CpSOO$^\bullet$) formed by the following reaction:

$$\mathrm{CpS^\bullet + O_2 \rightleftharpoons CpSOO^\bullet} \qquad (17)$$

This behaviour is in agreement with that of other thiols pulse irradiated in the presence of oxygen. In fact, upon the irradiation of glutathione, cysteine and 2-mercaptoethanol under similar experimental conditions, the resulting transient spectra are all characterised by an absorption with a maximum around 540–560 nm, which has been attributed to the corresponding sulphur peroxyl radicals.[27,28]

Increasing the pH to 9.8, significant changes were induced in the transient absorption spectrum recorded only few microseconds after the pulse (Figure 5(b)). The absorption band at $\lambda \approx 550$ is still present, whereas a more intense absorption at $\lambda \approx 410$ nm is observed instead of the weak band at $\approx 350$ nm. The two absorption

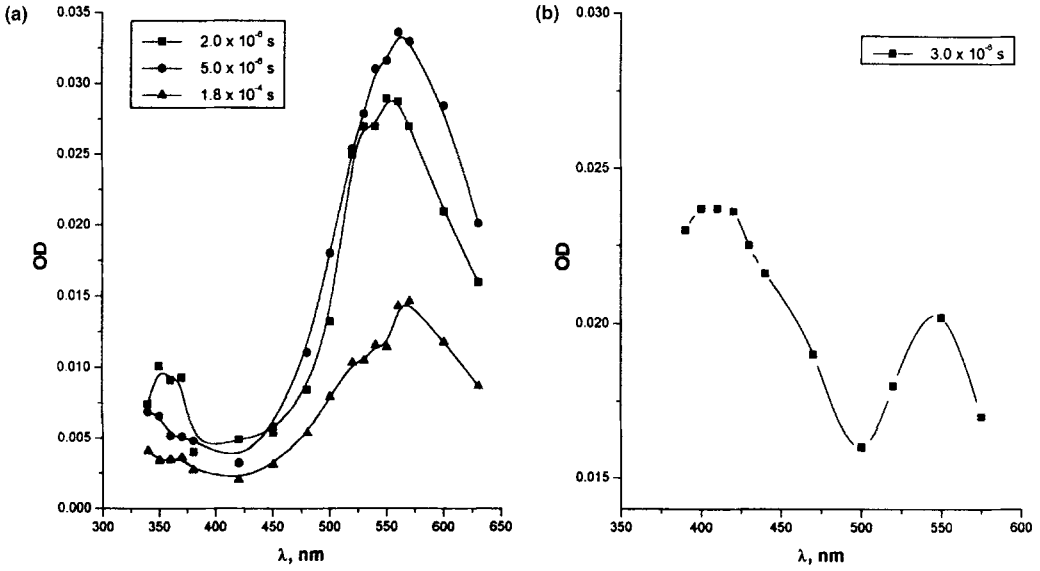


FIGURE 5 Time-resolved absorption spectra in the pulse radiolysis of $N_2O/O_2$ (60 : 40) saturated CpSH solutions (1 mM) at: (a) pH 7.0; (b) pH 9.8. Path length = 0.5 dm; dose ≈ 13 Gy.

maxima at 410 and 550 nm are ascribed to the disulfide radical anion, $CpSSCp^{-\bullet}$, and sulphur peroxyl radical, $CpSOO^{\bullet}$, respectively. Therefore, it is evident that under aerobic condition and at basic pH, the thiyl radical, $CpS^{\bullet}$, will react with oxygen (reaction (17)) in competition with the thiolate ion $CpS^{-}$ (reaction (5)).

No detailed kinetic analysis has been carried out on the radicals at 420 and 550 nm. However, in both cases the decays were observed to obey first order kinetics with different rates, pointing thus to different origins of the two transient absorptions. In analogy with what observed for other thiols,[29,30] the exponential decay at 420 nm could be ascribed to the electron transfer reaction from $CpSSCp^{-\bullet}$ to oxygen:

$$CpSSCp^{\bullet-} + O_2 \rightleftharpoons CpSSCp + O_2^{\bullet-} \qquad (18)$$

At 550 nm the disappearance of $CpSOO^{\bullet}$ could involve an oxygen-atom transfer to the parent thiol (reaction (19))[31,32] or a thermal conversion of $CpSOO^{\bullet}$ to the thermodynamically more stable $CpSO_2^{\bullet}$ sulphonyl radical (reaction (20)):[33,34]

$$CpSOO^{\bullet} \xrightarrow{+CpSH} CpSO^{\bullet} + CpSOH \qquad (19)$$

$$CpSOO^{\bullet} \longrightarrow CpSO_2^{\bullet} \qquad (20)$$

## 2.2 *Captopril/Cu(II) System*

Figure 6 shows the transient spectra of CpSH/Cu(II) (4:1) at pH 7.0 and 9.8, obtained by pulse-irradiated solutions containing 60% $N_2O$ and 40% $O_2$.

In both cases the spectra are characterised, immediately after the pulse, by a band at around 370 nm, which disappears while a new one appears at $\lambda \approx 560$ nm.

In the presence of Cu(II), the $(CpS)_n$–Cu(I) complex has been hypothesised to react with $^{\bullet}OH$ by reaction (15) giving an $^{\bullet}OH$-complex adduct absorbing at 370 nm (see Figure 4). When oxygen is present, the weak absorption in the above region, observed 2 μs after the pulse, can be still ascribed to the $^{\bullet}OH$-complex adduct primarily formed. The absorption at $\lambda \approx 560$ nm, which develops 5 μs after the pulse, may arise from the oxygen addition to the above $^{\bullet}OH$ adduct. Hence, the formation of a sulphur peroxyl type radical such as $CpS(OH)(OO^{\bullet})Cu(I)$ is likely to take place.

## 3 Raman Spectra

To achieve a better insight into the copper–CpSH interactions and to identify the groups involved in

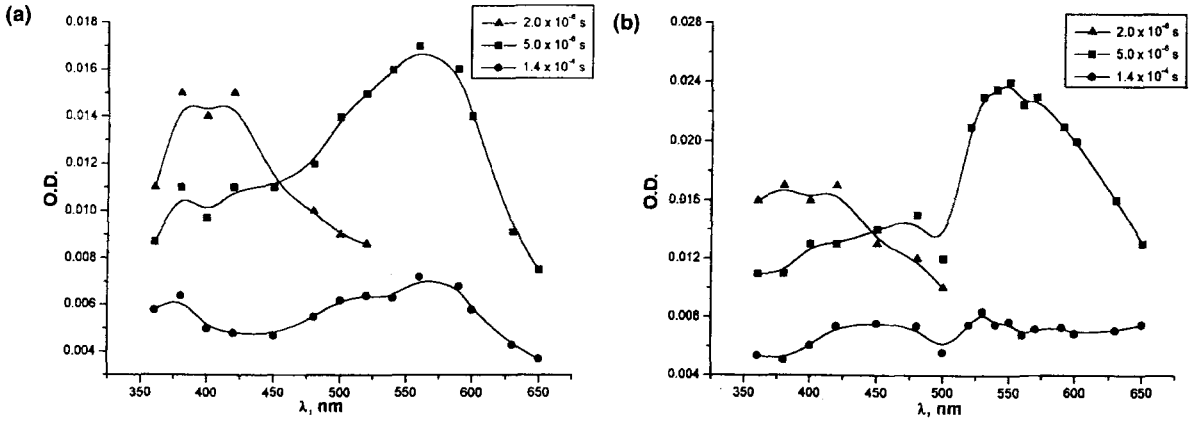


FIGURE 6 Time-resolved absorption spectra in the pulse radiolysis of $N_2O/O_2$ (60:40) saturated solutions of Cu(II)/CpSH (1:4) at: (a) pH 7.0; (b) pH 9.8. [Cu(II)] = 0.25 mM, [CpSH] = 1 mM. Path length = 0.5 dm; dose ≈ 13 Gy.

the eventual coordination, both CpSH alone and in the presence of copper(II) (metal/ligand 1 : 4) was investigated by Raman spectroscopy at two different pH values.

Figure 7 shows the Raman spectra of CpSH at pH 7.0 and 9.8, while Table I summarises the wavenumbers and assignments of the more significant bands. From a comparison of the spectra only some little differences are evident. In particular, the amide I band, due to the peptidic group stretching of CpSH, changes both position and shape. This band is conformation sensitive[35] and its wavenumber decreases from 1624 to 1621 $cm^{-1}$ with increasing pH, indicating that some modifications in the strength of inter-molecular hydrogen bonds involving the carbonyl group take place.

The carboxylic group is deprotonated at both pH values ($pK_a \approx 3.5$)[14] but the $\nu_a COO^-$ and $\nu_s COO^-$ stretching modes are not clearly visible because the former is completely overlapped by the amide I band (1600–1670 $cm^{-1}$) and the latter, partially, by the $\delta(CH_2, CH_3)$ band ($\approx 1450\ cm^{-1}$).

The 2600–2500 $cm^{-1}$ spectral region bears information concerning the –SH group of CpSH. In both spectra a band at $\approx 2570\ cm^{-1}$ is observed, its intensity decreasing (by $\approx 50\%$) when the pH increases from 7.0 to 9.8. This band arises from the stretching mode of the S–H bond,[36] which is present at both pH values, although the concentration of the thiol group is halved at pH 9.8 ($pK_{SH} = 9.7$).

In the presence of copper(II) ions, some significant changes in the Raman spectra at both pH values indicate the formation of a dithiol species, CpSSCp, as well as the coordination of CpSH with the metal (Figure 8 and Table I). In fact, a new band, due to the stretching of the –S–S– bond,[36] appears at 510 $cm^{-1}$ and its intensity increases by about 30% going from pH 7.0 to 9.8, suggesting an increase of the CpSSCp concentration at basic pH.

The formation of CpSSCp is in agreement with the suggested reduction of copper(II) to copper(I) in the presence of thiol ligands (reaction (13)).[37] Hence, under our conditions almost all copper ions should be present as copper(I).

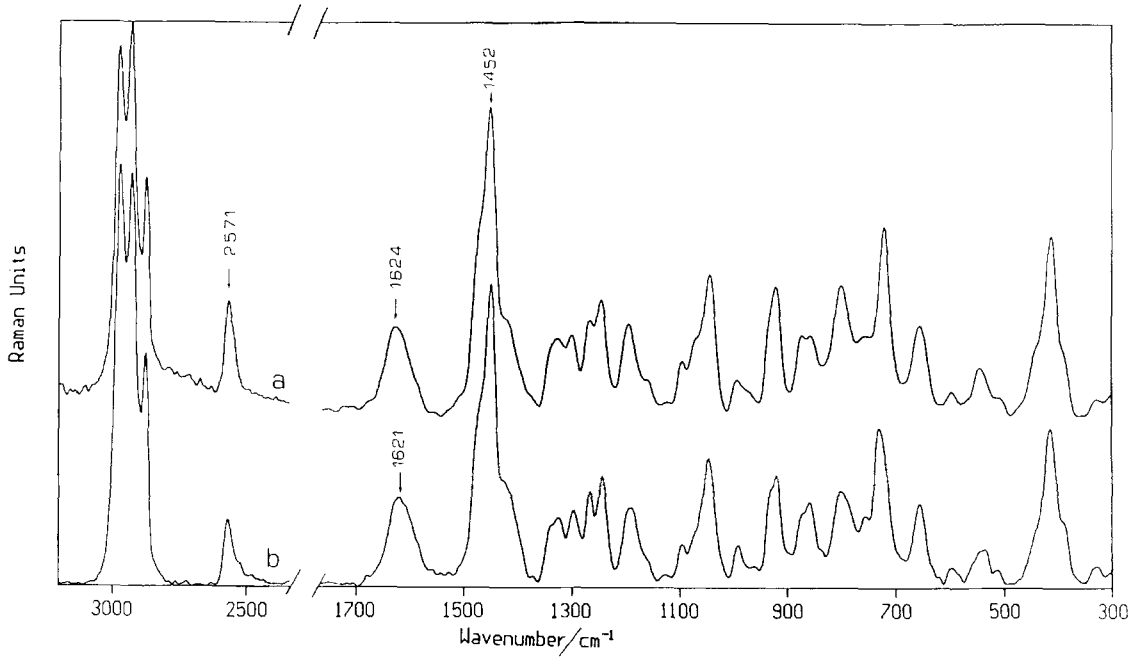


FIGURE 7 Raman spectra of CpSH at: (a) pH 7.0; (b) pH 9.8.

TABLE I Wavenumbers and assignments of the main Raman bands of CpSH and Cu(II)/CpSH (1 : 4) system at different pH

| Wavenumber, $cm^{-1}$ | | | | Assignment[a] |
|---|---|---|---|---|
| CpSH | | CpSH + Cu(II) | | |
| pH 7.0 | pH 9.8 | pH 7.0 | pH 9.8 | |
| 2571 m | 2571 w | — | — | $\nu$S–H |
| — | — | 1664 vw | — | $\nu$C=O Amide I |
| 1624 m, br | 1621 m | 1621 m | 1624 w, sh | $\nu$C=O Amide I |
| — | — | 1606 m, sh | 1606 m, br | $\nu$C=O Amide I |
| — | — | 1553 w | — | $\nu_a COO^-$ |
| 1452 s | 1451 s | 1452 s | 1451 s | $\delta$ ($CH_2$, $CH_3$) |
| 1418 m, sh | 1418 m, sh | 1415 m | 1416 m | $\delta$ C–H + $\nu_s COO^-$ |
| 1330 w, br | 1333 w, sh | 1333 w, br | 1333 w, sh | $\nu$C–N |
| — | 1325 w | — | 1323 w | $\nu$C–N |
| 1300 w | 1298 w | 1301 w | 1303 w | $\delta$C–H |
| 1194 m | 1189 m | 1197 m | 1194 w | $\delta$C–H |
| 1098 w, sh | 1097 w, sh | 1099 w | 1099 w | $\nu_{skeletal}$ |
| 1046 m | 1048 m | 1046 m, br | 1048 m, br | $\nu_{skeletal}$ |
| 998 vw | 992 vw | 996 s | 994 s | $\nu_{skeletal}$ |
| 921 m | 921 m | 924 m | 923 m | $\nu_{skeletal}$ |
| 874 w | 875 w, sh | 876 m | 876 m | r $CH_2$ |
| 856 w | 859 w | 856 m, sh | 850 w, sh | r $CH_2$ |
| 801 m | 802 m | 787 m | 791 w, br | r $CH_2$ |
| 723 s | 732 s | 727 s, br | 729 s, br | r $CH_2$ |
| — | — | 661 m | 660 m | $\nu$C–S |
| 657 m | 656 w | 656 m, sh | 656 w, sh | $\nu$C–S |
| — | — | 510 w | 510 m | $\nu$S–S |
| — | — | 450 m | 451 m | $\nu$Cu–O |
| 415 s | 416 s | 48 s | 420 s | $\nu_{skeletal}$ |

[a]Assignments: $\nu$ = stretching ($\nu_a$ and $\nu_s$ = asymmetric and symmetric stretching, respectively), $\delta$ = in plane deformation, and r = rocking. Intensities: w = weak, m = medium, s = strong, v = very, sh = shoulder, br = broad.

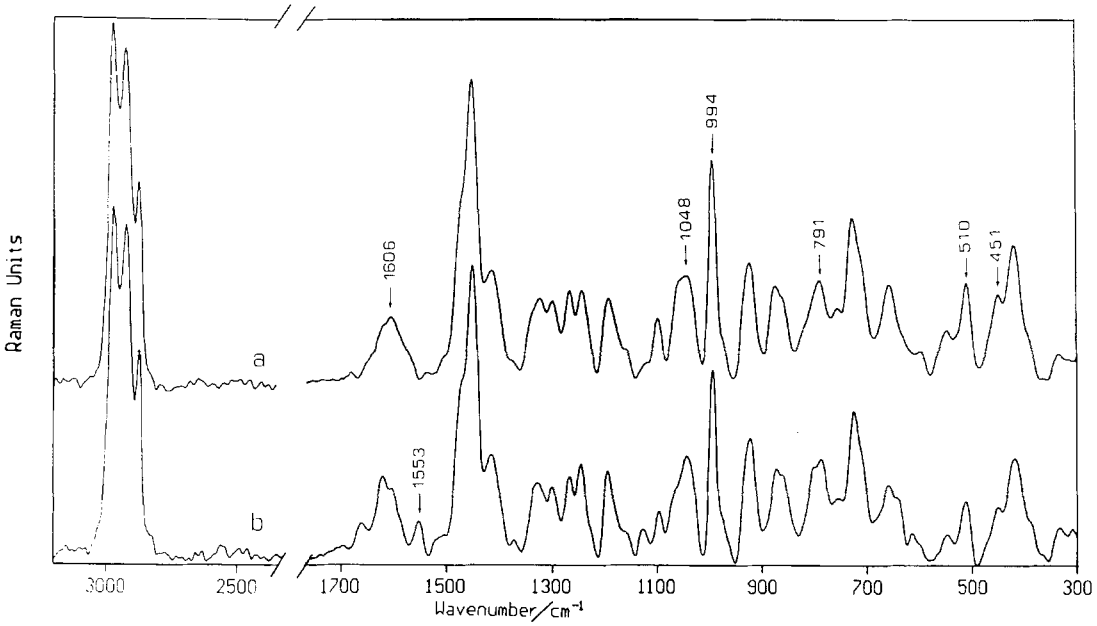


FIGURE 8 Raman spectra of Cu(II)/CpSH (1 : 4) at: (a) pH 7.0; (b) pH 9.8.

As far as the formation of a $(CpS)_n$–Cu(I) complex is concerned, some indications of the groups involved in the coordination can be inferred from the Raman spectra.

CpSH can coordinate metal ions via three possible sites: the thiol sulphur, the carbonyl oxygen and the carboxylate oxygen. The sulphur atom appears to be involved in the metal coordination both at pH 9.8 and 7.0. In fact, the band at $\approx 2570\,cm^{-1}$, due to the S–H stretching, disappears as a consequence of interaction with copper while changes are observed in the C–S stretching region (600–670 $cm^{-1}$) (Figure 8 and Table I).

In the presence of copper ions, the amide I band, visible at 1621 $cm^{-1}$ in the free ligand spectrum (Figure 7) shifts to 1606 $cm^{-1}$; this wavenumber decrease can be interpreted as a direct evidence of the involvement of the peptide group in the metal binding.

The formation of a bond involving an oxygen atom and the metal ion is also suggested by the presence of a new band at $\approx 450\,cm^{-1}$. This band arises from the Cu–O stretching vibration and its intensity decreases by $\approx 25\%$ when the pH decreases from 9.8 to 7.0.[38] This behaviour, as well as that observed for the $\nu$S–S band, suggests that the presence of the CpSSCp and $(CpS)_n$–Cu(I) species might be more relevant at alkaline pH. In other words, it means that the equilibria (13) and (14) are more shifted towards the products.

The ring closure on the carbonyl oxygen and the sulphur atom is further supported by the intensity and frequency changes of the bands ascribed to the skeletal vibrations (i.e. 1048, 994, 876, 791 and 729 $cm^{-1}$ at pH 9.8), among which the strong band at 994 $cm^{-1}$ can be assumed as a clear marker for metal chelation.

The coordination of the proline N-atom to Cu(I) seems unlikely, because both this N-atom cannot be protonated and because no new bands, corresponding to $\nu$M–N, appear over the 400–550 $cm^{-1}$ spectral range.[39]

As far as the carboxyl group is concerned, the coordination of Cu(I) through this group can be inferred only at pH 7.0, where a new band, due to the $COO^-$ asymmetric stretching, is visible at 1553 $cm^{-1}$. Conversely, at pH 9.8 the above band is still overlapped by the amide I band and does not shift to lower frequencies.

In conclusion, the vibrational data suggest the formation of two different $(CpS)_n$–Cu(I) complexes at pH 7.0 and 9.8, since the –SH and $>C{=}O$ peptidic groups are involved in metal chelation at both pH values, the $COO^-$ group instead only at neutral pH. Figure 9 reports the suggested structures of these two complexes. At pH 9.8 the formation of a monomeric species where two molecules of CpSH are coordinated to the Cu(I) ion is likely (Figure 9(a)), whereas at pH 7.0 the Cu(I) complex has probably the structure of a polymeric chain (Figure 9(b)), although one cannot completely rule out the presence, also at this pH, of the monomeric complex. This result agrees well with the data reported in the literature about the zinc–CpSH complexes: also in that case the complex structure depends on the pH value. At pH < 6 the Zn(II) ion is coordinated by three

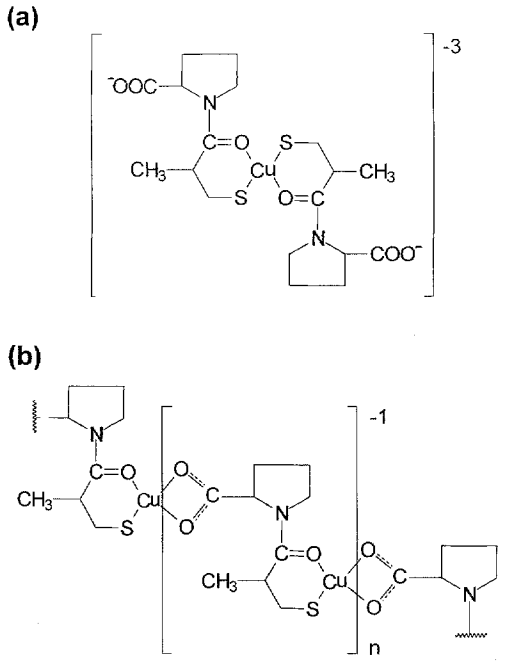


FIGURE 9 Proposed structures of the $(CpS)_n$–Cu(I) complexes at pH (a) 9.8 and (b) 7.0.

sites (carbonyl, carboxyl and thiol groups), whereas at pH > 6 the complex formation involves only two atoms (carbonyl oxygen and sulphur).[40,41]

## CONCLUSIONS

The presence of the thiol group in the CpSH structure is crucial in determining the radical scavenging action of the molecule. The oxidation of CpSH by $^{\bullet}OH$ radicals is affected by the protonation state of the thiol moiety, since the $^{\bullet}OH$ attack occurs mainly at the above site. At physiological pH, the –SH group is predominantly in the non-ionised form and the thiyl radical will be the main intermediate transient product. The thiyl radicals are known to possess mild oxidising properties and appear to be, potentially, much less harmful than $^{\bullet}OH$.

In the absence and in the presence of oxygen the thiyl radicals, $CpS^{\bullet}$, are converted to the corresponding disulphide, CpSSCp, and sulphur peroxyl radicals, $CpSOO^{\bullet}$, respectively. The latter is not a good hydrogen abstractor, although it does undergo rapid electron transfer reaction.[42] It is yet unclear whether this radical is reactive enough to be a damaging species and, therefore, whether it plays a role in oxygen-induced damage.

At pH close to the $pK_a$ of the –SH group, $CpS^{\bullet}$ is mainly conjugated as a reducing $CpSSCp^{-\bullet}$ species, as shown by the characteristic transient spectrum and by the experiments with PNAP. In the presence of oxygen, in addition to the sulphur peroxyl radicals, superoxide anion is formed by the fast electron transfer from $CpSSCp^{-\bullet}$. $O_2^{-\bullet}$, which has a selective reactivity, will have limited, if any, damaging effect if no catalytic metal ions are available.

In the presence of Cu(II) ions, the thiol group of CpSH is presumably involved in a one-electron reduction of cupric ions with consequent formation of the CpSSCp disulphide as well as in the Cu(I) chelation. In this contest, Raman spectroscopy results are useful to support the above mechanisms, since both CpSSCp and $(CpS)_n$–Cu(I) are distinguishable through selected well visible marker bands. It is noteworthy that the Cu(I) complex is still active as $^{\bullet}OH$ radical scavenger and in the reaction involving oxygen.

The pulse radiolysis and Raman spectra support the recently reported suggestion that the antioxidant mechanism of CpSH, in addition to is scavenging ability, is also related to the formation of a CpSH/Cu complex with a SOD-mimicking activity.[43,44] This specific anti-free radical effect can be related to the protection afforded by CpSH in the reperfusion induced myocardial injury caused, at least in part, by free radicals derived by oxygen, including $O_2^{-\bullet}$.

However, at this stage, it is difficult to speculate on the predominant mechanism (i.e. free radical scavenging or metal chelation) involved in the protective role of CpSH. In the literature there are contrasting data on the extent of the protection afforded by thiol- and non-thiol-containing ACE inhibitors and on the role of free –SH moiety.[22,45] Nevertheless, there is a generally recognised agreement on the value of the presence of the thiol group in CpSH with regard to both ACE inhibition and antioxidant capacity.

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