

Transport of the Cu(II) Bound with Histidine-Containing Tripeptides to Cysteine. Coordination Mode and Exchangeability of Cu(II) in the Complexes

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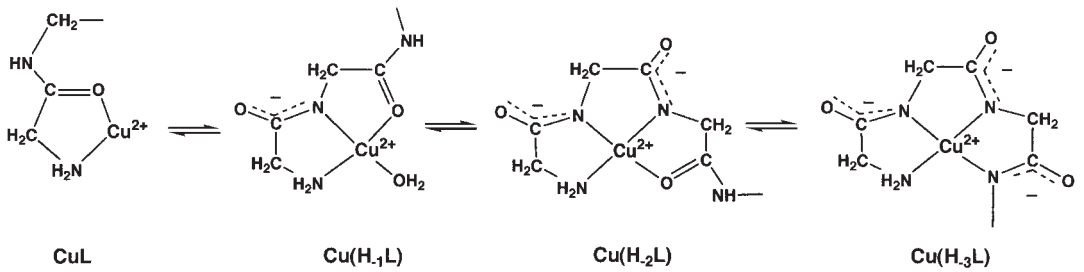
The transport of Cu(II) complexed with histidine-containing tripeptides ($Cu(H_{-i}L)$, L = HisGlyGly ($i = 2$), GlyHisGly ($i = 1$), and GlyGlyHis ($i = 2$)) to cysteine was examined by a stopped-flow spectrophotometric method. The S → Cu(II) charge transfer (LMCT) bands at 335 nm and 390 nm were used as probes for tracing the reaction. Primarily formed was the ternary $Cu(H_{-1}L)(Cys^-)$ complex. The rate of the $Cu(H_{-1}L)(Cys^-)$ formation depended on the affinity of Cu(II) for the donor atoms at the fourth binding site of $Cu(H_{-2}L)$. $Cu(H_{-1}L)(Cys^-)$ subsequently reacted with free Cys^- to yield a binary complex, $Cu(Cys^-)_2$. The rate of $Cu(H_{-1}L)(Cys^-)$ formation was generally faster than that of conversion from $Cu(H_{-1}L)(Cys^-)$ to $Cu(Cys^-)_2$. An exception was found in the reaction with $Cu(H_{-2}GlyGlyHis)$, where the relation $k_{1+} < k_{2+}$ existed. The ternary complex, $Cu(H_{-1}HisGlyGly)(Cys^-)$, was too labile to be detect by the conventional stopped-flow methods. Probably, $Cu(H_{-1}HisGlyGly)(Cys^-)$ upon forming changed spontaneously to $Cu(HisGlyGly)(Cys^-)$, in which the N-terminal His residue coordinated to the Cu(II) via the amino and imidazole nitrogens, and rapidly changed to $Cu(Cys^-)_2$.

It is well-known that naturally occurring peptides can coordinate to heavy-metal ions, such as Cu(II), to form stable chelate complexes. Amide groups in simple peptides coordinate to Cu(II) in a fashion with deprotonation of the amide nitrogens.[1] It is relatively easy to establish the site of coordination, because X-ray crystallographic results of single crystals may be extrapolated to species in solution with confidence.[2] The Cu(II) stepwise forms several kinds of peptide complexes, abbreviated as CuL, $Cu(H_{-1}L)$, $Cu(H_{-2}L)$, and $Cu(H_{-3}L)$, depending on the pH.[3] The Cu(II) first anchors the terminal amino nitrogen, and then coordinates to the carbonyl oxygen of the amide group, forming a chelate complex, abbreviated as CuL;[4] otherwise mentioned, Cu in the complexes denotes Cu(II). In weakly acidic solutions between pH 5–6, the amide group, coordinated to the Cu(II) of CuL, is deprotonated and the deprotonated-amide nitrogen replaces the carbonyl oxygen forming the $Cu(H_{-1}L)$ species with a Cu–N^- bond and a Cu–NH_2 bond.[4] As the pH increases, successive deprotonation of the neighboring amide group and exchange of the donor from the oxygen to the deprotonated amide-nitrogen occur to form $Cu(H_{-2}L)$ with N,N^-,N^-,O^--donors, and finally the $Cu(H_{-3}L)$ species with N,N^-,N^-,N^--donors (Scheme 1). The thus-formed fused-chelate complexes are thermodynamically stable, as studied by the pH-titration methods.[5]

Peptides, which have side chains with coordination ability, form Cu(II) complexes in a slightly different mode. A typical example is observed in His-containing peptides. Donor atoms in the CuL species for the HisGlyGly complex are considered to be the terminal amino and imidazole nitrogens, because the imidazole nitrogen has a stronger affinity for Cu(II) than the carbonyl oxygen. As the pH increases, the peptide bond between His and Gly in the CuL is deprotonated, and the deprotonated-amide nitrogen replaces the imidazole nitrogen, binding to the Cu(II) to form the $Cu(H_{-1}L)$ species. Similarly, GlyHisGly and GlyGlyHis can form the $Cu(H_{-1}L)$ and $Cu(H_{-2}L)$ species involving N,N^-,N(Im),O^--donors and N,N^-,N^-,N(Im)-donors, respectively (Scheme 2).[6,7]

The histidyl residue has been recognized as an important



Scheme 1. Equilibrium for the deprotonation of CuL and coordination structure of the $Cu(H_{-i}L)$ species ($i = 1$, 2, or 3); L = tetrapeptide or higher.

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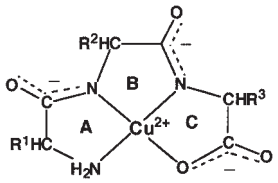
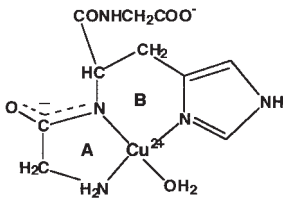
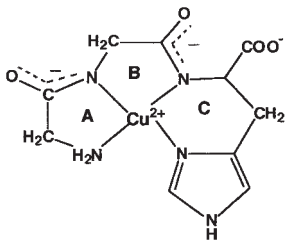


$Cu(H_{-i}L)$	R^1	R^2	R^3	λ_{max}/nm (ε/M^{-1} cm^{-1})
$Cu(H_{-2}GlyGlyGly)$	H	H	H	552 (152)
$Cu(H_{-2}GlyGlyLeu)$	H	H	$CH_2CH(CH_3)_2$	541 (166)
$Cu(H_{-2}GlyLeuGly)$	H	$CH_2CH(CH_3)$	H	547 (156)
$Cu(H_{-2}LeuGlyGly)$	$CH_2CH(CH_3)_2$	H	H	548 (162)
$Cu(H_{-2}HisGlyGly)$	CH_2Im	H	H	555 (131)



λ_{max}/nm (ε/M^{-1} cm^{-1})
$Cu(H_{-1}GlyHisGly)$ 595 (56.0)



λ_{max}/nm (ε/M^{-1} cm^{-1})
$Cu(H_{-2}GlyGlyHis)$ 523 (100)

Scheme 2. Coordination structure of the Cu(II) complexes of His- and Leu-containing tripeptides at pH 8.7.

metal binding site in peptides and proteins in biological processes, including metal-ion storage and transport.[8–10] Albumin was first identified as the plasma protein most likely to bind ionic copper, and could bind about 17% of Cu(II).[11] The binding site of Cu(II) is considered to be located at the amino-terminus involving a histidine at the third position. For example, the amino acid sequences at the amino-terminus are as follows: Asp–Ala–His–Lys– for human and Asp–Thr–His–Lys– for bovine albumins.[12] The Cu(II) bound to albumin is transported to histidine as $Cu(His)_2$, which is reduced to Cu(I) and transported into the liver cells.[13,14] In this process, His is considered to participate in the formation of a ternary complex, Cu(albumin)-(His), as an intermediate. In this connection, extensive studies were carried out using the $Cu(H_{-2}GlyGlyHis)$/His system as a model for Cu(II) transport in blood.[15,16]

We have been studying Cu(II) transport from $Cu(H_{-i}L)$ complexes to CysH via a ternary complex, $Cu(H_{-1}L)(Cys^-)$, where Cys^- denotes cysteinate and $i = 1$, 2, or 3.[17–19] The $S \rightarrow Cu(II)$ charge transfer (LMCT) absorption in the UV region was used as a probe for pursuing the ligand-exchange. It is very valuable to study the transport of Cu(II) from the GlyGlyHis complex to CysH, because, if the ternary $Cu(H_{-1}GlyGlyHis)(Cys^-)$ complex could be formed, it is easily confirmed by its $S \rightarrow Cu(II)$ CT absorption. The ligand-exchange reaction in the $Cu(H_{-i}L)$ species, ($i = 1$, 2, or 3), begins at the fourth binding site in the chelate ring **C**; the carboxylate O in $Cu(H_{-2}GlyGlyGly)$ and the imidazole N in $Cu(H_{-2}GlyGlyHis)$ occupies the fourth binding site. Here, the chelate rings in $Cu(H_{-i}L)$ are termed as **A**, **B** and **C** from the amino-terminus. The thiolate S^- enters first in the fourth binding site of the Cu(II). Subsequently, the amino nitrogen of the coordinated Cys^- replaces the deprotonated-amide nitrogen, coordinating with the Cu(II) to form the ternary complex. Accordingly, affinities of the fourth ligand in $Cu(H_{-2}L)$ for the Cu(II) are expected to play an important role in determining the rate of $Cu(H_{-1}L)(Cys^-)$ formation.

In this paper, were reported on the relation between the mode of coordination and the pathway of the transport of the Cu(II) bound with His-containing tripeptides, including HisGlyGly, GlyHisGly, and GlyGlyHis, to CysH, compared with those of complexes of Leu-containing tripeptides.

Experimental

Materials. Histidine-containing tripeptides were prepared by the conventional solution technique,[20–22] and leucine-containing peptides were purchased from BACHEM Feinchemikalien AG. (Switzerland). Those were pure, as checked by liquid chromatography. Copper(II) perchlorate, $Cu(ClO_4)_2 \cdot 6H_2O$, from G. Frederick Smith Chem. Co. (Columbus, Oh), was used after recrystallization from hot water. L-Cysteine was a product of Sigma Chemical Co. (St. Louis, Mo). Other chemicals were the purest of commercially available and used without further purification.

Preparation of Sample Solutions. A stock solution of Cu(II) was prepared by dissolving $Cu(ClO_4)_2 \cdot 6H_2O$ in purified water. The water used was once deionized and doubly distilled from all glass apparatus; the first distillation was from alkaline permanganate. The Cu(II) solution was standardized by titration with 0.01 M EDTA (1 M = 1 mol dm^{-3}) with murexide as an indicator.[23] Solutions of the peptide complexes were freshly prepared using aliquots of the standardized Cu(II) solution with a 4 mole % excess peptide at pH 8.7 in a 0.01 M borate buffer. Solutions of CysH were freshly prepared prior to spectroscopic measurements. The ionic strength (I) was maintained at 0.1 M with $NaClO_4$.

Stopped-Flow Spectrophotometric Measurement. Absorbance changes at single wavelength were recorded and analyzed on a computerized Union RA-401 stopped-flow spectrophotometer equipped with a 5 mm quartz cell. A solution of 1.10×10^{-3} M

$Cu(H_{-i}L)$ and five equivalents CysH was equilibrated at 25 °C under nitrogen in a vessel with a water jacket. After equilibration for 20 min, the reaction was initiated by mixing both solutions under nitrogen at 8 kg/cm^2.

The absorbance changes were recorded at intervals of either 5 nm or 10 nm over the range from 250 nm to 700 nm. The absorption spectrum was prepared by a point-by-point plot of the absorbance, an average of seven runs, against the wavelength. The dead time (DT) of the instrument, determined by the reaction of ascorbate with 2,4-dichlorophenol-indophenol, was 1.2 ms.[24] The spectrometer was calibrated by a previously reported method.[19]

Time-Dependent Distribution of the Transients. The concentrations of the Cu(II) and Cu(I) species during the reaction were calculated from absorbance–time plots at 265 nm, 330 nm and 390 nm. The ternary complex $Cu(H_{-1}L)(Cys^-)$ exhibited absorptions at 330 nm, and the binary complexes $Cu(Cys^-)_2$ at 330 nm and 390 nm. Their absorbances at 330 nm, $A^{ter}{}_{330}$ and $A^{bi}{}_{330}$, are represented as follows:

$$A^{ter}{}_{330} = \frac{(A_{330} - \beta A_{390})}{1 - (\beta/\alpha)}, \quad (1)$$

$$A^{bi}{}_{330} = 1 - A^{ter}{}_{330}, \quad (2)$$

where α and β are experimentally obtainable constants (9.00 ± 0.05 and 2.045 ± 0.005). Detailed procedures for calculating the concentrations of each copper species had been reported previously.[19]

Results

Absorption Spectrum of the Transients. The $Cu(H_{-i}L)$; ($i = 1$, 2), for His-containing peptides, afforded red-brown transients instantly upon mixing with CysH. The absorption spectra obtained at various times in the CysH reactions with $Cu(H_{-2}HisGlyGly)$, $Cu(H_{-1}GlyHisGly)$, and $Cu(H_{-2}GlyGlyHis)$ are shown in Figs. 1, 2, and 3.

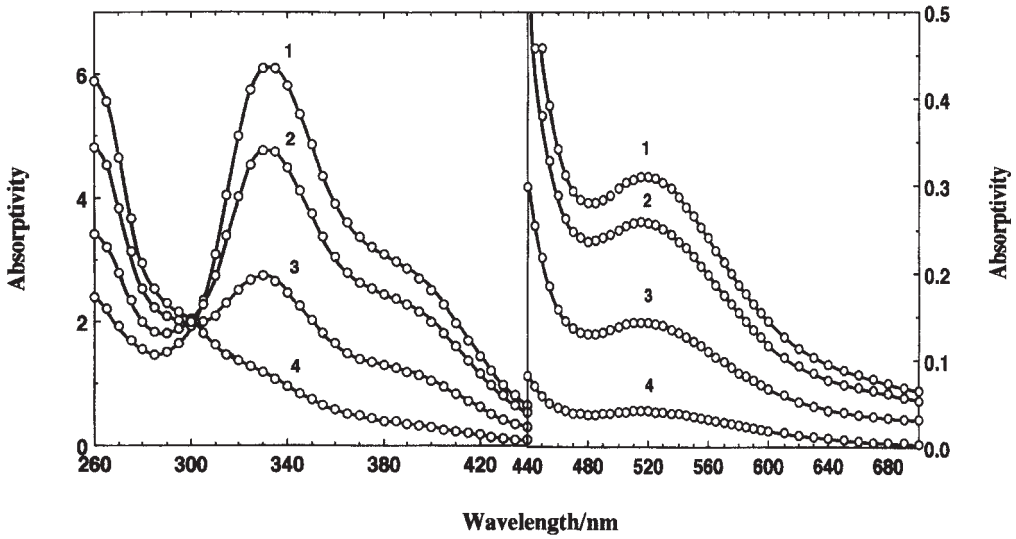


Fig. 1. Time-dependent absorption spectrum of the transients in the reaction of $Cu(H_{-2}HisGlyGly)$ with CysH at pH 8.7. [Cu(II)] = 4.98×10^{-4} M, [CysH] = 2.51×10^{-3} M, $I = 0.1$ M $NaClO_4$. 1, DT, 2, 500 ms, 3, 2 s, and 4, 10 s.

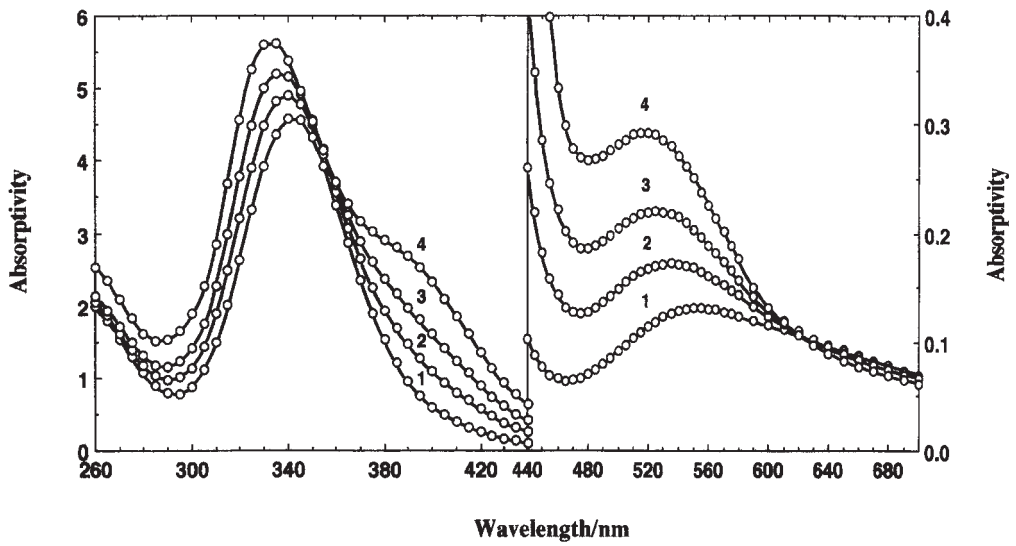


Fig. 2. Time-dependent absorption spectrum of the transients in the reaction of $Cu(H_{-1}GlyHisGly)$ with CysH at pH 8.7. [Cu(II)] = 4.98×10^{-4} M, [CysH] = 2.51×10^{-3} M, $I = 0.1$ M $NaClO_4$. 1, DT, 2, 20 ms, 3, 50 ms, and 4, 200 ms.

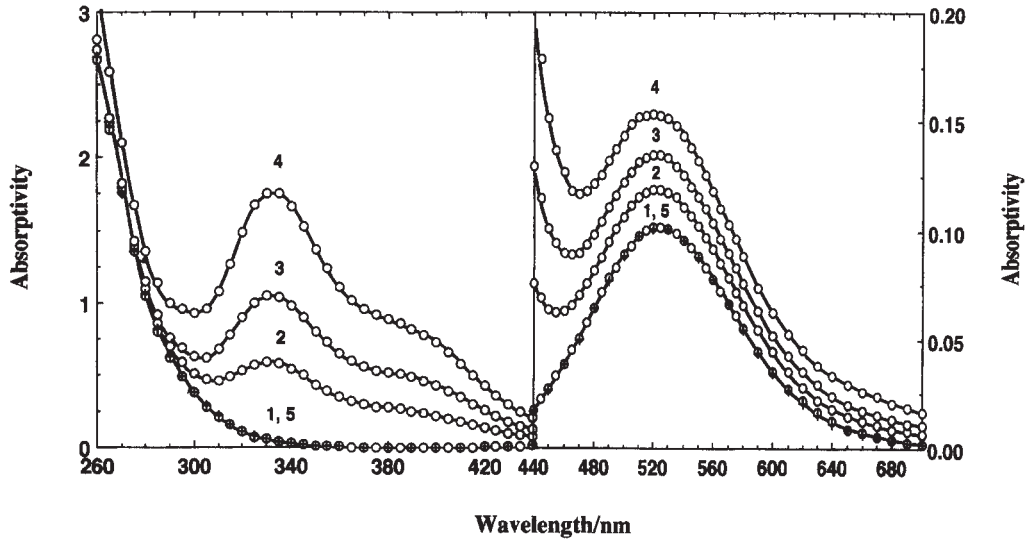


Fig. 3. Time-dependent absorption spectrum of the transients in the reaction of Cu(H_{-2}GlyGlyHis) with CysH at pH 8.7. [Cu(II)] = 5.16×10^{-4} M, [CysH] = 2.43×10^{-3} M, $I = 0.1$ M $NaClO_4$. 1, DT, 2, 1 s, 3, 2 s, 4, 5 s, and 5, Cu(H_{-2}GlyGlyHis).

Generally, Cu(H_{-i}L) reacts with CysH to first form a ternary complex, Cu(H_{-1}L)(Cys^-), which subsequently reacts with one molar CysH to yield a binary complex, Cu(Cys^-)$_2$.[17,19] Those two complexes are labile, but distinguished from each other by the absorption spectrum, as stated above. The main pathway for this consecutive reactions can be represented as follows:

$$\mathrm{Cu(H_{-}}_i\mathrm{L)} + \mathrm{Cys^-} + (i-1)\mathrm{H^+} \underset{k_{2-}}{\overset{k_{1+}}{\rightleftarrows}} \mathrm{Cu(H_{-1}L)(Cys^-)} \quad (3)$$

$$\mathrm{Cu(H_{-1}L)(Cys^-)} + \mathrm{Cys^-} + \mathrm{H^+} \overset{k_{2+}}{\rightarrow} \mathrm{Cu(Cys^-)_2} + \mathrm{L} \quad (4)$$

Cu(H_{-1}L)(Cys^-), Cu(Cys^-)$_2$, or both are reduced, depending on the pH, to the Cu(I) species.

Cu(H_{-2}HisGlyGly). At the beginning of the observation, Cu(H_{-2}HisGlyGly) afforded a transient with S → Cu(II) LMCT absorptions at 330 nm ($\varepsilon = 6000$) and 390 nm ($\varepsilon = 2880$), and d–d absorption at 525 nm ($\varepsilon = 300$), as shown in Fig. 1, where the vertical axis is expressed as mmolar absorptivity (absorbance/1×10^{-3} M Cu(II)/1 cm). The absorption spectrum was typical of Cu(Cys^-)$_2$. This was supported by its ESR spectrum. As the reaction progressed, the intensity of both the absorption and the ESR spectra decreased without changing their line-shape. Probably, the first transient Cu(H_{-1}HisGlyGly)(Cys^-), being labile, was converted to the Cu(Cys^-)$_2$ species immediately upon mixing Cu(H_{-2}HisGlyGly) and CysH solutions. Generally, the ternary complex Cu(H_{-1}glycinepeptide)(Cys^-), in which the N-terminal amino and neighboring deprotonated-amide group bind to Cu(II), is fairly stable enough to be detected by stopped-flow spectroscopic methods. The ternary complex produced in the Cu(H_{-1}HisGlyGly) reaction would have a different coordination structure from that of Cu(H_{-1}glycinepeptide)(Cys^-) species.

Cu(H_{-1}GlyHisGly). Since the fourth binding site of the Cu(II) in Cu(H_{-1}GlyHisGly) was occupied by exchangeable oxygen from water or hydroxide ion, as shown in Scheme 2, the reaction with CysH occurred rapidly. The first formed species may be the ternary complex, Cu(H_{-1}GlyHisGly)(Cys^-), which is subsequently converted to Cu(Cys^-)$_2$. The absorption bands at 345 nm and 570 nm could be assignable to the S → Cu(II) LMCT and d–d transition of Cu(II). The ternary Cu(H_{-1}L)(Cys^-) species, in which the L has a side chains in the middle of the peptide chain, e.g., GlyLeuGly, exhibit λ_{max} at 340–345 nm. As the reaction progressed, the absorption at 345 nm suffered a hypsochromic shift to 335 nm, and absorption around 390 nm newly appeared to increase. This indicates conversion from Cu(H_{-1}GlyHisGly)(Cys^-) to Cu(Cys^-)$_2$.

Cu(H_{-2}GlyGlyHis). The reaction of Cu(H_{-2}GlyGlyHis) with CysH occurred slowly. At the beginning, considerable amounts of the parent Cu(H_{-2}GlyGlyHis) complex remained, as shown in Fig. 3. The transient observed at 5 s, exhibiting the LMCT absorptions at 330 nm and 390 nm and the d–d tran-

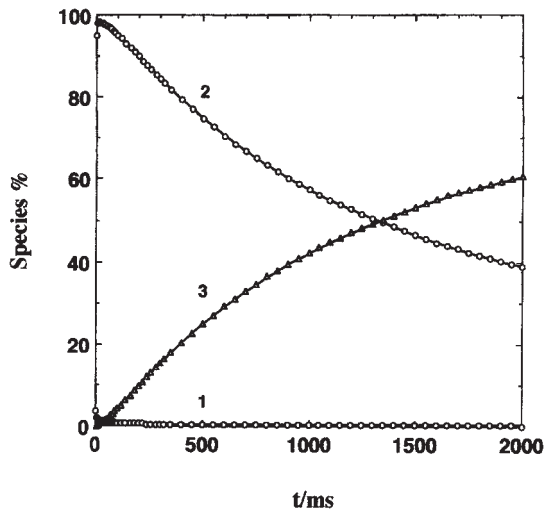


Fig. 4. Time-dependent distribution of copper species in the reaction of Cu(H_{-2}HisGlyGly) with CysH at pH 8.7. 1; Cu(HisGlyGly)(Cys^-), 2; Cu(Cys^-)$_2$, and 3; Cu(I) species. Conditions same as under Fig. 1.

sition at 525 nm, was identified to $Cu(Cys^-)_2$. It was suggested that the $Cu(H_{-1}GlyGlyHis)(Cys^-)$ upon forming was likely to change at a relatively fast rate to $Cu(Cys^-)_2$, which was successively reduced to Cu(I) species. The maximal amounts of $Cu(Cys^-)_2$, estimated from the intensity of the spectrum, was at most 30% of the total copper at 5 s.

Distribution of Cu(II) Species during the Reaction. It has been revealed that the bulky side chain of peptides in $Cu(H_{-i}L)$ either accelerates or retards the ligand-exchange reaction.[25] The distribution of copper species during the reaction with $Cu(H_{-2}HisGlyGly)$, $Cu(H_{-1}GlyHisGly)$, and $Cu(H_{-2}GlyGlyHis)$ was calculated as a function of time, compared with those for $Cu(H_{-2}LeuGlyGly)$, $Cu(H_{-1}GlyLeuGly)$, and $Cu(H_{-2}GlyGlyLeu)$ (Figs. 4–9).

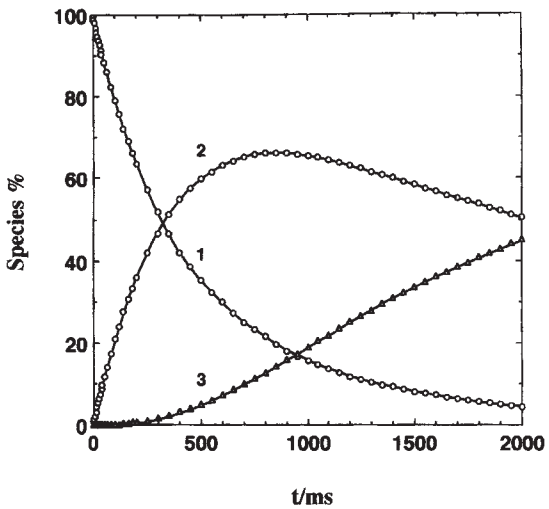


Fig. 5. Time-dependent distribution of copper species in the reaction of $Cu(H_{-2}LeuGlyGly)$ with CysH at pH 8.7. 1; $Cu(H_{-1}LeuGlyGly)(Cys^-)$, 2; $Cu(Cys^-)_2$, and 3; Cu(I) species. $[Cu(II)] = 5.03 \times 10^{-4}$ M, $[CysH] = 2.50 \times 10^{-3}$ M, $I = 0.1$ M $NaClO_4$.

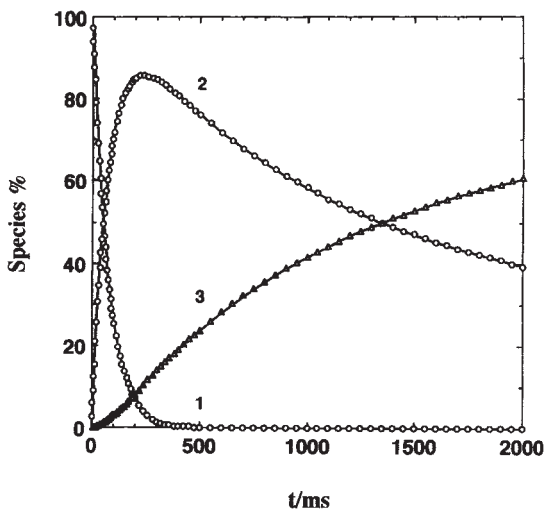


Fig. 6. Time-dependent distribution of copper species in the reaction of $Cu(H_{-1}GlyHisGly)$ with CysH at pH 8.7. 1; $Cu(H_{-1}GlyHisGly)(Cys^-)$, 2; $Cu(Cys^-)_2$, and 3; Cu(I) species. Conditions same as under Fig. 2.

$Cu(H_{-2}HisGlyGly)$ and $Cu(H_{-2}LeuGlyGly)$. As predicted from Fig. 1, both the rate constants, k_{1+} and k_{2+}, in Eqs. 3 and 4 were large enough to change the parent $Cu(H_{-2}HisGlyGly)$ complex completely to $Cu(Cys^-)_2$ within the dead time (DT) of the instrument (ca. 1.2 ms). The chelate-ring **A**, constructed by the N-terminal His residue, in $Cu(H_{-1}HisGlyGly)(Cys^-)$ was kinetically labile and rapidly opened to yield $Cu(Cys^-)_2$, as shown in Fig. 4. In contrast, the ring **A** in $Cu(H_{-1}LeuGlyGly)(Cys^-)$ was relatively stable, so that the k_2 could be determined (0.9×10^3 M^{-1} s^{-1}).[25] The maximal amount for the $Cu(Cys^-)_2$ evaluated was approximately 70% of the total copper at 800 ms in Fig. 5.

$Cu(H_{-1}GlyHisGly)$ and $Cu(H_{-2}GlyLeuGly)$. In the reac-

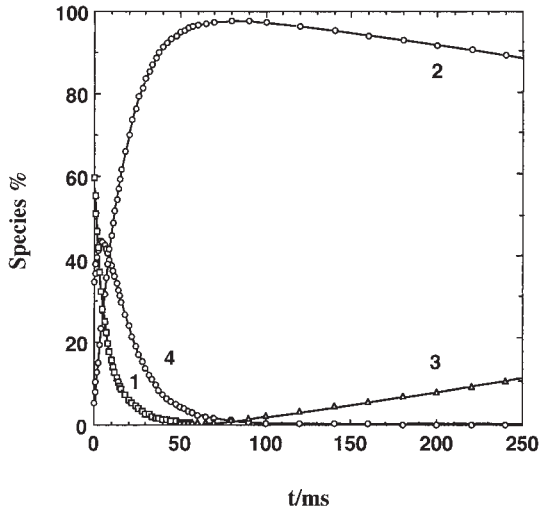


Fig. 7. Time-dependent distribution of copper species in the reaction of $Cu(H_{-2}GlyLeuGly)$ with CysH at pH 8.7. 1; $Cu(H_{-2}GlyLeuGly)$, 2; $Cu(H_{-1}GlyLeuGly)(Cys^-)$, 3; $Cu(Cys^-)_2$, and 4; Cu(I) species. $[Cu(II)] = 5.03 \times 10^{-4}$ M, $[CysH] = 2.50 \times 10^{-3}$ M, $I = 0.1$ M $NaClO_4$.

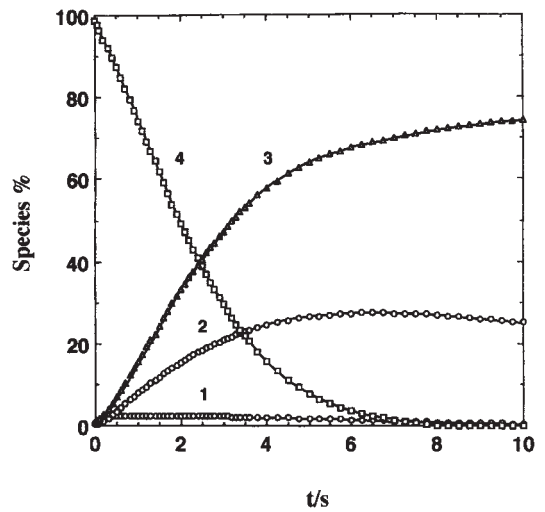


Fig. 8. Time-dependent distribution of copper species in the reaction of $Cu(H_{-2}GlyGlyHis)$ with CysH at pH 8.7. 1; $Cu(H_{-1}GlyGlyHis)(Cys^-)$, 2; $Cu(Cys^-)_2$, 3; Cu(I) species, and 4: $Cu(H_{-2}GlyGlyHis)$. Conditions same as under Fig. 3.

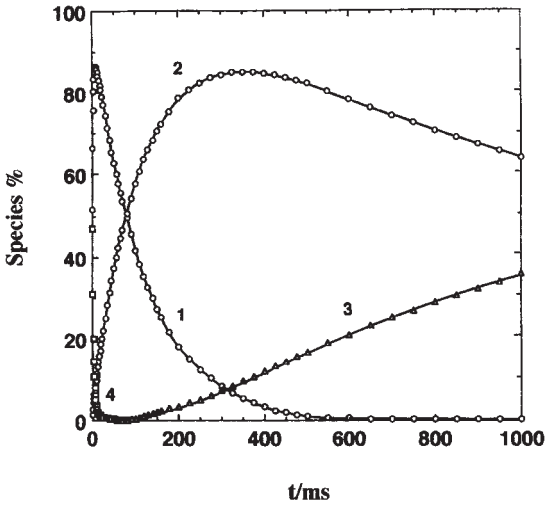


Fig. 9. Time-dependent distribution of copper species in the reaction of Cu(H_{-2}GlyGlyLeu) with CysH at pH 8.7. 1; Cu(H_{-2}GlyGlyLeu)(Cys^-), 2; $Cu(Cys^-)_2$, and 3; Cu(I) species. $[Cu(II)] = 5.03 \times 10^{-4}$ M, $[CysH] = 2.50 \times 10^{-3}$ M, $I = 0.1$ M $NaClO_4$.

tion of Cu(H_{-2}GlyLeuGly) with CysH, the isobutyl group attached to the chelate ring **B** not only retarded the formation of Cu(H_{-1}GlyLeuGly)(Cys^-) from Cu(H_{-2}GlyLeuGly), but also accelerated the formation of $Cu(Cys^-)_2$ from the ternary species. Those were clarified by the species distribution curve shown in Fig. 7, which indicates that $Cu(Cys^-)_2$ occupied more than 95% of the total copper at 80 ms. Cu(H_{-1}GlyHisGly) has a 5-6-membered fused-chelate structure and the fourth binding site of the Cu(II) is occupied by the kinetically exchangeable oxygen from the coordination water. Then, the formation of the Cu(H_{-1}GlyHisGly)(Cys^-) species was very rapid, as shown in Fig. 6. The side chain in the ring **B** appeared to slightly enhance the $Cu(Cys^-)_2$ formation as compared with the Cu(H_{-2}GlyGlyGly).

Cu(H_{-2}GlyGlyHis) and Cu(H_{-2}GlyGlyLeu). A bulky isobutyl group in Cu(H_{-2}GlyGlyLeu) slightly retards opening the chelate ring **C**.[25] Cu(H_{-2}GlyGlyHis), involving the imidazole nitrogen in the fourth binding site, reacted at a slower rate with CysH. A small amount of Cu(H_{-1}GlyGlyHis)(Cys^-), less than 3% of the total copper, was formed in the reaction, as shown in Fig. 8. In addition, since the rate constant k_{2+} was bigger than k_{1+}, the ternary complex Cu(H_{-1}GlyGlyHis)(Cys^-) upon forming spontaneously changed to $Cu(Cys^-)_2$. This is the reason why the spectrum of the ternary complex could not be observed at any time over the reaction period. Furthermore, as $Cu(Cys^-)_2$ formed, it was likely to suffer reduction, and thus amount was at most 30% of the total copper at 5–6 s after the start of reaction. The formation of $Cu(Cys^-)_2$ appeared to progress in parallel with its reduction.

Initial increases in the species distribution curves for the Cu(H_{-1}L)(Cys^-) species obeyed a second-order rate expression. From the curves, the apparent second-order rate constants, k'_{1+}, for each reaction system were tentatively estimated. The results are summarized in Table 1. Here, "v. rapid" means that k'_{1+} is bigger than an order of magnitude of 10^6 M^{-1} s^{-1}.

Table 1. Apparent Second-Order Rate Constants for the Reaction of Cu(H_{-i}L) with CysH at pH 8.7

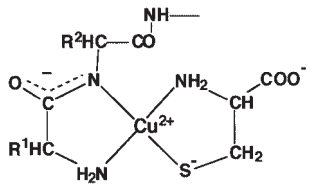
Cu(H_{-i}L)	$k_{1+}/10^3$ M^{-1} s^{-1}	$k_{2+}/10^3$ M^{-1} s^{-1}
Cu(H_{-2}GlyGlyGly)	v. rapid[a)]	3.3[13]
Cu(H_{-2}HisGlyGly)	v. rapid	v. rapid
Cu(H_{-2}LeuGlyGly)	v. rapid	0.9[13]
Cu(H_{-1}GlyHisGly)	v. rapid	6.9
Cu(H_{-2}GlyLeuGly)	130[13]	11[13]
Cu(H_{-2}GlyGlyHis)	0.04	3–4
Cu(H_{-2}GlyGlyLeu)	300[13]	3.0[13]

a) Very rapid; $k > \sim 10^6$ M^{-1} s^{-1}.

Discussion

Absorption Spectrum of the Ternary Complex, Cu(H_{-1}L)(Cys^-). The Cu(H_{-i}L) species examined, except for Cu(H_{-2}HisGlyGly), formed ternary Cu(H_{-1}L)(Cys^-) complexes. The amino acid residue at N-terminus coordinated to Cu(II) via the amino and the neighboring deprotonated-amide nitrogens, constructing a five-membered chelate ring (Scheme 3). The absorption spectrum of the Cu(H_{-1}L)(Cys^-) could be resolved to three transition bands by a previously reported method.[26] The results are summarized in Table 2. The absorption bands at 330–340 nm, assignable to a $\sigma(S) \rightarrow Cu(II)$ CT band, were flanked at lower energy (400 nm) by a weaker $\pi(S) \rightarrow Cu(II)$ CT band. The side-chain in the ring **B** tended to shift the $\sigma(S) \rightarrow Cu(II)$ CT band to lower energy (600–700 cm^{-1}).

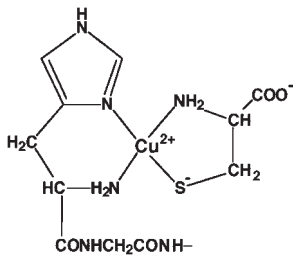
In a reaction with Cu(H_{-2}HisGlyGly), the first transient was identified as the binary $Cu(Cys^-)_2$ complex, based on its spectral parameters. The Cu(H_{-2}HisGlyGly), exhibiting d–d transition of Cu(II) at $\lambda_{max} = 555$ nm (Scheme 2), is considered to have a coordination structure involving N,N^-,N^-,O^--donors, as referred to the Billo' rule.[27] If the His-residue in the ring **A** conserves this coordination mode, the ternary complex, Cu(H_{-1}HisGlyGly)(Cys^-), should be detected. However, Cu(H_{-2}HisGlyGly) was changed completely to $Cu(Cys^-)_2$ upon mixing with CysH. It is predicted that the chelate ring **A** in Cu(H_{-1}HisGlyGly)(Cys^-) is kinetically labile to rapidly dissociate through an intramolecular rearrangment. Probably, the chelate ring **A** is rearranged to a labile form, which is probably the histamine-like coordination structure (Scheme 4). When the chelate rings **A** and **B** participate in a fused-chelate of the ternary complex, the complex is fairly stable. A monodentate *N*-acetyl-penicillamine (Aps) could form a ternary complex Cu(H_{-2}HisGlyGly)(Aps^-) with a $S \rightarrow Cu(II)$ CT band at 360 nm. Both the chelate rings **A** and **B** are likely to be conserved in Cu(H_{-2}HisGlyGly)(Aps^-), where the His coordinates to Cu(II) via the terminal amino and two neighboring deprotonated amide nitrogens.[28]



Scheme 3. Coordination structure for the ternary complex Cu(H_{-1}L)(Cys^-).

Table 2. Parameter of the Absorption Spectrum for the Ternary $Cu(H_{-1}L)(Cys^{-})$ and Binary $Cu(Cys^{-})_2$ Complexes

Complex	λ_{max}/nm (ε/M^{-1} cm^{-1})		
	$\sigma(S) \rightarrow Cu(II)$	$\pi(S) \rightarrow Cu(II)$	d–d
$Cu(H_{-1}GlyGly)(Cys^{-})$	333 (4240)	406 (193)	542 (162)
$Cu(H_{-1}GlyGlyGly)(Cys^{-})$	335 (4210)	398 (347)	542 (141)
$Cu(H_{-1}HisGlyGly)(Cys^{-})$	not detected		
$Cu(H_{-1}LeuGlyGly)(Cys^{-})$	333 (4450)	383 (312)	538 (147)
$Cu(H_{-1}GlyHisGly)(Cys^{-})$	342 (4520)	402 (247)	565 (131)
$Cu(H_{-1}GlyLeuGly)(Cys^{-})$	342 (4770)	408 (62.5)	577 (151)
$Cu(H_{-1}GlyGlyLeu)(Cys^{-})$	334 (4250)	382 (419)	536 (144)
$Cu(H_{-1}GlyHisGly)(Acs^{-})$	346 (5310)	405 (288)	553 (157)
$Cu(Cys^{-})_2$	334 (6170)	391 (2760)	526 (351)



Scheme 4. Proposed coordination structure for the ternary complex $Cu(H_{-1}HGG)(Cys^{-})$.

Effects of Side-Chain of Amino-Acid Residue on the Ligand-Exchange. In a previous paper, we described that the formation of the $Cu(H_{-1}L)(Cys^{-})$ species from $Cu(H_{-2}L)$ is a sequential reaction composing of (3a) and (3b):[19,29]

$$Cu(H_{-2}L) + Cys^{-} \underset{k_{11-}}{\overset{k_{11+}}{\rightleftarrows}} Cu(H_{-2}L)(Cys^{*-}), \quad (3a)$$

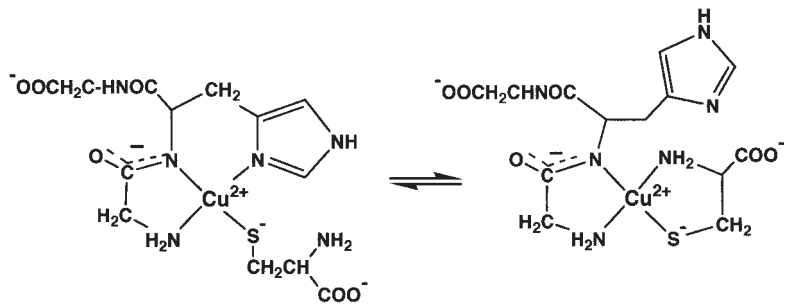
$$Cu(H_{-2}L)(Cys^{*-}) + H^{+} \underset{k_{12-}}{\overset{k_{12+}}{\rightleftarrows}} Cu(H_{-1}L)(Cys^{-}). \quad (3b)$$

A ligand replacement in $Cu(H_{-2}L)$ begins primarily at the carboxylate end. Firstly, the Cys^{-} replaces the fourth donor of $Cu(H_{-2}L)$ with the thiolate S, forming a ternary complex, $Cu(H_{-2}L)(Cys^{*-})$, in which Cys^{*-} works as a monodentate. In this reaction, the chelate ring **C** is dissociated. Next, the amino-nitrogen of the Cys^{*-} would replace the deprotonated amide-nitrogen to form $Cu(H_{-1}L)(Cys^{-})$. By this reaction, the chelate ring **B** is opened.

The affinity of Cu(II) for the fourth donor controlled the rate of the $Cu(H_{-1}L)(Cys^{-})$ formation. Since the fourth site in $Cu(H_{-1}L)$, except for $Cu(H_{-2}GlyGlyHis)$, is occupied by the exchangeable oxygen, the rate constant k_{1+} is order of 10^5 M^{-1} s^{-1} or bigger. On the other hand, $Cu(H_{-2}GlyGlyHis)$, involving the imidazole nitrogen in the fourth site, was sluggish toward ligand-exchange, and the rate constants was arranged as follows; $k_{1+} < k_{2+}$. Then, a sequence of reactions, $Cu(H_{-2}GlyGlyHis) \rightarrow Cu(H_{-1}GlyGlyHis)(Cys^{-}) \rightarrow Cu(Cys^{-})_2$, appears to occur simultaneously. The amount of the ternary complex was less than 5% of the total copper.

A feature of the reaction between $Cu(H_{-1}GlyHisGly)$ and CysH was a little complicated. The formation of $Cu(H_{-1}L)(Cys)$ is very rapid, because the fourth binding site in $Cu(H_{-1}GlyHisGly)$ is occupied by kinetically exchangeable oxygen. The k_{1+} is probably 10^6 M^{-1} s^{-1} or bigger. The coordinated imidazole nitrogen in $Cu(H_{-2}GlyHisGly)(Cys^{*-})$ would resist being exchanged with the amino nitrogen of Cys^{*-} (Scheme 5).

Generally, conversion from $Cu(H_{-2}L)(Cys^{*-})$ to the $Cu(H_{-1}L)(Cys^{-})$ species was accompanied by protonation of the coordinated amide nitrogen. In contrast, reaction 3b in $Cu(H_{-1}GlyHisGly)(Cys^{*-})$ does not involve protonation. Then, an assumption could be proposed that the imidazole nitrogen in $Cu(H_{-1}GlyHisGly)(Cys^{-})$ is capable of replacing the amino nitrogen of Cys^{-} to back to $Cu(H_{-1}GlyHisGly)(Cys^{*-})$. The overall equilibrium constant, K_1, (k_{1+}/k_{1-}), between $Cu(H_{-1}GlyHisGly)$ and CysH was one or two orders of magnitude smaller than other system, and evaluated approximately 10^4 M^{-1}.[28] $Cu(H_{-2}GlyHisGly)(Cys^{*-})$ and $Cu(H_{-1}GlyHisGly)(Cys^{-})$ could not distinguish from each other, based on their absorption spectra. This is because $Cu(H_{-2}Gly-$



Scheme 5. Equilibrium between $Cu(H_{-1}GlyHisGly)(Cys^{*-})$ and $Cu(H_{-1}GlyHisGly)(Cys^{-})$.

HisGly)(Acs$^-$), having an analogous coordination structure to Cu(H$_{-2}$GlyHisGly)(Cys^{*-}), is shown to exhibit a similar spectrum to Cu(H$_{-1}$GlyHisGly)(Cys$^-$); where Acs$^-$ denoted a monodentate *N*-acetylcysteinate.

Bulky side chains without coordinating ability also modify the feature of the ligand-exchange reaction. An isobutyl group in Leu-containing peptides appeared to retard the ligand-exchange when it was involved in the chelate ring dissociated by CysH. The k_{1+} value for Cu(H$_{-2}$GlyGlyLeu) was one order of magnitude smaller than that of Cu(H$_{-2}$GlyGlyGly). A similar result was observed in the k_{2+} of Cu(H$_{-2}$LeuGlyGly)(Cys$^-$) compared with Cu(H$_{-2}$GlyGlyGly)(Cys$^-$).[25] As reported previously concerning the GlyLeuGly complex, the isobutyl group in ring **B** not only decreases the k_{1+} value, but also increases the k_{2+}.

Conclusion

The ligand-exchangeability of the Cu(II) in Cu(H$_{-i}$L) ($i = 1$ for GlyHisGly and $i = 2$ for other tripeptides) with CysH was determined by the coordinating ability of the fourth donors and the position of the side chains in the peptide backbone. Rings **C** and **B** of a 5-5-5 membered fused chelate ring in Cu(H$_{-i}$L) were successively opened by Cys$^-$, and a ternary complex, Cu(H$_{-1}$L)(Cys$^-$), was formed. The rate of Cu(H$_{-1}$L)(Cys$^-$) formation was controlled primarily by the affinity of the Cu(II) for the fourth donor atom involved in ring **C**. In Cu(H$_{-2}$GlyGlyHis), since kinetically stable imidazole nitrogen occupied the fourth site, the formation of the Cu(H$_{-1}$L)(Cys$^-$) species was slow. The rate constant k_{1+} was smaller than k_{2+} in the formation of Cu(Cys$^-$)$_2$ from Cu(H$_{-1}$GlyGlyHis)(Cys$^-$). A species distribution curve reveals that Cu(H$_{-2}$GlyGlyHis) and Cu(Cys$^-$)$_2$ were major species in the reaction, and that Cu(H$_{-2}$GlyGlyHis)(Cys$^-$) was less than 5% of the total copper. Cu(H$_{-1}$GlyHisGly) reacted with CysH, initially forming the ternary complex Cu(H$_{-1}$GlyHisGly)(Cys^{*-}). The formation of a ternary complex, Cu(H$_{-1}$GlyHisGly)(Cys$^-$), from Cu(H$_{-1}$GlyHisGly)(Cys^{*-}) may involve an intramolecular ligand-exchange between the imidazole nitrogen of His and the amino nitrogen of Cys^{*-} in Cu(H$_{-1}$GlyHisGly)(Cys^{*-}). The ternary complex of HisGlyGly with CysH was extremely labile, so much so that it could not be detected by stopped-flow techniques. Probably, the N-terminal histidyl residue, coordinating to Cu(II) via the amino and imidazole nitrogens, formed a kinetically labile complex with a histamine-like structure. The position of the side chains, with or without coordination ability, was important in determining the reaction rate. The side chains in the chelate ring **C** retard the ligand-exchange, as observed in the Cu(H$_{-1}$GlyGlyLeu) reaction. The side chains in chelate-ring **B** not only retarded Cu(H$_{-1}$GlyLeuGly)(Cys$^-$) formation, but also stimulated conversion from Cu(H$_{-1}$GlyLeuGly)(Cys$^-$) to Cu(Cys$^-$)$_2$.

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3 Abbreviation used; L: free tripeptide molecule, $H_2NCHR^1CO–NHCHR^2CO–NHCHR^3COO^-$ for tripeptides, Gly: glycine, His: histidine, CysH: cysteine, Cys$^-$: cysteinate.

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