Stopped-Flow Spectroscopic Studies on the Ligand-Exchange Reaction of Cu–(Glycine-Peptide) Complexes, Cu($H_{-i}L$), with Cysteine. Cu(II) Transport and Characterization of the Intermediate Ternary Complexes Cu($H_{-1}L$)(Cys^-); L = Glycylglycine ($i = 1$), Triglycine ($i = 2$), Tetraglycine ($i = 2$ or 3), and Pentaglycine ($i = 2$ or 3)

Akira Hanaki,*,# Manabu Hiraoka, Toshikazu Abe, Yasuhiro Funahashi,† and Akira Odani††

Department of Applied Chemistry, Faculty of Engineering, Shizuoka University, Jyohoku, Hamamatsu 432-8561

†Department Applied Chemistry, Nagoya Institute of Technology, Syowa-ku, Nagoya 466-0061

††Research Center for Materials Science, Nagoya University, Chikusa-ku, Nagoya 464-8602

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Cu(II) complexes of glycine-peptides, abbreviated as Cu($H_{-i}L$): L = glycylglycine, triglycine, tetraglycine, and pentaglycine ($i = 1$–3), react with cysteine to form ternary complexes, Cu($H_{-1}L$)(Cys^-), as first intermediates. The spectral parameters of the ternary complexes, which were similar irrespective of the peptides, were as follows: $\lambda_{max} = 332 \pm 1$ nm ($\varepsilon = 4250 \pm 50$ $M^{-1}\,cm^{-1}$), $g_{\parallel} = 2.170 \pm 0.005$, $g_{\perp} = 2.00 \pm 0.05$, and $A_{\parallel} = (2.05 \pm 0.01) \times 10^{-4}$ cm^{-1}, indicating that the ternary complexes have identical coordination structures: the Cu(II) coordinates with the peptides via the nitrogens from the terminal amino and the neighboring deprotonated-amide group, and with cysteine via the amino nitrogen and the thiolate sulfur. Based on the absorbance-time curves, the concentrations of each Cu(II) and Cu(I) species during the reaction were calculated. The species distribution curve clearly visualized the pathway of the Cu(II) transport from Cu($H_{-i}L$) to Cu($Cys^-)_2$ via Cu($H_{-1}L$)(Cys^-). The rate of Cu($H_{-1}L$)(Cys^-) formation, which was evaluated from the initial increase in the species distribution curve, depended on the coordination modes of the Cu($H_{-i}L$) complexes. Both the Cu($H_{-1}L$), involving N,N^-,O^-,O* donors, and the Cu($H_{-2}L$), involving N,N^-,N^-,O^-* donors, rapidly formed Cu($H_{-1}L$)(Cys^-) complexes, where the donors asterisked represent the fourth ligand in the Cu($H_{-i}L$) complexes. The second-order rate constant, k_{1+}, was on the order of 10^6 $M^{-1}\,s^{-1}$, or bigger. The Cu($H_{-3}L$), involving N,N^-,N^-,N^-* donors, reacted relatively slowly; k_{1+} was on the order of 10^4 $M^{-1}\,s^{-1}$. Those results indicate that the affinity of the Cu(II) for the fourth donor in Cu($H_{-i}L$) determines the rate of metal-transport.

Most potent Cu(II)-chelators of biolgical interest are peptides that donate nitrogen atoms, derived from amino, amide, and imidazole groups, for constructing the metal binding site. One of the most popular binding sites is the amino-terminus of the peptide chain, in which nitrogens from an amino group and two or three neighboring deprotonated-amide groups bind to the Cu(II).[1] X-ray crystallographic results of single crystals support this coordination mode in solution.[2,3] Such coordination results in complexes with a violet-red–blue color (biuret test) that persists in solution as well as a solid. Thus, the amino terminus of proteins, which has a high affinity for Cu(II), is considered to be a first site for receiving the incoming metal ion in metal transport.[4]

The complex formation of Cu(II) with simple peptides (HL)[5] has been extensively studied.[1,6–8] The peptide can form several kinds of Cu(II) complexes, abbreviated as CuL, Cu($H_{-1}L$), Cu($H_{-2}L$), and Cu($H_{-3}L$), depending on the pH (Scheme 1). The amide group coordinates to Cu(II) in a fashion with deprotonation of the amido-nitrogen. In weakly acidic solutions at around pH 5, the metal ion is bound to the amino nitrogen and the carbonyl oxygen of the neighboring amide group to form CuL, which is less stable than amino acid complexes.[9] Between pH 5–6, the bound amide group is deprotonated. The deprotonated amide nitrogen replaces the carbonyl oxygen to bind to the Cu(II) forming the Cu($H_{-1}L$) species with a Cu–N^- bond, as well as the Cu–NH_2 bond.[6,7] The $CuH_{-1}L$ has a 5-5 membered fused-chelate structure ($\lambda_{max} = \sim 620$ nm) with N,N^-,O donors. The formation of a fused-chelate ring results in wrapping of the Cu(II) in the peptide backbone, thus stabilizing the complex.[1,6–8] As the pH increases, successive deprotonation of the neighboring amide group and the exchange of donor from the oxygen to the nitrogen occur. Thus, the Cu($H_{-2}L$) species with N,N^-,N^-,O-donors ($\lambda_{max} = \sim 550$ nm), and finally the Cu($H_{-3}L$) species with N,N^-,N^-,N^--donors ($\lambda_{max} = \sim 510$ nm) are formed. The fused-chelate complexes have been shown to be very stable in solution, as studied by pH-titration methods.[10] The peptide complexes, such as Cu($H_{-2}L$) and Cu($H_{-3}L$), are thermodynamically stable, but it is still uncertain whether the Cu(II) within the peptide complex is kinetically stable or labile. The kinetic stability of metal ions within the complex should play an important role in its

Present address: National Institute of Radiological Sciences, Anagawa 4-9, Inage-ku, Chiba 432-8561

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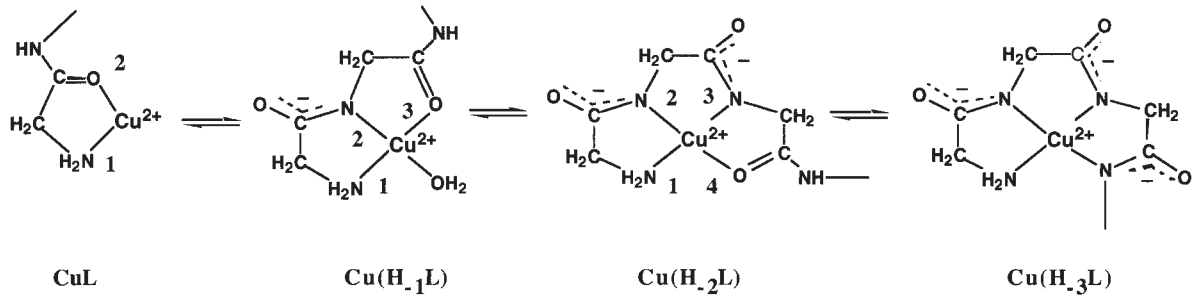


Scheme 1. Coordination structures of the $Cu(H_{-i}L)$ species ($i = 1$, 2, or 3).

transport and distribution.

The kinetic stability of Cu(II) in peptide complexes could be assessed by the rate constant of the ligand-exchange reaction.[11] The ligand exchange in $Cu(H_{-i}L)$ is a complicated reaction involving multi-intermediates. In order to understand the feature of the pathway of metal-ion transport, a proper substrate for detecting and determining the intermediate should be selected. We have been studying Cu(II) transport from $Cu(H_{-i}L)$ to cysteine (CysH) or penicillamine (PesH).[12–14] The Cu(II)–S^-(aliphatic thiolate) transition band at $\lambda_{max} =$ 330–340 nm can be used as a probe for pursueing the ligand-exchange, because, when the complexes with a Cu(II)–S^-(thiolate) bond have been formed, the metal ion is transferred into spectroscopically different environments. In this paper, we describe the exchangeability of the Cu(II) in the $Cu(H_{-i}L)$ species, where L were glycine-peptides, including GlyGly (G2), GlyGlyGly (G3), GlyGlyGlyGly (G4), and GlyGlyGlyGlyGly (G5), based on the results from experiments on a ligand-exchange with CysH. The simple Gly-peptides were valuable in fundamentally understanding how the Cu(II) wrapped in the peptide backbone was released by CysH. We first demonstrated evidence for the formation of the ternary complex, $Cu(II)(H_{-1}L)(Cys^-)$, and its coordination mode and structure in solution. Here, otherwise stated, the copper ion in the ternary and binary complexes involving Cys^- denotes Cu(II). Secondly, we visualized the pathway of the Cu(II) transport from $Cu(H_{-i}L)$ to $Cu(Cys^-)_2$ via $Cu(H_{-1}L)(Cys^-)$. Finally, the relation between the Cu(II)-exchangeability and the coordination mode was elucidated.

Experimental

Materials. All of the glycine-peptides, products from BACHEM Feinchemikalien AG. (Switzerland), were pure, as checked by chromatography, and used without further purification. Copper(II) perchlorate, $Cu(ClO_4)_2 \cdot 6H_2O$, from G. Frederick Smith Chem. Co. (Columbus, Oh), was purified by recrystallization. L-Cysteine was a product of Sigma Chemical Co. (St. Louis, Mo). All 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, which was deionized once and doubly distilled from all glass apparatus, and the first distillation from alkaline permanganate. The solution was standardized by titration with 0.01 M EDTA (1 M = 1 mol dm^{-3}) with murexide as an indicator.[15] Solutions of the Cu(II) complexes were freshly prepared using aliquots of the standardized Cu(II) solution with a 3 mole % excess peptide to ensure complex formation. Solutions of CysH were freshly prepared prior to spectroscopic measurements. The ionic strength (I) was maintained at 0.1 M with $NaClO_4$ for a measurement of the absorption spectrum and at 0.5 M with $NaNO_3$ for an ESR measurement.

Spectrophotometric Measurement. Single-wavelength measurements of the absorbance against time were carried out at 25 °C and $I = 0.1$ M $NaClO_4$, and analyzed on a computerized Union RA-401 stopped-flow spectrophotometer equipped with a 5 mm quarz cell. The solutions of the 1.00×10^{-3} M $Cu(H_{-i}L)$ and of four-molar equivalents CysH were equilibrated at 25 °C in a water-jacketted vessel under nitrogen. After equilibration for 20 min, the reaction was started by mixing both solutions at 8 kg/cm^2 N_2.

The absorbance was recorded at intervals of either 5 nm or 10 nm over the range from 250 nm to 700 nm. The absorption spectrum of the solution at a certain time was obtained by a point-by-point plot of the absorbance against the wavelength. The absorbance was an average of at least eight runs. The dead time (DT) of the instrument determined by the reaction of ascorbate with 2,4-dichlorophenolindophenol was 1.2 ms.[16]

The spectrometer was calibrated against the emission at 656.3 nm from a D_2 lump, and standardized by a $Cu(H_{-2}G3)$ solution ($\varepsilon = 155$ M^{-1} cm^{-1}), which was freshly prepared at pH 9.5.

ESR Measurement. The ESR spectrum was recorded on a JEOL JES-RE-1X spectrometer with 100 kHz field modulation. The g value and hyperfine constant of the spectrum were calculated by a comparison with a standard Mn(II) doped in MgO at room temperature and with 1,1-diphenyl-2-picrylhydrazyl (DPPH) at 77 K. A continuous-flow method was applied to the measutrements at room temperature by using a JES-SM-1 mixing apparatus with a M07-K04 sample mixer and a JES-LC-01 flow cell. The concentration of Cu(II) was 3.0×10^{-3} M in 0.05 M borate buffer at pH 9.3 and $I = 0.5$ M $NaNO_3$. The dead time was controlled by changing the flow rate of the sample solutions. The dead time estimated at a flow rate of 3.2 mL/s was approximately 7 ms, and that at 2.2 mL/s was 10 ms. For measurements at 77 K, the transients were quenched by a stopped-flow and rapid-freezing technique reported previously.[17] The concentration of Cu(II) was 1.25×10^{-3} M in a 0.05 M borate buffer at pH 8.2–10.2 and $I =$ 0.5 M $NaNO_3$. The minimum quenching time was estimated to be 4 ms, based on the rate of Ni–EDTA/triethylenetetramine reaction.[17]

Results

UV and Visible Absorption Spectrum of the Reaction Intermediates. a) $CuH_{-1}L$ and $CuH_{-2}L$: The reactions of $Cu(H_{-1}G2)$ and $Cu(H_{-2}G3)$ species with CysH were conducted at pH 9.3. Upon mixing both solutions, a red-brown color appeared instantly, and faded within a few seconds

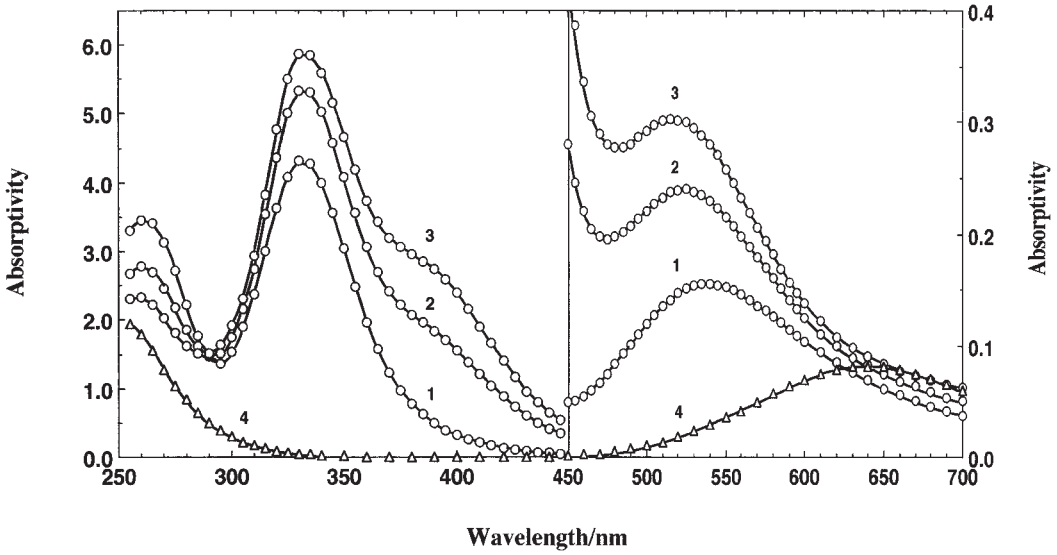


Fig. 1. Time-dependent spectral changes on addition of Cu(H_{-1}G2) to CysH at pH 9.3. (1), dead time, (2), 100 ms, (3), 400 ms, and (4), Cu(H_{-1}G2). [Cu(II)] = 4.92×10^{-4} M and [CysH] = 2.05×10^{-3} M (4.17 equiv.), 0.01 M borate buffer (I = 0.1 M $NaClO_4$), T = 25 °C.

thereafter. In the Cu(H_{-1}G2) reaction, the absorption spectra at the start; i. e., within DT, at 100 ms, and 400 ms, are shown in Fig. 1, where the vertical axis is expressed as the absorptivity per 1 mM Cu(II)/1 cm cell. The formation of the first intermediate was extremely rapid. The spectrum at the beginning exhibited λ_{max} at 333 nm and 535 nm. The absorption bands at 333 nm and around 535 nm were assignable to the S → Cu(II) charge transfer (LMCT) and the d–d transition of Cu(II), respectively, and the ratio of the absorbances at 330 nm and 390 nm ($R = A_{330}/A_{390}$) was approximately 9.00. A stopped-flow molar-ratio plot of the first transient at 330 nm clearly exhibits the stoichiometric relation of [CysH]/[Cu(II)] = 1.0, as shown in Fig. 2. Provided that the peptide remains

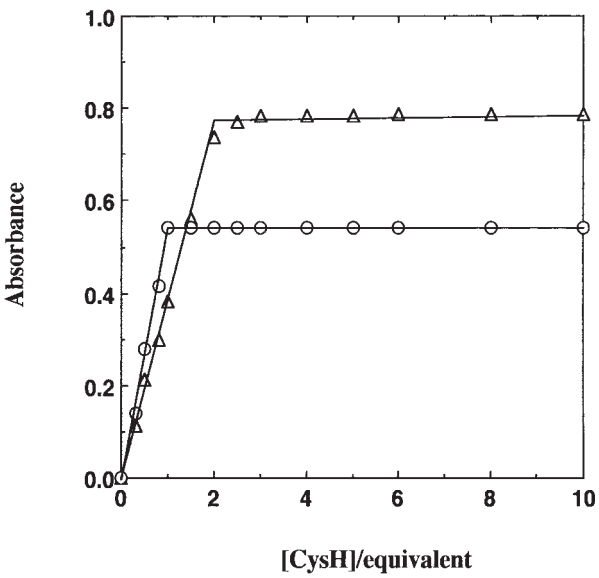


Fig. 2. Stopped-flow molar ratio plots for the first transients produced in the reactions of CysH with Cu(H_{-1}G2) at pH 9.5 (○) and $Cu(Gly)_2$ (△). [Cu(II)] = 4.92×10^{-4} M, 0.01 M borate buffer (I = 0.1 M $KClO_4$), T = 25 °C.

complexed with Cu(II), the formation of the ternary Cu(H_{-1}G2)(Cys^-) complex is suggested. The Cu(H_{-2}G3) also formed a ternary complex, Cu(H_{-1}G3)(Cys^-).

As the reaction proceeded, a new LMCT absorption at around 390 nm, in addition to the 333 nm band, appeared and increased thereafter untill 400 ms. This absorption spectrum was characteristic of a second transient. The absorption specrtum at 400 ms, exhibiting LMCT absorptions at 332 nm and 390 nm ($R = A_{330}/A_{390}$ = 2.04) and the d–d absorption around 520 nm, was assignable to the binary complex $Cu(II)(Cys)_2$, as compared with the absorption spectrum of the first transient in the $Cu(Gly)_2$/CysH system. The stoichiometric relation of [CysH]/[Cu(II)] = 2.0 exists in the $Cu(Gly)_2$/CysH system, as shown in Fig. 2. A family of successive spectra for the initial 400 ms showed an isosbestic point at 290 nm.

b) CuH$_{-3}$L: The Cu(H_{-3}G5) species occupies approximately 95% of the total Cu(II) at pH 9.3.[18,19] Upon mixing Cu(H_{-3}G5) with four-molar equivalents of CysH, a red-brown color appeared. The absorption spectra of the reaction mixtures for the initial 600 ms are shown in Fig. 3, and the absorbance–time plots at 265 nm, 330 nm, and 390 nm in Fig. 4. At the beginning of the observation, the intensity of A_{330} (absorbance at 330 nm) was very weak, indicating that the formation of the first transient, probably Cu(H_{-1}G5)(Cys^-), is sluggish. The A_{265} reachaed the maximum at 50 ms, where the spectrum exhibited a LMCT absorption at 332 nm, but not at 390 nm, and the d–d transition at 535 nm, and $R \fallingdotseq 9.00$. The A_{330} values increased thereafter, and a new absorption at around 390 nm appeared and increased. Those bands reached maxima at 500–600 ms ($R \fallingdotseq 2.04$), while the A_{265} was mimimal at 600 ms. The spectrum at 500–600 ms, with the LMCT bands at 332 nm and around 390 nm and the d–d band at 515 nm, is assignable to the binary $Cu(Cys^-)_2$ complex. At the stage from 100 ms to 500 ms, the R value changed from 9.00 to 2.04, and a family of spectra exhibited an isosbestic point at 285 nm. This indicates successsive transformations of two transients,

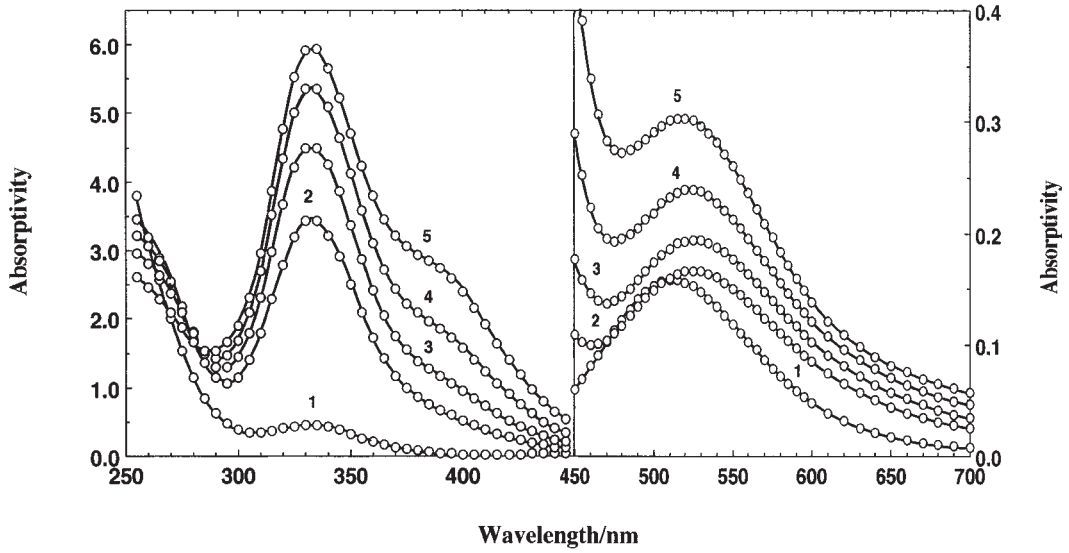


Fig. 3. Time-dependent spectral changes on addition of Cu(H_{-3}G5) to CysH at pH 9.3. (1), dead time, (2), 50 ms, (3), 100 ms, (4), 200 ms, and (5) 600 ms. [Cu(II)] = 5.17×10^{-4} M and [CysH] = 1.94×10^{-3} M (3.75 equiv.), 0.01 M borate buffer (I = 0.1 M $KClO_4$), T = 25 °C.

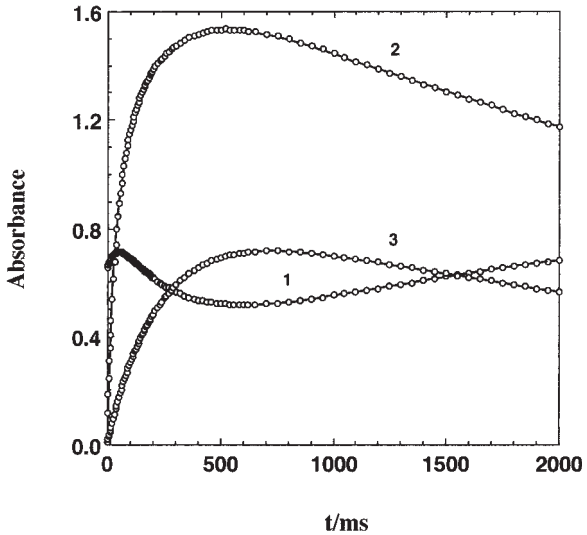


Fig. 4. Absorbance–time plots for the reaction of Cu(H_{-3}G5) with CysH. (1), 265 nm, (2), 330 nm, and (3), 390 nm. Conditions same as under Fig. 3.

from Cu(H_{-1}G5)(Cys^-) to Cu(Cys^-)$_2$. Both transients were paramagnetic, as determined by ESR spectroscopy, as described later.

Beyond 600 ms, the intensities of both LMCT and d–d absorptions, as well as the ESR signal, decreased, while the A_{265} otherwise increased, and thereby the R value began to increase bigger than 2.04. The A_{265} was likely to be indicative of a reduction of the Cu(II) species. The family of the spectra in this stage showed an isosbestic point at 300 nm.

Method Determining the Concentrations of Intermediates. The transport of Cu(II) from Cu(H_{-i}L) to CysH is considered to consist of reactions (1), (2):

$$\mathrm{Cu(H_{-}{}_{i}L) + Cys^- + (}i-1)\mathrm{H^+ \overset{k_{1+}}{\rightleftarrows} Cu(H_{-1}L)(Cys^-)}. \quad (1)$$

The Cu(H_{-1}L)(Cys^-) produced in reaction (1) either changes to Cu(Cys^-)$_2$, or is reduced to the Cu(I) species.

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

The Cu(Cys^-)$_2$ is also likely to be reduced to the Cu(I) species. The Cu(II) complex of polyaminocarboxylic acid is shown to react with CysH, finally forming the Cu(I) species in the same manner.[20]

The copper species in the reaction system primarily contain four species. The total concentration, $[Cu]_o$, is expressed by

$$\begin{aligned}[Cu]_o = {} & [Cu(H_{-i}L)] + [Cu(H_{-1}L)(Cys^-)] \\ & + [Cu(Cys^-)_2] + [Cu(I)\ \text{species}].\end{aligned} \quad (3)$$

In the reactions with the Cu(H_{-1}L) and Cu(H_{-2}L) species, those parent complexes are completely consumed at the begining of the observation, as stated above. At the initial stage, where the Cu(I) species does not exist, $[Cu]_o$ is the sum of [Cu(H_{-1}L)(Cys^-)] and [Cu(Cys^-)$_2$]. Then, the observed absorbance, A_{obsd}, can be represented by

$$A_{obsd} = \varepsilon^{ter}[Cu(H_{-1}L)(Cys^-)] + \varepsilon^{bi}[Cu(Cys^-)_2], \quad (4)$$

where ε^{ter} and ε^{bi} represent the molar absorptivities for Cu(H_{-1}L)(Cys^-) and Cu(Cys^-)$_2$, respectivity. At the begining of the observation, where $[Cu]_o$ = [Cu(II)(H_{-1}L)(Cys^-)], the molar absorptivity ε^{ter} can be obtainaed as $\varepsilon^{ter} = A_{obsd}/[Cu]_o$. The ε^{bi} is similarly obtainable in same manner in the Cu(Gly)$_2$/CysH reaction.

As the reaction proceeds, Cu(Cys^-)$_2$ is progessively formed. A_{obsd} at 330 nm can be resolved to A^{ter} and A^{bi} by

$$A^{ter}{}_{330} = \frac{(A_{obsd} - \beta A_{390})}{1 - (\beta/\alpha)} \quad (5)$$

and

$$A^{bi}{}_{330} = A_{obsd} - A^{ter}{}_{330}. \quad (6)$$

Here, α and β represent the R values for the ternary and binary

complexes, respectively. Both are experimentally available, as mentioned above: $\alpha = 9.00 \pm 0.05$ and $\beta = 2.045 \pm 0.005$. As the amounts of the Cu(I) species increase progressively, the β value increases over 2.045. In this case, A_{obsd} has to be corrected in order to avoid a contribution from the 265 nm band.[21]

Since in the reaction system involving the G4 or G5 complexes, considerable amounts of the parent $Cu(H_{-3}L)$ complex exist at the initial stage, Eq. 3 has to be rewritten as

$$[Cu]_o = [Cu(H_{-1}L)(Cys^-)] + [Cu(Cys^-)_2] + [Cu(H_{-3}L)]. \quad (7)$$

$Cu(H_{-3}L)$ does not exhibit a critical absorption in the UV region. ε^{ter} was determined by successive approximations while referring to those for $Cu(H_{-1}G2)(Cys^-)$ and $Cu(H_{-1}G3)(Cys^-)$. The ε^{ter} estimated for $Cu(H_{-1}G5)(Cys^-)$ was 4280 $M^{-1}\,cm^{-1}$, as is shown later.

In order to assess the distribution of transients during the reaction, the concentrations of the four copper species, including the $Cu(H_{-i}L)$, $Cu(H_{-1}L)(Cys^-)$, $Cu(Cys^-)_2$, and Cu(I) species, were clculated and plotted as a function of time. The distribution curves in Figs. 5 and 6, calculated in the reactions of $Cu(H_{-1}G2)$ and $Cu(H_{-i}G5)$ at pH 8.0 and pH 10.0, clearly reveal a sequence of the Cu(II) transport from the $Cu(H_{-i}L)$ to $Cu(Cys^-)_2$ via $Cu(H_{-1}L)(Cys^-)$. The $Cu(H_{-1}G2)$ has changed quantitatively to the ternary complex within DT, while the $Cu(H_{-3}G5)$ reacts at a fairly slow rate.

As shown in Figs. 5 and 6, the $Cu(H_{-1}L)(Cys^-)$ upon forming was rapidly reduced at pH 8. The rate of reduction for $Cu(Cys^-)_2$, estimated in the $Cu(Gly)_2$/CysH system, depended on the pH. The half lives of $Cu(Cys^-)_2$, evaluated from an A_{390}–time plot, were 250 ms at pH 8.0 and 15 s at pH 10. Over pH 10, the reactions (1), (2), and the reduction of $Cu(Cys^-)_2$ occurred in sequence.

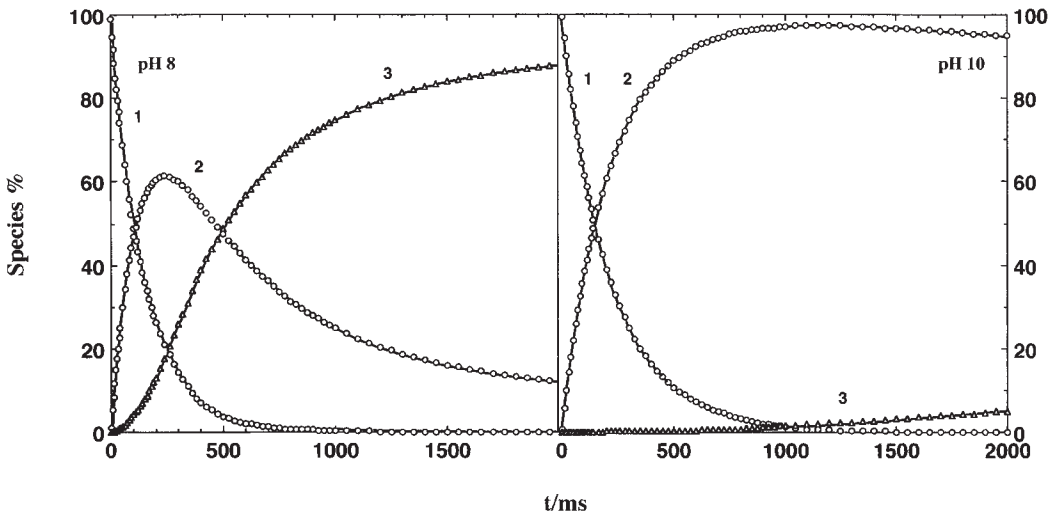


Fig. 5. Time-dependent distribution of the copper species produced in the reaction of $Cu(H_{-1}G2)$ with CysH at pH 8.0 and pH 10.0. (1), $Cu(H_{-1}G2)(Cys^-)$, (2), $Cu(Cys^-)_2$, and (3), Cu(I)Z. $[Cu(II)] = 2.56 \times 10^{-4}$ M, $[CysH] = 1.04 \times 10^{-3}$ M (4.08 equiv.), $T = 25$ °C, 0.01 M borate buffer ($I = 0.1$ M $NaClO_4$).

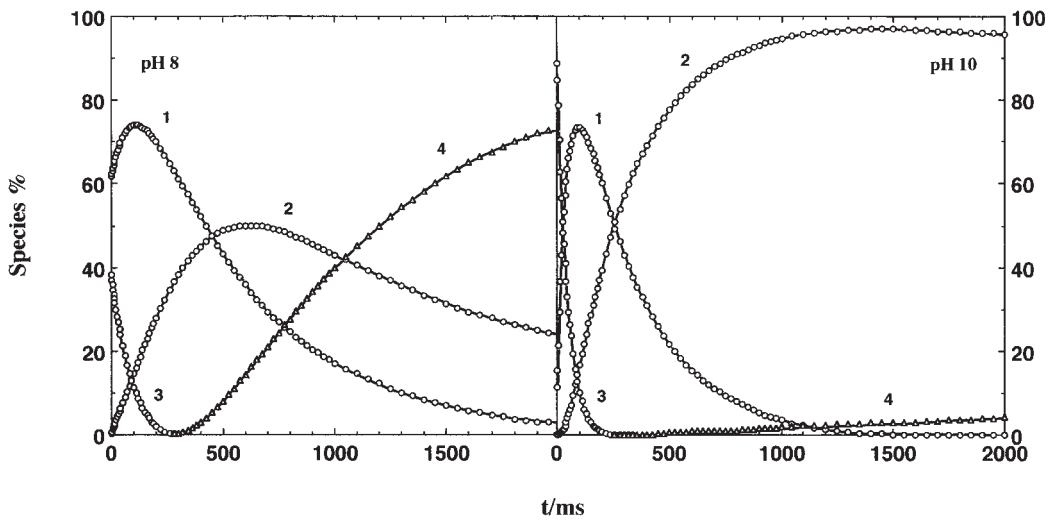


Fig. 6. Time-depended distribution of the copper species produced in the reaction of $Cu(H_{-3}G5)$ with CysH at pH 8.0 and pH 10.0. (1), $Cu(H_{-1}G5)(Cys^-)$, (2), $Cu(Cys^-)_2$, (3), $Cu(H_{-3}G5)$, and (4), Cu(I)Z. $[Cu(II)] = 2.46 \times 10^{-4}$ M, $[CysH] = 1.01 \times 10^{-3}$ M (4.12 equiv.), $T = 25$ °C, 0.01 M borate buffer ($I = 0.1$ M $NaClO_4$).

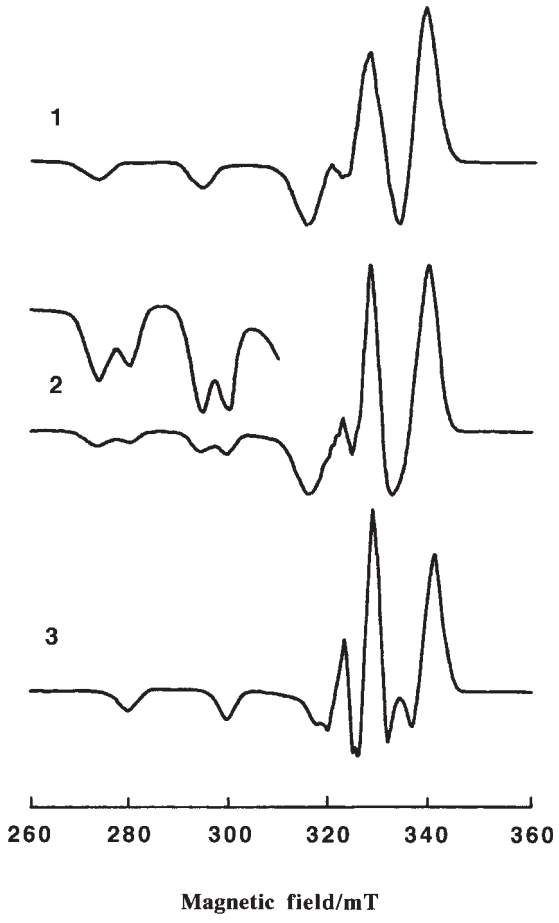


Fig. 7. ESR spectra (at 77 K) of the transient in the reaction of $Cu(H_{-3}G5)$ with CysH. $[Cu(II)] = 1.25 \times 10^{-3}$ M, $[CysH] = 5.00 \times 10^{-3}$ M, (1), pH 8.2, (2), pH 10.2, and (3), $Cu(Cys^-)_2$ (pH 10.2).

ESR Spectrum of the Intermediates. The ESR spectra at 77 K of the transients were measured by the stopped-flow and rapid-quenching method, and those at room temperature by the continuous-flow method. The spectra at 77 K at $[CysH]/[Cu(H_{-3}G5)] = 4$ are shown in Fig. 7. The spectrum at pH 8.2 was typical of the Cu(II) with D_{4h} symmetry, revealing the formation of a single species, i.e., the ternary complex, with $g_\parallel = 2.18$ and $A_\parallel = 2.00 \times 10^{-4}$ cm^{-1}. The first transients in the cysteine reactions with the G2, G3, G4, G6, and G8 complexes also exhibited ESR spectra with similar parameters and line-shapes ($g_\parallel = 2.18 \pm 0.01$ and $A_\parallel = (2.00 \pm 0.05) \times 10^{-4}$ cm^{-1}), indicating that the Cu(II) is likely to locate in the same coordination environment. As the pH increased, the line shape of the spectrum became complicated. At pH 10, the spectrum in the $g_\parallel$ component was split into two; one is assignable to the ternary complex $Cu(H_{-1}G5)(Cys^-)$ and another the binary $(Cys^-)_2$ complex, with $g_\parallel = 2.14$ and $A_\parallel = 1.92 \times 10^{-4}$ cm^{-1}.

The spectrum of the transients at room temperature was obtained at 10 ms and 20 ms after the start of the reaction. The spectra at $[CysH]/[Cu(H_{-3}G5)] = 1.25$ ($t = 10$ ms) and 4.0 ($t = 20$ ms) are shown in Fig. 8. The spectrum at 10 ms was typical of Cu(II) consisting of four hyperfine lines with an additional ligand hyperfine splitting of seven lines, which was indicative of an N_3 donor set; two are derived from the G5 moiety and one is from Cys^-. The line-shape of the spectrum 2 is complicated, probably because of contamination of $Cu(Cys^-)_2$ with a smaller g_0 value.

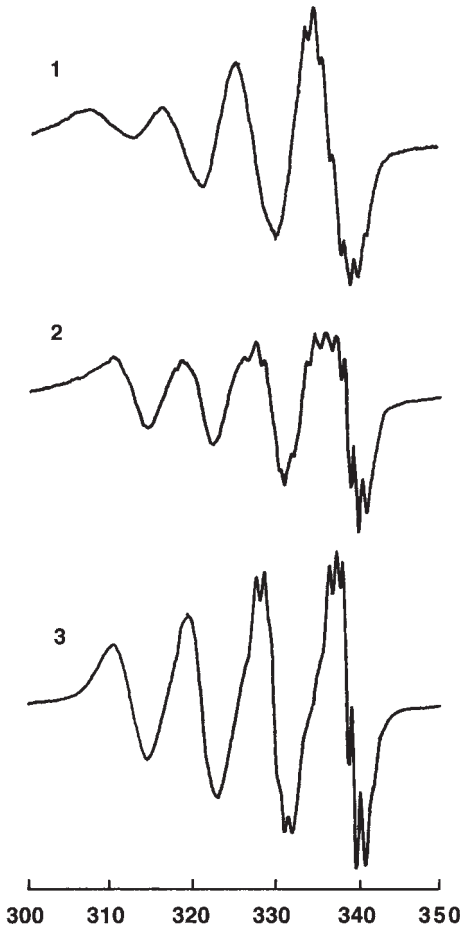


Fig. 8. ESR spectra (at room temperature) of the transient in the reaction of $Cu(H_{-3}G5)$ with CysH at pH 9.3 and $[Cu(II)] = 5.1 \times 10^{-3}$ M. (1), 1.25 equivalent CysH, $t = 10$ ms, (2), 4.0 equivalent CysH, $t = 20$ ms, and (3), $Cu(Cys^-)_2$.

The parameters of the ESR spectrum for the $Cu(H_{-1}G5)(Cys^-)$ complex were as follows: $g_\parallel = 2.175$, $A_\parallel = 2.00 \times 10^{-4}$ cm^{-1}, and $g_\perp = 2.045$, by using the Kivelson and McConnell's equation,[22,23] $g_0 = (g_\parallel + 2g_\perp)/3$, and $A_0 = (Ag_\parallel + 2A_\perp)/3$.

Cu(II)-Ion Exchangeability and Rate of Ligand-Exchange. The exchangeability of Cu(II) is considerded to be related to both the reaction rate and the equilibrium constant. The initial increases in the distribution curves for the $Cu(H_{-1}L)(Cys^-)$ in Figs. 5 and 6 fitted the second-order kinetic expression. This indicates that the reaction was first order to the parent $Cu(H_{-i}L)$ complexes and to CysH. Both $Cu(H_{-1}G2)$ and $Cu(H_{-2}G3)$ reacted with CysH, forming $Cu(H_{-1}L)(Cys^-)$ at an unmeasureably rapid rate. In slightly alkaline solutions, the G4 and G5 complexes exist as equilibrium mixtures, which are composed of the $Cu(H_{-2}L)$ and $Cu(H_{-3}L)$ species. The $Cu(H_{-2}L)$ species suffered a rapid li-

Table 1. Apparent Second-Order Rate Constants k_{1+} for the Reactions of Cu(H$_{-3}$G4) and Cu(H$_{-3}$G5) with Cysteine[a)]

Parent Complex	pH 8.5	pH 9.5	pH 10.5
Cu(H$_{-3}$G4)	n.d. (Cu(H$_{-2}$L)/80%)	1.20×10^4 (Cu(H$_{-3}$L)/70%)	1.20×10^4 (Cu(H$_{-3}$L)/96%)
Cu(H$_{-3}$G5)	9.85×10^3 (Cu(H$_{-3}$L)/74%)	1.50×10^4 (Cu(H$_{-3}$L)/97%)	2.40×10^4 (Cu(H$_{-3}$L)/97%)

$[Cu(II)]_o = 2.46 \times 10^{-5}$ M, $[CysH]_o = 4.00 \times [Cu(II)]_o$. a) In parenthesis is shown main species and its amount.

gand-exchange as did Cu(H$_{-1}$G2) and Cu(H$_{-2}$G3), while the Cu(H$_{-3}$L) was relatively sluggish. Those were suported by species distribution curves. In the pH region where the Cu(H$_{-3}$L) was predominant, the apparent k_{1+} values were estimated, as shown in Table 1.[24] The k_{1+} value for Cu(H$_{-3}$G4) was comparativly smaller than that of Cu(H$_{-3}$G5), probably because negative charges of the carboxylate group in the Cu(H$_{-3}$L) hindered the attack of Cys$^-$ on the Cu(II). The carboxylate group in Cu(H$_{-3}$G4) occupies the nearest position on the Cu(II). The exchangeability of the Cu(II) in the Cu(H$_{-3}$L) would depends on the coordination environment, as well as the coordination mode.

Discussion

Absorption Spectrum of the Ternary Complex, (Cys$^-$)Cu(H$_{-1}$G5). The ternary Cu(H$_{-1}$G5)(Cys$^-$) complex was predominant in the ligand-exchange reaction from 30 ms to 180 ms. The average of the absorptivity of Cu(H$_{-1}$G5)(Cys$^-$), each obtained at 50 ms, 75 ms, 100 ms, 125 ms, 150 ms, and 175 ms, was plotted against the wavelength to prepare the absorption spectrum. The relative errors, 2σ/(mean value), for a best fit were less than 2% around λ_{max} The thus-obtained spectrum could be resolved into three transition bands by a previously reported method.[25] The 333 nm absorption assignable to a $\sigma(S) \rightarrow$ Cu CT band was flanked at lower energy (404 nm) by a weaker $\pi(S) \rightarrow$ Cu CT band. The parameters from electronic absorption and the ESR spectra of the ternary complexes are summarized in Table 2. The results indicate that those complexes have identical coordination modes and structures, where the peptide works as a bidentate ligand donating the nitrogens from the terminal amino and the neighboring deprotonated-amide groups, and the cysteine donates the amino nitrogen and thiolate sulfur also working as the bidentate ligand.

Metal-Ion Exchangeability of CuH$_{-i}$L. The Cu(II) in Cu(H$_{-i}$L) was shown to be transported to CysH via the ternary complex, Cu(H$_{-1}$L)(Cys$^-$). Firstly, the CysH would replace the fourth donor atom of Cu(H$_{-i}$L) with the thiolate S, forming a ternary complex, Cu(H$_{-2}$L)(Cys^{*-}), in which Cys^{*-} denotes the monodentate Cys$^-$.[14] Nextly, the Cu(H$_{-2}$L)(Cys^{*-}) would change to Cu(H$_{-1}$L)(Cys$^-$) through intramolecular rearrangements (Scheme 2). A sequence of reactions can be represented by the following reactions:

$$Cu(H_{-i}L) + Cys^- + (i-1)H^+ \underset{k_{11-}}{\overset{k_{11+}}{\rightleftarrows}} Cu(H_{-2}L)(Cys^{*-}), \quad (8)$$

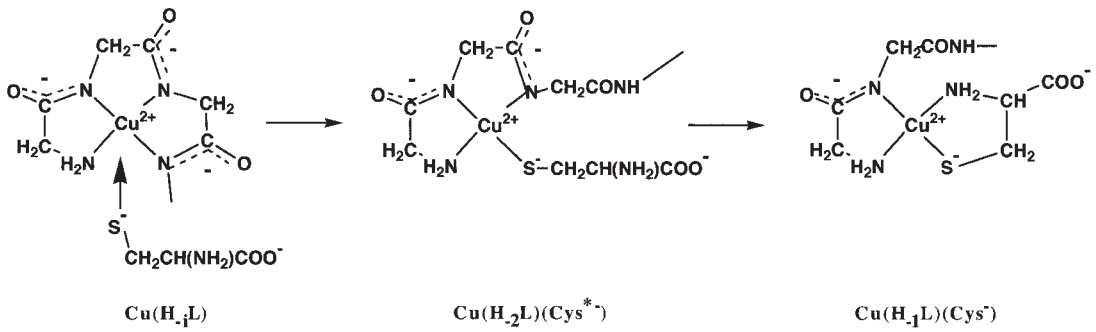
$$Cu(H_{-2}L)(Cys^{*-}) + H^+ \underset{k_{12-}}{\overset{k_{12+}}{\rightleftarrows}} Cu(H_{-1}L)(Cys^-). \quad (9)$$

The ternary Cu(H$_{-2}$L)(Acys$^-$) and Cu(H$_{-2}$L)(GS$^-$) complexes, analogous to the Cu(H$_{-2}$L)(Cys^{*-}),[12,13] exhibit strong CT absorption at 327 nm (L = G3 with N,N$^-$,N$^-$ donors) and 342 nm (L = G2 with N,N$^-$,O donors),[26] where Acys$^-$ and GS$^-$ denote *N*-acetylcysteinate and glutathionate, respectively. Since the Cys^{*-} in Cu(H$_{-2}$L)(Cys^{*-}) undergoes

Table 2. Spectral Parameters for the Ternary Complexes, (Cys$^-$)Cu(II)(H$_{-1}$L), and the Binary Complex, Cu(II)(Cys$^-$)$_2$[a)]

Complex	Absorpion Spectrum λ_{max}/nm (ε/M^{-1} cm^{-1})			ESR $g_\parallel$	$A_\parallel/10^{-4}$ cm^{-1}	$g_\perp$
Cu(H$_{-1}$G2)(Cys$^-$)	332 (4250)	406 (193)	542 (162)	2.179	1.97	2.04
Cu(H$_{-1}$G3)(Cys$^-$)	333 (4200)	405 (194)	535 (150)	2.174	2.01	2.05
Cu(H$_{-1}$G5)(Cys$^-$)	333 (4200)	404 (194)	540 (150)	2.175	2.00	2.04
Cu(Cys$^-$)$_2$	334 (6170)	391 (276)	526 (351)	2.139	1.92	2.04

a) The absorption and ESR spectra were measured at pH 9.3 and pH 9.5, respectively.



Scheme 2. Transport of Cu(II) from Cu(H$_{-i}$L) to Cu(H$_{-1}$L)(Cys$^-$) via Cu(H$_{-2}$L)(Cys^{*-}).

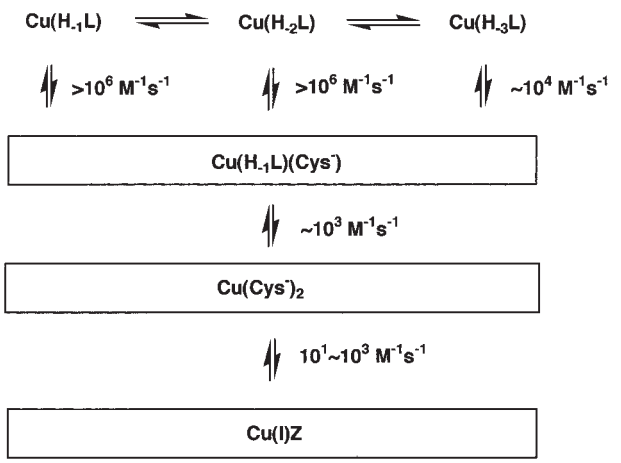
a fast ring closure to yield $Cu(H_{-1}L)(Cys^-)$, $Cu(H_{-2}L)(Cys^{*-})$ could not be detected, irrespective of the coordination modes of L.

The k_{11+} value for $Cu(H_{-3}G5)$ was $\sim 5 \times 10^5$ $M^{-1}\,s^{-1}$, while those in $Cu(H_{-1}G2)$ and $Cu(H_{-2}G3)$ were probably 10^7 $M^{-1}\,s^{-1}$. The equilibrium constants (K) of Eq. 8, defined as k_{11+}/k_{11-}, were $10^{4.5}$ for $Cu(H_{-1}G2)(Acys^-)$ and $10^{2.5}$ for $Cu(H_{-2}G3)(Acys^-)$. That for $Cu(H_{-2}G5)(Acys^-)$ was less than 10. The ternary $Cu(H_{-2}G5)(Cys^{*-})$ complex, which easily dissociates back to $Cu(H_{-3}G5)$, is hard to change to $Cu(H_{-1}G5)(Cys^-)$, so that the k_{1+} in Eq. 1 had a magnitude of 10^4 $M^{-1}\,s^{-1}$ or less. On the other hand, $CuH_{-2}L$ and $CuH_{-1}L$ complexes, having bigger K values, can promptly form the $Cu(H_{-1}L)(Cys^-)$ species at $k_{1+} > 10^6$ $M^{-1}\,s^{-1}$. Thus, the K is considered to play an important role in determining the Cu(II) exchangeability in $Cu(H_{-i}L)$, and a fairly good linear-relationship exists between the equilibrium constant in Eq. 8 and the rate constant in Eq. 1.[13] The kinetic data reveal that the affinity of Cu(II) for the the fourth ligand in $Cu(H_{-i}L)$ is important in determining the metal-ion exchangeability.

Conclusion

The transport of the Cu(II) in $Cu(H_{-i}L)$ (L = G2–G5, i = 1, 2, and 3) to CysH begins at the fourth binding site in $Cu(H_{-i}L)$, and the affinity of Cu(II) for the fourth donor determines the rate of its replacement with the thiolates S. The $Cu(H_{-1}L)$ and $Cu(H_{-2}L)$ complexs, in which the oxygen from the coordinated water/hydroxide and carbonyl/carboxylate occupy the fourth site, respectively, were labile toward the ligand-exchange. On the contrary, ($CuH_{-3}L$), with the nitrogen from a deprotonated-amide group in the fourth site, was less labile toward the ligand-exchange. Referring to the pK_c values (6.0, 6.9, and 8.0)[19] of the deprotonation for $Cu(H_{-i}G5)$, the major species at pH 7 is $Cu(H_{-2}G5)$, so that the Cu(II) is easily and rapidly transported to CysH (Scheme 3).

The amino-terminus of peptides and proteins give a potent binding site for Cu(II), and the amide groups coordinate to Cu(II) in a fashions with deprotonation of the amide nitrogens. This coordination mode takes a unique role in stabilizing the Cu(II) complex. Albumin and transcuprein are identified as the plasma proteins most likely to be acceptors to the incoming copper ion.[4] It is considered that the N-terminus of those plasma proteins surves as a Cu(II) binding site of high affinity, and that the Cu(II) accepted by those proteins is transported to a certain chelator such as histidine,[27,28] and into the cell across the cell membrances with the reduction to Cu(I). D-Penicillamine (β,β-dimethyl-β-mercapto-D-alanine) is the first practical and effective oral therapeutic agent for Wilson's disease.[29] The results from stopped-flow spectroscopic studies demonstrate that the characteristic spectral properties of the Cu(II)–S(thiolate) bond can be used as a probe for studying metal-ion transport.



Scheme 3. Pathway of the Cu(II) transport from $Cu(H_{-i}L)$ to CysH.

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$$A_{330} = A'_{330} - \sum_{i=1}^{n} p_i (A'_{265} - A_{265(min)})^i, \quad (10)$$

where both A'_{330} and A'_{265} denote the observed values, and $A_{265(min)}$ the absorbance at the minimum level. The terms p_i (real number) and i (integer) were experimentally estimated so as to be level off at $R = 2.045 \pm 0.005$. The i value used was 2.

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