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Structural Studies of Copper(II)-Hippuryl-L-histidyl-L-leucine(HHL) Complex by NMR Methods

Seongran Lee, Jihyun Jun and Hoshik Won*

Department of Applied Chemistry, Hanyang University, Ansan 425-791 Korea
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Abstract : Hippuryl-L-histidyl-L-leucine(HHL) is widely used as a substrate of angiotensin converting enzyme(ACE) cleaving the neurotransmitter angiotensin(I) to the octapeptide angiotensin(II). The structure of the substrate molecules should provide information regarding the geometric requirements of the ACE active site. For the purpose of determination of in vivo reaction, metallo(Cu, Zn)-HHL complexes were synthesized and the degree of complex formation were identified by MALDI-TOF, ESI mass spectrometric analysis. In addition, the pH-dependent species distribution curves were obtained by potentiometric titration. Nitrogen atoms of imidazole ring and oxygen atom of caboxylate groups in the peptide chain were observed to be participated in the metal complex formation. After purification of complexes further structural characterization were made by utilizing UV-Vis, electrochemical methods and NMR. Complete NMR signal assignments were carried out by using 2D-spectrum techniques COSY, TOCSY, NOESY, HETCOR. A complex that two imidazole and carboxylate groups are asymmetrically participating to coordination mode was predicted to the solution-state structure of Cu(II)-HHL_2 based on ^{13}C-NMR signal assignment and NOE information.
Keywords: HHL, Complex, NMR

INTRODUCTION

Hippuryl-L-histidyl-L-leucine(HHL) can act as a substrate for the angiotensin converting enzyme(ACE).[1] The angiotensins are peptides that act as vasoconstricting agents(causing blood vessels to narrow). Angiontensin II is formed from angiotensin I in the blood by the enzyme, ACE.[2,3] Angiotensin II is a very potent chemical that causes the muscles surrounding blood vessels to contract and thereby narrows the blood vessels. The

* To whom correspondence should be addressed. E-mail : hswon@hanyang.ac.kr

narrowing of the vessels increases the pressure within the vessels and can cause high blood pressure (hypertension). ACE inhibitors are medications that slow
the activity of the enzyme, which decreases the production of angiotensin II. As a result, the blood vessels enlarge or dilate, and the blood pressure is reduced. This lower blood pressure makes it easier for the heart to pump blood and can improve the function of a failing heart. In addition, the progression of kidney disease due to high blood pressure or diabetes is slowed.[4] Kinetic studies of the inhibition of ACE are often carried out using HHL as the substrate.

The coordination mode of peptides towards cations of transitional metal has been widely studied because of its biological importance. The metal ion is located in a matrix constructed by the polypeptide chain surrounding it and bound with the functional group from the chain in most case histidine imidazole.[5] The imidazole ring is a metal binding site in metalloproteins and thus has profound effects on their biological actions. In this view, imidazole containing ligands have been investigated to mimic structural features of enzymes.[6] The complex formation processes are generally characterized by the successive deprotonation and coordination of amide nitrogen. With the increase of pH, further deprotonation of the peptide groups and donor exchange from oxygen to nitrogen occur successively. Depending on the metal to ligand ratio, metal complexes may give rise to steric strain and effect stability and coordination structure. In most case, the copper(II) ion is first anchored by on N-terminal amino group, but N-terminal benzoyl residue prevents metal ions from being anchored to the N-terminal proton of molecule, first anchor site may be N(3)-imidazole ring of histidyl residue.[7]

Copper(II) ion is one of the more involved ions in protein-mediated biological processes. For this reason a wide range of Cu(II)-HHL complex mimicking the functionality ACE active site have been studies. The anchoring sites for the Cu^{2+} ion in peptide is a competition between the NH_2 and the COOH terminal groups, the deprotonated peptide NH in addition to the imidazole ring.[8] The study was performed by integrating several spectroscopic techniques(UV-Vis, CV, ^{1}H, ^{13}C and ^{2}D NMR), combined with potentiometric determinations and computer modeling optimization.

EXPERIMENTAL SECTION

Materials

A tripeptide ligand HHL(hippuryl-L-histidyl-L-leucine, MW=429.28) using as a substrate of ACE(Angiotensin converting enzyme) from Aldrich was used in metal complexation without further purification. Cu(II), Cu(I), Zn(II)-HHL complexes were prepared by using $Cu(NO_3)_2$, $ZnCl_2$, CuCl. All reagents prepared by dissolving in deionized water.

Potentiometry

The stability constants of the proton and metal complexes were calculated from pH titration curves obtained at 25℃ with a Titronic universal (SCHOTT) automatic titration system and a CONSORT C831 pH meter. The ligand concentration was 2×10^{-3}mol · dm^{-3} and the metal ion to ligand ratio was 1:2. The ionic strength was adjusted to 0.1mol · dm^{-3} with KNO_3 in each case. The titrations were performed over the range pH 2.5-11.0 with NaOH solution. Stability constants were calculated with the aid of a BEST program.[9] The overall stability constants β for species $Cu_pH_qL_r$ is defined by the following equation. Where p, q, and r denote the moles of Cu(II), H and L in $Cu_pH_qL_r$.[10]

$$pCu + qH + rL \xleftrightarrow{\beta_{pqr}} Cu_pH_qL_r$$

$$\beta_{pqr} = \frac{[Cu_pH_qL_r]}{[Cu]^p[H]^q[L]^r}$$

Spectroscopic measurements

The UV-Vis absorption spectra of copper(II) complexes were recorded on a Spectraview2000(K-mac) in the same concentration range as used for pH titration. ^1H, ^{13}C and 2D NMR spectra were acquired on a Varian Mercury 300MHz. All pH values were adjusted with NaOD. After NMR experiments, ESI-Mass spectra were averaged with each acquired from 920 m/z. Cyclic voltammograms were recorded under the EG&G Prinston Applied Research potentiostat and galvanostat model 265A.

RESULTS AND DISCUSSION

Metal-ligand solution precipitated to addition of sodium hydroxide, approximately between pH 5.5-7.5. Precipitation start at bound to imidazole N-nitrogen, while complete redissolution occurs during to the dissociation of the second peptide hydrogen. Precipitation of metal hydroxide was prevented to using auxiliary complexing agent. Ammonia buffer which not only fixes the pH, but serves to complex the metal ion keep it in solution. In order to compare with Cu-HHL complex and Cu(II)-ammonia, cyclic voltammetry method was used.(Fig. 1) Cyclic voltammetry is used to characterize the redox behavior of compounds and to elucidate the kinitics of electrode reaction. Cu(II)-HHL and Cu(II)-ammonia complex was measured each reduction potentials -0.11V, -0.145V. Cu(II)-ammonia complex is larger reducing power than Cu(II)-HHL complex because Cu(II)-HHL complex is binding to imidazole ring.

The ^{1}H, ^{13}C signals of the ligand HHL were assigned by 2D NMR spectra (COSY, TOCSY, NOESY) and by comparing the chemical shifts of metal complexes. The various ratio of metal to HHL complexes were obtained from NMR experiments, the most stable structure was observed in $CuHHL_2$ complex. The chemical shifts of ligand HHL and copper(II)-HHL_2 complex were presented in Table 1. Due to paramagnetic character of copper(II), ligand peaks of $CuHHL_2$ complex were shifted upfield, downfield and broadened.[11] Especially nitrogen of imidazole ring was affected by paramagnetic, the peaks were shifted downfield. α-carbon of leucine in c-terminal was shifted upfield (Fig 2).

In order to get some coordination modes of copper(II)-HHL complexes, UV-Vis absorption spectra were obtained at different metal to ligand ratios. The set of spectra of solutions containing the copper(II) ion and HHL, at various metal to ligand ratios and at conditions of $C_L=2\times10^{-3}M$ with pH 10.5, were presented Fig. 3. Absorption spectra of the copper(II) complexes were measured in the range 250-700nm and the existence of the usual d-d band(around 550nm) was observed in all cases. Especially, UV-Vis absorption of Cu(II)-HHL complex were measured in 575nm.

From the potentiometric titration data only two logK values were evaluated for HHL(Fig4). The values obtained $\log K_1^H$=6.6(1), $\log K_2^H$=2.78. They were consistent of one imidazole nitrogen and one carboxylate oxygen present in the molecule. The hydrolysis

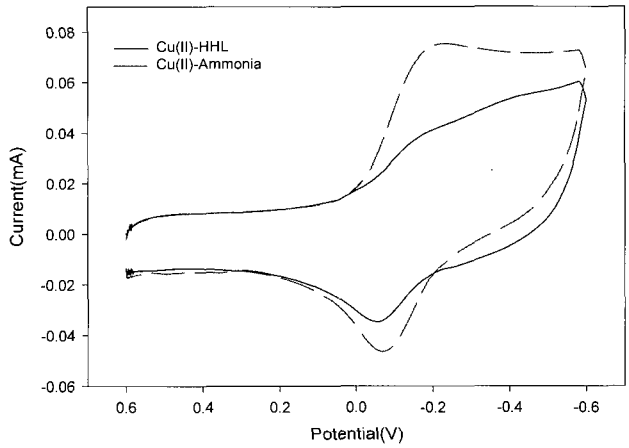

Fig. 1. Current-Potential curve of Cu(II)-ammonia and Cu(II)-HHL complex.

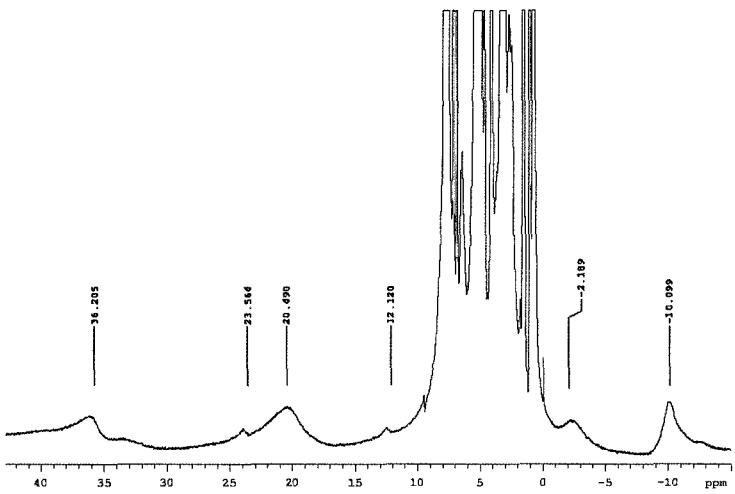

Fig. 2. ^{1}H-NMR spectrum of Cu(II)-HHL 1:2 complex exhibiting paramagnetic chemical shifts arising from the Cu(II) binding.

Table 1. ^{1}H and ^{13}C-NMR chemical shifts of the shifted signal of metal complex.

Complex (ppm) / Assignment	Free-ligand (HHL)		Cu(II)-HHL_2		Zn(II)-HHL		Cu(I)-HHL	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Leu CH_3	0.77	21.78	0.75	21.70	0.75	21.86	0.77	21.87
	0.81	23.99	0.81	23.95	0.81	23.91	0.82	23.92
Leu CH	1.18	25.58	1.05	25.39	1.10	25.55	1.2	25.56
CH_2	1.55	42.06	1.54	41.81	1.54	42.18	1.54	41.99
Leu α-carbon	4.17	54.90	4.15	54.86	4.16	54.91	4.16	54.98
His-CH_2	3.07	29.56	3.06	29.49	3.06	29.66	3.07	29.46
His α-carbon	4.65	54.58	4.65	54.57	4.65	54.53	4.67	54.5
Imidazole	7.23	136.71	7.12	137.05	7.16	136.89	7.28	
(2H,4H)	6.86	118.38	6.84	118.19	6.87	118.20	6.88	
Hip CH_2	4.10	44.48	4.10	.	4.09	44.65	4.10	44.66
	7.87	128.4	7.84	128.66	7.86	128.33	7.86	128.43
Hip benzene ring	7.57	129.8	7.56	129.89	7.56	129.77	7.58	129.86
	7.66	133.74	7.64	133.79	7.66	133.58	7.66	133.56
		180.23		180.64		180.19		.
Carbonyl		172.54		172.9		172.58		172.54
Carbon		171.88		172.14		172.01		171.99
		171.40		171.9		171.56		171.57
		133.71		134.11		134.13		
Quaternary Carbon		133.61		133.45		133.82		

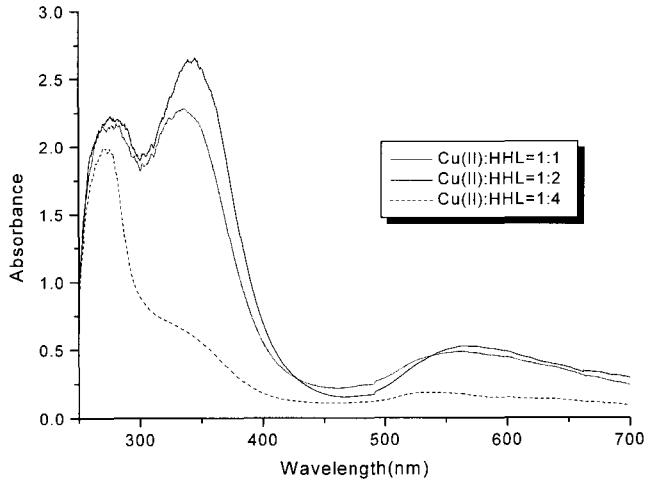

Fig. 3. UV-Vis absorption spectra of various ratio of metal ion to ligand complexes.

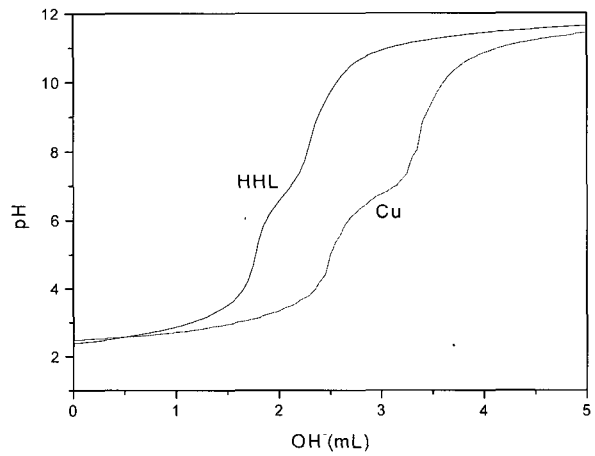

Fig. 4. Potentiometric titrtation curves for ligand HHL(H_2L) and copper-HHL_2 complex as a function of add OH.

Table 2. Stability constants of the copper(II)-HHL complex at 25℃ and I=0.1mol · $dm^{-3}(KNO_3)$

Species	log β	Max %	At pH
HL^0	6.6	122.12	3.5
H_2L^+	9.38	170.76	2.0
CuL^+	6.405	94.34	5.2
CuL_2	9.225	15.5	7.2
$CuLH_{-1}$	-1.405	97.64	12

process occurred in the alkaline solution, due to the excess of hydroxide ions added during the forward titration. In this condition, each protonation step or deprotonation step was easily recognised by the comparison of the logK values. They were attributed to the amino and imidazole nitrogen, respectively, belonging to the first histidyl residue. The second protonation step is close to the carboxyl group. Their stability constants are presented in table 2. Several complex species were considered to be present in solution(pH 2-10) and the best model, fitting potentiometric data, account for the following species, [CuL^+], [CuL_2], [$CuLH_{-1}$].[12-14] For the metal to ligand ratios used, all other species introduced were discarded during the evaluation of logβ by BEST program. From the solution species distribution curves(Fig 5), it can be seen that the neutral species CuL^+ is the major species in the range of pH 5-6.5.

CONCLUSION

The results obtained from the combined potentiometric and spectroscopic studies on the copper(II) complexes. In case of copper(II), it was found that [CuL^+], [$CuLH_{-1}$], [CuL_2] are the main species in all cases. The metal ion is coordinated primarily via the histidyl residue of imidazole nitrogen and carbonyl oxygen donors and this species exits only in low

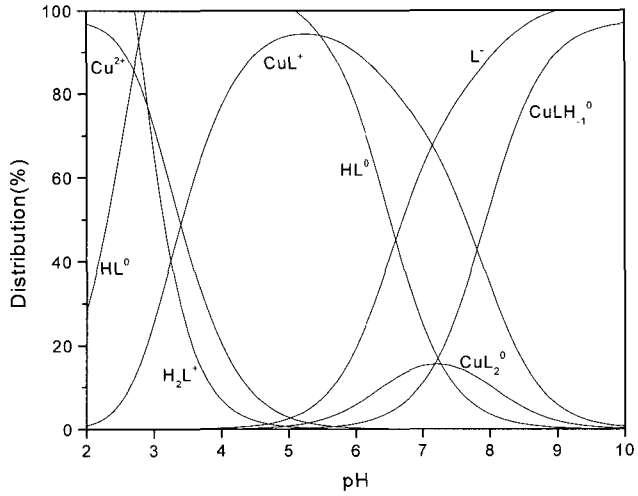

Fig. 5. Species distribution curves as a function of pH calculated by using the stability constants for the Cu-HHL_2 complex.($C_{CU}^{2+}=1.2\times10^{-3}$mol · dm^{-3}, $C_{HHL}=2.5\times10^{-3}$mol · dm^{-3})

concentration. The system found by copper(II) and HHL is not soluble a neutral pH values with the low copper(II) concentrations present in physiological conditions, the species formed by copper(II) with HHL might be a reliable model for copper(II) complex by protein chains biological fluids.

In NMR experiments condition, due to paramagnetic character of Cu(II), ^1H spectrum signals of Cu(II)-HHL complex occur to shifting. It was first affected by the paramagnetic broadening. At higher pH dimer complex was formed, having strong paramagnetic interaction between the copper(II). The copper(II) complex present considerably higher stability constants than other metals.

The Histidyl residues are among the most common metal binding sites of peptides and their presence has a significant impact on the coordination chemistry of peptide molecules. The metal complexes are used to mimic the binding sites of various metalloenzyme, and they have also been used as enzyme inhibitors. These findings will serve as basic

information for understanding metal-peptide interaction involved in such biological actions as metal ion transport, peptide hormone receptor binding, enzyme mechanisms.

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