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Specific Interactions of Cu^{2+} Ions with Fragments of Envelope Protein of Hepatitis B Virus

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ABSTRACT

Potentiometric and spectroscopic (EPR, CD, and absorption spectra) data have shown that a fragment of envelope proteins of the hepatitis B virus could be very specific binding molecules for Cu^{2+} ions using arginine lateral NH_2 donor sites. The presence of Pro and Asp residues makes Arg binding not only very specific, but also very efficient.

INTRODUCTION

Synthetic peptide analogs of envelope proteins of the hepatitis B virus (HBV) are used to identify antigenic determinants which induce immunity to this particular pathogen [1, 2] and to detect the antibodies (anti-S and anti-pre-S) in sera of hepatitis B patients [3]. Immunization of rabbits with mixtures of synthetic fragments [S(140–146), pre-S2 (12–22), pre-S1 (12–32)] of HBV envelope proteins elicits cells and antibodies recognizing the native hepatitis B serum antigen (HBsAg) as well as homologous peptides [4]. The biological activity of the peptide fragments mentioned above does not seem to need the involvement of any metal ion, although the role of metals, including copper ions, in immunological processes is well known [5]. The large amount of experimental data indicates a copper requirement of the immune system, although the molecular mechanisms of copper involvement are still barely understood.

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The amino acid sequences of the peptide analogs of HBV envelope proteins like peptide S (Ps, Thr-Lys-Pro-Thr-Asp-Gly-Asn-Gly) and pre-S2 (pS2, Leu-Gln-Asp-Pro-Arg-Val-Arg-Gly-Leu-Thr-Leu) do not contain any efficient amino acid residue like His which could act as a specific binding site for Cu^{2+} ions. The presence of breakpoint Pro residue [6–8] and Arg residues with the lateral NH_2 groups could, however, lead to quite specific and efficient interactions of the title peptides with Cu^{2+} ions [9]. The Pro residue, which acts as a "breakpoint" to metal ion coordination, allows involvement of the lateral group, even if its donor atom is far away from the main-chain donor system to which metal ion is anchored [8]. The presence of Asp residue close to the N-terminal amino group may also increase the specificity in metal–peptide interactions due to involvement of the β-carboxylate group in metal ion binding [10, 11].

In this work, we have studied Cu^{2+} binding by PS and pS2 peptides. To solve the coordination equilibria formed between undecapeptide pre-S2 and Cu^{2+}, we have also studied its shorter fragment, Leu-Gln-Asp-Pro-Arg (pSS2).

EXPERIMENTAL

Synthesis of PS, pS2, and pSS2 Peptides

Three peptides were synthesized manually by the solid-phase method [4]. The purity of the final products was checked by the TLC and HPLC methods on a BECKMAN model 338 chromatograph. Amino acid analysis was performed on a BECKMAN model 121 analyzer and optical rotations on a PERKIN-ELMER model 141 polarimeter. The purity of the peptides was also checked by potentiometric titrations.

Potentiometric Studies

The stability constants of the H^+ and Cu^{2+} complexes were calculated from the pH titration data obtained at 25°C with a MOLSPIN automatic titration system. Alkali was added from a 0.1 cm^3 micrometer syringe which has been calibrated by both weight titrations and titration of standardized materials. The titrations were performed with a micro combined glass–calomel electrode (Russell) calibrated in hydrogen ion concentrations using HNO_3 [12]. The ionic strength was adjusted to 0.1 $mol \cdot dm^{-3}$ (KNO_3), the ligand concentration to 10^{-3} $mol \cdot dm^{-3}$, and a Cu^{2+}-to-ligand ratio of 1:1 was used. Stability constants were calculated with the SUPERQUAD computer program [13]. The standard deviations reported were calculated by assuming error randomness. The pH for the titrations ranged from 3.5 to 11.5, except PS for which the available pH range was limited to values between 3 and 7.

Spectroscopic Measurements

Absorption spectra was recorded with a BECKMAN DU 650 spectrophotometer. Electron paramagnetic resonance (EPR) measurements were carried out on a BRUKER ESP 300E spectrometer at *X*-band (9.3 GHz) at 120 K in 1:2 ethanediol–water solutions. Circular dichroism (CD) spectra were obtained with a JASCO J-600 spectropolarimeter in the 200–750 nm range. The results are expressed as $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$. The $\Delta\varepsilon$ values were evaluated for the maximum concentration of the particular species obtained from the potentiometric data

calculations. Concentrations used in the spectroscopic measurements were similar to those used in the potentiometric titrations.

RESULTS AND DISCUSSION

Ligands

Protonation constants for PS, pS2, and pSS2 are shown in Table 1. All three peptides have three protonation sites centered at the N-terminal amino group, and two carboxylates, that of the C-terminal and the β-COOH of Asp residue. The stepwise log K constant values for both carboxylate (log $K \sim 2.3$–3.4) are very close to each other, and cannot, therefore, be assigned to the protonation of any particular carboxylate oxygen, while assignment of β_{HL} to the protonation of the amino N is unambiguous (log $\beta_{HL} \sim 7.4$–7.6). Carboxyl protonation equilibria will, in general, not be important when considering the coordination of Cu^{2+} ions which occurs above pH 4.

Cu^{2+} Complexes

A peptide having no competitive binding site in the amino acid side-chains usually begins its coordination to the metal ion via the N-terminal amino group. Its nitrogen acts as an anchoring site, and as pH increases above 5, the Cu^{2+} ion promotes the ionization of protons from successive peptide nitrogens, with formation Cu-N^- bonds, until the complex $CuH_{-3}L$ (a 4N coordinated species) is formed [14, 15]. Stability constant calculations suggest that the most simple coordination model exists for the Cu^{2+}-PS system. Proline, as the only naturally occurring amino acid containing a secondary nitrogen atom, is unable to form a

TABLE 1. Stability Constants of Complexes of H^+ and Cu^{2+} with PS pS2 and pSS2 Peptides at 25°C and I = 0.1 mol·dm^{-3} (KNO_3)

	log β [−log K]		
(a) H^+ Complexes	HL	H_2L	H_3L
PS	7.38(2)	11.01(2) [3.63]	14.37(2) [3.36]
pS2	7.40(4)	11.33(7) [3.93]	13.58(10) [2.25]
pSS2	7.59(2)	11.43(3) [3.84]	14.80(3) [3.37]

	log β [−log K]				
(b) Cu^{2+} Complexes	CuL	$CuH_{-1}L$	$CuH_{-2}L$	$CuH_{-3}L$	$CuH_{-4}L$
PS	4.66(2)	−0.39(1) [5.05]			
pS2		0.18(1)	−5.88(3) [6.06]	−15.13(5) [9.25]	−25.92(7) [10.79]
pSS2		0.53(1)	−5.35(2) [5.88]	−14.89(3) [9.54]	

Cu-N^- bond, and therefore it induces a break in the normal mode of coordination occurring for regular peptides like tetraalanine [6–8]. Thus, the Pro residue inserted into the third position in PS breaks the successive coordination to its amide nitrogen to the Cu^{2+} ion. This allows the formation of only two species in the pH range 3–7, CuL and $CuH_{-1}L$ (Fig. 1a) which, according to spectroscopic data, are 1N and 2N complexes with $\{NH_2CO\}$ and $\{NH_2N^-\}$ coordination modes, respectively (Table 2). There is no involvement of the lateral group of Arg within the pH range up to 7. The measurements above pH 7 were not possible due to some foaming of solutions and a bad electrode response.

pS2 and pSS2 peptides also contain Pro residue inserted in their sequences. In both cases, however, Pro occupies the fourth position just after the Asp residue. According to earlier studies [10, 11], the ^{3}Asp side-chain β-carboxylate may block the Cu^{2+} binding to the successive donor on its C-terminal side. Thus, the major Cu^{2+} coordination should occur with an N-terminal tripeptide fragment Leu-Gln-Asp via $\{NH_2N^-, N^-, \beta\text{-}COO^-\}$ donor set. The potentiometric and spectroscopic results indicate that these donors set as likely binding sites at pH below 9. Two major complexes are formed between pH 4 and 9, $CuH_{-1}L$ and $CuH_{-2}L$ (Table 1, Fig. 1b, c). The very stable $CuH_{-2}L$ species corresponds to the $\{NH_2, 2\times N^-, \beta\text{-}COO^-\}$ coordination set. This is clearly seen in the CD spectra of this species (Table 2) which, besides the d–d transitions centered at ~550–560 nm (3N species [10, 15]), exhibit three charge transfer bands at ~310, ~270, and ~240 nm, which indicate the binding of amide nitrogen, amine nitrogen, and carboxylate, respectively [10, 15]. In simple peptide with ^{3}Asp residue, the $CuH_{-2}L$ complex is the only species observed at pH above 5.5 [10]. However, in both pSS2 and pS2 peptides, a pH increase above 8 leads to the formation of one or two other species, respectively (Tables 1, 2, Fig. 1). The pK formation of the $CuH_{-3}L$ complexes (9.2–9.5) could correspond to the deprotonation of an equatorially bound water molecule [14], but as mentioned above, the coordination of β-COO^- in the fourth equatorial position around Cu^{2+} excludes such a possibility [10]. The formation of the $CuH_{-3}L$ complex with pSS2 leads to slight changes in the UV region of CD spectra. The negative band at ~240 nm increases its intensity about two times. Since the changes in the d–d and the other charge transfer bands is not very distinct, the likely cause of the variation observed around 240 nm is the slight change in the coordination mode around Cu^{2+}, e.g., additional binding at the axial position. The only possible donor bound to Cu^{2+} which gives a charge transfer transition in this region is the lateral arginine nitrogen donor [9, 16]. There are two Arg residues in the pS2 sequence at positions 5 and 7, and only one, next to breakpoint ^{4}Pro, in pSS2. Because both peptides form the same $CuH_{-3}L$ species with very similar log K values (Table 1), it is the ^{5}Arg which could be involved in Cu^{2+} binding via its lateral nitrogen donor. However, the parallel process of an axially bound water molecule cannot be excluded. The increase of pH in the Cu^{2+}-pSS2 system even above 11 does not cause any other complex deprotonation (Fig. 1b), and the $CuH_{-3}L$ species is the only complex found up to pH 11.5.

In the case of a Cu^{2+}-pS2 system about pH 10, the new species $CuH_{-4}L$ is clearly seen in the potentiometric data calculations, as well as in the CD spectra (Fig. 1c, Table 2). The pK of the $CuH_{-3}L$ complex deprotonation of 10.8 could correspond to the deprotonation of the axially bound water molecule. However, a very distinct variation of the CD spectra in the region of 245 nm suggests the

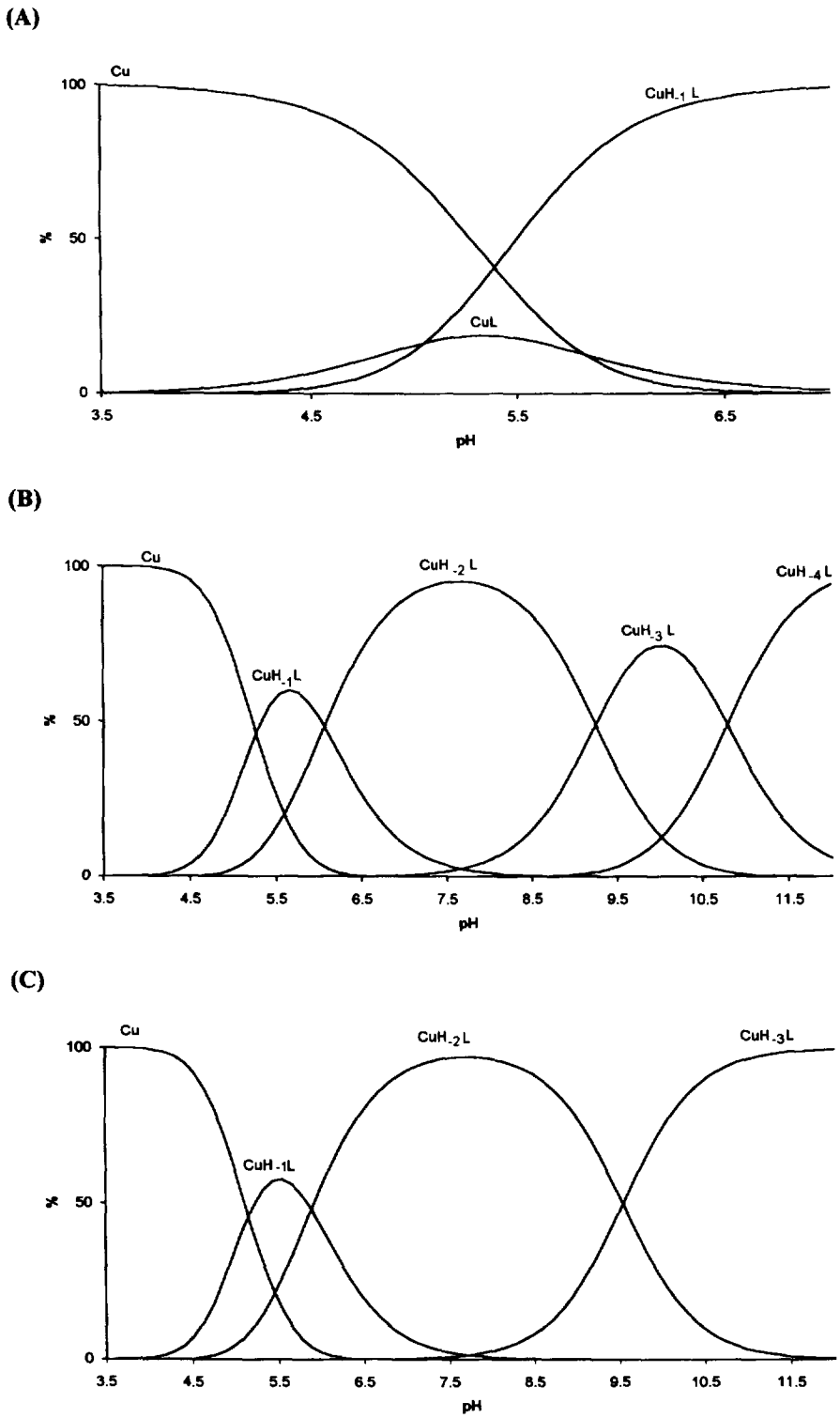

FIGURE 1. Species distribution curves for (a) Cu^{2+}-PS, (b) Cu^{2+}-pS2, and (c) Cu^{2+}-pSS2 systems. The metal-to-ligand molar ratio is 1:1, metal concentration is 10^{-3} mol·dm^{-3}.

TABLE 2. Spectroscopic Parameters for Cu^{2+} Complexes of PS, pS2, and pSS2 Peptides

Peptide	Species	UV-Vis λ [nm] (ε)	CD λ [nm] ($\Delta\varepsilon$)	EPR $g_{\parallel}$	EPR $A_{\parallel}$ ($cm^{-1} \cdot 10^4$)
PS	$CuH_{-1}L$	655 (110)	670 (−0.215)[a]	2.2499	172.5
			317 (0.494)[b,c]		
			227 (−12.200)[f]		
pS2	$CuH_{-2}L$	544 (179)	565 (−0.632)[a]	2.2025	202.0
			311 (0.760)[b]		
			271 (−2.511)[c]		
			235 (−1.356)[d]		
			227 (−2.635)[f]		
	$CuH_{-4}L$	542 (182)	552 (−1.025)[a]	2.2010	205.0
			312 (1.548)[b]		
			274 (−2.451)[c]		
			244 (4.091)[c]		
pSS2	$CuH_{-2}L$	547 (118)	554 (−0.354)[a]	2.2064	200.0
		285 (1679)[s]	309 (1.020)[b]		
			270 (−2.613)[c]		
			236 (−0.822)[d]		
	$CuH_{-3}L$	547 (124)	560 (−0.528)[a]	2.2035	203.0
		290 (1540)[s]	307 (1.020)[b]		
			269 (−2.688)[c]		
			234 (−1.311)[d,e]		

[a] B + E, d–d transitions.
[b] $N^- \Rightarrow Cu^{2+}$, charge transfer (CT) transition.
[c] $NH_2 \Rightarrow Cu^{2+}$, CT transition.
[d] β-$COO^- \Rightarrow Cu^{2+}$, CT transition.
[e] Lateral Arg $NH_2 \Rightarrow Cu^{2+}$, transition.
[f] Intraligand transitions.
[s] Shoulder.

involvement of the second Arg lateral nitrogen rather than the water deprotonation. The negative Cotton effects observed for the $CuH_{-3}L$ complex around 240 nm change into a very strong positive band when the $CuH_{-4}L$ species becomes a major complex in solution (Fig. 1c, Table 2). This is very convincing evidence for the variation of the binding mode in the Cu^{2+}-pS2 system, taking into account the very clear appearance of the new charge transfer band at 244 nm, which most likely corresponds to the lateral ^{7}Arg nitrogen-to-metal transition [9, 16]. The distinct variations of the $\Delta\varepsilon$ values for other transitions also indicate the major change in the ligand position placed around the metal ion. Only coordination of a new donor may explain all these changes (Table 2), i.e., the binding of lateral NH_2 of ^{7}Arg to the equatorial site of the Cu^{2+} complex substituting β-COO^- group. This unusual binding ability of the pS2 peptide derives from a very unique amino acid sequence with ^{3}Asp, ^{4}Pro, and two arginines situated at the C-terminal side of Pro residue. Although the coordination of ^{5}Arg lateral nitrogen is less evident from the spectroscopic data, a comparison of the pSS2 binding pattern to those of Ala-Ala-Asp-X-peptides [10] allows us to suggest some role for this residue in the metal ion binding. The involvement of ^{7}Arg in coordination to the Cu^{2+} ion seems to be well docu-

mented, both by calculations of the potentiometric titration data as well as CD spectroscopy.

CONCLUSIONS

A very unique sequence of peptide fragments of HBV peptide envelope proteins leads to very specific and effective binding of the Cu^{2+} ion. The possibility of involvement of lateral donors of arginines can be used by natural systems to cross-link the protein surface with the receptor site quite effectively and specifically. Proline, which acts as a "breakpoint" residue when inserted inside the peptide sequence, leads to very original coordination patterns of natural peptides, allowing a virtually unlimited possibility to use the metal ion to mediate the interactions with other natural molecules in a very efficient and specific way.

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