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Specific Interactions of Cu^{2+} Ions with Enantiomers of Hepatitis B Virus Surface Antigen 140–146 Region

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Potentiometric and spectroscopic studies on Cu(II) interactions with 140–146 fragment of the hepatitis B virus antigen have shown that the basic binding sites of metal ion are centered at a peptide N-terminal donor system and the side chain donor atoms are not competing in the metal ion coordination.

Key words: peptide, Cu(II), enantiomer, HBV, protonation constants

Our earlier work on the Cu^{2+} coordination with fragments of envelope protein of hepatitis B virus (HBV) [1] have shown that some peptide sequences of HBV can bind metal ion efficiently in very specific manner. The presence of proline residue within the peptide sequence, which acts as a break-point residue [1–4], usually may lead to very specific interactions of the adjacent functions including lateral NH_2 groups of Lys or Arg. The envelope proteins of HBV span the lipid bilayer of the virus and are involved in some critical functions including receptor binding, viral assembly and secretion. Thus, they become the major targets for the immune-directed elimination. The 140–146 amino acid sequence of HBV is a surface antigen region belonging to one of the five major hydrophilic regions that contain B-cell epitope cluster [5]. Short peptide sequences of protein antigens can be used as effective immunogens inducing antibodies able to neutralize native proteins [6]. The studies on immunological properties of the (140–146) region peptide fragments have shown that L- and D-peptides may induce different immunological response. The L-peptide was found to be more effective in inducing the production of antibodies in the sera, while D-peptide evoked stronger response in the mucosal tissues [7]. The differences in biological response to enantiomeric peptides derive from the different structural properties of the respective peptides. It is very likely, that also the binding abilities of both type of peptides will be different from each other. The effect of a substitution of L on D-amino acid residue on binding ability is rarely studied and it inspired us to study this problem on two enantiomers of Thr-Lys-Pro-Thr-Asp-Gly-Asn (140–146 region of HBV surface antigen)

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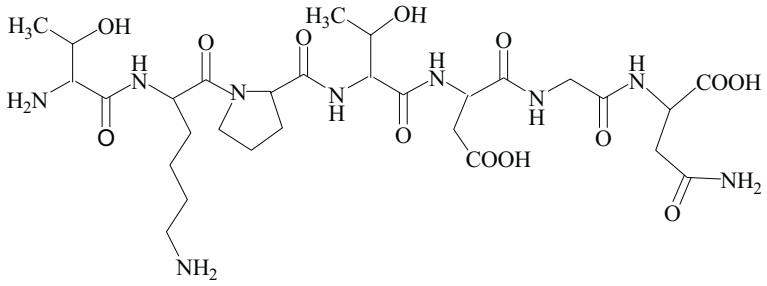
containing all L (enantiomer L) and all D (enantiomer D) amino acid residues. Five other peptides were also studied for comparison.

The results obtained for metal ion interactions could be useful also in the discussion of the structural features of enantiomers in biological tests.

Scheme 1



Thr-Lys-Pro-Thr-Asp-Gly-Asn

EXPERIMENTAL

Potentiometric measurements: Stability constants for proton, Cu(II) complexes were calculated from titration curves carried out at 25°C using sample volumes of 1.5 cm^3. Alkali was added from 0.250 cm^3 micrometer syringe, which was calibrated by both weight titration and the titration of standard materials. Ligand concentration was 1×10^{-3} mol dm^{-3}. The metal-to-ligand ratios were 1:1 and 1:1.5 for heptapeptides. For tri- and tetrapeptides the metal-to-ligand ratios were 1:2 and 1:1.5. The pH-metric titrations were performed at 25°C in 0.1 mol dm^{-3} KNO_3 on a MOLSPIN pH-meter system using a Russel CMAW 711 semi-micro combined electrode calibrated in hydrogen ion concentrations using HNO_3 [8]. Three titrations were performed for each molar ratio, and the SUPERQUAD computer program was used for stability constant calculations [9]. Standard deviations quoted were computed by SUPERQUAD, and refer to random errors only.

Spectroscopic studies: Absorption spectra were recorded on a Beckman DU 650 spectrophotometer. Circular dichroism (CD) spectra were recorded on a Jasco J 720 spectropolarimeter in the 850–230 nm range. EPR spectra were recorded on a Bruker ESP 300E spectrometer at X-band frequency (9.4 GHz) at 120 K. Metal concentration in CD, UV-VIS spectroscopic measurements was adjusted to 1×10^{-3} mol dm^{-3} and metal to ligand ratios were 1:1. Metal concentration in EPR spectroscopic measurement was adjusted to 3×10^{-3} mol dm^{-3} and metal to ligand ratios were 1:1 for heptapeptides and 2:1 for tri- and tetrapeptides. The spectroscopic parameters were obtained at the maximum concentration of the particular species as indicated by the potentiometric calculations.

Peptide synthesis: The details of peptide synthesis and analytical procedures are given in [7]. The purity of all peptides was verified additionally by potentiometric titrations.

RESULTS AND DISCUSSION

Protonation constants. L- and D-enantiomer of Thr-Lys-Pro-Thr-Asp-Gly-Asn heptapeptide (peptide 1 and peptide 5) behave as H_4L acids with protonation constants given in Table 1, together with the assignment of particular values to peptide functions. The data for other peptides studied are also collected in Table 1. The com-

Table 1. Protonation and complex-formation constants of peptides at 25°C and I = 0.1 mol dm^{-3} KNO_3.

	Peptide 1	**Peptide 2**	**Peptide 3**	**Peptide 4**	**Peptide 5**	**Peptide 6**	**Peptide 7**
HL	10.39±0.01	10.16±0.01	10.35±0.02	10.08±0.02	10.41±0.01	10.95±0.02	10.33±0.02
H_2L	17.42±0.01	17.28±0.02	17.47±0.03	17.22±0.03	17.38±0.02	20.54±0.02	17.52±0.03
H_3L	21.27±0.02	21.30±0.02	22.17±0.04	21.89±0.04	21.76±0.02	27.64±0.03	21.30±0.04
H_4L	24.23±0.02	24.29±0.02	–	–	25.08±0.02	31.66±0.04	–
pK^{Arg}	–	–	–	–	–	10.95	–
pK^{Lys}	10.39	10.16	10.35	10.08	10.41	9.56	10.33
pK^{NH_2}	7.03	7.12	7.12	7.14	6.97	7.10	7.19
pK^{COO}	3.85	4.02	4.70	4.67	4.38	4.02	3.78
pK^{COO}	2.96	2.99	–	–	3.32	–	–
log β							
CuH_2L	–	–	–	–	–	24.89±0.04	
CuHL	14.86±0.03	14.83±0.04	15.38±0.02	14.65±0.02	14.76±0.06	20.01±0.01	15.44±0.03
CuL	10.68±0.01	10.14±0.01	10.40±0.01	9.74±0.01	10.12±0.01	11.99±0.01	10.86±0.01
$CuH_{-1}L$	1.68±0.01	1.34±0.02	1.83±0.02	0.92±0.02	1.51±0.01	2.69±0.01	1.82±0.03
pK^{amide}	4.18	4.69	4.98	4.91	4.64	4.88	4.58
pK^{Lys}	9.00	8.80	8.57	8.82	8.61	8.02	9.04
pK^{Arg}	–	–	–	–	–	9.30	–

parison of the pK values for particular peptides indicate slight differences of the basicity of the main binding group, an N-terminal NH_2. The more distinct differences are observed for protonation of the β-COO^- of asp5. The distinct increase of pK values are observed for Thr-Lys-Pro-Thr-Asp-Gly-Asn-NH_2 (peptide 3) and D-Thr-Lys-Pro-Thr-Asp-Gly-Asn-NH_2 (peptide 4), in which C-terminal Asn residue ends with amide instead of carboxylate function.

Cu(II) complexes. The set of the complex species obtained from the calculations based on potentiometric titration curves for L-enantiomeric heptapeptide is the same as that of its N-terminal tripeptide fragment Thr-Lys-Pro (peptide 7) (Table 1). Very similar species distribution curves obtained for Cu(II) with hepta- and tri-peptide (Fig. 1) indicate very close binding abilities of both ligands, *i.e.* their almost identical binding modes and the stabilities of the particular species. As Pro acts as a breakpoint residue, *i.e.* it does not take part in the metal ion binding [1,4], the binding donor set involved in the Cu(II) ion coordination originates from two N-terminal amino acids, Thr and Lys. The CD spectra exhibit two charge transfer bands around 315 and 270 nm, which correspond to $N^-_{amid} \rightarrow$ Cu(II) and $NH_2 \rightarrow$ Cu(II) transitions. Thus, the major CuL species is the 2N complex with $\{NH_2,N^-\}$ coordination mode. This binding mode is also supported by EPR parameters and the energy of the d-d transition (around 650 nm) observed in the absorption spectra [1–4,10]. Thus, none of the amino acid residue being on the C-terminal side of Pro serves any donor to bind metal ion. It is interesting to note, however, that CuL complex with amide analogue of heptapeptide (peptide 2, Scheme 1) is distinctly weaker than that with free C-terminal carboxylate (Fig. 2, Table 1). This may suggest some interactions of the distant COO^- with metal ion.

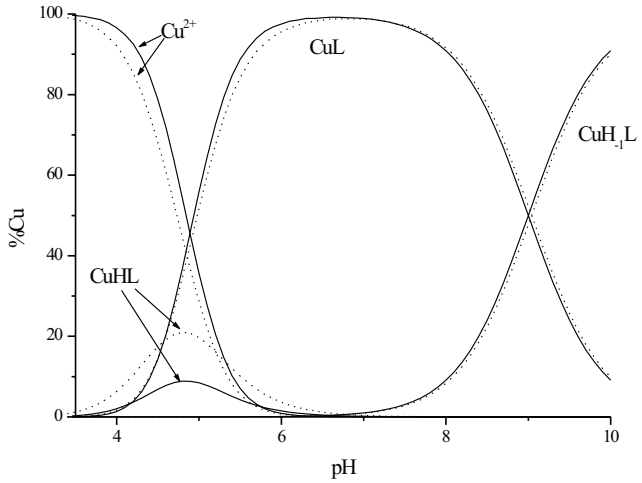


Figure 1. Species distribution curves for Cu(II)-peptide 1 (solid line) and Cu(II)-peptide 7 (dotted line) complexes at 25°C and $I = 0.1$ mol dm^{-3} KNO_3. Ligand concentration 1×10^{-3} mol dm^{-3}. Ligand to metal ratio for peptide 1 – 1.5:1 and for peptide 7 – 2:1.

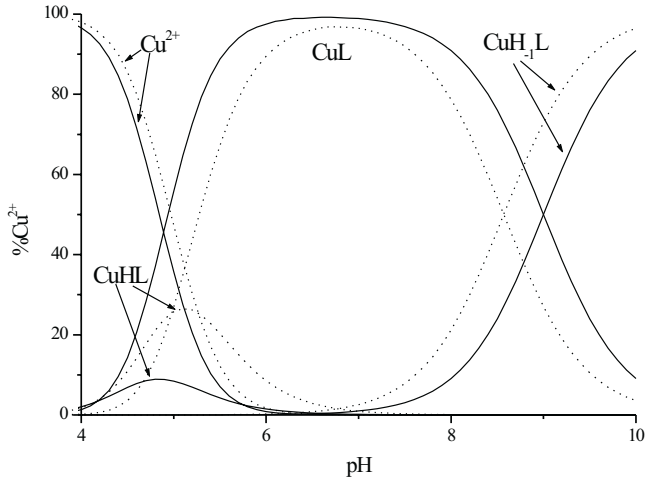


Figure 2. Species distribution curves for Cu(II)-peptide 1 (solid line) and Cu(II)-peptide 2 (dotted line) complexes at 25°C and $I = 0.1$ mol dm^{-3} KNO_3. Ligand concentration 1×10^{-3} mol dm^{-3}. Ligand to metal ratio 1.5:1.

The comparison of the Thr-Lys-Pro (peptide 7) tripeptide with Thr-Lys-Pro-Arg (peptide 6) analogue clearly indicate that the involvement of the donor set of the residue placed on the C-terminal side of Pro is, however, likely (see also [1,10,11]). The species distributions for Cu(II)–Thr-Lys-Pro and Cu(II)–Thr-Lys-Pro-Arg systems look similar (Fig. 3). However, careful considerations of stability constants indicate the involvement of Arg side chain NH_2 moiety in the direct interaction with metal ion. According to the stability constants calculated from the potentiometric curves, Thr-Lys-Pro-Arg forms four complex species (Fig. 3, Table 1). The CuHL species, a major complex in the pH range 5–7.5, according to the spectroscopic data (Table 2), is a 2N complex with the binding mode, $\{NH_2,N^-\}$, as 2N species in the case of tripeptide analogue. Above pH 7.5 CuHL undergoes deprotonation (log K = 8.02) and then above pH 8 CuL dissociates another proton (log K = 9.30) to give $CuH_{-1}L$ species. The latter deprotonation process is found for all complexes studied and it corresponds to proton dissociation from lateral NH_3^+ of Lys2 (pK = 9.56). The former proton dissociation (CuHL $\rightarrow$ CuL) has much lower log K value than side chain Arg NH_2 (8.02 *vs*. 10.95, Table 1). The distinct lowering of the protonation constant strongly suggests the effective involvement of the Arg lateral nitrogen donor in metal ion binding. The involvement of this nitrogen in the metal ion coordination is also seen in the changes of the CD spectra when pH increases above 9 (Table 2).

In the other peptide studied in this work the potential binding sites are Asp5 β-COO^-, Asn terminal carboxylate and its lateral amide moiety. Only C-terminal carboxylate seems to have some impact on the complex stability.

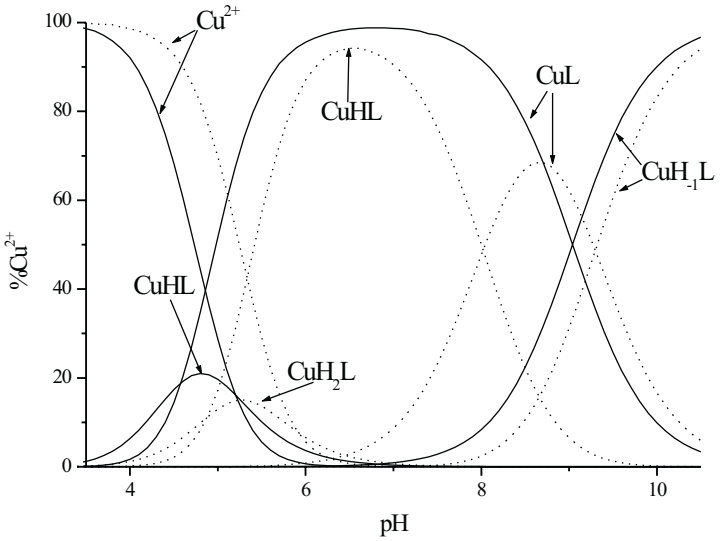


Figure 3. Species distribution curves for Cu(II)-peptide 7 (solid line) and Cu(II)-peptide 6 (dotted line) complexes at 25°C and $I = 0.1$ mol dm^{-3} KNO_3. Ligand concentration 1×10^{-3} mol dm^{-3}. Ligand to metal ratio 2:1.

The effect of enantiomeric substitutions: The set of Cu(II) complexes formed with both heptapeptides is exactly the same. There are three complexes formed, CuHL, CuL and $CuH_{-1}L$ with metal ion bound to N-terminal fragment of a peptide ligand. As mentioned above, the presence of the Pro3 breaks a consecutive coordination of Cu(II) to third and fourth amide nitrogen, thus the binding mode in the complexes are $\{NH_2,C=O\}$ for CuHL and $\{NH_2,N^-,C=O\}$ for CuL and $CuH_{-1}L$ species. The comparison of the species distribution curves obtained for L- and D-enantiomers (decrease of the free metal concentration) indicates that L-enantiomer is slightly stronger ligand than its D-analogue (Fig. 4). The comparison of the binding stabilities of the D-enantiomer with those of D-Thr-Lys-Pro-Thr-Asp-Gly-Asn (peptide 2) shows that the major contribution to lower the complex stability derives from first D-amino acid residue. The species distributions obtained for the Cu(II) complexes with D-enantiomer and heptapeptide having all L-amino acids except N-terminal Thr are almost identical (Fig. 4).

CONCLUSIONS

The heptapeptide 140–146 fragment of the hepatitis B virus surface antigen is relatively effective ligand for Cu(II) ions. The basic binding site is located on the N-terminal fragment of the peptide. The substitution of the L-amino acid residues to D-enantiomer has distinct although not very large effect on the complex stabilities.

Table 2. Spectroscopic parameters for Cu(II) complexes of peptides.

	UV-Vis		CD		EPR		UV-Vis		CD		EPR	
Species	λ nm	ε $mol^{-1}\ dm^3\ cm^{-1}$	λ nm	Δε $mol^{-1}\ dm^3\ cm^{-1}$	$A_{\|\|}$ $cm^1\times10^4$	$g_{\|\|}$	λ nm	ε $mol^{-1}\ dm^3\ cm^{-1}$	λ nm	Δε $mol^{-1}\ dm^3\ cm^{-1}$	$A_{\|\|}$ $cm^1\times10^4$	$g_{\|\|}$
	Thr-Lys-Pro-Thr-Asp-Gly-Asn (Peptide 1)						**D-Thr-Lys-Pro-Thr-Asp-Gly-Asn (Peptide 2)**					
CuL	670	116	695	–0.547	167	2.251	670	128	694	–0.499	163	2.254
			534	–0.051sh.					585	–0.047sh.		
			316	+0.598					313	+1.327		
			270	–1.140sh.					275	–0.897		
									247	+1.129		
$CuH_{-1}L$	638	96	720	–0.270	166	2.246	570	126	504	–0.498	169	2.239
			537	–0.147					330	–0.696		
			305	+0.330					285	–1.164		
			273	–0.329					254	+2.269		
	Thr-Lys-Pro-Thr-Asp-Gly-Asn-NH_2 (Peptide 3)						**D-Thr-Lys-Pro-Thr-Asp-Gly-Asn-NH_2 (Peptide 4)**					
CuL	663	114	692	–0.5600	164	2.257	668	116	692	–0.419	168	2.251
			568	–0.10sh.					551	–0.161		
			314	+0.697					312	+1.005		
			270	–1.026					273	–0.610		
									245	+0.967		
$CuH_{-1}L$	596	87	628	+0.135	169	2.245	600	95	638	+0.091	154	2.243
			510	–0.331					513	–0.245		

Table 2 (continuation)

			333	–0.274			333	–0.496
			282	–0.144			278	–0.182
			258	+0.649			248	+1.431
D-Thr-D-Lys-D-Pro-D-Thr-D-Asp-D-Gly-D-Asn (Peptide 5)								
CuL	655	111	704	+0.467	166	2.254		
			313	–0.510				
			264	+0.803				
CuH$_{-1}$L	638	91	693	+0.351	171	251		
			554	–0.330				
			313	+0.881				
			281	–0.095				
Thr-Lys-Pro (Peptide 7)								
CuL	640	107	681	–0.266	175	2.249		
			316	+0.349				
			270	–0.791				
CuH$_{-1}$L	640	122	704	–0.262	172	2.252		
			520	–0.148				
			311	+0.464				
			268	–0.100				

Table 2 (continuation)

Thr-Lys-Pro-Arg (Peptide 6)						
CuHL	648	126	702	–0.211	169	2.256
			575	–0.211		
			317	+0.648		
			268	–1.034		
CuL	633	135	671	–0.400	139	2.251
			567	–0.328		
			315	+0.917		
			268	–1.566		
$CuH_{-1}L$	641	140	720	–0.273	151	2.248
			534	–0.129		
			305	+0.328		
			268	–0.396		

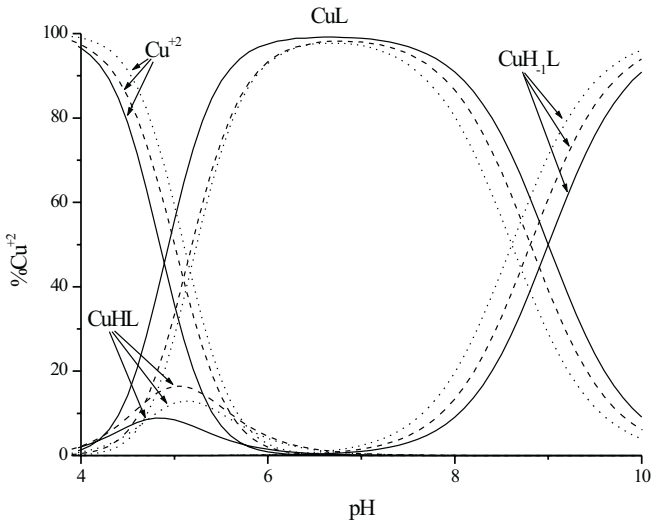


Figure 4. Species distribution curves for Cu(II)-peptide 1 (solid line), Cu(II)-peptide 2 (dotted line) and Cu(II)-peptide 5 (dashed line) complexes at 25°C and $I = 0.1$ mol dm^{-3} KNO_3. Ligand concentration 1×10^{-3} mol dm^{-3}. Ligand to metal ratio 1.5:1.

The side chain donor system present on the C-terminal side of the Pro3 is not competing in metal ion binding, although C-terminal carboxylate has a strong effect on the formation of the CuHL and CuL species.

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