Characterization of the Copper(II)- and Nickel(II)-Transport Site of Human Serum Albumin. Studies of Copper(II) and Nickel(II) Binding to Peptide 1–24 of Human Serum Albumin by ^{13}C and ^{1}H NMR Spectroscopy†

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ABSTRACT: As a basis for understanding the role of albumin in the transport of metal ions, detailed investigations have been carried out to elucidate the structure of Ni(II)- and Cu(II)-binding site of the peptide residue corresponding to the NH_2-terminal peptide fragment 1–24 of human serum albumin by ^{1}H and ^{13}C NMR spectroscopy. These studies have been conducted in aqueous medium at different pH values and at different ligand/metal ratios. The results show the following: (i) Diamagnetic Ni(II) complex and paramagnetic Cu(II) complex are in slow exchange rate on the NMR time scale. (ii) Titration results of Ni(II)-bound form of peptide 1–24 show the presence of a 1:1 complex in the wide pH range (6.0–11.0), and the same stoichiometry is proposed for Cu(II) as well. (iii) Analysis of the spectra suggests that both Ni(II) and Cu(II) have one specific binding site at the NH_2-terminal tripeptide segment (Asp^1-Ala^2-His^3...) involving the Asp^1 α-NH_2, His^3 N(1) imidazole, two deprotonated peptide nitrogens (Ala^2 NH and His^3 NH), and the Asp^1 COO^- group. (iv) Complexation of Ni(II) and Cu(II) causes conformational change near the metal-binding site of the polypeptide chain, but there is no other binding group involved besides those in the first three residues.

Human serum albumin possesses a specific binding site for both Cu(II) and Ni(II). Copper(II) and Ni(II) bound to albumin are known to be the transport forms of these metals in blood (Bearn & Kunkel, 1954; Neumann & Sass-Kortsak, 1967; Lau & Sarkar, 1971; Lucassen & Sarkar, 1979; Sarkar, 1980, 1981, 1983a,b). The metal-transport site of serum albumin is one of the most extensively studied metal-binding sites of proteins. The major contribution in our knowledge of this site is due to a series of investigations conducted in the laboratories of Peters (Peters, 1960; Peters & Hawn, 1967; Peters & Blumenstock, 1969; Shearer et al., 1967) and Gurd (Shearer et al., 1967; Bradshaw et al., 1968), as well as in our own laboratory (Arena et al., 1979; Camerman et al., 1976; Glennon & Sarkar, 1982a; Iyer et al., 1978; Kruck et al., 1976; Lau & Sarkar, 1975, 1981; Lau et al., 1974; Laussac & Sarkar, 1980; Rakhit & Sarkar, 1981; Sarkar, 1983a,b).

Initially, the Cu(II)-transport site was suggested to involve the α-amino nitrogen, two intervening peptide nitrogens, and the imidazole nitrogen of the histidine residue in position 3 (Peters & Blumenstock, 1969). Subsequent investigations were carried out in our laboratory with simple tripeptide molecules, e.g., glycylglycyl-L-histidine and glycylglycyl-L-histidine *N*-methylamide which were designed to mimic the proposed Cu(II)-transport site of human serum albumin (HSA).[1] The results showed many similarities between HSA and these peptides in their Cu(II)-binding properties (Camerman et al., 1976; Kruck et al., 1976; Lau & Sarkar, 1975; Lau et al., 1974). Interestingly, the X-ray structure analysis of the Cu(II)–GGHNMA complex showed a distorted square-planar structure involving four nitrogen ligands as had been proposed for the Cu(II)-transport site of albumin (Camerman et al., 1976). We then synthesized the NH_2-terminal native sequence tripeptide of HSA, L-aspartyl-L-alanyl-L-histidine *N*-methylamide, and reported its Cu(II)-binding properties (Iyer et al., 1978). Again, a considerable similarity was evident between the native sequence peptide and the designed peptides in their Cu(II)-binding characteristics, but from the point of Cu(II)-binding affinity and dissociation, the native sequence peptide resembled closer to the protein albumin. This posed the question as to whether Cu(II)-binding site indeed involved any additional group(s) besides the four nitrogen ligands already proposed. Studies with ^{13}C NMR spectroscopy of the Cu(II) binding to AAHNMA provided the first indication of the involvement of the β-carboxyl side chain of the aspartyl residue (Laussac & Sarkar, 1980a).

Investigations of the Ni(II)-binding to HSA and AAHNMA revealed that the Ni(II)-binding site is also located at the NH_2-terminal of the protein (Glennon & Sarkar, 1982a). Like Cu(II), Ni(II) showed the lack of specificity of binding to dog serum albumin, implicating the importance of the histidine residue in the third position (Appleton & Sarkar, 1971; Glennon et al., 1983). The characteristic visible absorption spectra of HSA–Cu(II) were generated by adding Cu(II) to a solution of HSA–Ni(II), which raised the possibility of an overlapping binding site for both metals on the same protein (Glennon & Sarkar, 1982a). ^{13}C NMR and ^{1}H NMR investigations of the Ni(II) binding to the native sequence tripeptide revealed a similar structure of the Ni(II)-binding site as that of the Cu(II) binding to AAHNMA (Laussac & Sarkar, 1980a,b).

The native sequence tripeptide provided only the bare metal-binding site devoid of any other adjacent residues. From these studies, one does not know the influence of the neighboring residues and the conformation of this region of the polypeptide upon complexation. It is also difficult to obtain such information experimenting with the intact protein mol-

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[1] Abbreviations: HSA, human serum albumin; P_{24}, NH_2-terminal peptide 1–24 of human serum albumin; GGHNMA, glycylglycyl-L-histidine *N*-methylamide; AAHNMA, L-aspartyl-L-alanyl-L-histidine *N*-methylamide; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

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ecule in native state. The large scale isolation of peptide 1–24 fragment of HSA has enabled us to undertake a complete analysis of the Cu(II) and Ni(II) binding to this polypeptide fragment by ^{13}C and ^{1}H NMR spectroscopy. The utility of ^{13}C and ^{1}H NMR to study metal binding to large biological molecules is well recognized since the position and width of the resonance lines are sensitive to electronic changes which can occur within the molecular framework. Results show that both these metals bind to P_{24} in 1:1 stoichiometry. They both form pentacoordinated structure involving α-NH_2 nitrogen, two peptide nitrogens, an imidazole nitrogen from histidine-3, and the carboxyl oxygen from the side chain residue of the NH_2-terminal aspartic acid residue. There is no other binding group involved besides those in the first three residues of the protein.

Experimental Procedures

Materials. The P_{24} was obtained by controlled peptic digestion of HSA and subsequent purification of the fragment by Sephadex G-25 and Celex-D anion-exchange chromatography. Detailed procedure will be published elsewhere. $NiCl_2 \cdot 6H_2O$ and $CuCl_2 \cdot 2H_2O$ were purchased from Fisher Scientific Co. (Ottawa, Ontario, Canada), and sodium 4,4-dimethyl-4-silapentane-1-sulfonate was from Aldrich Chemical Co. (Milwaukee, WI). D_2O (99.8% D) from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France) was used as a solvent.

Instrumentation. DCl and NaOD solutions were used to adjust the pH which was measured with a Corning pH meter, Model 12, and an Ingold microelectrode. The pH values are not corrected for deuterium isotope effects.

Solutions with variable metal concentrations were made up by micropipetting from a concentrated D_2O solution of the anhydrous nickel(II) and copper(II) chloride. Nicolet 360 and Bruker WM 250 spectrometers operating in the Fourier transform mode were used for ^{1}H and ^{13}C spectra. The high-field ^{1}H NMR experiments were performed by using the Nicolet 360-MHz facility at the Toronto Biomedical NMR Center. Samples were examined at 23 ± 1 °C. The usual ^{1}H spectrometer conditions were 2200 Hz sweep width, 1 s cycle time, 16K data points, and 50 scans. Carbon-13 NMR spectra were recorded by using the equipment of the Laboratoire de Chimie de Coordination in Toulouse, France, at 62.8 MHz with broad band proton decoupling and quadrature detection. The usual conditions were 15 000 Hz sweep width, 1 s cycle time, 20 μs pulse width, 16K data points, and 50 000 scans for a 10^{-2} M solution of P_{24}–Ni(II).

Both ^{1}H and ^{13}C chemical shifts were measured (in ppm) with DSS for external reference calibration; the signal for the external reference was then recorded in the computer of the spectrometer.

Results and Discussion

The majority of the ^{1}H and ^{13}C signals arising from the atoms of the backbone and of the side chains of P_{24} have been assigned. This has been achieved by jointly using the pH-dependent chemical shifts, the determination of the characteristic pK values, the combined use of multiple and selective proton-decoupled ^{1}H and ^{13}C NMR spectra, and the comparison of the spectra with the NH_2-terminal tripeptide segment of HSA (Laussac & Sarkar, 1980a). The NH_2-terminal peptide 1–24 of HSA contains many functional groups which are potential ligands for Ni(II) and Cu(II). These include the α-NH_2, the NH_2's of $Lys^{4,12,20}$, the C-terminal Leu^{24} COO^-, COO^- of $Glu^{6,16,17}$ and $Asp^{1,13}$, imidazole nitrogens of $His^{3,9}$, and the amide nitrogens of the peptide groups. However, the complexation study gives evidence that the binding sites of both metals are the same and involve primarily the first three NH_2-terminal residues. For this reason and for the sake of clarity, only the resonances corresponding to the first three amino acids will be taken into consideration.

Table I: Comparison of ^{1}H NMR Data δ and $-\Delta^a$ for P_{24}–Ni(II) and AAHNMA–Ni(II) in D_2O at pH 8.0

	P_{24}/Ni(II) (1/1)			AAHNMA/Ni(II) (1/1)
resonance	δ (unbound)	δ (bound)	$-\Delta$	$-\Delta$
Asp^1 CHα	3.82	3.58	0.24	0.28
Ala^2 CHα	4.24	3.68	0.56	0.60
His^3 CHα	4.55	4.08	0.47	0.51
Asp^1 CH$_2\beta$	2.54	2.43	0.11	0.10
	2.66	2.60	0.06	
Ala^2 CH$_3$	1.35	1.30	0.05	0.05
His^3 CH$_2\beta$	3.00	2.85	0.15	0.11
		2.89	0.11	
Im C(2)H	7.80	7.56	0.24	0.27
Im C(4)H	6.99	6.94	0.05	0.10

$^a \Delta = \delta$ (bound) $- \delta$ (unbound).

Table II: Comparison of ^{13}C NMR Data δ and Δ^a for P_{24}–Ni(II) and AAHNMA–Ni(II) in D_2O at pH 7.2

	P_{24}/Ni(II) (1/1)			AAHNMA/Ni(II) (1/1)
reso-nance	δ (unbound)	δ (bound)	Δ	Δ
Asp^1 COO^-	176.50	187.35	10.85	10.48
Asp^1 CO	171.35	178.37	7.02	6.74
Ala^2 CO	174.50	179.04	4.54	4.79
His^3 CO	172.58			2.02
Im^3 C(2)	135.50	136.60	1.10	1.05
Im^3 C(4)	116.95	114.36	−2.59	−2.57
Im^3 C(5)	131.60	133.31	1.71	1.51

$^a \Delta = \delta$ (bound) $- \delta$ (unbound).

The ^{1}H and ^{13}C chemical shifts parameters corresponding to the metal-free and -bound ligand for the first three amino acid residues of P_{24} are reported in Table I and Table II parallel to those obtained with AAHNMA at the same pH.

Nickel(II)-Transport Site. (i) ^{1}H NMR of P_{24}–Nickel(II) Complex. Gradual addition of Ni(II) to the peptide near physiological pH results in selective shifting of the resonances and extensive changes throughout the whole ^{1}H NMR spectrum. The most striking observation comes from the aromatic spectrum shown in Figure 1. In the region 6.8–7.8 ppm, the increasing addition of Ni(II) to the P_{24} results in a progressive decrease of intensity for both C(2)H and C(4)H downfield resonances due to His^3 imidazole protons and in the gradual appearance of two new resonances, at 7.56 and 6.94 ppm, respectively. The remaining resonances and especially the two upfield peaks corresponding to the imidazole protons of the second histidine residue are unaffected by Ni(II) complexation. The Ni(II) is therefore in slow exchange since only the intensity changes are observed, rather than continuous shifts in peak position. A similar behavior was observed with the tripeptide AAHNMA (Laussac & Sarkar, 1980b). Since the system is in slow exchange, it is possible to unequivocally assign the proton resonances in the P_{24}–Ni(II) complex. Furthermore, it can be seen in Figure 1 that increasing Ni(II) concentration induces an increase in intensity of the new resonances until a P_{24}/Ni(II) ratio equals to one where no peaks corresponding to free peptide are detected. This clearly shows

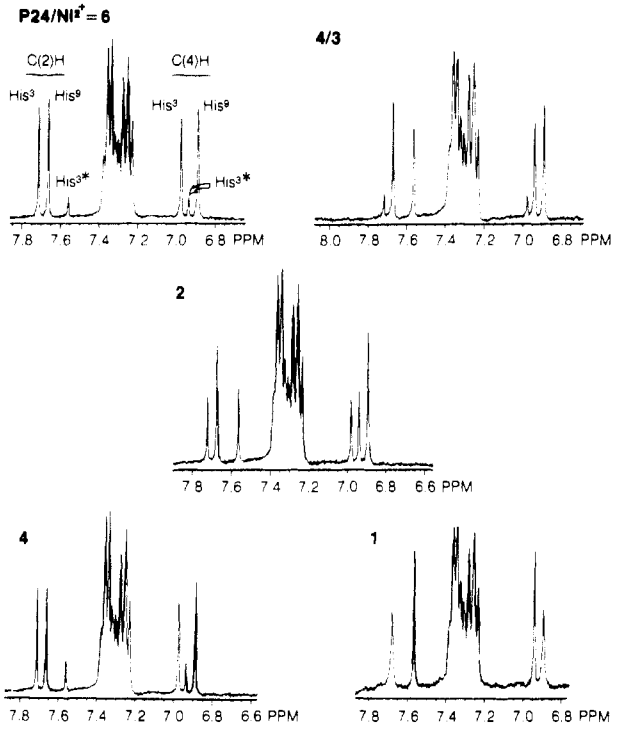

FIGURE 1: Effect of increasing P_{24}/Ni(II) ratios (=6, 4, 2, 4/3, and 1) on the 360-MHz ^1H NMR spectrum of P_{24} at pH 7.2. Part corresponding to the downfield region.

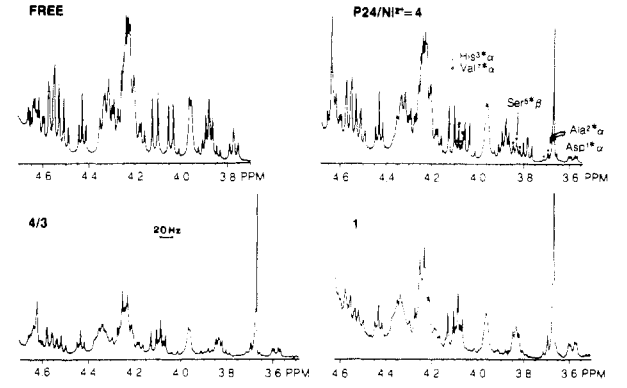

FIGURE 2: α-CH region of the 360-MHz ^1H NMR spectrum of P_{24} at pH 8.00 and for different P_{24}/Ni(II) ratios (=4, 4/3, and 1).

that P_{24}–Ni(II) complex possesses a 1:1 stoichiometry. Similar results are observed with other spectra as well.

Figure 2 shows the spectra corresponding to the CαH resonances at pH 8.00 and for different P_{24}/Ni(II) ratios. In this region, when Ni(II) is added to the solution, most of the resonances remain unshifted upon complexation but there is a significant shift in the frequencies of several other resonances. Of importance, a characteristic quadruplet structure corresponding to one of the Ala CαH proton shifts to 3.68 ppm, and a triplet signal shifts to 4.08 ppm, while a doublet of doublet shifts to 3.58 ppm. Each of these multiplet structures has been identified by homonuclear decoupling experiments and correspond to Ala^2* CαH, His^3* CαH, and Asp^1* CαH, respectively.[2] Furthermore, as observed for the imidazole protons, addition of increasing amounts of Ni(II) produces an increasing intensity of the different peaks until a 1:1 stoichiometry with a concomitant decrease and finally disappearance of the resonances corresponding to the free ligand. More surprising is the behavior of Ser^5* CβH$_2$ and Val^7* CαH resonances. The former was shifted a little upfield (0.05 ppm) upon Ni(II) binding with a small variation in coupling constant of ca. 1 Hz, while the Ser^5 CαH resonance was not affected, and the latter was shifted downfield by 0.03 ppm without any change in coupling constant. These observations could be interpreted in terms of conformational effects due to complexation.

Analysis of the β-CH$_2$ region (2.2–3.2 ppm) was also facilitated by the slow rate of exchange we observed between P_{24}–Ni(II) and metal-free P_{24}. Similarly, addition of less than stoichiometric amounts of Ni(II) to a solution of P_{24} results in a decrease of the intensity of the resonances for free P_{24} with a proportional appearance of a new set of resonances. At a P_{24}/Ni(II) ratio of 1/1, the ^1H NMR spectrum does not indicate any resonances due to metal-free P_{24}. Decoupling of the Asp^1* CαH protons at 3.58 ppm collapsed the corresponding Asp^1* CβH$_2$ resonances to an AB pattern at 2.43 and 2.60 ppm, respectively. Similarly, by homonuclear irradiation of the His^3* CαH signal at 4.08 ppm, the His^3* CβH$_2$ ABX multiplet coalesces into two singlets at 2.85 and 2.89 ppm, respectively. Additional changes were observed in this region; indeed, a new set of resonances centered at 2.92 ppm can be encountered. Decoupling experiments clearly show that this signal is spin coupled to the resonance at 1.58 ppm. This observation unequivocally assigns the signal at 2.92 ppm to the CδH$_2$ group of Arg^{10}* residue. This behavior seems to be similar to that observed for the Ser^5* CβH$_2$ and Val^7* CαH resonances.

The high-field region from 0 to 2 ppm is the least affected. Upon addition of Ni(II) ion, only a new doublet signal appears at 1.30 ppm, whose intensity increases with the amount of Ni(II) added, at the expense of the signal assigned to the Ala CβH$_3$ atoms in free P_{24} at 1.35 ppm. The effect of irradiating the quadruplet centered at 3.68 ppm and corresponding to Ala^2* CαH reduced the doublet at 1.30 ppm to a singlet peak which in turn is attributed to Ala^2* CβH$_3$.

In Table I, the ^1H values show very clearly that the direction (upfield) and the magnitude of the chemical shifts which occur for P_{24} and AAHNMA upon binding of the peptide to the Ni(II) ion are the same. This strongly suggests that these resonances with altered chemical shifts should reflect the general region where the metal binds and that both ligands have the same coordinating sites. Furthermore, as observed earlier, results of the magnetic moment measurement with the tripeptide fragment are consistent with a diamagnetic Ni(II) complex (Laussac & Sarkar, 1980b). Thus, the same chemical shift behavior encountered with both ligands and the absence of line broadening established that the coordination of P_{24} to Ni(II) leads to a low-spin diamagnetic Ni(II) complex.

At pH 8.0, specific metal donor sites such as NH$_2$-terminal, Asp^1 COO$^-$, and imidazole of P_{24} are actually deprotonated, and it appears that the upfield chemical shift values for Asp^1 CαH, Asp^1 CβH$_2$, His^3 C(2)H, and His^3 C(4)H reflect only the effect due to complexation. However, the largest upfield shift is observed for Ala^2 CαH (0.56 ppm) and His^3 CαH (0.46 ppm); these large upfield shifts may have been caused by (i) deprotonation of the Ala^2 NH and His^3 NH nitrogen atoms and (ii) complexation of Ni(II) ion to the deprotonated nitrogens [Ni(II) promotes the ionization of peptide hydrogens from peptides with the formation of deprotonated N(peptide)–metal bonds (Nakon & Angelici, 1974)]. So, it would be expected that both the deprotonated nitrogen atoms will be bonded to the metal ion. Observing the considerable similarity in the binding properties of P_{24}–Ni(II) and those of AAHNMA–Ni(II), the above ^1H results suggest that Ni(II) forms a complex with this peptide involving the α-NH$_2$ ter-

[2] Asterisk denotes the resonances affected by complexation.

minal, N(1) imidazole of the third histidine residue, and two deprotonated peptide nitrogens (Ala^2 NH and His^3 NH). The ^{13}C data will show us definitively the involvement of the Asp^1 COO^- group.

The study in the pH range 6.0–11.0 shows that the resonances for bound ligands are clearly observed and unaffected, indicating the presence of the same complex in this wide pH range. The only change comes from the Arg^{10} * $C\alpha H_2$ group which, in basic solution, adopts the same chemical shift as in free peptide, while Ser^5 * $C\beta H_2$ and Val^7 * $C\alpha H$ are unaffected. We analyzed these data by conformational change effects and conclude that the pH had no effect on the conformation near the metal center but can produce some modifications outside the binding site.

There is no indication of any line broadening when bound and unbound forms are both present. In these circumstances, the upper limit on the exchange rate in the "well-observed" case can be mathematically described by the condition

$$2\pi(\Delta\nu_M)\tau_M \gg 1 \quad (1)$$

where $\Delta\nu_M$ is the ^1H chemical shift difference (in Hz) between bound and free ligand and τ_M is the lifetime of the ligand bound to the metal ion (McLaughlin & Leigh, 1973). This condition refers to all individual resonances, and it is applied to the different $\Delta\nu_M$ values. The smallest chemical shift perturbation induced by Ni(II) was found to be ca. 20 Hz at 360 MHz for the His^3 C(4)H resonance. From this chemical shift separation, we estimate that the upper limit of the lifetime of the P_{24}–Ni(II) complex is $\tau_M \gg 10^{-2}$ s, and consequently, the exchange rate or the dissociation rate (k_{diss}) of the metal between these species is estimated to $k_{diss} \ll 100\ s^{-1}$.

(ii) ^{13}C NMR of P_{24}–Nickel(II) Complex. Major influence on the position of a carbon resonance line appears to be the distance of the carbon atom from the metal center while carbon atoms far from the metal-binding site remain unshifted or experience only a small shift upon metal complexation due, for example, to changes in molecular conformation. In either case, the primary change in magnetic environment will be in the immediate vicinity of the metal.

When Ni(II) is added to P_{24} solution, there is a significant perturbation in the frequencies arising from several groups in this peptide. Counter to the proton resonance, complexation produces a general downfield shift except for the C(4) carbon of the imidazole residue which is shifted upfield.

The most dramatic and exciting changes are observed for the CO and the COO^- resonances in the low-field part of the spectrum. The effects of the presence of Ni(II) on the 62.8-MHz ^{13}C NMR spectra of P_{24} are shown in Figure 3 at physiological pH. The fact that a new set of resonances are observed and that increasing additions of Ni(II) increase the intensity of these resonances with a proportional disappearance of some resonances corresponding to the free ligand indicates that the P_{24} is in slow exchange on the ^{13}C NMR chemical shift time scale between its free and complexed states [i.e., for some of the carbons in P_{24}, the rate of dissociation (k_{diss}) of the P_{24}/Ni(II) complex is much slower than their chemical shift differences in the bound and unbound form (eq 1)]. Furthermore, at a P_{24}–Ni(II) ratio of 1/1, the ^{13}C NMR spectrum does not indicate any resonances due to metal-free P_{24} as shown in Figure 3. These results strongly suggest that we have a 1/1 complex and confirm the ^1H NMR data.

We observe that the largest shift resulting from coordination of Ni(II) occurs for the Asp^1 * COO^- group (Δ = 10.85 ppm) which provides strong evidence that the carboxyl group is involved in the metal binding. Note that at this pH (7.2) the magnitude of this deshielding effect is due only to complexation and not to deprotonation. The Asp^1 * CO resonance was also strongly affected. Its resonance (labeled by a filled box) was deshielded by 7.02 ppm relative to free ligand and had a chemical shift comparable to that found in AAHNMA (Table II). Similarly, the Ala^2 * CO is greatly affected, and its position (labeled by a filled star in Figure 3) moves downfield by 4.54 ppm, while the His^3 * CO was not detected due to its position in the crowded CO region. On the basis of these observations, we suggest that all the groups whose resonances are perturbed by Ni(II) are due to complexation with Asp^1 * COO^- as well as the groups being in close proximity to (Asp^1 * CO, Ala^2 * CO), the coordination site.

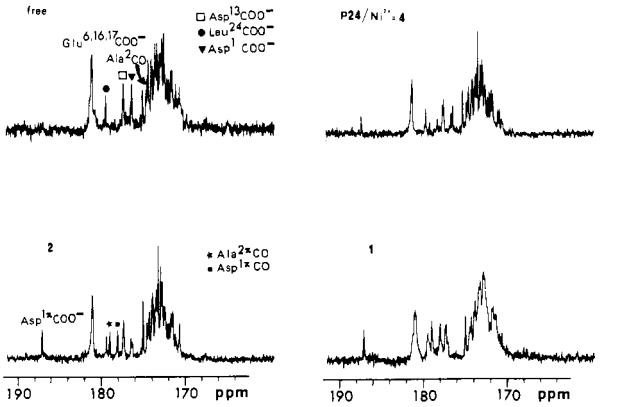

FIGURE 3: Portion of the ^{13}C NMR spectrum (62.8 MHz) obtained in D_2O at pH 7.2 showing the resonances of P_{24} and P_{24}/Ni(II) at different molar ratios (=4, 2, and 1). Part corresponding to the COO^- and CO region.

Similarly, the carbon resonances belonging to the aromatic residues show an interesting behavior upon Ni(II) binding. While all the peaks corresponding to both $Phe^{11,19}$ rings are not affected by metal, the C(2), C(4), and C(5) imidazole carbon resonances show a very large change in chemical shift upon complexation. Furthermore, only one imidazole residue is affected, suggesting that the remaining imidazole ring is remote from the coordination site. Both C(2) and C(5) carbons move downfield (Δ = 1.10 and 1.71, respectively), while C(4) moves upfield upon complexation (Δ = −2.59). Shifts of similar magnitude and sign have been observed with the native sequence tripeptide as can be seen in Table II, suggesting the binding involvement of the histidine in the third position in P_{24}.

No attempts were made to follow the perturbations caused by Ni(II) in the aliphatic region due to the complexity of the spectrum caused by the overlap of resonances at the frequencies used.

As we observed in Table I for ^1H results, Table II shows very clearly that the direction (mainly downfield) and the magnitude for all the different carbon chemical shifts are the same in both systems, suggesting unambiguously identical binding sites for the two ligands.

Copper(II)-Transport Site. The presence of an unpaired electron in a molecule gives rise to large shifts and broadening effects of the NMR resonances of nearby nuclei. If the rates of dissociation (k_{diss}) of the transition metal ion from the ligand are in the NMR slow-exchange limit on the chemical shift time scale (which are of the order of $10–10^3\ s^{-1}$), the resultant shifted and broadened resonances will be observed separate from the resonances of the diamagnetic ligand. As Cu(II) ions generally exhibit a relatively long relaxation time for the unpaired electron ($\tau_s^{-1} \sim 10^8\ s^{-1}$) (Swift & Connick, 1962), its dominant effect is line broadening; the relaxation in the bound sphere is so efficient that $1/T_{2m}^2 \gg \Delta\omega_M^2$, and one can assume that $\Delta\omega_M$, the shift between the resonances of free and

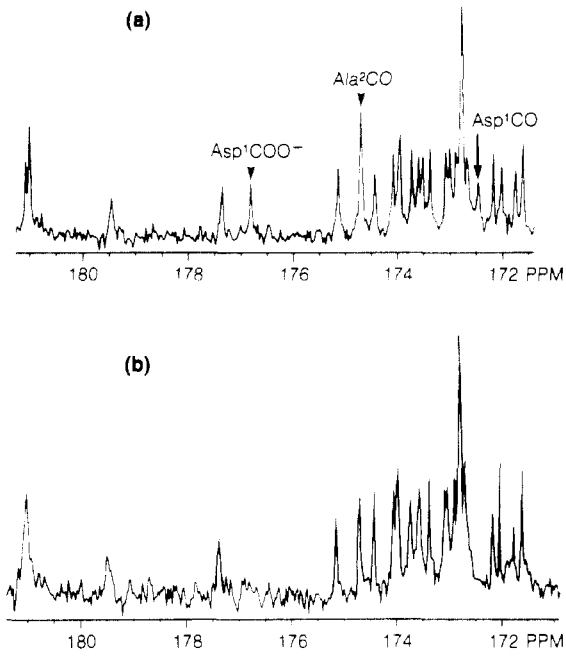

FIGURE 4: ^{13}C NMR spectra (90.6 MHz) of P_{24} in D_2O solution at pH 7.5, corresponding to the COO^- and CO region. Spectrum a is the free peptide, and spectrum b is of P_{24} in the presence of Cu(II) [P_{24}/Cu(II) = 2.5].

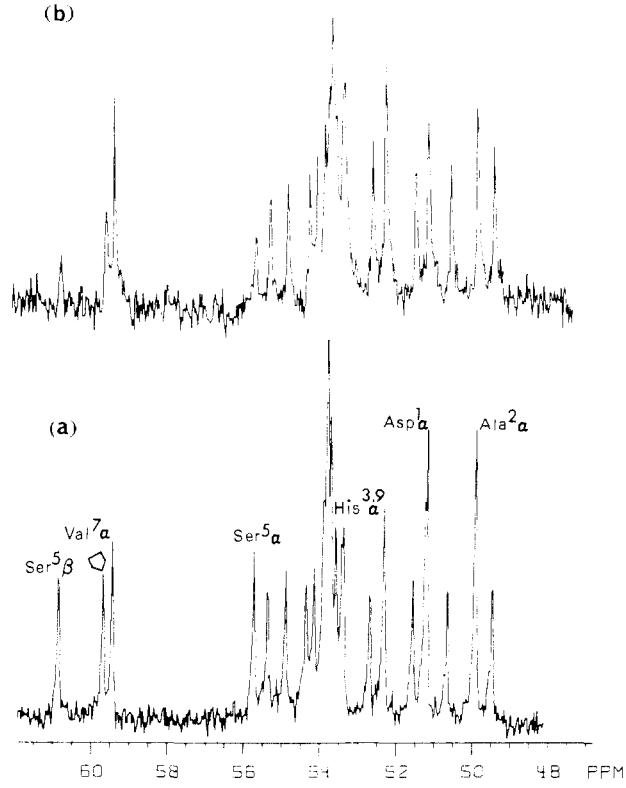

FIGURE 5: ^{13}C NMR spectra (90.6 MHz) of P_{24} in D_2O solution at pH 7.5, corresponding to the α-CH region. Spectrum a is of the free peptide, and spectrum b is of P_{24} in the presence of Cu(II) [P_{24}/Cu(II) = 2.5].

bound ligand, is negligible. This is consistent with different observations on Cu(II)-containing systems (Beattie et al., 1976; Laussac & Sarkar, 1980a; Laussac et al., 1983). Theory of the paramagnetic line broadening of NMR spectra undergoing chemical exchange has been treated elegantly by Swift & Connick (1962) and by Luz & Meiboom (1964). We refer the readers to those papers for further details.

As has been generally encountered, the large paramagnetic broadening observed in the presence of small amounts of Cu(II) indicates that the metal ions are effectively exchanging between the various donor sites. In these conditions, each resonance corresponds to the weighted average of unbound and bound ligand. The broadening studies rely on "fast-exchange" conditions.

NMR spectra obtained from the complexation studies of P_{24} (M_r 2743) with Cu(II) show a rather curious and unusual behavior of a Cu(II)-bound peptide. Indeed, unlike the other peptide–Cu(II) complexes, it is necessary to use a very low ligand/Cu(II) ratio to detect change in intensity, broadening, or disappearance of certain resonances. The most interesting results are derived from the ^{13}C spectra. At P_{24} to Cu(II) ratio as low as 10/1, there is no evidence of a change in ^{13}C for different pH values. But at high levels of copper (P_{24}/Cu(II) = 2.5) the peaks of some carbons are selectively broadened beyond detection or show a slowing down of the intensity of the resonances indicating a close proximity to the paramagnetic Cu(II) ion. Striking observations are encountered in Figure 4 corresponding to the low-field part of the ^{13}C NMR spectrum at physiological pH. From this spectrum, it is clear that Cu(II induces a complete disappearance of the various carbons. For example, the main interesting observation is that the peaks corresponding to Asp^1 COO^- and to Asp^1 CO at 176.5 and 172.5 ppm, respectively, disappear completely in the noise. Similarly, the signal at 174.7 ppm corresponding to two $Ala^{2,8}$ CO or $Ala^{2,21}$ CO carbons are seen to be affected due to the paramagnetic effect of the metal ion by the slowing down of this intensity resonance.

Addition of Cu(II) ions to a solution of P_{24} produces also significant changes in the aromatic region. The C(4) and C(5) imidazole carbons exhibit remarkably large change. However, due to the small chemical shift difference (ca. 0.3 ppm) between C(4) and C(5) resonances of both imidazole residues, it was difficult to see which peak was affected. Nevertheless, the upfield C(5) resonance seems to have disappeared in the noise and can be attributed to His^3 C(5).

In the same way, as shown in Figure 5, the resonances associated with the Cα carbons exhibit some interesting change. Upon addition of Cu(II) to P_{24}, the intensity of the ^{13}C NMR signals from the Ala^2 Cα (49.9 ppm) and from the Asp^1 Cα (51.2 ppm) decreased to approximately half of its signal value, while the peak at 53.7 ppm from the $His^{3,9}$ Cα residues is greatly affected. Similar results are observed with the corresponding methylene and methyl carbons spectrum. Interesting is the fact that, between pH ca. 5.5 and pH 10, the extent of the Cu(II)-induced broadening is independent of pH. The behavior of the resonances in the downfield region of Figure 5 is quite striking. There is considerable alteration of the peaks corresponding to Ser^5 Cα (55.7 ppm), Val^7 Cα (59.7 ppm), and Ser^5 Cβ (60.9 ppm). These results show the possibility that the hydroxyl group of the serine-5 and the side chain of the valine-7 residues are located sufficiently close to the Cu(II), as judged from the change of its carbon resonances when going from free to complexed ligand (cf. Figure 5). However, the orientation of the ϵ-ammonium moiety of Lys^4 must be such that this residue is located far from the metal center, because peaks associated with lysine are not affected in spectra upon complexation. These interesting observations agree well with data obtained from the Ni(II) complexation studies (see above).

The present studies have revealed some significant and here-to-unknown properties which may have important relevance in studying the paramagnetic behavior of Cu(II) by NMR spectrscopy. The overall data obtained and the unusual behavior of P_{24} bound to Cu(II), i.e., use of a high concen-

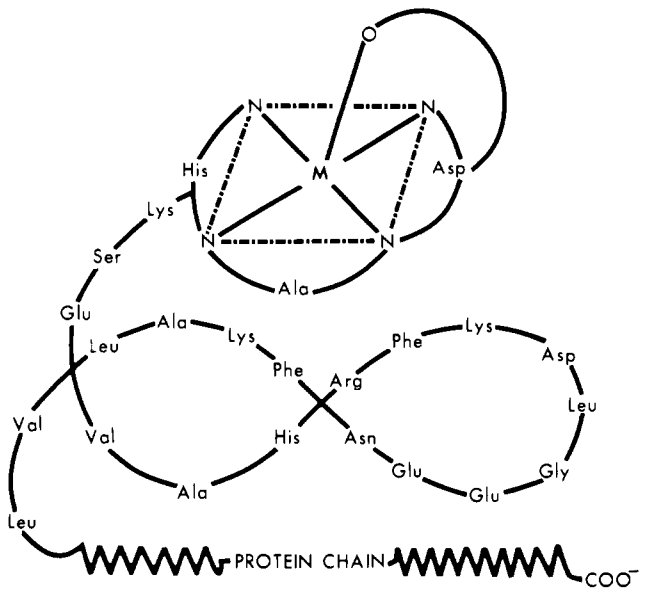

FIGURE 6: Proposed structure of the Ni(II)- and Cu(II)-binding site of human serum albumin.

tration of Cu(II) ion to detect modification of the spectra, strongly suggest that the system is in slow exchange as encountered with the diamagnetic Ni(II) study. This observation reveals for the first time, with this kind of biological ligand, that it is necessary to use a very low peptide/Cu(II) ratio to observe spectral changes. Recently, a similar observation has also been made with a cyclic peptide (Robert et al., 1983a,b). In this case, two sites are clearly observed corresponding to the complexed and free ligands and show an ideal case of a very slow process in Cu(II)-bound peptides. In the present work, exchange is faster but, nevertheless, slower than any results encountered to date; i.e., our system is in a "quasi"-slow-exchange condition. The line widths are governed by the exchange rate $1/\tau_M$, which is equal to the dissociation rate constant, k_{diss}, of the P_{24}–Cu(II) complex. Thus, at this pH, the Cu(II) ions are tightly bound to the P_{24}, and very slow dissociation or second-order ligand exchange occurs. This is consistent with our observations on the Ni(II)–complex ($k_{diss} \ll 100\ s^{-1}$) and with kinetic information on Cu(II)–peptide and Cu(II)–protein systems (Lau & Sarkar, 1975; Sarkar et al., 1976; M. Tabata and B. Sarkar, unpublished results). Unfortunately, no structural information can be obtained from NMR results about the distances of the amino acid nuclei to the Cu(II) ion in the complex; however, the selectivity in signal change can be used qualitatively to locate the binding site of paramagnetic Cu(II) ions. Thus, it appears on the basis of these spectra that, at high level of Cu(II), only a few residues are sufficiently close to Cu(II) to undergo paramagnetic effect. These residues, which correspond to Asp^1, Ala^2, and His^3, provide ligands to the Cu(II) ion in P_{24}.

Conclusion

The rate of dissociation of the metal from the complexes is slow on the NMR time scale, and we estimate that the lifetime of Ni(II) complex is ca. 1 s at room temperature, while with Cu(II), the lifetime is certainly lower. The results of the ^1H and ^{13}C NMR experiments show that P_{24} interacts with Ni(II) and Cu(II) to yield a complex with a unique spectrum in the wide pH range. There is no evidence for multiple coordination binding sites or the binding of more than one metal atom per molecule. The stoichiometry of Ni(II) to P_{24} is 1:1, and we propose the same stoichiometry for Cu(II).

The results reported above suggest that low-spin Ni(II) binds at the same metal ion binding site on P_{24} as does paramagnetic Cu(II). Five groups are clearly implicated by the ^1H and ^{13}C NMR data: (1) the α-NH_2 terminal, (2) the N(1) imidazole of the third residue, (3) two deprotonated peptide nitrogens (Ala^2 NH and His^3 NH), and (4) Asp^1 COO^- as shown in Figure 6. Apart from some local change, the structure appears to remain relatively unaltered by complexation. However, the Ser^5, Val^7, and Arg^{10} residues seem to be affected, while no modification of peaks corresponding to Lys^4 were detected in our spectra. Thus, it appears that complexation of Cu(II) and Ni(II) affects some residues near the metal but not as metal ligands, either via direct through space effects or via a local conformational change.

Registry No. Asp, 56-84-8; Ala, 56-41-7; His, 71-00-1; AAHNMA, 66277-14-3; Cu, 7440-50-8; Ni, 7440-02-0.

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Interaction of Prothrombin with Factor Va–Phospholipid Complexes†

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ABSTRACT: The effects of factor Va and the phospholipid-binding fragment of factor Va [factor Va light chain (LC), M_r 80000] on the binding of prothrombin, factor X, and factor Xa to phospholipid vesicles are reported. Equilibrium binding experiments were performed that utilized large-volume vesicles, which can be removed from the bulk solution by centrifugation. Factor Va decreased the dissociation constant of the prothrombin–phospholipid complex 50-fold, from 2.0×10^{-7} M to 4.0×10^{-9} M. For the factor X–phospholipid complex the decrease was 60-fold (1.8×10^{-7} M to 3.0×10^{-9} M) and for factor Xa, 160-fold (1.6×10^{-7} M to 1.0×10^{-9} M). The ratios of moles of protein bound to moles of total added factor Va at saturation of phospholipid–bound factor Va indicate an 1:1 stoichiometric complex of either factor Xa, factor X, or prothrombin and phospholipid-bound factor Va. In the presence of factor Va LC, the dissociation constants of factor Xa– and prothrombin–phospholipid complexes were increased, while the maximal protein-binding capacities of the vesicles were not affected by factor Va LC. The data suggest a competitive interaction between factor Xa and factor Va LC binding as well as between prothrombin and factor Va LC binding at the phospholipid surface. From this, it is concluded that the phospholipid-binding fragment of factor Va alone does not serve as the binding site for interactions of factor Xa and prothrombin with factor Va.

Conversion of prothrombin into thrombin by factor Xa is most efficient in the presence of factor Va and membranes containing acidic phospholipids. Rosing et al. (1980) demonstrated that phospholipids lower the K_m for prothrombin, while factor Va increases the V_{max} of thrombin formation.

Equilibrium binding experiments employing various techniques have indicated that the interaction of prothrombin and factor Xa with acidic phospholipid is mediated by calcium ions (Nelsestuen & Lim, 1977; Mayer et al., 1983). However, factor Va associates with membranes directly by interacting with the polar head groups of the acidic phospholipids (van de Waart et al., 1983).

It is well appreciated that a phospholipid surface is required for a proper assembly of the proteins involved in prothrombin activation. The formation of the catalytic unit, a stoichiometric (1:1) factor Xa–factor Va complex with a dissociation constant of 3×10^{-9} M, is stimulated by acidic phospholipids, resulting in a phospholipid-bound factor Xa–factor Va complex with a dissociation constant of approximately 10^{-10} M (Nesheim et al., 1979; Lindhout et al., 1982).

Localization of the prothrombinase complex on a phospholipid surface per se, as facilitated by factor Va, does not explain the effect of factor Va on the catalytic efficiency of factor Xa. To that end, a role of factor Va in the formation of the enzyme–substrate complex was proposed (Esmon & Jackson, 1974; Rosing et al., 1980). However, quantitative studies of the interaction of prothrombin with factor Va–phospholipid complexes have not been reported.

In a recent report, Tracy & Mann (1983) described experiments indicating that the interaction of factor Va with the platelet surface is mediated primarily through factor Va LC,[1] the 80000-dalton subunit of factor Va. This observation is consistent with our results using artificially prepared phospholipid membranes (van de Waart et al., 1983). Tracy & Mann (1983) suggested that the 80000-dalton subunit of factor Va alone provides the binding site for the interaction of factor Xa with platelet-bound factor Va. If artifically prepared phospholipid membranes are a suitable model for the biological membrane surface (platelets), then these results are not in agreement with our observation that the calcium-mediated interaction between the subunits of factor Va is required for factor Xa binding (Lindhout et al., 1982).

In this paper, we describe experiments indicating that the binding of prothrombin to membranes containing acidic phospholipids is facilitated by factor Va. We further demonstrate that the phospholipid-binding subunit of factor Va

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[1] Abbreviation: LC, light chain.

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