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Further characterization of the *N*-terminal copper(II)- and nickel(II)-binding motif of proteins

Studies of metal binding to chicken serum albumin and the native sequence peptide

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We have investigated the Cu(II)- and Ni(II)-binding properties of chicken serum albumin (CSA) and of the native sequence tripeptide derived from the *N*-terminus of this protein. Spectrophotometric and equilibrium dialysis experiments demonstrate that Cu(II) and Ni(II) bind non-specifically at the *N*-terminus of CSA. Proton displacement studies show that the histidine residue in the fourth position of the protein does not appear to participate in the binding of the two metals. Consistent results were obtained with the native sequence tripeptide L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide. The results presented here demonstrate that neither the glutamic acid residue in the third position nor the histidine in the fourth position participate in the binding of Cu(II) and Ni(II) to CSA. It is known, however, that a number of other albumins with a histidine residue in the third position possess high-affinity Cu(II)- and Ni(II)-binding sites. Our results provide further evidence that the *N*-terminal Cu(II)/Ni(II)-binding motif requires a histidine at the third position in order to bind Cu(II) and Ni(II) specifically.

INTRODUCTION

Albumin is the major transport protein in blood [1]. The transport of metals such as Cu(II) and Ni(II) in the blood is largely accomplished by serum albumin [2–4]. Studies undertaken with bovine, human and rat serum albumins (BSA, HSA and RSA respectively) have shown the presence of a high-affinity binding site for one Cu(II) ion [4]. The elucidation of the *N*-terminal amino acid sequence of BSA by Shearer *et al.* [5] allowed Peters & Blumenstock [6] to propose a model for the metal-binding site. The final characterization of the metal-transport site of HSA was carried out by Laussac & Sarkar [7], who established the participation of α-NH_2, two intervening peptide nitrogen atoms, the imidazole nitrogen atom of the histidine residue in the third position, and the side chain carboxyl group of Asp-1 in a penta-coordinated structure.

A number of studies have suggested that a histidine residue in the third position is essential for specific metal binding. The non-specific binding of Cu(II) and Ni(II) to dog serum albumin (DSA), for instance, was demonstrated by Appleton & Sarkar [8]. Sequence analysis of the *N*-terminal 24-residue peptide fragment of DSA revealed a tyrosine residue in the third position in place of the histidine residue [9]. As a consequence of this mutation the binding site is transformed from a specific to a non-specific one. This demonstration clearly established the inability of tyrosine to act as a specific ligand at this metal-binding site and the relative importance of the histidine residue in the third position for Cu(II) and Ni(II) binding.

The Cu(II)-binding characteristics of the tripeptide glycyl-L-histidyl-L-lysine have been intensively investigated. This tripeptide, which shows a growth-modulating effect in cultured cells, can be co-isolated with copper and with iron from plasma in association with albumin and α-globulin. Studies from this laboratory have shown that, although this tripeptide does bind Cu(II), under physiological conditions it does so in bis complexes and in ternary complexes with albumin [10,11]. The stability of the mono complex is much lower than that of the comparable complex formed by a peptide with histidine in the third position [10].

Chicken serum albumin (CSA) differs from both HSA and DSA in that it has glutamic acid at the third position [12]. This is due to an insertion in the protein sequence when compared with HSA, as opposed to the substitution observed in DSA. The histidine residue present in the third position in HSA has thus been shifted to the fourth position in CSA. CSA therefore provides an opportunity to study a protein in which histidine has been moved to the fourth position and in which the third position is occupied by a negatively charged amino acid, glutamate. The studies presented here reveal that the insertion of glutamate into the third position, with the concomittant shifting of histidine to the fourth position, results in only non-specific binding of Cu(II) and Ni(II) to the *N*-terminal sequence of CSA.

EXPERIMENTAL

Materials

CSA and HSA (Fraction V powder), NaCl, Tris and acrylamide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. NaOH, $CuCl_2$, HCl, 1,2-dimethoxyethane and methanol were obtained from Fisher Scientific Co., Don Mills, Ont., Canada. $NiCl_2$ was obtained from BDH Inc., Toronto, Ont., Canada. ^{63}Ni was obtained from Amersham Canada Ltd., Oakville, Ont., Canada. *N*-Benzoxycarbonyl-L-alanine *N*-hydroxysuccinimide ester and L-glutamic acid α-benzyl ester were obtained from Bachem Sales Inc., Torrance, CA, U.S.A. Anhydrous ether and *N*-ethylmorpholine were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and triethylamine was obtained from Eastman Kodak Co., Rochester, NY, U.S.A.

Purification

Fraction V powder of CSA was subjected to charcoal treatment to remove contaminating fatty acids and other lipids [13]. The

Abbreviations used: HSA, human serum albumin; RSA, rat serum albumin; DSA, dog serum albumin; CSA, chicken serum albumin; PSA, pig serum albumin.

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treated CSA was fractionated twice on a Sephadex G-100 column equilibrated in 50 mM-Tris/HCl, pH 7.4. A non-denaturing 7% polyacrylamide gel demonstrated that the isolated CSA was more than 95% monomer.

Spectrophotometry

The visible absorption spectra of the Cu(II)–CSA and Ni(II)–CSA complexes were determined on a Hitachi U-3210 spectrophotometer at 25 °C. The spectral measurements on the 0.43 mM-CSA solutions in 0.15 M-NaCl were obtained at several pH values over the range 300–800 nm. The difference spectrum for CSA in the presence of 1 equivalent of Cu(II) or Ni(II) was obtained by using as reference a CSA solution of the same concentration and pH, but free of metal.

Proton displacement

Titrations were performed under an argon atmosphere using a Radiometer titrator model TTT60 and a PHM 60 Research pH meter. The CSA solution was prepared at a concentration of 8.78 μM in 0.15 M-NaCl and was adjusted to the required pH. Following the addition of 1 molar equivalent of Cu(II) as $CuCl_2$, the protons released were titrated with 5.48 mM-NaOH. Titrations undertaken after consecutive additions of a total of 4 molar equivalents of Cu(II) at each of four pH values determined the mol of protons displaced by each additional equivalent at each pH.

Equilibrium dialysis

Binding of ^{63}Ni(II) to CSA was determined by equilibrium dialysis. One compartment contained 0.1 mM-CSA or 0.09 mM-HSA and ^{63}Ni(II), with the ^{63}Ni(II)/albumin ratio increasing from 0.2 to 2.2 in 0.1 M-*N*-ethylmorpholine, pH 7.5, in 0.06 M-NaCl. The second compartment contained 0.1 M-*N*-ethylmorpholine, pH 7.5, in 0.06 M-NaCl. The total volume in each compartment was 1.5 ml. Following 6 days of equilibration at 4 °C with shaking, equal aliquots from each pair of compartments were counted for ^{63}Ni(II) radioactivity in a Beckman LS8100 liquid scintillation counter.

Titrations

All titrations were carried out with a Radiometer PHM64 pH meter, TTT60 titrator and ABU80 autoburette, at 25 °C, under an argon atmosphere. A solution of 0.1 M-NaOH, prepared carbonate-free and stored under argon, was standardized by titration against potassium hydrogen phthalate. Analytical-grade $CuCl_2$ and $NiCl_2$ were dissolved in 1 mM-HCl and standardized by complexometric titration against EDTA using murexide as indicator. All solutions were made 0.15 M in NaCl, and the starting pH of the ligand solution was lowered with known amounts of HCl. Solutions were titrated with 0.010 M-NaOH from pH 2.1 to 11.0. Peptide concentrations ranged from 0.40 to 1.6 mM, and metal ion concentrations varied from 0.081 to 0.34 mM. The molar ratio of peptide to metal ranged from 2.39:1 to 10.0:1 in the metal variation titrations, and from 1.93:1 to 7.69:1 in the ligand variation titrations.

Processing of titration data and calculations of the stability constants and species distribution were carried out using a series of computer programs developed in this laboratory [14–16].

Synthesis of the native sequence peptide L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide

In order to mimic the *N*-terminal sequence as part of the polypeptide chain, it was necessary to block the *C*-terminus of the peptide with *N*-methylamide. This procedure significantly complicated the synthesis of this peptide.

To a stirred mixture of *N*-benzoxycarbonyl-L-alanine *N*-hydroxysuccinimide ester (2.97 g) and L-glutamic acid α-benzyl ester (2.2 g) in anhydrous 1,2-dimethoxyethane (70 ml), triethylamine (1.1 ml) was added and the mixture was stirred overnight. After filtration, the solvent was removed *in vacuo*. The residue was diluted with water and the pH adjusted to approx. 4 at 4 °C. The precipitated solid was filtered, washed with water and dried (yield 3.0 g; m.p. 141–143 °C).

Into a solution of *N*-benzoxycarbonyl-L-alanyl-L-glutamic acid α-benzyl ester (2.8 g) in methanol (200 ml) at 4 °C was bubbled methylamine gas for 3 h. I.r. and n.m.r. spectra showed the reaction to be complete, and specific for the terminal carboxyl group. Solvent removal *in vacuo* gave *N*-benzoxycarbonyl-L-alanyl-L-glutamic acid *N*-methylamide as a white solid. This was dissolved in methanol (100 ml). Acetic acid (6 ml) and 10% Pd/C were added and the mixture was hydrogenated for 4 h. The catalyst was filtered and the solvent removed *in vacuo*. Methanol was added to the residue and evaporated. This was repeated three times. The resulting gummy material, upon trituration with ether, solidified to give L-alanyl-L-glutamic acid *N*-methylamide. It was filtered, washed with anhydrous ether and dried (yield 1.2 g).

A mixture of the above dipeptide and *N*-benzoxycarbonyl-β-t-butyl-L-aspartic acid *N*-hydroxysuccinimide ester (0.73 g) in anhydrous 1,2-dimethoxyethane (50 ml) with triethylamine (0.2 ml) was stirred overnight. After filtration, the solvent was removed *in vacuo*. This was repeated three times. The residue was diluted with water (150 ml) and the pH adjusted to $\sim$ 4 at 4 °C. The precipitated white solid was filtered, washed with water and dried (yield 0.7 g).

N-Benzyoxycarbonyl-β-t-butyl-L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide (0.7 g) was dissolved in methanol (100 ml). Acetic acid (2 ml) and 10% Pd/C were added, and the mixture was hydrogenated for 4 h. The crude product obtained after the filtration, methanol extraction and trituration (0.45 g) was cooled in an ice bath and treated with anhydrous HCl in 1,2-dimethoxyethane (6.5 M, 25 ml). After a few minutes, the ice bath was removed and the mixture was stirred at room temperature (20–22 °C) for 2 h. Anhydrous ether (200 ml) was added and the precipitated solid was filtered, washed with anhydrous ether and dried *in vacuo* over KOH (yield 0.4 g). The tripeptide L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide was purified on a silica column in propan-2-ol/water/acetic acid (44:5:1, by vol.). The purity of the tripeptide was established using t.l.c., amino acid analysis and ^{1}H- and ^{13}C-n.m.r.

RESULTS

Spectrophotometry

Fig. 1 shows the spectra of Cu(II)–CSA as a function of pH. As the pH increased, the absorption maximum gradually shifted

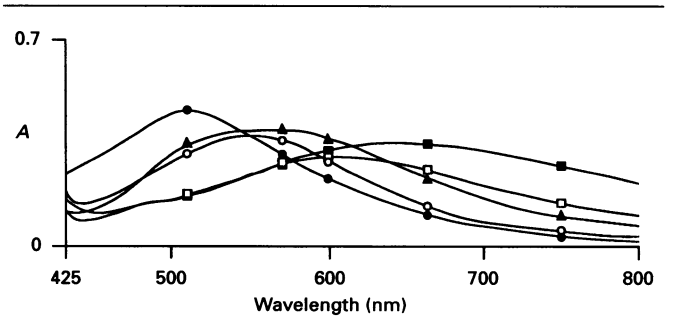

Fig. 1. Visible spectra of CSA in the presence of one equivalent of Cu(II) as a function of pH

■, pH 6.5; □, pH 8.4; ▲, pH 9.3; ○, pH 10.5; ●, pH 11.2.

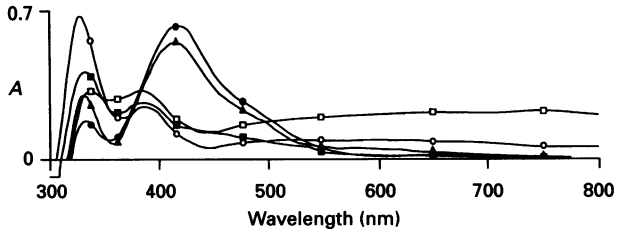

Fig. 2. Visible spectra of CSA in the presence of one equivalent of Ni(II) as a function of pH

□, pH 5.6; ■, pH 7.4; ○, pH 9.3; ▲, pH 10.5; ●, pH 11.2.

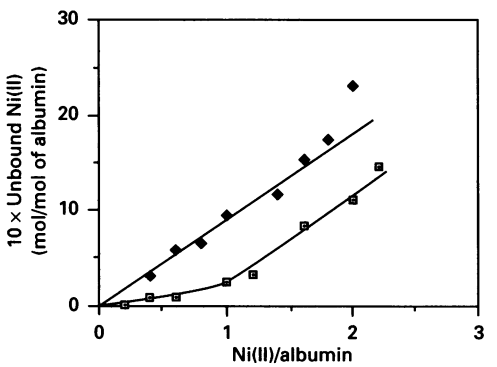

Fig. 3. Equilibrium dialysis of CSA and HSA at pH 7.5 against increasing molar equivalents of Ni(II)

⊡, HSA; ◆, CSA.

from 647 nm at pH 6.5 to 511 nm at pH 11.2. The shift was accelerated above pH 8.4 and was complete at pH 11.2. This gradual shifting of the absorption maximum is typical of non-specific binding of Cu(II) to the albumin [8]. The broadness of the peaks indicates that the sites of binding of Cu(II) to CSA are heterogeneous.

The spectra of Ni(II)–CSA as a function of pH are shown in Fig. 2. At pH 5.6 there were two peaks, one at 340 nm and one at 390 nm. This indicates an octahedral co-ordination for Ni(II) and is consistent with non-specific binding of the metal. As the pH increased to 9.3, the absorbance at 340 nm increased while that at 390 nm decreased slightly, thus indicating that the binding of Ni(II) to CSA is non-specific and heterogeneous. As the pH is increased to 10.5 and 11.2, the absorption maximum shifted to 425 nm, suggesting the presence of a planar co-ordination. The results of Ni(II) binding to CSA are very similar to those found with DSA, in which non-specific binding of Ni(II) to the albumin is also observed [17].

Equilibrium dialysis

CSA and HSA were dialysed against various concentrations of Ni(II) for 6 days at 4 °C. The results are shown in Fig. 3, in which unbound Ni(II) (mol/mol of CSA and HSA) is plotted against the total Ni(II)/albumin ratio. For HSA, the ratio of unbound Ni(II) to albumin increased at a slow rate at Ni(II)/albumin ratios of 0–1. The increase in the slope of this plot at values greater than 1 mol of Ni(II)/mol of albumin indicates that HSA possesses a single specific binding site for 1 mol of Ni(II).

The amount of unbound Ni(II) was much higher for CSA when the Ni(II)/albumin ratio was less than 1. Furthermore, there was no change in the slope of unbound Ni(II) at Ni(II)/albumin ratios greater than 1. This indicates that the first binding site for Ni(II) on CSA is very non-specific compared with the first Ni(II)-binding site on HSA.

Table 1. Proton displacements from HSA and CSA by $CuCl_2$

Values shown are for each equivalent addition at various pH values. HSA data are taken from ref. [8].

Molar addition of $CuCl_2$	Proton displacement			
	pH 5.72	pH 6.53	pH 8.00	pH 9.00
First				
HSA	2.5	2.9	2.6	2.3
CSA	1.8	1.5	1.7	1.6
Second				
HSA	1.8	2.2	1.2	0.8
CSA	1.4	1.5	1.7	1.8
Third				
HSA	1.1	1.4	1.6	1.9
CSA	1.2	1.3	1.6	2.2
Fourth				
HSA	1.0	1.1	1.4	2.2
CSA	0.8	1.2	1.6	2.4

Table 2. Log stability constants (log *βpqr*) of complex species M*p*H*q*A*r*

M = Cu(II) or Ni(II); A = L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide. Measurements were in 0.15 M-NaCl at 25 °C.

			log *βpqr*	
p	*q*	*r*	Cu(II)	Ni(II)
1	1	1	17.34	16.33
1	0	1	13.17	12.04
1	−1	1	7.58	6.62
1	−2	1	1.11	0.17
1	−3	1	−7.03	−8.14
1	2	2	32.70	30.90

Proton displacement

The results of the proton displacement due to the first Cu(II) atom binding to CSA and HSA, measured at pH 5.72, 6.53, 8.0 and 9.0, are shown in Table 1. The results for CSA were different from those of HSA, but were very similar to those obtained for DSA, indicating that the ligands responsible for Cu(II) binding to CSA are similar to those responsible for Cu(II) binding in DSA.

Native sequence peptide

In order to better characterize the binding of Cu(II) and Ni(II) to the *N*-terminal binding site of CSA, the native sequence tripeptide L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide was utilized. Binding of Cu(II) and Ni(II) to the native sequence peptide was characterized by analytical potentiometry and spectroscopy.

The equilibrium which occurs between the anionic peptide (A), metal (M) and protons (H) can be represented by the general equilibrium equation:

$$pM + qH + rA = MpHqAr \quad (1)$$

where *p*, *q* and *r* are the stoichiometric quantities of M, H and A respectively.

The stabilities of the species formed are represented by the stoichiometric equilibrium constant *βpqr*, expressed in terms of

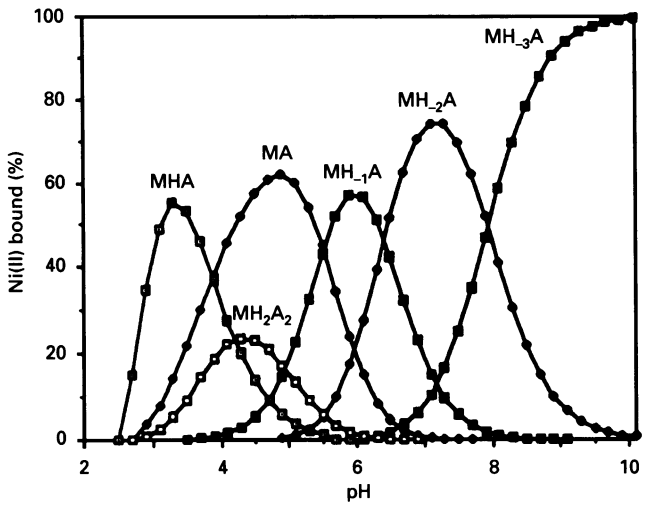

Fig. 4. Species distribution of L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide bound to Ni(II) as a function of pH

⊡, MHA; ◆, MA; ◘, $MH_{-1}A$; ◇, $MH_{-2}A$; ■, $MH_{-3}A$; □, MH_2A_2; where M = metal, A = anionic peptide and H = proton.

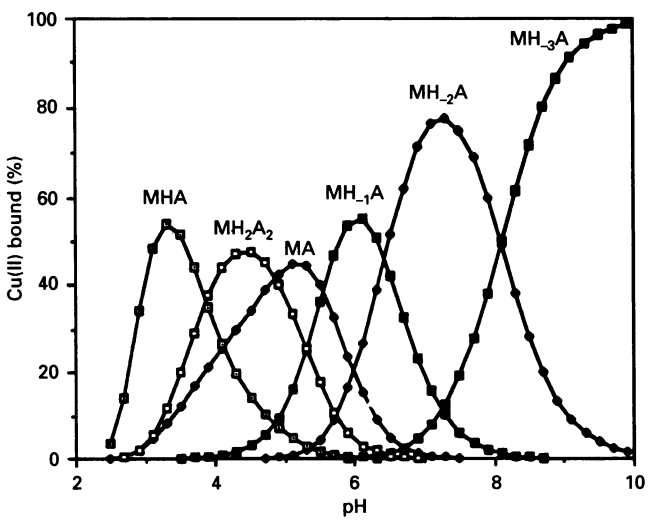

Fig. 5. Species distribution of L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide bound to Cu(II) as a function of pH

⊡, MHA; ◆, MA; ◘, $MH_{-1}A$; ◇, $MH_{-2}A$; ■, $MH_{-3}A$; □, MH_2A_2, where M = metal, A = anionic peptide and H = proton.

concentrations at constant ionic strength, temperature and pressure:

$$\beta pqr = \frac{M_pH_qA_r}{[M]^p[H]^q[A]^r} \quad (2)$$

The functions used to obtain values for the unbound portion of metal ion M and ligand A at any specified pH values from titration data have been described previously [14]. Using these data, the stoichiometric equilibrium constants ($\log \beta pqr$) for the species of Cu(II) and Ni(II) were obtained (Table 2). The distribution of species of the native sequence tripeptide L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide with Cu(II) or Ni(II) with varying pH was determined from titration data by the least-squares method using the best mathematical fit as described earlier [14]. The distribution of species as a function of pH is shown in Figs. 4 and 5. The species distribution with both Cu(II) and Ni(II) appear very similar, with the exception of the bis complex, which is favoured with Ni(II) compared with Cu(II). Binding of metal at low pH probably involves the carboxyl groups of the tripeptide as they are easily deprotonated at lower pH values. As the pH is increased, no single predominant species is observed until high pH (≈ 10). Spectroscopy was utilized to characterize the metal–peptide species present at pH 10. The Ni(II) complex had a $\lambda_{max.}$ of 405 nm and the Cu(II) complex had a $\lambda_{max.}$ of 520 nm (results not shown).

DISCUSSION

Examination of the *N*-terminal sequences of albumins from various species (Fig. 6) indicates that whereas RSA, BSA and HSA have histidine in the third position, DSA and pig serum albumin (PSA) have tyrosine substituted for histidine. It has been demonstrated previously that the *N*-termini of RSA, BSA and HSA are able to bind Cu(II) and Ni(II) with high specificity [4]. However, mutation of histidine to tyrosine, as in DSA, results in a change from a specific metal-binding site to a non-specific one [8,9], due to the inability of tyrosine to participate as a metal-binding ligand [18,19]. In the case of CSA, the sequence is unchanged from that of HSA except for the insertion of a glutamic acid residue at the third position, thus displacing the histidine residue to the fourth position.

The *N*-terminal sequence of CSA thus could be predicted to bind Cu(II) and Ni(II) specifically in two possible manners. The glutamic acid side-chain carboxyl group could provide a ligand to replace the imidazole group at the third position; because of the carboxyl group's negative charge, it may have a high affinity for the bivalent metals. The second possibility is that the imidazole group of the histidine residue could provide a ligand from the fourth position.

The data presented here indicate that, contrary to these predictions, Cu(II) and Ni(II) do not bind specifically at the *N*-terminus of CSA. The equilibrium dialysis of Ni(II) versus CSA indicates that there is no single specific binding site for Ni(II). Thus neither the glutamic acid residue at position three nor the histidine residue at position four are sufficient to create a high-affinity metal-binding site. The titration data of the peptide also support this finding of non-specificity, as a heterogeneous mixture of species was found at physiological pH, similar to what is observed with glycylglycyl-L-tyrosine *N*-methylamide [20].

Several conclusions can be reached from the spectral data regarding the ligands responsible for Cu(II) binding in CSA. Studies of pentapeptide binding to Cu(II) have shown that the relative effectiveness of nitrogen-containing ligands in lowering the $\lambda_{max.}$ is α-amino nitrogen > peptide nitrogen > imidazole nitrogen [21]. For the tripeptide Gly-Gly-His and for HSA binding to Cu(II), the presence of histidine in the third position results in a $\lambda_{max.}$ of 525 nm [8,21]. Owing to the replacement of histidine by tyrosine in the third position of DSA and the resulting absence of the imidazole group in the co-ordination of

RSA	Glu	Ala		His	Lys	Ser	Glu	Ile	Ala	His	Arg	Phe	Lys	Asp	Leu
HSA	Asp	Ala		His	Lys	Ser	Glu	Val	Ala	His	Arg	Phe	Lys	Asp	Leu
BSA	Asp	Thr		His	Lys	Ser	Glu	Ile	Ala	His	Arg	Phe	Lys	Asp	Leu
PSA	Asp	Thr		Tyr	Lys	Ser	Glu	Ile	Ala	His	Arg	Phe	Lys	Asp	Leu
DSA	Glu	Ala		Tyr	Lys	Ser	Glu	Ile	Ala	His	Arg	Tyr	Asn	Asp	Leu
CSA	Asp	Ala	Glu	His	Lys	Ser	Glu	Ile	Ala	His	Arg	Tyr	Asn	Asp	Leu

Fig. 6. *N*-Terminal sequences of RSA, HSA, BSA, PSA, DSA and CSA

The first 14 amino acids of RSA [26], HSA [26], BSA [5], PSA [27] and DSA [9], and the first 15 amino acids of CSA [12], are shown. Spaces have been introduced in the sequences of the first five peptides in order to maximize sequence alignment.

Cu(II), there is a gradual shifting of $\lambda_{max.}$ as a function of pH until it reaches 520 nm at pH 11.0. This shift in $\lambda_{max.}$ reflects the increased relative importance of the α-amino nitrogen and the peptide nitrogens in the co-ordination of Cu(II) in DSA [8]. The data presented in the present paper indicate that the Cu(II)–CSA complex exhibits a similar broad $\lambda_{max.}$ shift at high pH. Therefore the α-amino nitrogen and the peptide nitrogens appear to have importance in the co-ordination of Cu(II) to CSA. These conclusions are supported by the proton displacement data, which show *N*-terminal metal-binding ligands in CSA as being similar to metal-binding ligands in DSA.

Studies of the *N*-methylamide derivative of glycylglycyl-L-tyrosine have shown that co-ordination of Ni(II) involves the α-amino nitrogen and three peptide nitrogens [17]. The species distribution of Cu(II)-L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide as a function of pH suggests successive deprotonation of the peptide backbone, as was observed with glycylglycyl-L-tyrosine *N*-methylamide. The high-pH Ni(II) complex has a $\lambda_{max.}$ of 405 nm, similar to that observed for glycylglycyl-L-tyrosine *N*-methylamide, which forms a planar complex involving the *N*-terminal nitrogen and three peptide nitrogens. The existence of this type of complex is further supported by the spectral features of the Cu(II) complex of L-aspartyl-L-alanyl-L-glutamic acid *N*-methylamide. The $\lambda_{max.}$ of 520 nm at high pH confirms the presence of nitrogen ligands in the complex similar to those in the Cu(II)–DSA complex.

It is clear that the side chains of tyrosine and glutamic acid in position three do not provide a specific square planar co-ordination geometry at the *N*-terminus of a protein. Moreover, the imidazole group from His-4 appears not to be involved in the co-ordination of the metal at the α-*N*-terminus of CSA. The data presented indicate that the insertion of glutamic acid into the third position of CSA and the resulting shift of histidine to the fourth position prevents the imidazole nitrogen from participating in the binding of the metal, rendering the *N*-terminus of CSA a non-specific site for Ni(II) and Cu(II). Additionally, the negative side chain of Asp in the first position of CSA is obviously insufficient by itself to confer specificity of metal binding, despite the fact that the carboxyl side chain of Asp-1 of HSA is involved in specific binding of Ni(II) and Cu(II) [7,22]. Consistent with this result, studies with Cu(II) and human α-fetoprotein have shown that the carboxyl side chain is not necessary for binding specificity. Human α-fetoprotein, with an *N*-terminal sequence of Thr-Leu-His, binds Cu(II) as avidly and specifically as HSA, forming a complex involving four nitrogens in the square plane [23–25]. Thus we can predict that a structure composed of a free *N*-terminal nitrogen, two intervening peptide nitrogens and an imidazole nitrogen of a histidine residue in position three represents a specific *N*-terminal Cu(II)/Ni(II)-binding motif in a protein.

Conclusions

The data presented here demonstrate the lack of a specific binding site for Cu(II) and Ni(II) in both CSA and the native sequence peptide at physiological pH. The metals appear to be complexed in a square planar configuration, with the *N*-terminal nitrogen and three backbone peptide nitrogens as ligands. This differs from the high-affinity metal-binding site of HSA, in which the metals are complexed in a penta-coordinated structure with the *N*-terminal nitrogen, two intervening peptide nitrogens, the imidazole nitrogen and an aspartyl carboxylate group as ligands. The co-ordination of metals in CSA appears to be the same as that in DSA. In DSA the third residue (histidine) has been mutated to a tyrosine, resulting in the absence of the imidazole nitrogen required for specific binding of Cu(II) and Ni(II). Thus there is no imidazole nitrogen available to bind metals. In CSA, although the glutamic acid residue present in the third position may participate in the binding of metals at physiological pH, it is not sufficient to form a specific metal-binding site. The displacement of histidine from the third to the fourth position alters the spatial orientation of the imidazole nitrogen and thus prevents this histidine from participating in the binding of Cu(II) or Ni(II) at the *N*-terminus. This demonstrates that the specific binding of Cu(II) or Ni(II) requires a precise co-ordination geometry, with histidine at the third position.

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