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QUARTERLY

Minireview

The impact of the amino-acid sequence on the specificity of copper(II) interactions with peptides having nonco-ordinating side-chains

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The review presents specific interactions that occur in complexes of Cu(II) ions with peptides composed only of amino acids with nonco-ordinating side chains. Three classes of such peptides are discussed. The first type (NSFRY analogues) is characterised by the presence of a specific combination of bulky and aromatic residues, leading to a formation of multiple weak interactions around Cu(II) that result in an extremely high stability of complexes. The second class is composed of complexes of vasopressins and oxytocins, achieving superstability through a pre-conformation in the peptide molecule. The third group are oligopeptides containing one or two proline residues. These peptides form exotic macrochelate loops with Cu(II) in a result of the break-point effect of Pro residues. Particular emphasis in the review was given to stability constants of complexes, compared to oligoglycine or oligoalanine peptides.

Peptides are very specific and effective ligands for a range of metal ions. They contain a variety of potential donor atoms and the complexes formed can exist in a variety of conformations. Among these metal ions, Cu(II) has been the most widely studied and seems to enter the most diversified range of chemical interactions [1].

Recently, most of the attention of bioinorganic chemists working on metallopeptides has been devoted to systems containing specific co-ordinating side chains, and, in particular, Xaa-Yaa-His sequences modelling the N-terminal binding site of serum albumin [2–5].

Peptides with nonco-ordinating side-chains have only three or four types of donor centers available for a metal ion. They are: amino or amide nitrogen and carbonyl or carboxyl oxygens. In general, such peptides behave like tetraalanine (Fig. 1). A number of variations, however, can occur when particular residues are present in the peptide sequence. For example, when the residue contains an aromatic ring (Phe or Tyr) hydrophobic interactions or ring stacking can influence the stabilities of the complexes formed as well as their structures. The interactions within a peptide molecule may stabilise a particular peptide conformation which in turn may have an essential influence on metal-peptide co-ordination equilibria, both in thermodynamic and structural sense. On the other hand, particular metal-assisted conformations may be of physiological importance, e.g. when a peptide hormone interacts with its receptor.

Abbreviations: ANF, atrial natriuretic factory; AVP, arginine vasopressin; OXT, oxytocin.

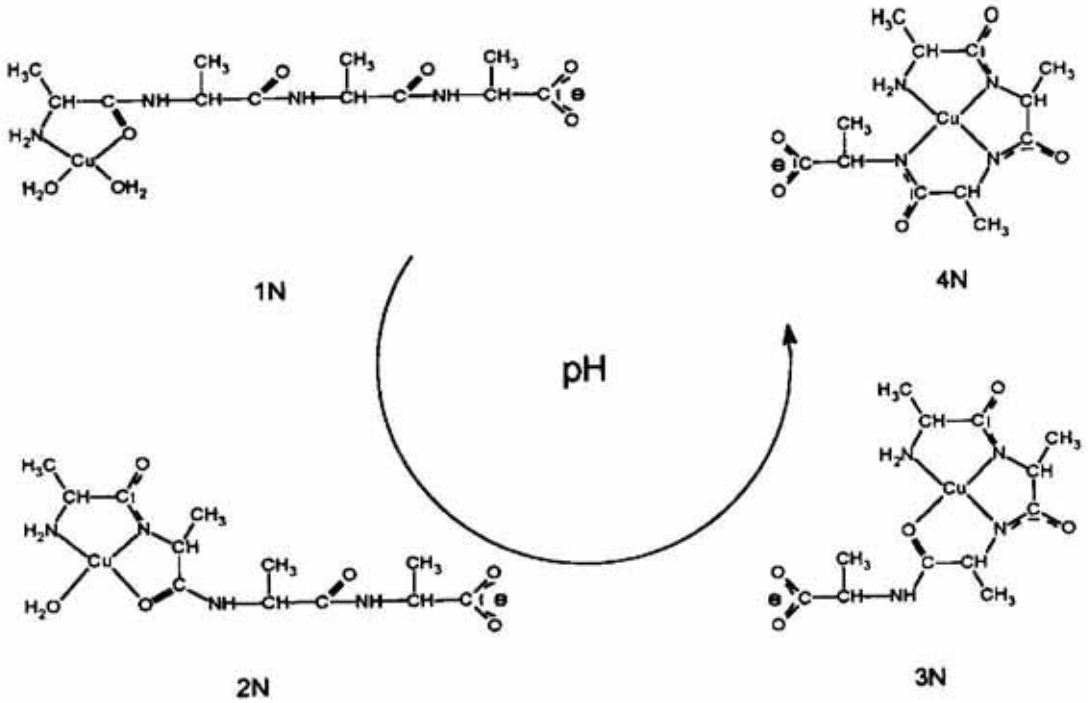

Figure 1. The stepwise complex formation between Cu(II) and tetraalanine.

The aim of this short review is the presentation of specific interactions in copper(II)-peptide systems, like unusual binding modes or very high complex stability that result from interactions other than the direct co-ordination of side-chain donors. In general, peptides with at least four amino-acid residues will be discussed. For a review of co-ordination properties of shorter peptides see [6].

Cu(II)-COMPLEXES WITH PEPTIDES COMPOSED OF GLYCINE AND ALANINE

The most important donor centre in such peptides is the N-terminal nitrogen, which is usually a primary NH_2 group. The N-terminal amino nitrogen acts as an anchoring binding site, preventing metal ion hydrolysis. The adjacent carbonyl oxygen is the second

Table 1. Values of log $^*K^a$ for Cu(II) complexes of simple tetra- and pentapeptides and Met-enkephalin.

Peptide	1N	2N	3N	4N	Ref.
Gly-Gly-Gly-Gly	–2.89	–8.39	–15.28	–24.57	[7]
Ala-Gly-Gly-Gly	–2.89	–8.75	–15.73	–24.99	[9]
Ala-Ala-Ala-Ala	–3.36	–8.58	–16.22	–25.48	[10]
Ala-Ala-Ala-Ala-Ala-NH_2	–3.11	–8.70	–16.44	–24.41	[11]
Gly-Gly-Gly-Gly-Gly	–2.66	–8.76	–15.76	–23.90	[9]
Ala-Gly-Gly-Gly-Gly	–2.58	–8.58	–15.58	–23.79	[9]
Gly-Gly-Gly-Gly-Ala	–2.63	–8.64	–15.69	–23.94	[9]
Tyr-Gly-Gly-Phe-Met	–2.86	–8.01	–15.13	–23.62	[18]

[a]For notation and definitions of constants see Annex.

donor completing the chelate ring [7]. As the pH is raised, the Cu(II) ion is able to deprotonate successive peptide nitrogens, forming Cu-N^- bonds, until eventually a $[CuH_{-3}L]^{2-}$ species (4N complex) is formed at about pH 9. Figure 2a presents a corresponding species

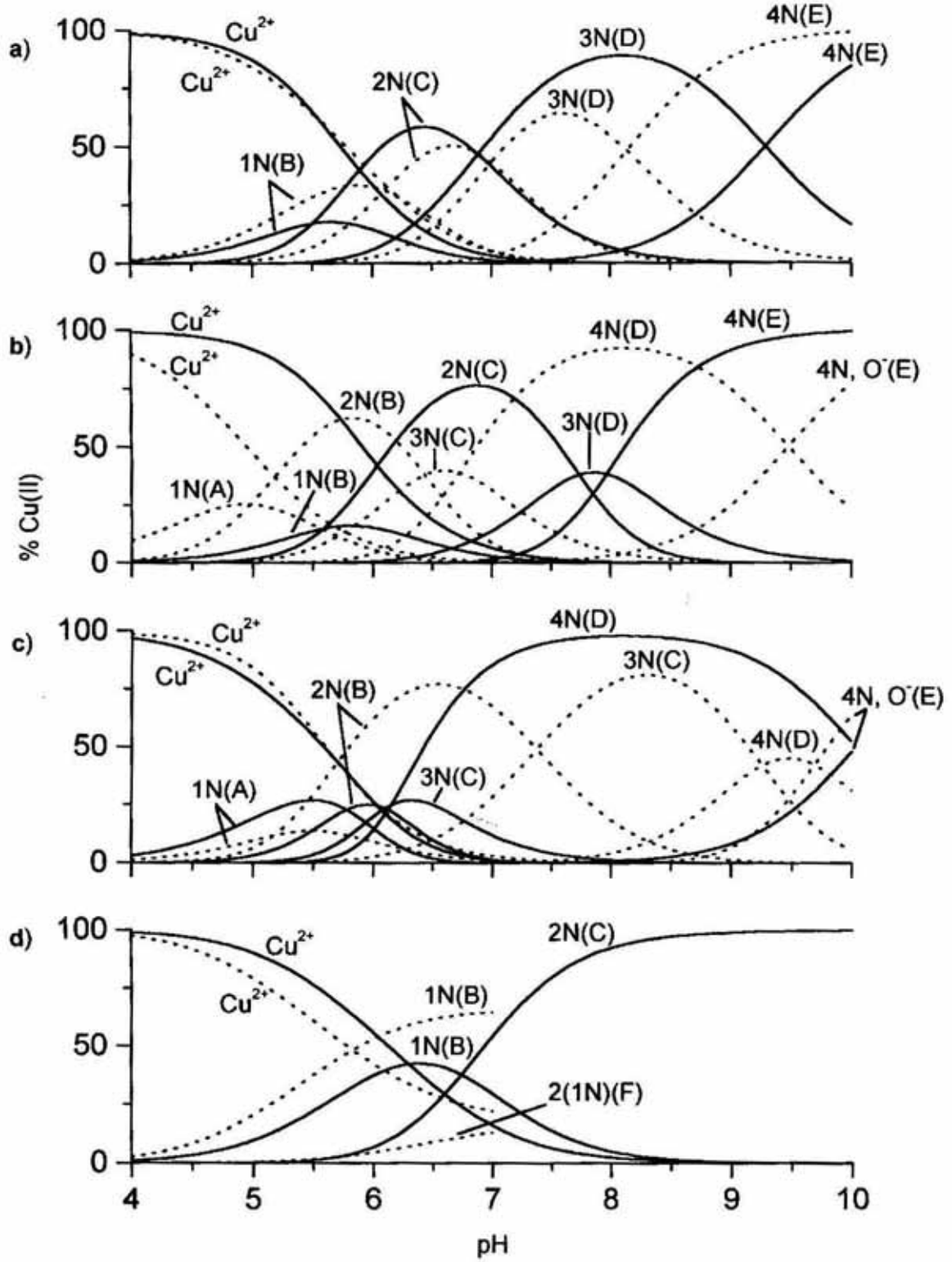

Figure 2. Species distribution curves for Cu(II) complexes with selected peptides (0.001 mol dm^{-3} peptide and Cu(II)); a: (—) GGGG, (- - -) GGGGG; b: (—) AAAAA-NH_2, (- - -) NSFRY-NH_2; c: (—) AVP, (- - -) Ala-AVP; d: (—) GPPGG, (- - -) GPGPG; A = CuHL, B = CuL, C = $CuH_{-1}L$, D = $CuH_{-2}L$, E = $CuH_{-3}L$, F = CuL_2.

Table 2. Values of log *K for Cu(II) complexes of the analogues of the atrial natriuretic factor (ANF) pentapeptide

Peptide	1N	2N	3N	4N	Ref.
NSFRY-NH_2	–1.93	–6.91	–13.35	–20.08	[11]
ASFRY-NH_2	–3.36	–8.26	–15.55	–21.52	[21]
NAFRY-NH_2	–2.23	–7.40	–14.24	–20.59	[21]
NSARY-NH_2	–2.07	–6.97	–13.93	–20.56	[21]
NSFAY-NH_2	–2.33	–7.24	–14.31	–20.88	[21]
NSFRA-NH_2	–2.84	–7.56	–14.65	–21.75	[21]
NSFRY-OH	–1.82	–6.89	–14.06	–20.47	[21]
AAAAA-NH_2	–3.11	–8.70	–16.44	–24.41	[11]

distribution diagram, and Fig. 1 provides the structures of particular complexes. Formulae and constants used throughout the text are explained in the Annex. The formation of stable 5-membered chelate rings by consecutive nitrogens is the driving force of the coordination process, lowering the pK value of the first amide nitrogen by as much as 10 log units [7, 8]. The deprotonations of particular amide nitrogens are well separated from each other in the simple members of this group of peptides, like tetraalanine. This indicates the lack of co-operativity in the binding process.

Table 1 contains protonation-corrected stability constants for Gly- and Ala-containing tetra- and pentapeptides. All-glycine peptides form stronger complexes than their alanine counterparts. Alanine methyl substituent is not bulky enough to directly interfere with the complex formation. Apparently, the flexibility of Gly residues reduces strain in chelate rings and thereby stabilises the complex. The extension of the peptide chain with an additional residue results in an increase of stability of the final 4N complex at the expense of the 3N species [7, 9–11]. This interesting effect has been interpreted as evidence for the presence of a particular conformation of the C-terminal part of the peptide in the 4N complex, stabilised indirectly by Cu(II) [12]. Unfortunately, the data on complexes of still longer Gly or Ala peptides, that might shed more light on such phenomena, are not available in the literature.

EFFECTS OF AROMATIC AND BULKY SUBSTITUENTS

Numerous studies on complexation of Cu(II) ions by aromatic and aliphatic, hydrophobic amino-acids and small peptides containing them indicated an increased complex stability through: (i) direct interaction between the Cu(II) ion and π-electron density of the ring, (ii) aromatic stacking, or (iii) hydrophobic interactions [1, 13–15]. The magnitudes of the effects observed were, however, moderate, seldom exceeding one log

Table 3. Values of log *K for Cu(II) complexes of arginine vasopressin (AVP) and its analogues

Log *K	1N	2N	3N	4N	Ref.
Ala-AVP	–2.87	–8.04	–15.42	–24.68	[23]
OXT	–2.14	–7.94	–14.24	–21.34	[23]
AVP	–2.48	–8.26	–14.36	–20.51	[22]
D-Val^4-AVP	–2.68	–8.06	–13.75	–22.17	[22]

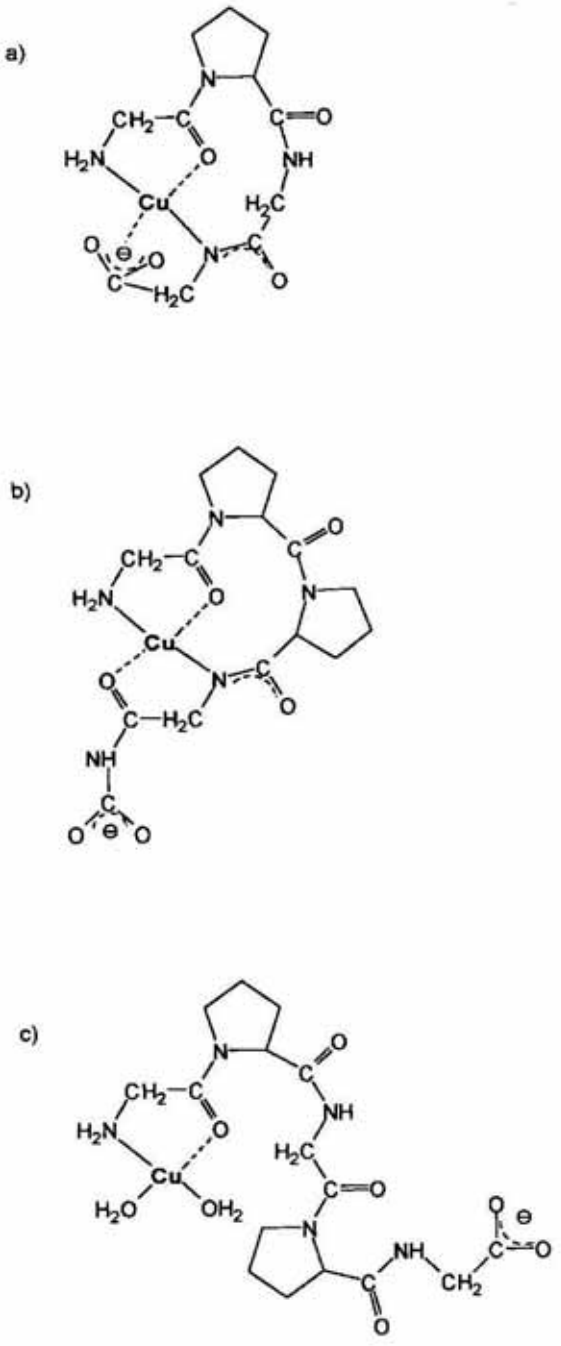

Figure 3. Selected structures of Cu(II) complexes of proline peptides; a: the $CuH_{-1}L$ complex of Gly-Pro-Gly-Gly; b: the $CuH_{-1}L$ complex of Gly-Pro-Pro-Gly-Gly; c: the CuL complex of Gly-Pro-Gly-Pro-Gly.

unit of stability gain. In longer peptides, like Phe-Phe-Phe and Phe-Phe-Ser-Asp-Lys [16], Tyr-Tyr-Tyr [17], or Tyr-Gly-Gly-Phe-Met (Met-enkephalin) [18] no stabilisation by aromatic residues was seen at all. The π interaction, although clearly seen in Cu(II) complexes of oligopeptides containing a single aromatic ring [19], contributes only marginally to their stability [20].

The C-terminal pentapeptide of ANF (atrial natriuretic factor, a blood pressure-regulating peptide), Asn-Ser-Phe-Arg-Tyr-NH_2, binds Cu(II) ions with a very high affinity (see Table 2 for stability data and Fig. 2b for speciation). The stability of the 4N complex of this peptide is only slightly lower from that of the albumin-like peptides, although the donor set is identical to pentaalanine and there is no pre-organisation present in the free peptide [11]. A subsequent study, employing systematic substitutions of individual residues with alanines, indicated the additivity of contributions of all five residues, as well as the N-terminal amide, to the stability gain [21]. The very efficient stabilisation of the 4N complex by the C-terminal residues (Arg and Tyr) is particularly surprising, as these residues do not interact directly with the Cu(II) ion, and no such effect was seen in the apparently similar Met-enkephalin [18] (cf. Tables 1 and 2).

The results of all these studies suggest that the complex-stabilising effects of non-bonding residues are very specific with respect to the sequence and/or neighbourhood of particular bulky residues. A Tyr-5 side-chain may have a strong and additive (so apparently **not** depending on sequence) contribution to stability in the ANF peptide. The same Tyr-5 has no effect at all on the corresponding complex with Met-enkephalin, thus suggesting that, in some more intricate sense, the sequence **was** crucial in the ANF peptide, after all.

PRE-CONFORMATION IN THE PEPTIDE MOLECULE

Arginine vasopressin (AVP) and oxytocin (OXT), important neurohypophyseal hormones, are nonapeptides. Their molecules contain a loop resulting from the disulphide bridge between cysteines in positions 1 and 6. We have found that this loop provides an excellent pre-formed co-ordination site for Cu(II) ions, with the stability increase for the 4N complex of about 4 orders of magnitude

Table 4. Values of log *K for Cu(II) complexes of selected peptides containing one or two proline residues

Log *K	1N	2N	Ref.
FPGG	–2.51	–9.29	[30]
GPGF	–3.18	–10.04	[30]
FGPF	–2.87	–7.50	[31]
FGPY	–2.74	–7.46	[31]
GPPGG	–2.92	–9.66	[33]
EPPGG	–2.79	–9.79	[33]
GPGPE	–1.96		[33]
GPGPQ	–2.56		[33]
GPKPG	–2.91	–9.37	[33]
RPKPQ	–2.82	–9.28	[33]
Substance P	–2.93	–9.55	[33]

over the complexes with oligoalanines having the same (amino + 3 amides) donor set [22, 23] (Table 3). This effect stems from a particular conformation of these hormones, locked into a ring by the disulfide bridge. Positions of the first 4 nitrogen atoms are apparently suited for Cu(II) co-ordination. An extension of the peptide at the N-terminus by a single Ala residue completely removes the stabilisation of all complexes [23] (Fig. 2c), and a substitution of an L-amino acid in position 4 of AVP with a D-residue decreases specifically the stability of the 4N complex [22]. This sensitivity of AVP and oxytocin to substitutions further reveals the subtlety of indirect conformational interactions between Cu(II) and peptides.

PROLINE BREAK-POINT

Proline is the only natural amino acid possessing a secondary amine. Prolyl residue introduced into the peptide chain in position 2, 3 or 4 does not have an amide proton that might be displaced by Cu(II). As a consequence, the stepwise co-ordination of consecutive amide nitrogens is no longer possible. Moreover, the Pro residue increases the propensity of the peptide chain to bend. We have coined a term "break-point" [24] for this phenomenon and investigated numerous examples of exotic co-ordination modes of Cu(II) in proline-containing peptides. The novel and important feature of co-ordination of prolyl peptides is the encouragement of formation of large macrochelate loops, with Cu(II) bound to the amino group and a distant donor. The latter might be either a main-chain amide or a donor group of a normally nonco-ordinating side chain, like the Tyr phenolate, the ε-amine of Lys or even the lateral carboxylate of the C-terminal glutamic acid.

In particular, in Pro-2 tetrapeptides, when Tyr or Lys are absent, the amide nitrogen of the fourth residue co-ordinates to Cu(II). When Tyr or Lys are present in position four, then their side-chains are involved in co-ordination. If, however, the formation of an appropriate macrochelate is impossible for sterical reasons (e.g. Tyr-1 or Tyr-3), then Cu(II) dimers with the (amino + phenolate) binding predominate, utilising a bent peptide molecule as a bridge between cupric ions. Pro-3 tetrapeptides behave similarly. Their complexes are more stable than Pro-2 species because, in their case, the Cu(II) ion is co-ordinated at the N-terminus by the (amino + amide) donor set, with the macrochelate-forming group occupying the third co-ordination site [25–31].

Pro-2-Pro-3 pentapeptides form the most stable macrochelates in their class (Table 4). This is due to a very rigid conformation imposed by these residues on the peptide, increasing the entropic contribution to complex stability [32, 33]. The exotic structure of the Gly-Pro-Pro-Gly-Gly complex of Cu(II) is presented in Fig. 3 and its speciation in Fig. 2d.

The behaviour of the Pro-2-Xaa-3-Pro-4 moiety in pentapeptides is the most unusual of all: in Gly-Pro-Gly-Pro-Gly there is no macrochelate at all. Only weak, amino-bound *mono* and *bis* complexes were found, with no evidence for e.g. C-terminal carboxylate co-ordination (Fig. 2d). However, in Gly-Pro-

Gly-Pro-Glu, the lateral carboxylate binds Cu(II), as evidenced both by spectroscopy and potentiometry (increase of complex stability by 0.6 log units, see Table 4). A Lys residue introduced in position 3 of such a pentapeptide readily co-ordinates, forming yet another macrochelate [33].

The potential importance of these interactions for the biological function of proline-containing neurohormones has been previously postulated [34]. The bent conformations assumed by many proline peptides upon Cu(II) binding through macrochelate formation resemble β-turns, which are believed to be essential for receptor binding by Pro-containing neurohormones and neuromodulators, like casomorphins or Substance P. In this way, Cu(II) co-ordination at the receptor may activate these bioligands.

A note on the mechanism of complex stabilisation

The differences in stabilities of metal complexes of peptides with the identical set of donors reflect the differences of the rate of nucleophilic attack of protons on the metal-bound nitrogens [35]. It is believed that the shielding exerted by bulky amino-acid residues slows down this proton-assisted hydrolysis and thereby stabilises the complex, and this has been indeed shown for the Ni(II) complex of Val-Ile-His-Asn [4]. The examples presented above indicate, however, that the bulkiness of a side-chain is not sufficient to stabilise the Cu(II) binding. Also, direct π interactions between aromatic rings and the metal ion have only limited effects on complex stability in longer peptides (although they may be crucial conformationally). Complexes of Cu(II) with oligopeptides, even in the absence of side-chains capable of direct participation in the binding, appear to be governed by very subtle factors, which are far from being fully explained. The idea emerging e.g. from our studies of the ANF peptides [11, 21] is the concept of "packaging" of particular side chains in specific sets, to provide global shielding to Cu(II), rather than specific effects, that would reproducibly manifest themselves for particular amino acids in particular positions.

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ANNEX

1. Protonation reaction of a ligand (L) below can be quantitatively described by the equilibrium constant, ***K***, as well as by the stability constant, β:

$$L^{2-} + H^+ \xleftrightarrow{K_1} HL^-$$

$$\beta_1 = K_1 = \frac{[HL^-]}{[L^{2-}][H^+]}$$

In a general case of a molecule binding **i** hydrogen ions:

$$\beta_i = \frac{[H_iL]}{[L][H^+]^i}$$

2. Similar constants can be defined for metal ion co-ordination:

$$M^{\alpha+} + H_2L \xleftrightarrow{K_1} MH_2L^{\alpha+}$$

$$\beta_1 = \frac{[MH_2L^{a+}]}{[M^{a+}][H^+]^2[L]}$$

In general, for a complex containing **i** metal ions, **j** hydrogen ions and **k** ligand molecules:

$$\beta_{ijk} = \frac{[M_iH_jL_k]}{[M]^i\,[H^+]^j\,[L]^k}$$

Note that for reactions involving proton displacement from amide groups by a metal ion **j** can assume negative values. This is because amide protons do not dissociate freely, and therefore cannot be introduced into the ligand formula.

Reaction of a metal ion with a ligand can be written as a proton competition reaction:

$$M^{a+} + H_nL \leftrightarrow [MH_jL]^{(a-n+j)^+} + \\ + (n-j)H^+$$

Equilibrium constant for such reactions is denoted $^*K$. Values for $^*K$ constants can be easily derived from stability constants:

$$\log {}^*K = \log(\beta(CuH_jL)) - \log(\beta(H_nL)).$$