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How non-bonding amino acid side-chains may enormously increase the stability of a Cu(II)–peptide complex

Wojciech Bal [a], Marcin Dyba [a], Franciszek Kasprzykowski [b], Henryk Kozłowski [a,*], Rafał Latajka [a], Leszek Łankiewicz [b], Zbigniew Maćkiewicz [b], Leslie D. Pettit [c]

[a] *Faculty of Chemistry, University of Wrocław, F. Joliot-Curie 14, 50383 Wrocław, Poland*
[b] *Faculty of Chemistry, University of Gdańsk, Gdańsk, Poland*
[c] *School of Chemistry, The University, Leeds LS2 9JT, UK*

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Abstract

A combined pH-metric and spectroscopic (UV–Vis, circular dichroism and electron paramagnetic resonance) study of Cu(II) binding to analogues of Asn–Ser–Phe–Arg–Tyr–NH_2 systematically substituted with Ala residues revealed the presence of indirect, additive conformational effects resulting in a very high stability enhancement for 4N complexes. The major contribution to the stability is exerted by non-binding side-chains of 4th and 5th amino acids. This effect is explained on the basis of spectroscopic data by the formation of a secondary fence shielding the Cu(II) binding site from the bulk of the solution. Such a structure, not reported previously, is of possible importance for the understanding of interactions of metal ions with proteins.

Keywords: Copper complexes; Peptide complexes

1. Introduction

Although much attention has been given to folding processes in proteins, and in particular, hydrophobic effects, these are only partially understood [1]. In particular, the existence of relations between the binding of metal ions to proteins and the local hydrophobicity at the binding site has only recently been recognised [2].

Cu(II) and a few other transition metal ions can form stable oligopeptide complexes. The process of coordination involves stepwise binding of deprotonated amide nitrogens. Typically, in the absence of His residues, the metal ion is anchored to the α-amino nitrogen of the N-terminal residue. Then, with increasing pH, subsequent amide nitrogens bind and the peptide molecule wraps around the metal ion (for reviews, see Refs. [3,4]). Such a process yields a range of complex species differing in the number of nitrogen atoms bound to the metal ion. For Cu(II) this number varies from 1 to 4 (in short, 1N to 4N complexes). Stabilities of respective complexes of various peptides are generally similar if further donor groups are absent. As an exception from this rule, arginine vasopressin (AVP) and several of its analogues have been found to form extremely stable 4N complexes with Cu(II) [5,6]. The stability gain, of 3–4 orders of magnitude, was attributed to a particular conformation of the N-terminal part of these peptides, locked into a 'tailored' Cu(II) site by the disulphide bridge between Cys-1 and Cys-6.

In our previous paper we reported the extreme stability enhancement in a Cu(II) complex of Asn–Ser–Phe–Arg–Tyr–NH_2 (NSFRY-NH_2) [7]. Although there is no conformational predisposition for metal binding in this peptide, the 4N complex of this peptide is about 10 times more stable than the 4N complex of AVP. We suggested that this enhancement resulted from a collapse of non-bonding side-chains, triggered by the bending of the peptide chain around the Cu(II) ion in the 4N complex. According to our interpretation, the side-chains shielded the coordination plane from the bulk of solution and thereby reduced accessibility of coordinated amide nitrogens to water molecules. This in turn slowed down the hydrolysis rate of metal–peptide bonds and consequently increased the stability of the complex. Further support for this concept was found in the solution structure of the Ni(II) complex of Val–Ile–His–Asn, where formation of a hydrophobic fence by Val and Ile side-chains was proposed to contribute to an analogous stability-enhancing mechanism [8]. In order to gain a better understanding of the metal ion-

* Corresponding author. Tel.: +48-71-204 251; fax: +48-71-3282 348; e-mail: henrykoz@wchuwr.chem.uni.wroc.pl

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assisted molecular ordering, we undertook the synthesis and coordination studies of a series of systematically substituted NSFRY analogues. The results of our investigations are the subject of this paper.

2. Experimental

2.1. Peptide synthesis

All peptides were synthesised by standard solid phase methods, as described previously [7].

2.2. Potentiometry

The protonation and stability constants of H^+ and Cu(II) complexes were determined at $I=0.1$ mol dm^{-3} (KNO_3) and 25°C using pH-metric titrations over the pH range 3–11.5 (Molspin automatic titrator, Molspin Ltd., Newcastle-upon-Tyne, UK) with NaOH as titrant. Changes in pH were monitored with a combined glass–calomel electrode calibrated daily in hydrogen concentrations by HNO_3 titrations [9]. Sample volumes were 1.5–2.0 ml. Ligands concentrations of 10^{-3} mol dm^{-3} and ligand:Cu(II) molar ratios of 1.05:1 were used. The data (3–5 titration curves per system, 100 experimental points each) were analysed using the Superquad program [10]. Standard deviations computed by Superquad refer to random errors only. They give, however, a good measure of the importance of a given species in solution.

2.3. Circular dichroism (CD)

CD spectra were recorded at 25°C on a Jasco J-600 spectropolarimeter. All spectra were recorded over the range of 200–750 nm, using 1 and 0.1 cm cuvettes. Peptide concentrations were 10^{-4} mol dm^{-3}. For complexation experiments, samples with 1.05:1 ligand-to-metal ratios were used. The concentration of Cu(II) was 10^{-3} or 10^{-4} mol dm^{-3}. Spectra are expressed in terms of $\Delta\varepsilon=\varepsilon_l-\varepsilon_r$, where ε_l and ε_r are molar absorption coefficients for left and right circularly polarised light, respectively.

2.4. Electronic absorption (UV–Vis)

Spectra were recorded on a Beckman DU-650 spectrophotometer over the spectral range of 200–900 nm in 1 cm cuvettes, using the same samples as in CD measurements.

2.5. Electron paramagnetic resonance (EPR)

EPR spectra were recorded at 120 K on a Bruker ESP 300E spectrometer at the X-band frequency (9.3 GHz). 1:2 ethanediol–water was used as a solvent in order to obtain homogeneity of frozen samples. Cu(II) concentrations of 3×10^{-3} mol dm^{-3} and 1.05:1 peptide:Cu(II) ratios were used.

3. Results and discussion

For clarity of discussion, the data for all relevant peptide analogues are presented and discussed, including the results presented in our previous account [7].

Table 1
Protonation of NSFRY analogues and stability constants of their cupric complexes

A Log	HL	H_2L	CuHL	CuL	$CuH_{-1}L$	$CuH_{-2}L$	$CuH_{-3}L$
NSFRY-NH_2 [a]	9.62	16.06	14.13	9.15	2.71	−4.02	−13.50
ASFRY-NH_2	9.73(1)	17.41(1)	14.05(5)	9.15(1)	1.86(1)	−4.11(1)	−14.60(1)
NAFRY-NH_2	9.55(1)	16.03(1)	13.80(2)	8.63(1)	1.79(2)	−4.56(1)	−14.82(2)
NSARY-NH_2	9.60(1)	16.05(1)	13.98(2)	9.08(1)	2.12(1)	−4.51(1)	−15.05(2)
NSFAY-NH_2	9.87(1)	16.55(1)	14.22(1)	9.31(1)	2.24(1)	−4.33(1)	−15.02(1)
NSFRA-NH_2	6.58(1)			3.74(5)	−0.98(7)	−8.06(1)	−15.20(1)
NSFRY-OH [b]	9.23(2)	15.87(1)	14.05(3)	8.98(1)	1.81(1)	−4.60(2)	−13.75(1)
AAAAA-NH_2 [a]	8.04			4.93	−0.66	−8.40	−16.37
B Peptide	$pK(NH_3)^+$	log $*K$(1N)	pK(2N)	pK(3N)	pK(4N)	pK(Tyr-OH) [c]	ΔpK(Tyr) [d]
NSFRY-NH_2	6.44	−1.93	5.02	6.44	6.73	9.48	−0.14
ASFRY-NH_2	7.68	−3.36	4.90	7.29	5.97	10.49	+0.76
NAFRY-NH_2	6.48	−2.23	5.17	6.84	6.35	10.26	+0.71
NSARY-NH_2	6.45	−2.07	4.90	6.96	6.63	10.54	+0.94
NSFAY-NH_2	6.68	−2.33	4.91	7.07	6.57	10.69	+0.82
NSFRA-NH_2	6.58	−2.84	4.72	7.08	7.14		
NSFRY-OH	6.64	−1.82	5.07	7.11	6.41	9.15	−0.08
AAAAA-NH_2	8.4	−3.11	−8.70	7.74	7.97		

[a] From Ref. [7].
[b] log β H_3L = 19.30(3).
[c] In the complex.
[d] pK_{Tyr} in the complex − pK_{Tyr} in the ligand.

3.1. Protonation of free peptides

All the peptides studied in this work (except for NSFRA-NH_2, devoid of a tyrosine residue) have two ionisable protons: one on the terminal amino group and another on the phenolic oxygen of Tyr side-chain. Macroscopic protonation constants obtained from potentiometry are separated by more than two log units and it can thus be safely assumed that they correspond to respective microconstants (see Table 1).

CD spectra of all the peptides studied exhibited similar patterns, characterised by a negative band below 200 nm ($\Delta\varepsilon$ from -10 to -20) and a positive band or shoulder around 220 nm ($\Delta\varepsilon$ from $+2$ to $+4$). Both bands originate from the amide chromophores. The observed pattern is believed to be a superposition of irregular and left-handed extended helix components [11]. The peptides thus assume a range of rapidly interconverting conformations and no defined structures are formed. The presence of aromatic chromophores only weakly perturbed this pattern by distorting shapes of bands related to peptidic chromophores rather than producing additional bands, with the exception of the L_a band of the deprotonated tyrosine ring, seen as a positive feature near 245 nm [12].

3.2. Spectroscopic characterisation of complexes—binding modes

The existence of complexes involving a single deprotonation of the ligand molecule was indicated by potentiometry for all the analogues. In some cases concentration of this species can be as high as 25% of total copper (Fig. 1). These complexes produced, however, only tentative shoulders at

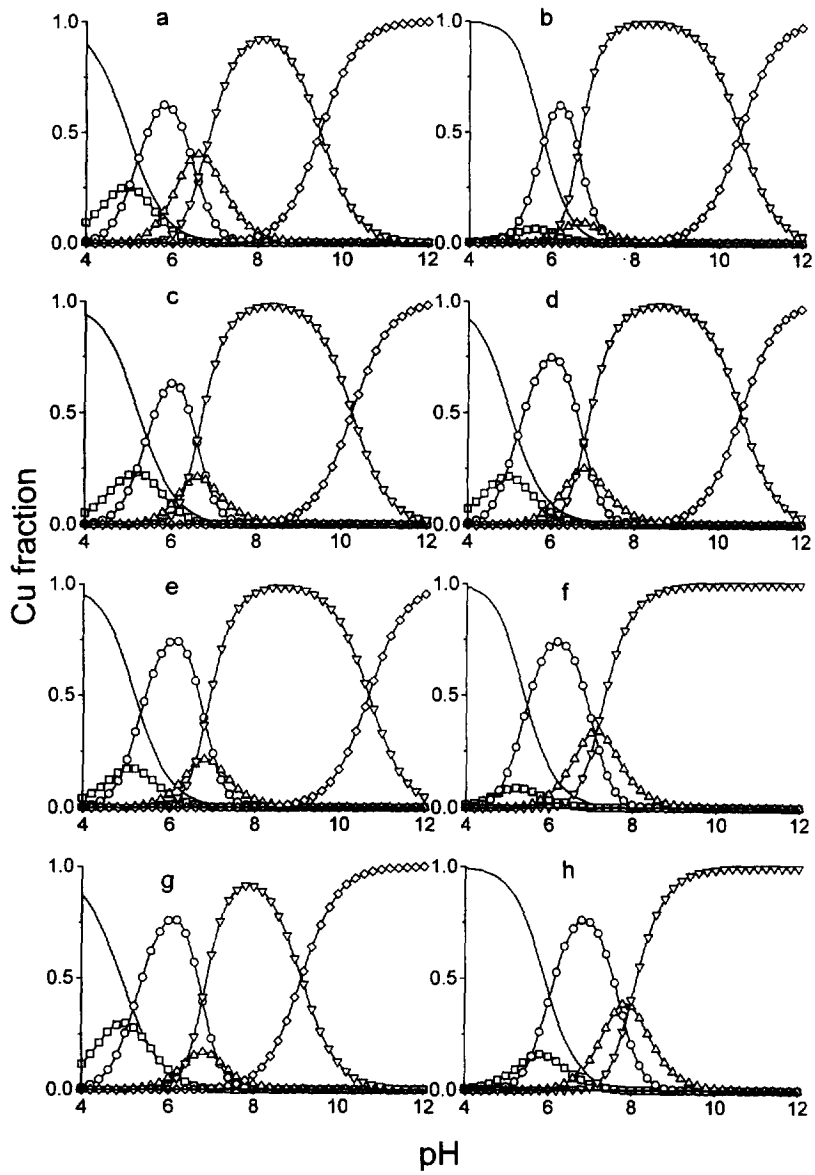

Fig. 1. Species distribution diagrams for Cu(II) complexes of all peptides discussed, $c_{Cu(II)} = c_L = 10^{-3}$ M: ———, Cu^{2+}; – □ –, 1N; – ○ –, 2N; – △ –, 3N; – ▽ –, 4N; – ◇ –, 4N, Tyr-O^-; (a) NSFRY-NH_2; (b) ASFRY-NH_2; (c) NAFRY-NH_2; (d) NSARY-NH_2; (e) NSFAY-NH_2; (f) NSFRA-NH_2; (g) NSFRY-OH; (h) AAAAA-NH_2.

Table 2
Spectroscopic data for 1N complexes

	EPR		Vis		pH
	g	A ($\times 10^4$ cm^{-1})	λ (nm)	ε (dm^3 mol^{-1} cm^{-1})	
NSFRY-OH	not detected		750sh	30	4.4
NSFRY-NH_2	not detected				
ASFRY-NH_2	not detected				
NAFRY-NH_2	2.33	156	746sh	16	4.5
NSARY-NH_2	2.34	164	790	17	4.1
NSFAY-NH_2	2.33	155	720sh	18	4.3
NSFRA-NH_2	2.33	163	750sh	30	4.4
AAAAA-NH_2	2.34	167	745sh	30	5.1

sh denotes a shoulder on the spectrum.

about 750 nm in absorption spectra (Table 2), and were not seen in CD spectra. This is typical for Cu(II)–peptide interactions [13]. However, good separation of resonance lines in EPR spectra often makes detection of 1N complexes possible (see Table 2). Comparison of EPR and d–d band parameters did not reveal any differences between Asn-1 and Ala-1 complexes and therefore excluded direct involvement of the lateral amide of Asn in coordination. We can therefore state that Cu(II) binding in the lowest pH complex in all systems discussed is of 1N type, similar to simple peptides [3,4].

Formation of the next species also involved a single deprotonation. Spectroscopic data collected in Table 3 are consistent with typical 2N (terminal amine + neighbouring amide) coordination [3,4]. These 2N complexes released two protons between pH 6 and 8 and transformed into 4N complexes, as evidenced by spectral parameters (Table 4). The intermediate 3N complexes, also present in the equilibrium, were not characterised spectroscopically owing to their relatively low abundance. An additional deprotonation, seen in Tyr-containing analogues occurred at the phenolic oxygen. This event introduced some changes into the spectra, but did not affect the overall coordination mode (Table 5).

3.3. Effects of amino acid substitutions on the stability gain in NSFRY-NH_2 analogues

The pK value of formation is a good measure of the relative stability of a particular complex species when coordination occurs via deprotonation. In the case of NSFRY-NH_2 analogues it properly describes the formation of 2N complexes. For 1N complexes pK cannot be defined, and the log $^*K$ value has to be used instead. Differences of these values between NSFRY-NH_2 and other analogues are collected in Table 6 to present the effect of amino acid substitutions on the formation of particular complexes. Calculation of distribution of Cu(II) between NSFRY-NH_2 and other analogues is an alternative method of presentation of stability differences, emphasising the overall binding capabilities of a given peptide, rather than comparing particular complexes. These pairwise distributions, presented as ratios, are collected in Fig. 2.

3.3.1. 1N complexes

The values in Table 6 indicate that the substitution of Asn-1 with Ala has the greatest effect on the stability of 1N species. A sizeable effect from Tyr-5 is also seen. Substitutions of other residues with alanines have smaller, but still uniformly diminishing impact on the stability of these complexes. Only the presence of the carboxylate rather than the amide at the C-end of the peptide molecule marginally enhances the complex stability.

3.3.2. 2N complexes

Amino acid substitutions have much smaller effect on the pK of formation of the 2N species. Comparison with AAAAA-NH_2 and other simple peptides indicates that the 1N → 2N transition in NSFRY-NH_2 analogues is less enhanced than the 1N or 4N formation, but still the 2N species form at a pH approximately 0.5 log units lower than with typical oligopeptides.

3.3.3. 3N complexes

The pK values of formation of 3N complexes presented in Table 1B are usually higher than the respective values of 4N complexes. This phenomenon is well known from complexes of His-containing peptides, and reflects cooperativity of binding of the 3rd and 4th nitrogens to Cu(II) [4].

3.3.4. 4N complexes

The cooperative transition from 2N to 4N complexes is the prominent feature of NSFRY-NH_2 and its analogues. The values collected in Table 6 clearly indicate that Asn-1 and Ser-2 do not significantly contribute to this effect. Contributions of Phe-3, Arg-4, and C-terminal amide are approximately equal, and the largest effect is conveyed by the Tyr-5 residue. Compared with AAAAA-NH_2, contributions of all amino acid residues in NSFRY-NH_2 to the lowering of the pK of this reaction are additive to within experimental error of stability constant determinations (-2.54 versus -2.46). Competition plots (Fig. 2) reveal another specific effect in the Tyr residue, seen only in NSFRY-NH_2 and NSFRY-OH. In other analogues complexation results in the elevation of pK_{Tyr} by 0.7–0.8 log units,

Table 3
Spectroscopic data for 2N complexes

	EPR		Vis		CD	
	g	A ($\times 10^4$ cm^{-1})	λ (nm)	ε (dm^3 mol^{-1} cm^{-1})	λ (nm)	$\Delta\varepsilon$ (dm^3 mol^{-1} cm^{-1})
NSFRY-NH_2	2.26	167	655	(65)	710	(−0.06) [a]
					537	(−0.05) [a]
					313	(+0.28) [b]
					268	(−0.57) [c]
					245	(+1.5) [d]
					217	(+4.3) [d]
					<190	(< −12) [d]
ASFRY-NH_2	2.25	179	644	(64)	710	(−0.26) [a]
					588sh	(−0.19) [a]
					312	(+0.82) [b]
					268sh	(−0.73) [c]
					235	(−3.5) [d]
					217	(+4.5) [d]
					190	(−11) [d]
NAFRY-NH_2	2.26	175	660	(42)	670	(+0.06) [a]
					539	(−0.08) [a]
					314	(+0.14) [b]
					275sh	(−0.7) [c]
					237	(−2.6) [d]
					217	(+3.8) [d]
					192	(−14) [d]
NSARY-NH_2	2.26	177	648	(66)	700	(−0.39) [a]
					560sh	(−0.15) [a]
					313	(+0.38) [b]
					269	(−0.54) [c]
					235	(−1.6) [d]
					220	(−2.0) [d]
					203	(+3.5) [d]
					192	(−13) [d]
NSFAY-NH_2	2.26	174	650	(91)	726	(−0.15) [a]
					624	(+0.03) [a]
					541	(−0.10) [a]
					315	(+0.38) [b]
					266sh	(−1.3) [c]
					236	(−4.3) [d]
					219	(+2.1) [d]
					191	(−10) [d]
NSFRA-NH_2	2.26	177	648	(80)	710	(−0.18) [a]
					560	(−0.23) [a]
					322	(+0.16) [b]
					271	(−1.2) [c]
					232	(−4.5) [d]
					215	(+3.0) [d]
					193	(−15) [d]
NSFRY-OH	2.25	172	660	(68)	730	(−0.22) [a]
					535	(−0.12) [a]
					310	(+0.37) [b]
					268sh	(−0.76) [c]
					234	(−3.0) [d]
					220	(+5.3) [d]
					200	(+11.5) [d]
					<190	(< −20) [d]
AAAAA-NH_2	2.25	179	652	(96)	685	(−0.32) [a]
					580sh	(−0.22) [a]
					303	(+0.64) [b]
					270	(−0.5) [c]
					244	(+1.4) [d]
					225	(−2.2) [d]
					191	(−11) [d]

sh denotes a shoulder on the spectrum
[a] d–d band.
[b] $N^- \rightarrow Cu(II)$ CT band.
[c] $NH_2 \rightarrow Cu(II)$ CT band.
[d] Intraligand band.

Table 4
Spectroscopy data for 4N complexes. Tyr side chain protonated or absent

	EPR		Vis		CD	
	g	A ($cm^{-1} \times 10^4$)	λ (nm)	ε ($dm^3\ mol^{-1}\ cm^{-1}$)	λ (nm)	$\Delta\varepsilon$ ($dm^3\ mol^{-1}\ cm^{-1}$)
NSFRY-NH_2	2.18	204	501	(131)	501	(−0.65) [a]
					307	(+0.17) [b]
					280	(−1.90) [c]
					252	(+3.8) [d]
					234	(−16.0) [d]
					212	(+46.0) [d]
					202	(+48.0) [d]
					<190	(< −50) [d]
ASFRY-NH_2	2.16	216	506	(147)	508	(−2.02) [a]
					309	(+1.05) [b]
					275	(−2.21) [c]
					251	(+3.15) [d]
					236	(−18.2) [d]
					213	(+40.0) [d]
					200	(+45.0) [d]
					<190	(< −58) [d]
NAFRY-NH_2	2.16	218	504	(132)	503	(−1.61) [a]
					309	(+0.68) [b]
					275	(−2.1) [c]
					250	(+2.4) [d]
					233	(−13.0) [d]
					213	(+24.0) [d]
					199	(+28.0) [d]
					<190	(< −33) [d]
NSARY-NH_2	2.17	216	504	(125)	507	(−1.14) [a]
					306	(+1.00) [b]
					273	(−2.8) [c]
					249	(+1.35) [d]
					231	(−19.0) [d]
					219sh	(−16) [d]
					199	(+6.0) [d]
					<190	(< −8) [d]
NSFAY-NH_2	2.16	212	508	(170)	510	(−1.81) [a]
					310	(+1.00) [b]
					276	(−3.00) [c]
					249	(+4.17) [d]
					232	(−19.0) [d]
					214	(+51.8) [d]
					197	(+87.5) [d]
					<190	(< −35) [d]
NSFRA-NH_2	2.17	215	514	(162)	530	(−1.80) [a]
					310	(+0.66) [b]
					277	(−3.0) [c]
					248	(+3.4) [d]
					235	(−3.64) [d]
					212	(+56.0) [d]
					<190	(< −70) [d]
NSFRY-OH	2.17	208	510	(120)	502	(−1.67) [a]
					307	(+0.82) [b]
					273	(−1.91) [c]
					248	(+1.45) [d]
					233	(−11.1) [d]
					214	(+21.3) [d]
					201	(+31.0) [d]
					<190	(< −30) [d]
AAAAA-NH_2	2.18	219	517	(194)	528	(−1.18) [a]
					304	(+0.84) [b]
					270	(−2.60) [c]
					242	(+3.5) [d]
					214	(+7.3) [d]
					<190	(< −35) [d]

[a] d–d band.
[b] $N^- \rightarrow Cu(II)$ CT band.
[c] $NH_2 \rightarrow Cu(II)$ CT band.
[d] Intraligand band.

Table 5
Spectroscopy data for 4N complexes. Tyr side chain deprotonated

	EPR		Vis		CD	
	g	A ($cm^{-1} \times 10^4$)	λ (nm)	ε ($dm^3\ mol^{-1}\ cm^{-1}$)	λ (nm)	$\Delta\varepsilon$ ($dm^3\ mol^{-1}\ cm^{-1}$)
$NSFRY\text{-}NH_2$	2.17	214	505	(138)	501	(−1.09) [a]
					305	(+0.09) [b]
					275	(−3.02) [c]
					252	(+4.4) [d]
					234	(−14.0) [d]
					213	(+51.0) [d]
					203	(+49.0) [d]
					<190	(<−50) [d]
$ASFRY\text{-}NH_2$	2.17	213	509	(156)	512	(−1.94) [a]
					306	(+0.88) [b]
					278	(−3.2) [c]
					249	(+3.4) [d]
					236	(−7.4) [d]
					213	(+48.0) [d]
					203sh	(+39.0) [d]
					<190	(<−60) [d]
$NAFRY\text{-}NH_2$	2.16	214	510	(134)	508	(−1.61) [a]
					304	(+0.97) [b]
					277	(−2.0) [c]
					250	(+3.05) [d]
					234	(−6.9) [d]
					213	(+30.0) [d]
					203sh	(+27.0) [d]
					<190	(<−50) [d]
$NSARY\text{-}NH_2$	2.17	212	510	(130)	514	(−1.16) [a]
					303	(+1.18) [b]
					273	(−2.5) [c]
					247	(+1.7) [d]
					230	(−14.9) [d]
					205	(+9.5) [d]
					192	(−25) [d]
$NSFAY\text{-}NH_2$	2.16	216	505	(176)	519	(−1.71) [a]
					306	(+1.18) [b]
					276	(−3.50) [c]
					247	(+4.8) [d]
					232	(−10.0) [d]
					214sh	(+49) [d]
					208sh	(+55.5) [d]
					202	(+60.0) [d]
					<190	(<−42) [d]
NSFRY-OH	2.16	215	510	(123)	502	(−1.82) [a]
					305	(+0.91) [b]
					272	(−2.48) [c]
					248	(+2.82) [d]
					233	(−8.7) [d]
					213	(+24.5) [d]
					206	(+26.5) [d]
					<190	(<−30) [d]

[a] d–d band.
[b] $N^- \rightarrow Cu(II)$ CT band.
[c] $NH_2 \rightarrow Cu(II)$ CT band.
[d] Intraligand band.

but in these two, this value is even slightly decreased, and deprotonation of the Tyr ring results in the increase by several times in the affinity to Cu(II).

The ultimate goal of this study was to explain the superstability of the 4N complex of $NSFRY\text{-}NH_2$ in structural terms and possibly to draw conclusions for the rational design of

Table 6
Differences in complex species stabilisation (log values) between NSFRY-NH_2 and analogues

Deviations from NSFRY-NH_2 values (in parentheses)	log *K 1N (−1.93)	pK 2N (5.02)	pK 3N+4N (13.17)	
ASFRY-NH_2	−1.43	−0.08	−0.09	Σ = −2.46
NAFRY-NH_2	−0.30	−0.15	−0.02	
NSARY-NH_2	−0.14	+0.12	−0.42	
NSFAY-NH_2	−0.40	+0.11	−0.47	
NSFRA-NH_2	−0.91	+0.30	−1.05	
NSFRY-OH	+0.11	−0.05	−0.41	
AAAAA-NH_2	−1.18	−0.57	−2.54	
AAAA-OH [a]	−1.43	−0.20	−3.71	

[a] From Ref. [19].

strong peptidic metal ion chelators. Early studies by Martin and coworkers [14] revealed that the Cu(II) complexes of di- and tripeptides, with three or four planar coordination sites of the Cu(II) ion occupied, have a single, negative d–d band in CD spectra. The $\Delta\varepsilon$ value for this band was found to be the additive function of particular amino acid residues in specific positions, e.g. $\Delta\varepsilon(AAA) = \Delta\varepsilon(AGG) + \Delta\varepsilon(GAG) + \Delta\varepsilon(GGA)$. Deviations from additivity in complexes of dipeptides and bis-complexes of amino acids have been successfully used by Yamauchi et al. as evidence for electrostatic and hydrophobic interactions between non-bonding side-chains [15].

Czarnecki and Margerum [16] reported that the introduction of a single chiral (Ala) residue into the terminal position of Cu(II)-complexed pentapeptide (GGGGA) produces a sizeable Cotton effect in the d–d band, despite the fact that the only chirality centre in the complex molecule is distant from the metal ion. This effect was explained by proposing the existence of hydrogen bonds between the peptide group of the fifth residue and the atoms of Cu(II)-bound residues. Their formation is possible on the basis of the known crystal structure of the Cu(II)–pentaglycine complex [17].

We verified the additivity principle for all five residues in NSFRY analogues (Table 7). The NSFRY-NH_2 deviates from additivity, but NSFRY-OH fulfils it when corrected for the C-terminal amidation, as do NSFAY-NH_2 and NSFRA-NH_2, where substitutions of one chiral residue with another in positions 4 and 5 do not affect the magnitude of the Cotton effect of the d–d band. This effect also agrees with the model of chirality transfer from these residues [16]. The hydrogen bonding scheme involves peptide chain atoms only, and is therefore sensitive primarily to the location of the α carbon, and not to the further atoms of the side-chain. The assumption of additivity stipulates the lack of interaction among side-chains of residues 1–3. This is in agreement with the stability results indicating that the side-chains of residues 1 and 2 do not contribute to the enhancement of the formation of 4N species, and thus do not interact with residues 3–5.

A large deviation from the additivity in magnitude of the d–d band occurs in NSFRY-NH_2, when all side-chains are present and the terminal carboxylate is substituted by the amide. Its probable direct cause is the notorious sensitivity of the Cotton effect to the conformation of the N-terminal residue [18]. The value of A_{II} for this complex is lower than for all Ala-substituted analogues (value for NSFRY-OH being second lowest), signifying slight distortion of the coor-

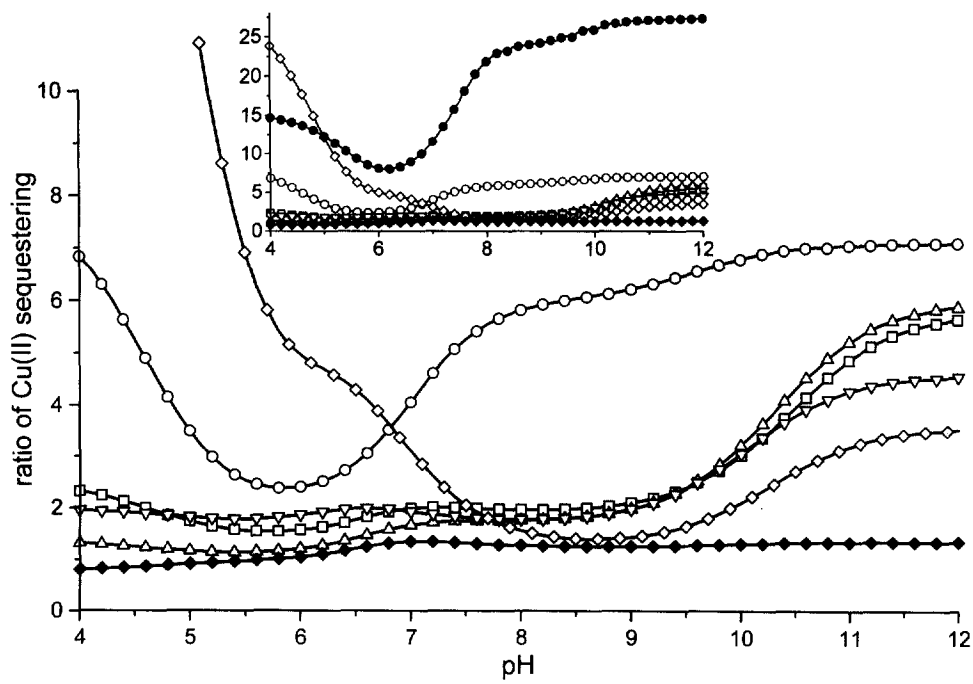

Fig. 2. Plot of pairwise ratios of Cu(II) sequestering, obtained by dividing total concentrations of Cu(II) bound to NSFRY-NH_2 by total concentrations of Cu(II) bound to a competing peptide. Calculations were done for 10^{-3} M Cu(II), NSFRY-NH_2 and a competing ligand: – ◇ –, ASFRY-NH_2; – ▽ –, NAFRY-NH_2; – △ –, NSARY-NH_2; – □ –, NSFAY-NH_2; – ○ –, NSFRA-NH_2; – ♦ –, NSFRY-OH; – ● –, AAAAA-NH_2.

Table 7
Effects of amino acid substitutions on the magnitude of the Cotton effect of d–d band of 4 N complexes with protonated Tyr residue

Substitution	Additive contribution [a]
A → N-1	+0.2
A → S-2	−0.2
A → F-3	−0.7
A → R-4	0
A → Y-5	0
–OH → –NH_2	−0.17 [b]

[a] $\Delta\varepsilon$ of the a-substituted peptide − $\Delta\varepsilon$ (NSFRY-NH_2)^{theor}; the latter calculated from the additivity principle: ASFRY-NH_2 + NAFRY-NH_2 + NSARY-NH_2 + NSFAY-NH_2 + NSFRA-NH_2 = 4 × (NSFRY-NH_2)^{theor} + AAAAA-NH_2.
[b] $\Delta\varepsilon$ (NSFRY-NH_2)^{theor} − $\Delta\varepsilon$ (NSFRY-OH) equal to $\Delta\varepsilon$ (AAAAA-NH_2) − $\Delta\varepsilon$ (AAAAA-OH) (the latter value from Ref. [15]).

dination plane in this complex. Finally, the C-terminal amidation results in the stability increase of 0.4 log units, but the substitution of Asn for Ala hardly affects relative stabilities of 2N through 4N complexes.

All the above effects can be accommodated in a model of the 4N complex in which the terminal amide group (or carboxylate, when present) is hydrogen bonded to the Cu(II)-bound amino nitrogen of residue 1. The amide, being bulkier than carboxylate, exerts greater steric strain on the Asn side-chain. Substitution of any residue with Ala allows this strain to be accommodated without a change in positions of Cu(II)-bonded nitrogens. It is, however, no longer possible for the more crowded complex of NSFRY-NH_2.

Inspection of far-UV CD spectra of 4N complexes reveals an unusual pattern with very intense, mostly positive bands between 200 and 240 nm present with Phe-containing analogues (Fig. 3). The probable mechanism responsible for this spectral feature is the coupling of Phe, and, to a lesser extent, of Tyr ring transitions with amide chromophores [20,21]. Three major aromatic bands can be found in this range: Tyr-L_a at 230 nm, Phe-L_b at 215 nm and Tyr-B at about 200 nm [21]. Therefore the CD spectra indicate that the Phe ring assumes a fixed conformation (necessary for effective coupling) in 4N complexes. Particularly large variations of magnitudes at 200 nm in the spectra of complexes of NSFAY-NH_2 (high) and NSARY-NH_2 (low) compared with other analogues may indicate interactions of the Tyr ring in the parent complex (NSFRY-NH_2): a π-positive bridge to Arg guanidinium group in the former case and edge-to-face interactions between both aromatic rings in the latter.

An insight into the environment of the Tyr residue can be obtained by analysing the effects of its deprotonation. The last column of Table 1B presents the impact of complexation on the pK of the Tyr ring. All Ala-substituted analogues have this value elevated by 0.7–0.9 log units, whereas NSFRY-NH_2–Cu(II) and NSFRY-OH–Cu(II) exhibit a slight lowering of this value. The former effect apparently reflects the decrease of the overall charge of the molecule in 4N com-

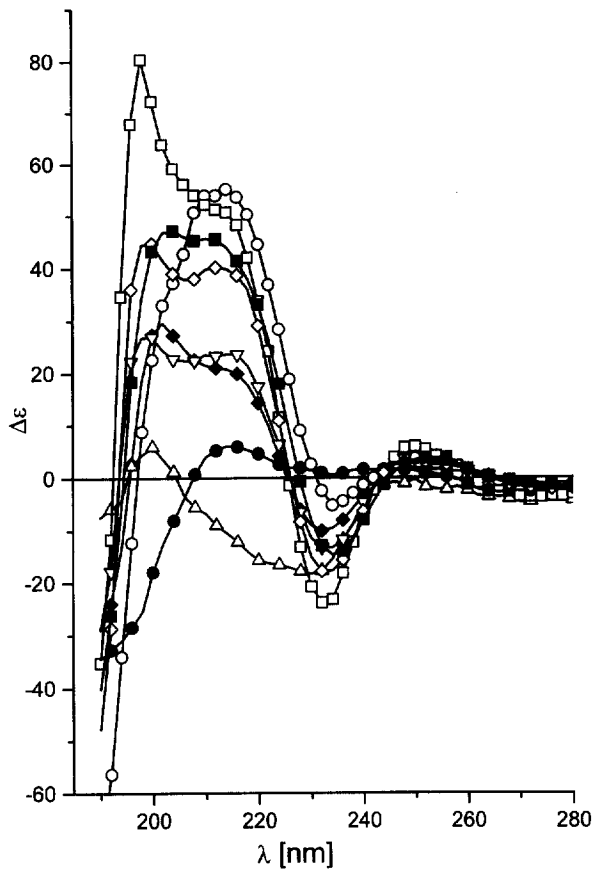

Fig. 3. Far-UV CD spectra of 4N complexes with Tyr residue protonated or absent: – ◇ –, ASFRY-NH_2; – ▽ –, NAFRY-NH_2; – △ –, NSARY-NH_2; – □ –, NSFAY-NH_2; – ○ –, NSFRA-NH_2; – ■ –, NSFRY-NH_2; – ● – , AAAAA-NH_2; – ◆ –, NSFRY-OH.

plexes by one, compared with free peptides, but the latter can only be explained by a formation of a salt bridge between the Tyr-O^- and the Arg guanidinium cation away from Cu(II). The change in complex conformation leading to this phenomenon must be very subtle, because it is triggered even by substitution of Ser-2 for Ala, and, e.g., CD spectra of 4N complexes of NAFRY-NH_2 and NSFRY-OH are almost identical. Plots in Fig. 2 show that this interaction increases the complex stability by 2–3 times. However, the phenolic oxygen is located away from the coordination site in all complexes, because its deprotonation does not influence the magnitude of CD of d–d bands significantly, except for the most sterically crowded, and thereby sensitive NSFRY-NH_2 complex. The positive influence of phenol deprotonation on complex stability is therefore probably due to overall charge lowering, stabilising the metal cation in the bound form.

Fig. 4 presents difference CD spectra demonstrating the impact of Tyr deprotonations on far-UV CD. Major effects are seen in the spectra of analogues with Ala substituted in positions 1, 3, and 4, supporting the involvement of these residues in the stabilisation of a particular Tyr ring conformation, as well as the presence of an interaction between residues 1 and 5.

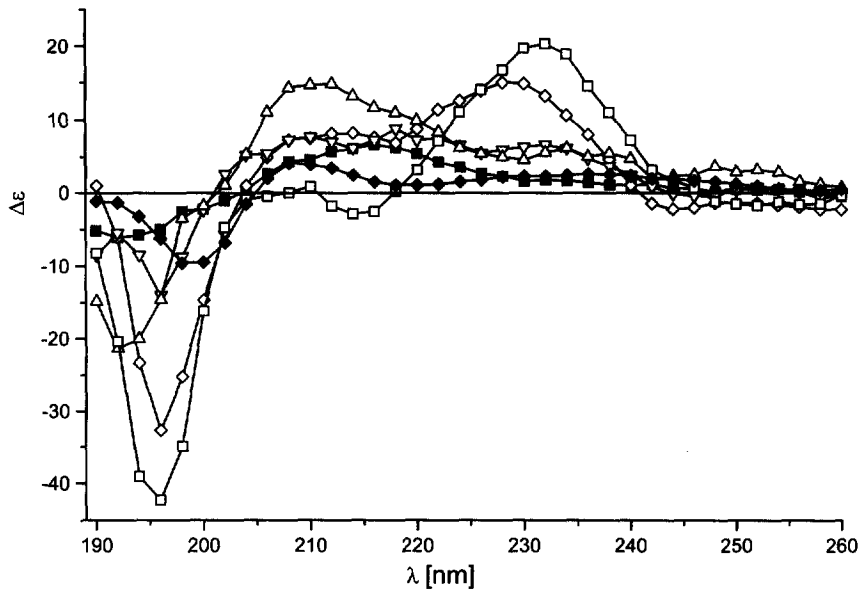

Fig. 4. Difference far-UV CD spectra of 4N complexes, i.e. the spectra of complexes with Tyr protonated (CuH_2L) subtracted from the spectra with Tyr deprotonated ($CuH_{-3}L$): – ◇ –, ASFRY-NH_2; – ▽ –, NAFRY-NH_2; – △ –, NSARY-NH_2; – □ –, NSFAY-NH_2; – ■ –, NSFRY-NH_2; – ♦ –, NSFRY-OH.

3.4. Model of the complex structure

We can now attempt to propose a view of the 4N complex of NSFRY-NH_2–Cu(II). There is a clear electronic effect from the Asn side-chain, seen also in the lowering of the amino p*K*, that increases the Cu–N bond strength, and also a secondary effect from Ser oxygen, stabilising 1N and 2N complexes. A fence around Cu(II) is provided by Asn and Phe side-chains. The 4N complex stabilisation also results from the formation of a main chain loop around Cu(II) by hydrogen bonding between polar atoms of residues 1 and 5. Such an effect is responsible for the stability gain in pentapeptide versus tetrapeptide complexes [22]. The most striking and novel effect, however, comes from the presence of the Arg and Tyr side-chains in positions 4 and 5, away from the Cu(II) site. The only explanation for this effect is the formation of a secondary fence above that formed by side-chains of residues 1–3. It is located over the coordination plane so that an edge-to-face interaction between Phe and Tyr chains is possible, but this is not required for the fence stability. In fact, all shielding contributions are additive rather than cooperative, as reflected by the values of p*K* (3N + 4N) in Table 6. The secondary fence has a greater effect on stability than the primary one, probably by effectively limiting the access of water molecules to Cu(II) over a larger surface.

4. Conclusions

Our study has shown for the first time that the non-bonding side-chains of amino acids not involved in metal binding can have a major impact on stability of a metal–peptide complex. This is accomplished by two conformational effects. One of these effects is the stabilisation of the pentapeptide loop around Cu(II) through interactions between residues 1 and 5. This effect seems to be generally exhibited by pentapeptides not containing histidine. Although it appears that in NSFRY analogues it is further augmented by contributions from specific side-chain interactions. The other effect, the formation of a secondary fence by Arg and Tyr residues is by far more general. It may provide new tools for rational design of metal-binding peptides, and may serve as a simple model for studying metal ion interactions with proteins. The combined electronic, conformational and hydrophobic effects amount to a stability gain exceeding that generated by a disulphide bridge in arginine vasopressin [5]. This finding reinforces the importance of individually weak and non-specific local interactions in folding processes and suggests a possibility that metal ions may influence them.

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