Cu^{II} Ion Coordination to an Unprotected Pentadecapeptide Containing Two His Residues: Competition Between the Terminal Amino and the Side-Chain Imidazole Nitrogen Donors

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The complex-formation equilibria of the pentadecapeptide TLEGTKKGHKLHLDY, the 114–128 protein fragment of SPARC, with the Cu^{II} ion have been investigated, at $I = 0.1$ mol·dm^{-3} (KNO_3) and $T = 298.2$ K. Protonation and complex-formation constants have been determined potentiometrically, and formation enthalpies measured by direct solution calorimetry; the complex-formation model and species stoichiometry have been carefully checked by means of UV/Vis absorption, CD and EPR spectroscopy. The structure hypotheses of the complex species are also based on detailed study of the ^1H and ^{13}C NMR spectra of the ligand in both the absence and presence of copper ions. The involvement in complex-formation of both the terminal amino and imidazole groups has been suggested and their specific behaviour at different pH values elucidated.

Introduction

SPARC (Secreted Protein, Acidic and Rich in Cysteine) is a 32 kD calcium-binding matricellular protein[1] secreted by many types of cells. It mediates the cell−matrix interactions without covering any primarily structural role:[2,3] it has counter-adhesive properties, thus altering the cell shape, it modulates growth factor activity and influences the cell-cycle. SPARC is the product of a single-copy gene; its sequence has been highly conserved both during vertebrate evolution and among species.[4] It is highly expressed during embryonic development while its expression in the adult is associated with tissue remodelling or renewal, also in response to injury. SPARC is a major product of many tumours,[5] most likely owing to its activity, and that of its fragments, in angiogenesis,[6] both in their free form and as copper complexes.[7]

Human SPARC consists of 286 amino acids, belonging to three distinct modules.[8] The *N*-terminal module (residues 1−52, after a 17 amino-acid signal sequence) is an acidic region, which binds 5−8 calcium ions with low affinity. It interacts with hydroxyapatite, being involved in the mineralization of cartilage and bones. The second module is a Cys-rich, follistatin-like domain (residues 53−137), in which all the 10 Cys residues are disulfide-bonded. A carbohydrate moiety is *N*-linked at Asn99. This module contains two bioactive peptides (residues 55−74 and 114−130), which enable SPARC to bind the copper ion.[7] The latter fragment, corresponding to the strongest Cu^{II}-binding site of the protein, contains the sequence Gly-His-Lys (GHK) (residues 121−123). GHK, a liver-cell growth factor,[9] is a well recognised Cu^{II} binding peptide;[10] it is present in the human serum and it most likely derives from SPARC hydrolysis.[7] Cu^{II}/GHK complexes proved to have angiogenic activity and to play an important role in wound healing.[9] Module III (residues 138−286) is largely α-helical and contains the extracellular high-affinity calcium binding sites. It is globular and contains two EF-hand motifs.

Recent investigations[11,12] into the complex-formation equilibria of protected $SPARC_{114-128}$ (Ac-TLEGTKKGHKLHLDY-NH_2), $SPARC_{122-126}$ (Ac-HKLHL-NH_2), $SPARC_{121-126}$ (Ac-GHKLHL-NH_2) and

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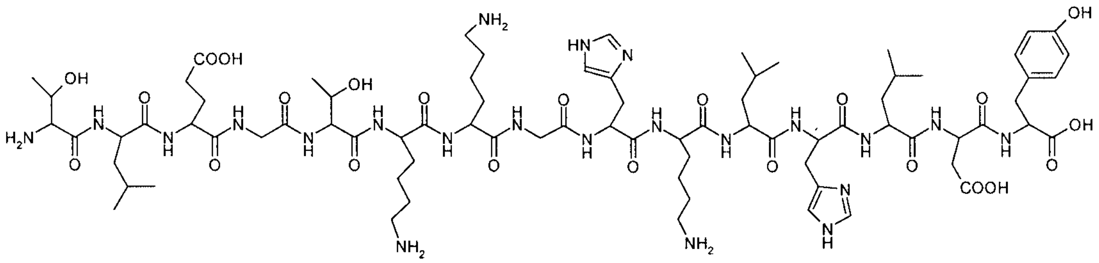

Figure 1. Structure of the ligand TLEGTKKGHKLHLDY.

$SPARC_{120-126}$ (Ac-KGHKLHL-NH_2) fragments with the Cu^{II} ion have shown the central role played by ^{122}His and ^{125}His at acidic pH. The copper-promoted deprotonation of three amido nitrogens has also been reported, at alkaline pH. Here the Cu^{II} complex-formation equilibria of the unprotected TLEGTKKGHKLHLDY pentadecapeptide (see Figure 1) have been investigated, at $I = 0.1$ mol·dm^{-3} (KNO_3) and $T = 298.2$ K, to gain new information on the role played in metal ion complexation by His residues belonging to relatively short peptides. In such cases the imidazole nitrogens can compete or cooperate with the terminal amino group for binding copper. In this context, an interesting recent report concerns the Prion Peptide fragment 106−126 ($PrP_{106-126}$),[13] which is neurotoxic in vitro and its activity is highly sensitive to the presence of copper, zinc or iron ions. In that system, the formation of a macrochelate structure at physiological pH has been suggested in which Cu^{II} is bound to both the terminal amino nitrogen and the imidazole ring of the ^{111}His residue. TLEGTKKGHKLHLDY is also a good model for the $PrP_{106-126}$ fragment. Protonation and complex-formation constants have been determined potentiometrically; formation enthalpies have been measured by direct solution calorimetry; complex-formation model and species stoichiometry have been carefully checked by means of UV/Vis absorption, CD and EPR spectroscopy. The proposed structures of the complex species are also based on a detailed study of the ^{1}H and ^{13}C NMR spectra of the ligand both in the absence and in the presence of copper ions.

Results

Ligand protonation constants are shown in Table 1 along with the corresponding thermodynamic quantities determined by calorimetry. It was not possible to distinguish between the two His or the three Lys residues. The terminal carboxylic group is deprotonated over the explored pH range and the corresponding equilibrium is not considered throughout. The thermodynamic results are in good agreement with literature data on analogous ligands,[11,12,14,15] within experimental errors.

Table 1. Protonation and Cu^{II} complex-formation thermodynamic parameters for TLEGTKKGHKLHLDY, at 298.2 K and $I = 0.1$ mol·dm^{-3} (KNO_3).

Species	Residue	Stoichiometry	logβ	log*K*	$-\Delta G^\circ$ (kJ·mol^{-1})	$-\Delta H^\circ$ (kJ·mol^{-1})	ΔS° (J·mol^{-1}·K^{-1})
LH	Lys	011	10.95(4)	10.9	62.5(2)	56(7)	20(23)
LH_2	Lys	012	21.61(3)	10.7	123.3(1)	99(2)	83(8)
LH_3	Lys	013	31.78(4)	10.2	181.3(2)	141(4)	136(13)
LH_4	Tyr	014	41.35(5)	9.6	235.9(3)	173(3)	211(11)
LH_5	terminal NH_2	015	48.65(6)	7.3	277.5(3)	209(3)	229(12)
LH_6	His	016	55.22(6)	6.6	315.0(3)	237(3)	261(12)
LH_7	His	017	61.07(6)	5.9	348.4(3)	261(4)	293(13)
LH_8	Glu	018	65.27(7)	4.2	372.3(4)	−	−
LH_9	Asp	019	68.94(7)	3.7	393.3(4)	−	−
MLH_6		116	59.76(9)		341.3(5)	−	−
MLH_5		115	55.21(3)	4.6	315.0(1)	243(5)	240(16)
MLH_4		114	49.85(7)	5.4	284.4(4)	247(4)	125(12)
MLH_3		113	42.37(7)	7.5	241.7(3)	257(4)	−53(15)
MLH_2		112	34.36(6)	8.0	196.0(5)	177(4)	63(14)
MLH		111	25.63(9)	8.7	146.2(6)	145(5)	4(16)
ML	Tyr	110	15.9(1)	9.7	90.9(6)	85(6)	21(21)
MLH_{-1}	Lys	11−1	5.8(1)	10.1	33.3(6)	74(9)	−137(30)
MLH_{-2}	Lys	11−2	−4.8(1)	10.6	−27.2(6)	5(12)	−109(39)
MLH_{-3}	Lys	11−3	−15.7(1)	10.9	−89.8(6)	−36(11)	−180(36)

Uncertainty on the last significant figure in parentheses, as given by the computation program

Table 1 also shows the whole set of thermodynamic parameters for complex-formation equilibria between the Cu^{II} ion and the pentadecapeptide. Ten mono complexes were detected in the explored pH range; all with different protonation degrees. The corresponding distribution diagram is reported in Figure 2. The ligand starts to bind the Cu^{II} ion at pH 3.5, forming the species MLH_6 and MLH_5. At pH > 4, MLH_4 is formed: it dominates the acidic pH range and is the main species at physiological pH. In the alkaline pH range (7.0−10.5) up to seven protons are subsequently lost by the MLH_4 complex due to deprotonation of some amido nitrogens and of basic side chains of Lys and Tyr residues.

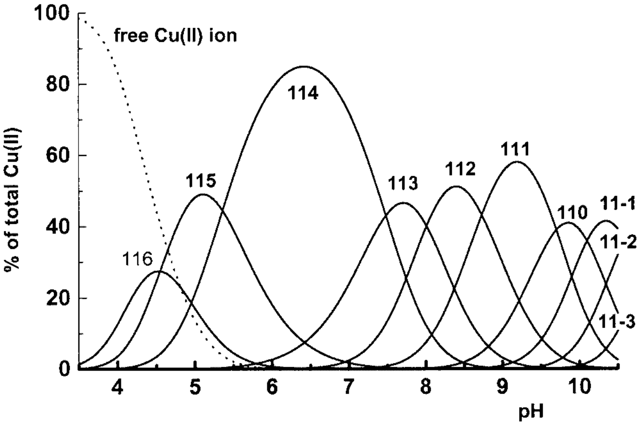

Figure 2. Distribution diagram of Cu^{II}/TLEGTKKGHKLHLDY complexes, at 298.2 K; $I = 0.1$ mol·dm^{-3} (KNO_3); [Cu^{II}] = 1.0×10^{-3} mol·dm^{-3}; [TLEGTKKGHKLHLDY] = 1.2×10^{-3} mol·dm^{-3}

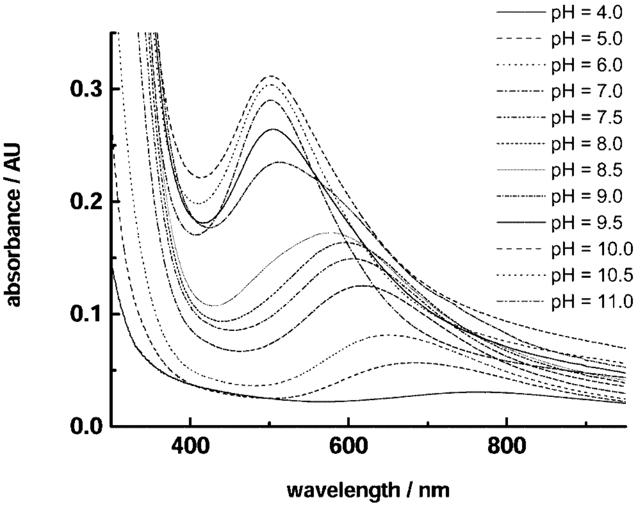

Figure 3. Absorption spectra of Cu^{II}/TLEGTKKGHKLHLDY solutions. [Cu^{II}] = 1.0×10^{-3} mol·dm^{-3}, [TLEGTKKGHKLHLDY] = 1.2×10^{-3} mol·dm^{-3}, $T = 298.2$ K, $I = 0.1$ mol·dm^{-3} (KNO_3).

Electronic spectra (Figure 3 and Table 2) confirm the presence of a complex species at a pH as low as 4: in fact at pH 4 the wavelength of maximum absorption is already lower than 800 nm. With increasing pH the d−d absorption band shifts towards lower wavelength values. This behaviour is typical of the coordination of successive nitrogen atoms to the metal ion. A noticeable change in the absorption spectrum is seen between pH 8.0 and 9.5, with the onset of a new absorption band with a maximum close to 500 nm.

The CD spectra show some typical absorption bands (Figure 4 and Table 2). A double band is present in the d−d region, from pH 5 on, which is typical of Cu^{II}/His coordination.[16] The two bands at 250−256 and 350 nm can be ascribed to a charge-transfer transition from imidazole π_2 and π_1 to copper.[17] The positive band at 316−320 nm is most likely due a charge-transfer transition from a deprotonated amido nitrogen to copper:[17] this band is clearly detected from pH 6.0 on.

$A_{\|}$ and $g_{\|}$ parameters of the EPR spectra are reported in Table 2. At pH 6 they are typical of a 2N complex; at pH 8 those of a 3N complex. From pH 10, the EPR data be-

Table 2. Spectroscopic parameters of Cu^{II}/TLEGTKKGHKLHLDY binary complex formation, at 298.2 K and $I = 0.1$ mol·dm^{-3} (KNO_3).

pH	UV/Vis λ/nm (ε/ $M^{-1}cm^{-1}$)	CD λ/nm (Δε/ $M^{-1}cm^{-1}$)	EPR $A_{\|}$ ($g_{\|}$)
4.0	765 (31)	234 (−1.47); 266 (0.07)	121 (2.50)
5.0	685 (58)	234 (−1.42); 256 (0.33); 670 (−0.11)	121 (2.42); 145 (2.29)
6.0	652 (83)	255 (0.33); 283 (−0.06); 317 (0.06); 699 (−0.17)	121 (2.40); 162 (2.27)
7.0	618 (128)	255 (0.58); 284 (−0.20); 316 (0.32); 701 (−0.20)	167 (2.27); 167 (2.22)
7.5	604 (153)	257 (0.50); 283 (−0.37); 319 (0.60); 350 (sh); 481 (−0.06); 695 (−0.19)	168 (2.27); 168 (2.22)
8.0	596 (168)	259 (sh); 282 (−0.46); 322 (0.89); 350 (sh); 495 (−0.15); 699 (−0.17)	177 (2.22)
8.5	518 (sh); 584 (176)	256 (sh); 282 (−0.42); 321 (1.02); 350 (sh); 504 (−0.37); 610 (0.09); 708 (−0.13)	179 (2.22)
9.0	509 (240); 584 (sh)	252 (sh); 283 (−0.23); 319 (1.05); 350 (sh); 505 (−0.73); 611 (0.23)	177 (2.22)
9.5	504 (271)	251 (sh); 283 (−0.03); 316 (1.26); 350 (sh); 504 (−1.25); 614 (0.37)	186 (2.21)
10.0	502 (319)	245 (sh); 314 (1.23); 505 (−1.38); 615 (0.39)	209 (2.18)
10.5	502 (311)	242 (6.38); 314 (1.31); 505 (−1.54); 616 (0.42)	205 (2.18)
11.0	502 (297)	243 (6.94); 312 (1.34); 505 (−1.66); 618 (0.42)	205 (2.18)
11.5	502 (288)	245 (7.50); 311 (1.27); 506 (−1.71); 620 (0.38)	205 (2.18)

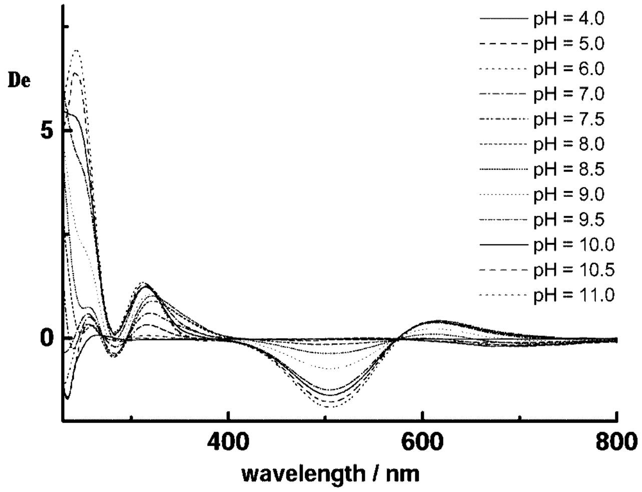

Figure 4. Circular dichroism spectra of Cu^{II}/TLEGTKKGHKLHLDY solutions. $[Cu^{II}] = 1.0 \times 10^{-3}$ mol·dm^{-3}, [TLEGTKKGHKLHLDY] = 1.2×10^{-3} mol·dm^{-3}, $T = 298.2$ K, $I = 0.1$ mol·dm^{-3} (KNO_3).

come typical of a 4N complex characterised by a strong ligand field. No evidence of binuclear species has been found over the whole pH range.

The ^{1}H NMR spectrum of TLEGTKKGHKLHLDY was recorded at both pH ≈ 6 and pH ≈ 10. At pH > 7, most amide protons were removed from the spectrum by chemical exchange. Thus, NMR studies at pH 10.4 were carried out in deuterium oxide (99.95%).

The complete assignment of the peptide at lower pH is reported in the Supplementary Material. None of the NMR spectroscopic data (temperature coefficients of the chemical shifts of amide protons, intramolecular NOEs and HN-H$_{\alpha}$ coupling constants) was consistent with the presence of secondary structure elements. Only the amide proton of ^{15}Tyr (see Supplementary Material), exhibits a different temperature coefficient compared to the other amide protons ($\Delta\delta/\Delta T = -4.3$ ppb/K), which is very close to values typical of a proton engaged in direct intramolecular or water-mediated hydrogen-bonding (head−tail interaction).

The complete assignment of the ^{13}C NMR spectrum of the peptide, recorded at alkaline pH, is given in the Supporting information (see footnote on the first page of this article).

Upon addition of copper, chemical shifts, line widths and spin−lattice relaxation rates were selectively affected. However, such effects could be observed only at relatively high Cu^{II}/peptide ratios (especially at pH > 7.0), much higher than is usual in NMR experiments on Cu^{II} complexes. As shown in Figure 5, the paramagnetic effects at pH 5.6 were mainly detected on ^{9}His and ^{12}His aromatic protons, on the His H$_{\beta}$ and on the ^{14}Asp H$_{\beta}$.

To observe signal broadening at pH 10.4 a large amount of copper was added to the system (Figure 6). The signals that underwent the largest broadening were those of His and ^{1}Thr residues, but, to a lesser extent, the ^{14}Asp, ^{6,7,10}Lys, and ^{15}Tyr residues were also affected. Similarly, the most affected relaxation rates were those of the His resi-

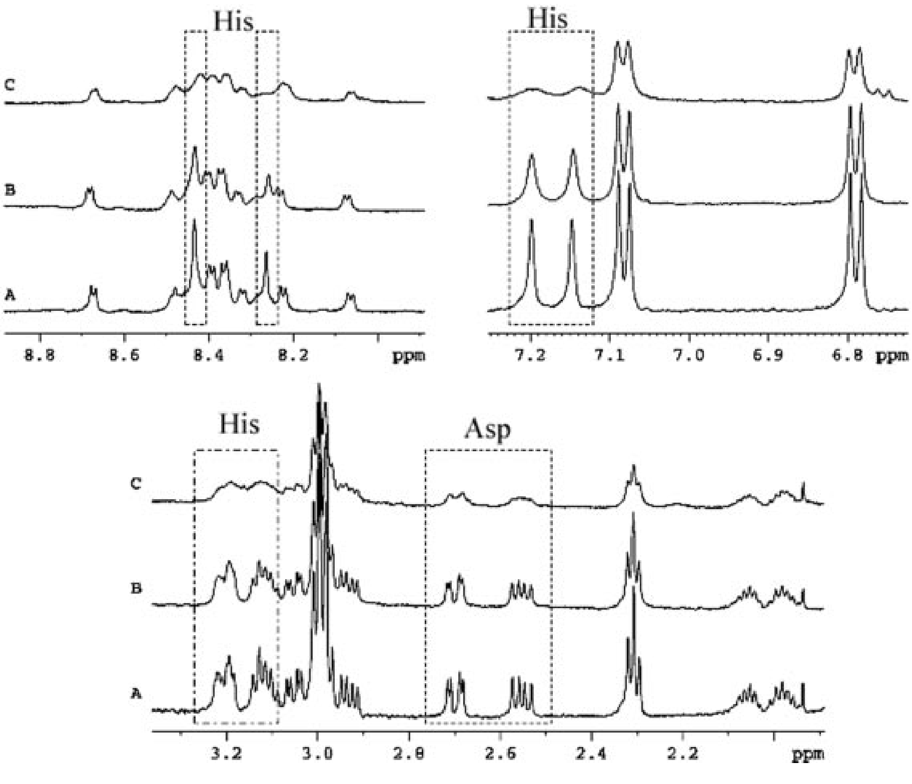

Figure 5. Selected regions of the ^{1}H NMR spectrum of TLEGTKKGHKLHLDY 2.5×10^{-3} mol·dm^{-3} in water at pH 5.6 (A traces) showing the effect of adding Cu^{II} 1.0×10^{-5} mol·dm^{-3} (B traces), 5.0 10^{-5} mol·dm^{-3} (C traces).

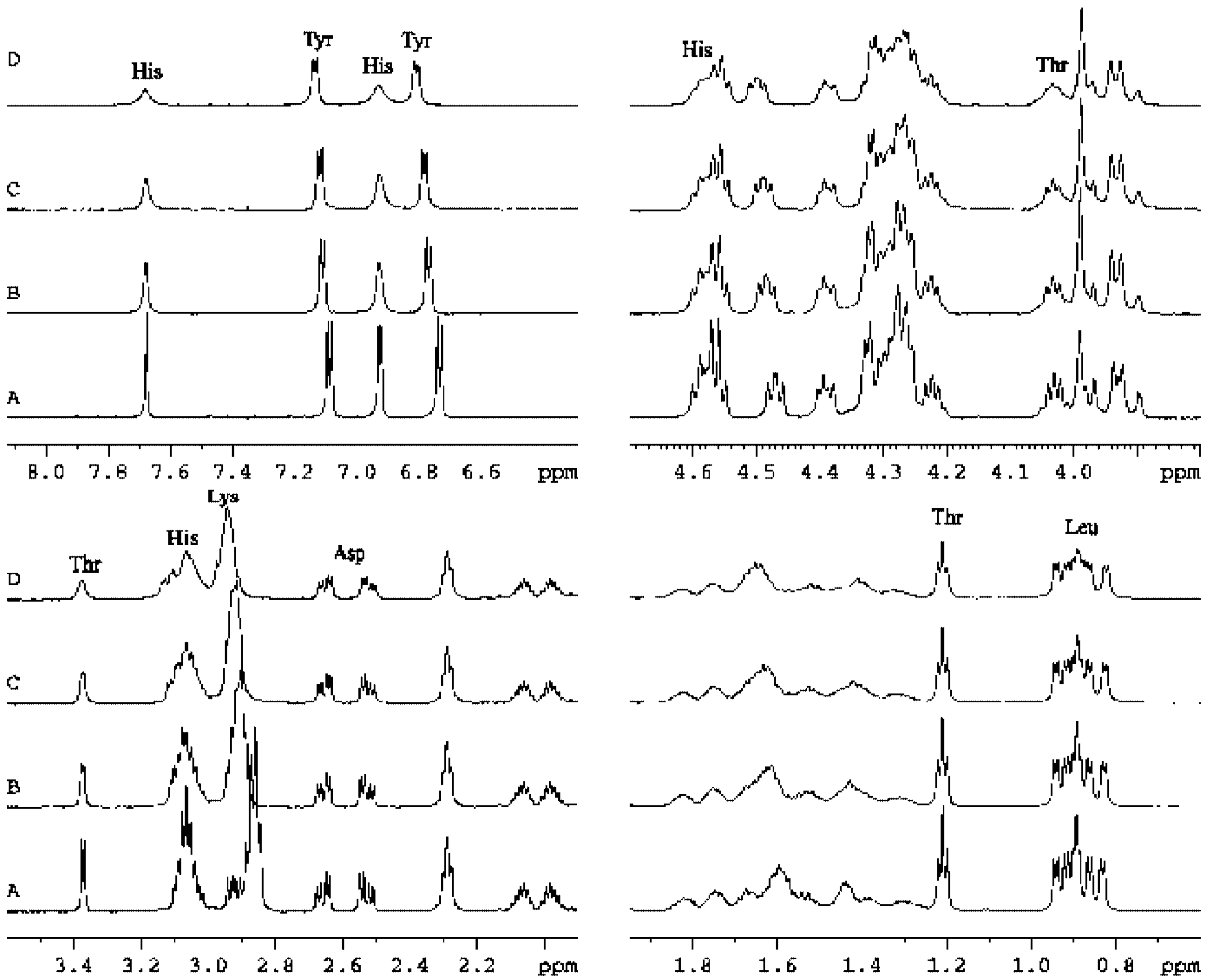

Figure 6. Selected regions of the ^{1}H NMR spectrum of TLEGTKKGHKLHLDY 4.0×10^{-3} mol·dm^{-3} in deuterium oxide at pH 10.4 (A traces) showing the effect of adding Cu^{II}: 1.6×10^{-4} mol·dm^{-3} (B traces), 3.3×10^{-4} mol·dm^{-3} (C traces), 8×10^{-4} mol·dm^{-3} (D traces).

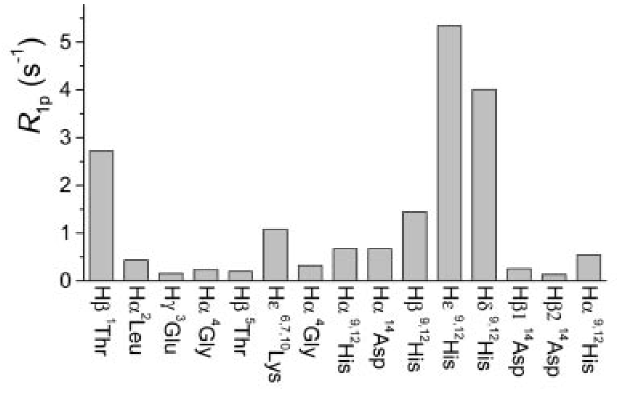

Figure 7. R_{1p} for selected protons of TLEGTKKGHKLHLDY (4.0×10^{-3} mol·dm^{-3}) in deuterium oxide at pH 10.5 after the addition of Cu^{II} (8.0×10^{-4} mol·dm^{-3}).

dues and the H_{α}, H_{β} of ^{1}Thr, with slight changes for protons of the ^{14}Asp, ^{6,7,10}Lys and ^{15}Tyr residues (Figure 7). The ^{1}H (Figure 6) and ^{13}C NMR spectra (see Supplementary Material) support the fact that copper perturbs two molecular regions: the *N*-terminal part of the peptide and the His regions. Overlapping ^{1}H NMR signals prevented differentiation of the ^{9}His and ^{12}His residues.

Discussion

TLEGTKKGHKLHLDY contains two different binding sites for copper ions: the terminal amino group and the two side-chain imidazole of its ^{9}His and ^{12}His residues. When the former is protected the two His residues behave as "anchoring" sites for copper at pH < 4.[11,12] Conversely, unprotected peptides can bind copper at approximately the same pH with their terminal amino and neighbouring carbonyl groups.[18] In both cases the metal can promote amido nitrogen ionisation, at pH < 7. The present ligand has both of the above capabilities: the two sites compete to bind copper, and binuclear species could also be formed. However, in the present study, where nearly equimolar concentrations of copper and TLEGTKKGHKLHLDY have been used, neither potentiometric nor spectroscopic evidence was found for binuclear species formation. To favour such complexes an excess of copper could have been used, but this was beyond the aim of the present work. However, reports of polynuclear copper/peptide complex formation are common, even in the presence of an excess of ligand.[10]

First of all it is interesting to compare the overall binding ability of the present peptide with that of its protected analogue.[11] The competition diagram reported in Figure 8 shows that the copper complexes with the unprotected peptide are favoured over the whole pH range. This suggests that the terminal amino group plays an important role in determining the behaviour of TLEGTKKGHKLHLDY as a copper ligand. In addition, the wavelength of maximum absorption in the electronic spectra is always slightly lower for the unprotected peptide (Table 2) compared with the protected one,[11] indicating a higher mean coordination number for the former ligand.

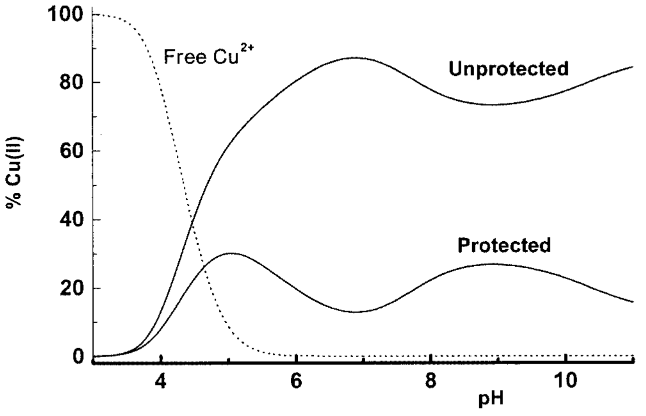

Figure 8. Competition diagram for Cu^{II} complex formation with TLEGTKKGHKLHLDY and Ac-TLEGTKKGHKLHLDY-NH_2. The concentrations were calculated by using the stability constants of Table 1 and ref.[11] at 298.2 K, $I = 0.1$ mol·dm^{-3} (KNO_3) and identical concentration of the three reagents: 1.0×10^{-3} mol·dm^{-3}.

The species detected at the most acidic pH is the MLH_6 complex (see Figure 2). EPR and UV/Vis data suggest that this is a 1N species where the metal ion is either bound to the terminal NH_2 or to one of the imidazole nitrogens; the three acidic groups (Asp and Glu side chains and terminal carboxylic) are deprotonated. Experimental data favour the second hypothesis. In fact, the computed step constant $\log\beta_{116} - \log\beta_{016} = 4.6$ (see Table 1), corresponding to the coordination of a protonated ligand to the Cu^{II} ion, is very close to that reported for the Cu^{II}/imidazole system (4.2) under the same experimental conditions.[15] Moreover, $\log\beta_{116}$, once the contribution due to terminal-NH_2 protonation has been subtracted ($59.8-7.3 = 52.5$, Table 1), is very close to the corresponding $\log\beta_{115}$ reported[11] for the protected ligand (52.0). Conversely, a 1N species where the Cu^{II} ion is chelated by the terminal-NH_2 and the neighbouring carbonyl groups could also be hypothesized. However, upon subtracting the constants for protonated sites from $\log\beta_{116}$ a value of 6.0 is obtained (see Table 1), which is significantly higher than the corresponding $\log\beta_{110}$ for the Cu^{II}/penta-alanylamide complex (4.9).[19] Finally, the wavelength of maximum absorption at pH 4 (765 nm, Table 2) is closer to that expected[20] for a $Cu-N_{Im}$ species (758 nm) than the mean value found[20] for a $Cu-N_{amino}$ complex (743 nm).

MLH_5 complex formation starts immediately after that of the MLH_6 species discussed above, at pHs still lower than 4. This complex reaches its highest concentration around pH 5, when it is the most abundant species in solution. The wavelength of maximum absorption in the UV/Vis spectra is blue-shifted with respect to pH 4, and the change in EPR parameters also point to a complex with additional nitrogen atoms bound to copper; the enthalpy of formation, minus the protonation contribution ($\Delta H^\circ_{115} - \Delta H^\circ_{015} = -34$ kJ·mol^{-1}, Table 1), is compatible with a 2N complex. Other structural hypotheses can be put forward for this species, the most likely of which is that TLEGTKKGHKLHLDY binds the metal ion with both imidazole nitrogens, as do its protected analogue[11] and its shorter fragments[12]. The corresponding $\log K$ (4.6, Table 1) is close to that already reported for the above systems and is compatible with deprotonation of the second imidazole nitrogen, promoted by the copper ion. CD spectra at pH 5 clearly show a positive band at 256 nm attributable to a π_2 charge-transfer transition from imidazole to copper.[17] The d−d band in the electronic spectra has its maximum at 685 nm (at pH 5), exactly the value expected for a $Cu-(N_{Im})_2$ complex.[20] Subtracting $\log K$ for the terminal amino group (7.3, Table 1) from $\log\beta_{115}$ gives 47.9, a value only slightly higher than that reported for the corresponding complex with the protected analogue (47.1).[11] This hypothesis is also in excellent agreement with the NMR spectroscopic data discussed below.

From pH 4.5 on a new species forms in solution: the MLH_4 complex, which reaches its maximum at pH 6.5, when it is almost the only species in solution. It is also the most abundant complex at neutral pH. Even though the EPR data remain those of a 2N complex a relevant blue-shift is registered in the electronic spectra when the pH changes from 5 to 7, suggesting the coordination of a further nitrogen atom, or at least the substitution of one nitrogen donor with another giving a higher ligand field. CD spectra show the onset of two new bands at 283 and 317 nm, which can be ascribed to a π_1 charge-transfer transition from imidazole to copper and to a charge-transfer transition from a deprotonated amido nitrogen to copper, respectively.[17] The $\log K$ (5.4, Table 1) is compatible with the deprotonation of both an amido and an amino nitrogen[18] promoted by the copper ion. It is difficult, at this point, to suggest a unique complex structure with the stoichiometry 114 that accounts for all of the above experimental results. EPR data suggest the simultaneous presence of different species with comparable concentrations. One possible structure is that already proposed for the corresponding complex formed by Ac-TLEGTKKGHKLHLDY-NH_2:[11] one imidazole and one neighbouring amido nitrogens bound to the equatorial plane of the copper complex. The second N_{Im} should be free, for steric reasons. This would explain the wavelength of maximum absorption in the electronic spectra (652 nm at pH 6, Table 2), which is close to the computed[20] value for this type of coordination (658 nm). This hypothesis also agrees with the EPR results. The complex stoichiometry requires that the terminal amino group is protonated. The $\Delta H°_{114}$ value, once the terminal amino group protonation is subtracted ($-247 + 36 = -211$ kJ·mol^{-1}, Table 1), becomes nearly identical to that found for the protected peptide (-212 kJ·mol^{-1}, ref.[11]). However, the MLH_4 complex formed by the unprotected ligand is much more stable than that reported for the protected peptide.[11] This can deduced by examining the competition diagram of Figure 8 and comparing logβ_{114} − logK_{amino} (42.5, Table 1) with the stability of the corresponding complex with the protected ligand (40.1, ref.[11]). The can be explained by the simultaneous formation, in the present system, of an additional complex species with the same stoichiometry but a different structure, i.e. involving the terminal amino group. The ligand should then form a macrochelate ring with at least one N_{Im} and the terminal amino group simultaneously bound to copper. Such a structure is similar to that previously reported for the $PrP_{106-126}$ peptide.[13]

The first complex to be formed in the alkaline pH range is MLH_3, which becomes the main complex at approximately pH 7.6. Four protons have already been released by the ligand at this stage, in addition to those of the acidic side chains: two from the imidazole side chains, one amido and the terminal amino. EPR data again suggest the presence in solution of two species. The corresponding log$K$ (7.5, Table 1) is very close to that found for the protonation of the terminal amino group (7.3, Table 1), but it is also compatible with an amido nitrogen deprotonation, promoted by copper. No new important signal appears in CD spectra when the pH is raised from 6.0 to 7.5; the N_{amido}−copper binding is confirmed by the band at 319 nm. The wavelength of maximum absorption at pH 7.5 (604 nm) is not far from the computed[20] average value for 3N complexes with various combinations of nitrogen atoms bound to copper (581−615 nm). The $-\Delta H°_{113}$ value also suggests a 3N coordination. Again, the simultaneous formation of two complex species with the same MLH_3 stoichiometry best explains the experimental results. One of the complexes should have the metal ion located at the His site of the ligand, as with the protected analogue,[11] with the terminal amino group not protonated and free. The second should be a macrochelate where the terminal amino, one imidazole and one amido nitrogen simultaneously bind the copper ion.

At more basic pHs the complex loses other protons, giving rise to species from MLH_2 to MLH_{-3}. The first two steps (species MLH_2 and MLH) are most likely due to the subsequent deprotonation of two further amido groups which bind the metal ion. The corresponding thermodynamic parameters support this hypothesis. The spectroscopic parameters are typical of a 4N species. The four last protons probably derive from the basic side chains of the ^{15}Tyr, ^{6}Lys, ^{7}Lys and ^{10}Lys residues. The corresponding log$K$s are practically identical to those of the free ligand (Table 1), while the spectroscopic data do not change from pH 10 on. These results suggest that no binding interaction is present in solution between the latter residues and the metal ion, as already found for the protected peptide.[11] It is worth noting that on raising the pH from 9 to 10 big changes are observed in all the registered spectra. In particular, in the CD spectrum the charge-transfer $N_{Im} \rightarrow Cu^{II}$ bands are no longer observed while a new intense band appears at 242−245 nm, which can be ascribed to a charge-transfer transition from the terminal amino group to copper. This result strongly suggests that complex structure in the most alkaline pH range is that typical[20] of any unprotected peptide: "four nitrogens, one amino and three deprotonated peptide nitrogens, occupy the four tetragonal positions about Cu^{2+}". Only weak interactions with other donor atoms should be present in the apical position. In such a structure the two imidazole rings should be free. Once again, the presence of another 4N species, where one N_{Im} and three amido nitrogens are bound to copper, cannot be excluded (see also below). However, this species, the main complex at alkaline pH in the case of the protected peptide,[11] should be present here only in lower amounts.

The effect of paramagnetic Cu^{II} on spin−lattice relaxation rates is measured by the value of the observed relaxation rates R_{1m} in presence of the metal ion. They are defined as:[21]

$$R_{1m} = \frac{p_f}{R_{1f}^{-1}} + \frac{p_b}{R_{1M}^{-1} + \tau_m} \tag{1}$$

where R_{1f} is the rate calculated in the absence of the metal, τ_m is the exchange lifetime from the bound to the free state and p_f and p_b are the molar fraction of the free and bound ligand, the relaxation of which is described by R_{1M}. Since the ligand occurs in the bulk and in the metal coordination sphere, exchange has to be considered. If the metal-bound ligand is much less concentrated than its free form a p_f of **1** can be assumed. Equation (1) can then be reduced:

$$R_{1m} - R_{1f} = R_{1p} = \frac{p_b}{R_{1M}^{-1} + \tau_m} \tag{2}$$

R_{1M} is usually accounted for by the Solomon equation[22] and is proportional to the inverse sixth power of the metal−nucleus distance. The obtained R_{1p} values are consistent with τ_m not dominating Equation (2), but the relatively high Cu^{II}/peptide ratios that can be reached without severe alteration of NMR parameters demonstrate that τ_m contributes to R_{1p} and, therefore, that an intermediate exchange region occurs ($\tau_m \cdot R^{-1}{}_{1M}$). Although the NMR spectroscopic data refer to a slight excess of ligand, and they cannot be directly transferred to the equimolar solutions discussed above, the distinct effect of Cu^{II} ions on the protons of both His residues (see Figure 5) strongly supports the involvement of two imidazoles in the metal coordination at acidic pHs. The obtained complex is similar to that formed by the protected peptide,[11] except for the involvement of the negatively charged carboxylate of adjacent ^{14}Asp in the coordination sphere. In addition, molecular mechanics calculations[23] performed on the Cu^{II}/TLEGTKKGHKLHLDY complex with the suggested $\{2N_{Im}, O^-\}$ donor set gave the low energy structures shown in Figure 9.

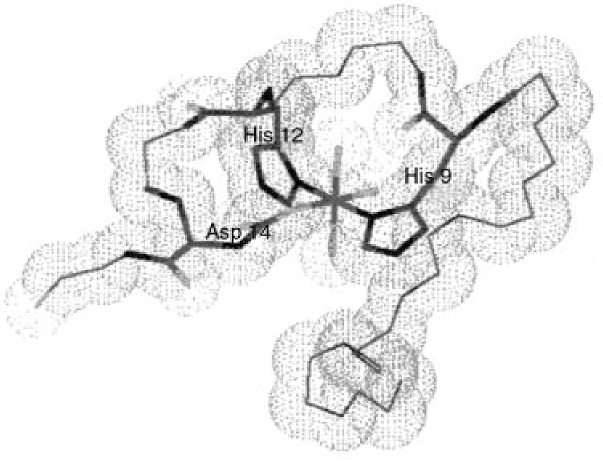

Figure 9. HYPERCHEM structure hypothesis for the Cu^{II}/TLEGTKKGHKLHLDY complex at pH 5.6.

At pH 10.4, ratios as high as 1:25 or 1:5 can be reached, which are consistent with slower off-rates arising from a more complicated exchange process. The observed spin−lattice relaxation rate enhancements (Figure 7) and line broadenings observed on the ^{1}H (Figure 6) and ^{13}C NMR spectra (see Supplementary Material) suggest the presence of two different complexes. In the first complex, copper is bound to ^{9}His and coordination takes place in the *N*-terminal direction, thus involving the ^{8}Gly and ^{7}Lys residues in the same way as with the protected peptide.[11] In the second complex the *N*-terminal group acts as an anchoring point and copper is bound to the amino nitrogen of ^{1}Thr and to the three amide nitrogens of the following residues (^{2}Leu, ^{3}Glu, ^{4}Gly). Moreover the effects measured on R_{1p} of ^{12}His, ^{14}Asp and ^{15}Tyr may result from the accumulation of the negative charges on the C-terminal side of the peptide (Tyr-O$^-$, Asp-COO$^-$) and their proximity to paramagnetic centre.

Experimental Section

TLEGTKKGHKLHLDY was synthesised manually on a 0.1 millimol scale by the solid-phase method, using the Fmoc/But amino acids,[24,25] according to the following procedure: (i) 5 and 15 min deprotection steps using 20% piperidine in DMF in the presence of 1% Triton; (ii) coupling reactions carried out with the protected amino acid diluted in DMF/NMP (NMP = *N*-methylpyrrolidone) (1:1, v/v) in the presence of 1% Triton, using DIC (1,3-diisopropylcarbodiimide) as the coupling reagent in the presence of HOBt (Fmoc-AA/DIC/HOBt, 1:1:1) for 1.5 h. The completeness of each coupling reaction was monitored by the chloranil and the ninhydrin tests.[26] The protected peptidyl resin was treated with a mixture of 95% trifluoroacetic acid (TFA), 2.5% water and 2.5% TIS (triisopropylsilane) for 1 h. The cleaved peptide was precipitated with diethyl ether and lyophilised. It was then purified by the solid-phase extraction (SPE) as described previously.[27] The product was homogeneous according to TLC and was judged to be more than 98% pure by HPLC (see Supplementary Material, Figure 3). The molecular weight was checked by FAB MS, using an AMD-604 spectrometer. The ion source was equipped with a Cs$^+$ gun. The energy of the Cs$^+$ ions was 12 keV.

Aliquots (2.5 cm^3) of sample solution, containing suitable amounts of the metal ion, the ligand, HNO_3 (when the initial pH value needed to be lowered) and KNO_3 (to adjust the ionic strength to 0.1 mol·dm^{-3}) were titrated potentiometrically with standard NaOH in a thermostatted potentiometric vessel (pH range: 3.5−11.0; T = 298.2 K). The metal ion concentration was close to $1\cdot10^{-3}$ mol·dm^{-3} and the metal-to-ligand ratio was 1:1.2. Two pairs of potentiometric titrations, for both protonation and complex-formation, were performed. Further experimental details, equipment and software used are reported elsewhere.[14] Thermodynamic data are given with an estimated accuracy reported as the uncertainty on the last significant figure.

CD, EPR and UV/Vis spectra were recorded on a JASCO J 715, on a Bruker ESP 300E (at, 120 K and 9.4 GHz) and on a Beckman DU 650 spectrometer, respectively. Sample solutions had the same composition as above; the pH was varied by addition of suitable amounts of standard NaOH or HCl, under potentiometric control. Spectra were recorded every 0.5 pH units.

Calorimetric titrations were performed with an Isoperibolic Calorimeter (Tronac) at 298.2 K, by addition of standard HNO_3 to a sample solution (2.5 cm^3) of the same composition as above. For each system two pairs of calorimetric titrations containing not less than 250 experimental points were used to calculate the thermodynamic quantities.

NMR spectra were performed at 14.1 T with a Bruker Avance 600 MHz Spectrometer at controlled temperatures (±0.1 K). Two different peptide solutions were prepared: the first with deionized water containing D_2O (10%), the second with deuterium oxide (99.95% from Merck), and both were carefully deoxygenated through a freezing/vacuum pumping/sealing/thawing procedure. The pH was adjusted to the desired values with either DCl or NaOD, and the required concentration of copper ions was achieved by using a stock solution of copper nitrate (Sigma Chemical Co.) in deuterium oxide. TSP-d_4, 3-(trimethylsilyl)-[2,2,3,3-d_4] propansulfonate, sodium salt, was used as an internal reference standard. The assignment of the spectra was accomplished with TOCSY, COSY, and NOESY 2D experiments. TOCSY spectra were recorded with a total spin-locking time of 75 ms using a MLEV-17 mixing sequence; NOESY spectra were obtained with standard pulse sequences at mixing times ranging between 50 and 400 ms. During all 2D experiments, water suppression was achieved by the Watergate method.[28] The spectral width of homonuclear 2D experiments was typically 6000 Hz in both F_1 and F_2 dimensions. Spin−lattice relaxation rates were measured with inversion recovery pulse sequences. The same sequence was also used to measure the single- or double-selective relaxation rates by means of suitably shaped π-pulses instead of the usual non-selective π-pulse. All rates were calculated by regression analysis of the initial recovery curves of longitudinal magnetization components, leading to errors not larger than ±3%. 2D $^{1}H-^{13}C$ shift correlation methods were used to detect and assign ^{13}C NMR spectra at relatively low concentrations. HMBC (multiple-bond heteronuclear multiple-quantum coherence) spectra were obtained with standard pulse sequences, over a spectral width of 6000 Hz in the F_2 dimension and 31700 Hz in the F_1 dimension a total of 1024 points were used in F_2 and 256 in F_1, which was zero filled once before processing; 8 transients were accumulated with a relaxation delay of 2.0 s for each of the 256 increments. A 5-mm broad-band probe was used to perform the ^{13}C NMR spectra, which were acquired with a FID composed of 32768 data points over a spectral width of 28700 Hz (acquisition time 0.570 s), a 90° pulse of 8.5 µs, relaxation delay of 5.0 s; the WALTZ16 broad-band decoupling method was used.[29,30]

Molecular structures were generated by the HYPERCHEM software package[23] implemented on a Pentium 120 MHz PC by using the ZINDO-1 semi-empirical method for charge calculations and the MM+ force field for molecular mechanics and dynamics calculations.

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