Metal Ion Effects on the *cis*/*trans* Isomerization Equilibrium of Proline in Short-Chain Peptides: A Solution NMR Study

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The effect of copper(II) ions on the probabilities of existence of the four detectable conformers of the tetrapeptide Tyr-Pro-Phe-Pro (β-casomorphin 4) in [$^2H_6$]DMSO was investigated by ^1H NMR spectroscopy. Integration of the Phe-NH signals provided the relative populations in the free state as tt/tc/ct/cc = *28:34:29:9 at 293 K (*c = cis, t = trans*). Copper(II) was shown to bind to all four isomers, yielding complexes with two different structures, depending on the conformation of Pro^2. The interpretation of paramagnetic relaxation rates of Pro^2-Hα signals provided the corresponding isomeric probabilities in the metal-bound state as 13:36:20:31. The observed stabilization of the conformation with the lowest probability of existence (*cc*) may be relevant for the biological role of copper and other metal ions.*

KEYWORDS:

bioinorganic chemistry · copper · NMR spectroscopy · peptides · proline

Introduction

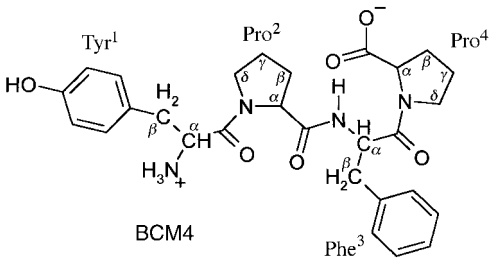
The effect of metal ions on peptide structure may profoundly influence the biological activities of the latter. Several earlier studies have shown that proline is a critical residue for secondary structure even in oligopeptides and also that it influences the interaction of the peptides with metal ions.

The main well-known feature of proline is that both *cis* and *trans* conformations are relatively stable at temperatures above 0 °C,[1] the energy barrier being large enough (> 20 kcal mol^{-1})[2] to yield relatively slow interchange rates. The *cis*/*trans* isomerization of proline-containing sequences has been shown to be relevant for (i) design of recognition sites,[3] (ii) protein folding,[4] (iii) formation and operation of channels by integral membrane proteins,[5, 6] and also (iv) mitosis regulation.[7]

The occurrence of *cis* or *trans* isomers is of course also relevant for natural or synthetic peptides having biological activity. The relatively high energy barrier and slow kinetics in fact impede binding of the "wrong" isomer to the receptor. Proteolytic enzymes, for example, selectively cleave the *trans* isomer.[8]

In order to ascertain whether metal ions may affect the *cis* ⇌ *trans* equilibrium, here we report NMR spectroscopic investigations of copper(II) ions interacting with Tyr-Pro-Phe-Pro (β-casomorphin 4, BCM4) that belongs to the family of exogenous opioid peptides isolated from enzymatic digests of the bovine milk protein β-casein.[9] This system has been chosen for the following reasons:

1. Several peptides having Tyr-Pro at the N terminus are highly active, particularly as releasing factors, neurotransmitters, or opiates in the central nervous system.
2. Copper is an essential element, found in relatively high concentrations in the brain, that, in its labile form, is generally complexed by amino acids or peptides.
3. Metal ions affect aminopeptidase-P, an exopetidase identified in pig kidney, capable of releasing the N-terminal amino acid from peptides with a penultimate proline residue.[10]



BCM4

Copper complexes of small peptides containing proline have already been the object of several spectroscopic and potentiometric investigations,[11–20] but, to our knowledge, without focusing on the effect of the metal on the *cis*/*trans* isomerization, most likely due to difficulties in detecting the effects on any single isomer. Here we show that high-field NMR spectroscopy allows to simultaneously investigate metal complex formation

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for any of the four stable isomers of a peptide with two rotationally hindered peptide bonds.

Results and Discussion

The NMR chemical shifts of BCM4 (10 mM in [D_6]DMSO) are summarized in Table 1. As expected and clearly shown in Figure 1, slow exchange allows to observe separate signals for each of the four isomers labeled *tt*, *tc*, *ct*, and *cc* (*c* = *cis*, *t* = *trans*). The completed assignments were based on the following:

1. Tyr and Phe residues were differentiated in ROESY spectra in which aromatic protons are connected to $H_{\beta\beta'}$;
2. Pro-$H_{\beta\beta'}$ and Pro-$H_{\gamma\gamma'}$ were assigned in COSY spectra;
3. *trans* or *cis* conformers were distinguished by the sequential NOE between Pro-H_δ or Pro-H_α with H_α of the preceding residue;
4. ROESY exchange cross peaks were connecting signals differing for the *trans* or *cis* conformation of the following proline;
5. all sets of signals could be subdivided into two groups by the *trans* or *cis* state of the following proline.

The probabilities of existence were determined by integrating the separated signals of Phe-NH groups (Figure 1), yielding *tt*/*tc*/*ct*/*cc* = 28:34:29:9 (at 293 K in [D_6]DMSO), in fairly good agreement with previous reports.[8, 24]

Upon addition of Cu^{II} (up to 1.3 μM), several signals were affected with consequent selective line broadening (also shown in Figure 1) and enhancement of longitudinal and transverse nuclear relaxation rates. The observed longitudinal paramagnetic relaxation rates for each isomer are summarized in Table 2 as R_{1p} values ($R_{1p} = 1/T_{1obs} - 1/T_{1free}$). Transverse paramagnetic relaxation rates are not included because, in most cases, they were too fast to be measured with accuracy. However, it was ascertained that R_{2p} was much faster than R_{1p} in all measurable cases (for example, R_{2p}/R_{1p} was measured as 12.2 for *tt*-^{3}Phe-H_α and as 9.8 for *ct*-Pro^2-H_α). The temperature dependence of R_{1p} is shown in Figure 2 for selected signals. Exchange averaging of relaxation rates in the metal-bound environment and in the bulk can be accounted for by Equation (1),[25]

$$R_{ip} = \frac{p_i}{(T_{ib} - \tau_b)} \quad (i = 1, 2) \quad (1)$$

where T_{ib}^{-1} is the nuclear relaxation rate of copper-bound protons, p_i is the fraction of metal-bound peptide, and τ_b^{-1} is the dissociation rate of the metal complex. In the case of a slow exchange regime ($\tau_b \gg T_{ib}$), $R_{1p} =$

Table 1. *^{1}H NMR chemical shifts (δ) and probabilities of existence (P*) of conformational isomers of BCM4 (10 mM in [D_6]DMSO at T = 293 K).*

	Conformation			
	tt	*tc*	*ct*	*cc*
Tyr^1-H_α	4.176	4.260	3.354	3.563
Tyr^1-H_β	2.835	2.929	2.581	2.949
	2.513	2.812	2.554	2.684
Tyr^1-H_{ortho}	7.074	7.124	6.917	6.924
Tyr^1-H_{meta}	6.700	6.700	6.700	6.700
Tyr^1-OH	9.257	9.257	9.257	9.257
Pro^2-H_α	4.370	4.571	3.583	3.911
Pro^2-H_β	1.951	1.860	1.636	1.460
		1.804		1.387
Pro^2-H_γ	1.746	1.628	1.512	1.173
Pro^2-H_δ	3.580	3.663	3.445	3.440
	3.187	3.217	3.174	3.238
Phe^3-NH	7.900	7.350	8.321	7.825
Phe^3-H_α	4.703	4.636	4.739	4.596
Phe^3-H_β	3.027	2.915	2.966	2.889
	2.818	2.841	2.874	2.838
Phe^3-H_{ortho}	7.292	7.230	7.282	–
Pro^4-H_α	4.361	3.661	4.381	3.810
Pro^4-H_β	2.008	2.008	2.143	1.938
Pro^4-H_γ	1.894	1.732	1.894	1.807
	1.859	1.405	1.859	
Pro^4-H_δ	3.637	3.414	3.637	3.611
	3.431	3.270	3.431	3.237
*P**	0.28	0.34	0.29	0.09

R_{2p} and R_{1p} increases with temperature. The temperature dependence and the observed R_{2p}/R_{1p} values therefore demonstrate that all four peptide ligands rapidly exchange between the free and metal-bound environments.[25] The Solomon – Bloembergen – Morgan (SBM) theory was therefore applied for interpretation of R_{1p} values of the four copper complexes.[26–28] The

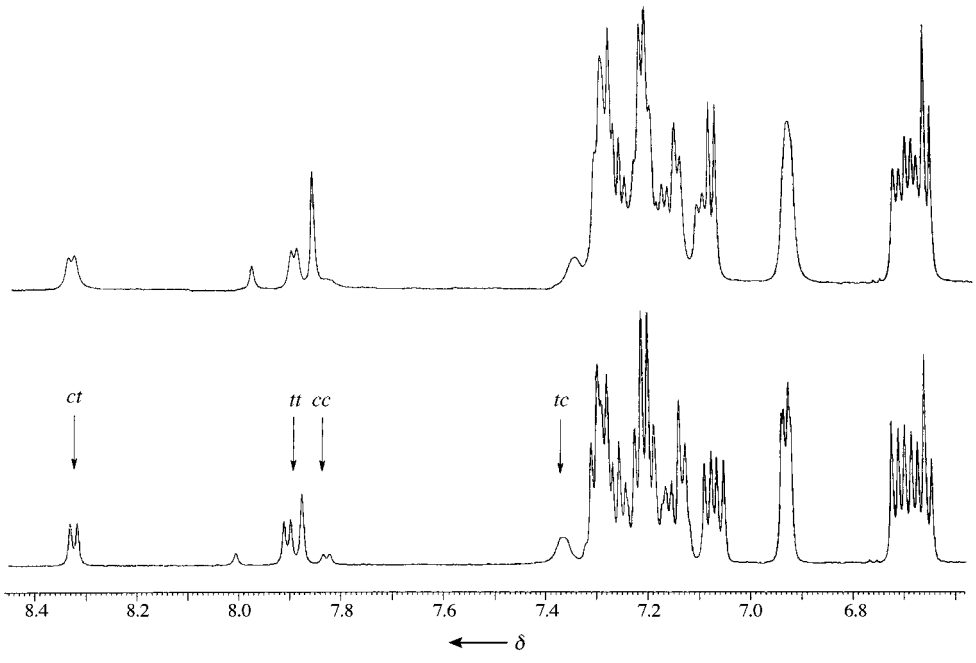

Figure 1. *Low-field regions of the 600-MHz ^{1}H NMR spectra of BCM4 (10 mM in [D_6]DMSO) before (lower trace) and after the addition of 1.3 μM Cu^{2+} (upper trace). The Phe-NH signals assigned to each of the four isomers are indicated by the arrows.*

Table 2. *^{1}H NMR paramagnetic relaxation rates R_{1p} measured for all conformational isomers of BCM4 in [D_6]DMSO in the presence of Cu^{2+} and metal–proton distances r in the corresponding copper complexes.*[a]

	R_{1p} [s^{-1}]				r [nm]			
	tt	*tc*	*ct*	*cc*	*tt*	*tc*	*ct*	*cc*
Tyr^1-H_α	1.012	2.478	3.460	5.024	0.275	0.281	0.241	0.244
Tyr^1-H_β	3.009	5.781	1.501	2.049	0.230	0.244	0.277	0.283
Tyr^1-$H_{\beta'}$		2.777	3.961			0.250	0.253	
Pro^2-H_α	2.274	5.372	3.460	4.702	0.241	0.247	0.241	0.246
Pro^2-H_δ	1.952	5.505			0.247	0.246		
Pro^2-$H_{\delta'}$	2.488	4.334			0.237	0.256		
Phe^3-NH			3.638	5.043			0.239	0.243
Phe^3-H_α	1.329	4.764	1.379	1.911	0.263	0.252	0.281	0.286
Phe^3-H_β	1.004	3.293	4.348	7.243	0.276	0.268	0.232	0.229
Phe^3-H_{ortho}	1.133	2.883			0.270	0.274		
Pro^4-H_α	1.064	2.760	1.377	1.871	0.273	0.276	0.281	0.287
Pro^4-H_δ	3.116	7.243	2.096	3.518	0.228	0.235	0.262	0.258
Pro^4-$H_{\delta'}$	1.121	2.325			0.271	0.284		

[a] [BCM4] = 10 mM, [Cu^{2+}] = 1.3 μM, T = 293 K.

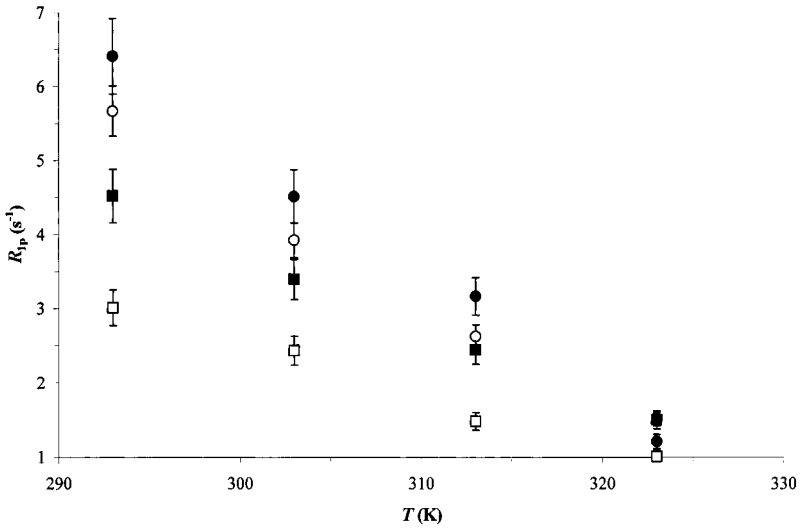

Figure 2. *Temperature dependencies of the paramagnetic contributions (R_{1p}) to the longitudinal relaxation rates of selected protons (● = tt-Pro^4-H_α, ○ = tc-Pro^2-H_α, ■ = ct-Tyr^1-H_α, □ = cc-Pro^2-H_α) of BCM4 (10 mM in [D_6]DMSO) in the presence of 1.3 μM Cu^{2+}.*

value of $R_{2p}/R_{1p} \gg 1$ thus verified is consistent with a dominant contribution to the longitudinal paramagnetic relaxation rate from the electron–nucleus dipole–dipole interaction; R_{1p} values therefore provide structure-sensitive parameters by using the following simplified equation [Eq. (2)];[29–32]

$$R_{1p} = 0.1\, p_b \frac{\gamma_I^2 \gamma_S^2 \hbar^2}{r^6} \left\{ \frac{3\,\tau_R}{1 + \omega_I^2 \tau_R^2} \right\} \quad (2)$$

where p_b is the fraction of metal-bound ligand, γ_I and γ_S are the nuclear and electron magnetogyric ratios, respectively, r is the metal–hydrogen distance, ω_I is the nuclear Larmor frequency, and τ_R is the motional correlation time of the complex.

Occurrence of four isomers in a solution containing an exceedingly small concentration of copper is very likely to yield several 1:1 and 1:2 species in equilibrium. Thus, Equation (3) must be considered for interpreting the longitudinal paramagnetic relaxation rates:

$$R_{1p} = 0.3\, \gamma_I^2 \gamma_S^2 \hbar^2 \sum_k \frac{p_{kB}}{r_k^6} \left\{ \frac{3\,\tau_{kR}}{1 + \omega_I^2 \tau_{kR}^2} \right\} \quad (3)$$

where r_k and τ_{kR} are the metal–nucleus distance and the rotational correlation time in the k-th species having the probability of existence p_{kB}.

Some insights into the composition of the mixture were obtained by measuring the d–d band energy in UV/Vis experiments. In copper(II)-peptide solutions, binding of amide nitrogen atoms causes a stepwise blue shift and energy increase of the d–d band.[11] The visible spectra recorded for a solution containing 1 mM Cu^{II} in [D_6]DMSO in the presence of BCM4 (1 to 400 mM) where all showing a d–d transition centered at 656 nm with $\varepsilon_{mol} \sim 30-40$. These findings exclude binding of more than two nitrogen atoms to copper in any case since $\varepsilon_{mol} >$ 100 would be obtained for d–d bands centered at 510–580 nm.

Further information was gained by measuring the proton spin–lattice relaxation rate of DMSO in a copper(II) solution upon addition of increasing concentrations of peptide (Figure 3). The effect of BCM4 was compared with that of carnosine (β-alanyl-L-histidine), which is well known to form 1:2 complexes at high ligand/copper ratios.[29, 31] The fast relaxation rate measured in a 0.2 mM solution of copper perchlorate is slowed by the addition of ligand as a consequence of solvent displacement from the metal coordination sphere. It is apparent that BCM4 is not as effective as carnosine in reducing the solvent relaxation rate, such that solvent molecules are retained in the coordination shell even at an exceedingly high ligand/metal ratio of 100:1. It was therefore concluded that 1:1 complexes are the prevailing species in our case; this is also supported by the observation that bis-complexes are not formed in measurable concentration in solutions containing a relatively high concentration of simple tripeptides.[33]

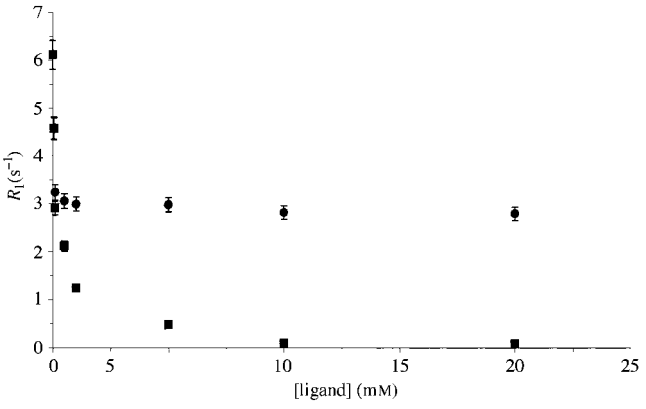

Figure 3. *Spin–lattice relaxation rates (at T = 300 K) of DMSO protons in the presence of 0.2 mM Cu^{2+} measured at increasing concentrations of either BCM4 (●) or carnosine (■).*

The obtained R_{1p} values can be grouped according to the conformation of Pro^2, thus suggesting the structure of the metal complex to be determined by the *cis* or *trans* conformation of this residue. The main differences between the two groups involve the high-field Tyr^1-H$_{\beta'}$, both Pro^2-H$_\delta$, Phe^3-NH, Phe^3-H$_{ortho}$, and the high-field Pro^4-H$_{\delta'}$ signals. Paramagnetic relaxation rates allow a qualitative delineation of metal-binding groups. The absence of paramagnetic effects on Phe^3-NH in the *tt* and *tc* isomers is a consequence of the binding of the ionized amide nitrogen atom to copper. In these isomers, as well as in the others, the metal is also coordinated by the terminal NH_2 and the carboxylate groups. These findings are consistent with earlier observations for several X-$_{trans}$Pro-X′ sequences at the N terminus of short-chain peptides.[12–14, 18] However, it is evident that the X-$_{cis}$Pro-X′ sequence behaves differently since the X′ amide group cannot contribute to binding the metal center.

In order to verify this interpretation, a hydrated copper ion was linked to the NH_2 group in molecular models of either Tyr-$_{trans}$Pro-Phe or Tyr-$_{cis}$Pro-Phe, calculated with the HYPERCHEM molecular graphics package.[23] The two complexes were then subjected to 25 ps of restrained molecular dynamics (MD) at 300 K with the MM+ force field. Five structures per picosecond were sampled over the MD run, some of which were superimposed as shown in Figure 4. It turned out that only the *trans* isomer allows a favorable coordinating location for the amide nitrogen atom.

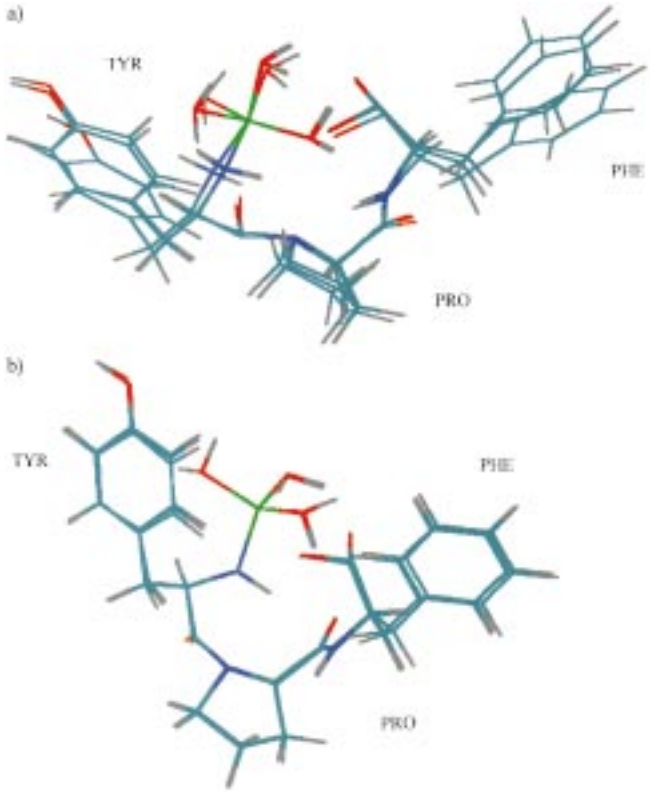

Figure 4. *Superposition of three low-energy stick models of the copper complexes of a) Tyr-$_{trans}$Pro-Phe and b) Tyr-$_{cis}$Pro-Phe. The copper ion is drawn in green and the coordination sphere has been completed by water molecules. All other atoms are in the CPK colors.*

When structural information is sought, *r* values can be only obtained from R_{1p} values, provided that p_b and τ_R are independently known. The rotational correlation time was evaluated by the frequency dependence of paramagnetic relaxation rates. In fact, measuring R_{1p} at three Larmor frequencies (600, 500, and 200 MHz) unambiguously provided $\tau_R = 0.20 \pm 0.01$ ns at 293 K. This value agrees with the one measured for the free peptide in the same solvent. The ratio between nonselective (nsel) and selective (sel) relaxation rates of NH and H$_\alpha$ protons was in fact measured as $R^{nsel}/R^{sel} = 1.17$ at 600 MHz, yielding $\tau_R = 0.20 \pm 0.02$ ns at 293 K.[22]

As for p_b, the usual approximation, $p_b = [Cu^{2+}]_{total}/[ligand]_{total}$, may be misleading for BCM4 which exists in four isomeric species. Maintenance of the same fractional populations as in the free state is not obvious; moreover, the exceedingly small fraction of copper-bound peptide (ca. 0.01) prevents a direct calculation of isomeric populations in the NMR spectra. The following procedure was therefore applied. Substitution of the correlation time and other constant values yields $p_{xx}/r^6 = 2.95 \times 10^{44} R_{1p}$ (p_{xx} is the probability of existence of any metal-bound isomer). This equation allowed the calculation of the iso-R_{1p} curves for Pro^2-H$_\alpha$ shown in Figure 5. The plots indicate that

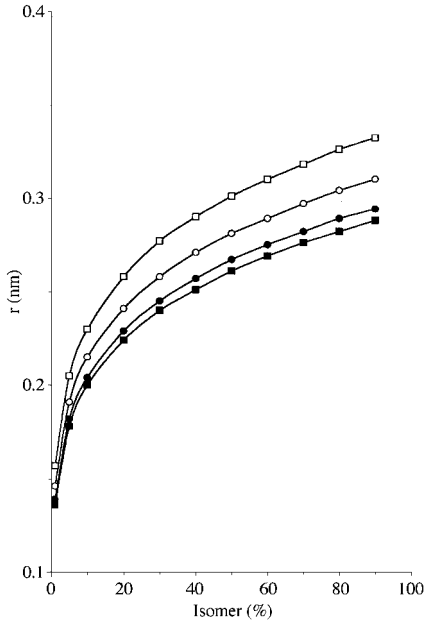

Figure 5. *Iso-R$_{1p}$ curves calculated for Pro^2-H$_\alpha$ of the four isomers of BCM4 in the presence of copper (□ = tt, ■ = tc, ○ = ct, ● = cc).*

populations lower than 10% (as for *cc* in the free state) would provide distances shorter than 0.2 nm that are not consistent with any molecular model. It follows that isomeric populations are not equal in the free and metal-bound states. It was, however, considered that:

a) the distance between copper and Pro^2-H$_\alpha$ does not change in *tt*– and *tc* – metal complexes, hence $p_{tc}/p_{tt} = R_{1p}(tc)/R_{1p}(tt) = 2.82$;
b) the distance between copper and Pro^2-H$_\alpha$ does not change in *ct* – and *cc* – metal complexes, hence $p_{cc}/p_{ct} = R_{1p}(cc)/R_{1p}(ct) = 1.51$;

c) $p_{tt} + p_{tc} + p_{ct} + p_{cc} = 1$, hence $p_{ct} = 0.40 - 1.52\,p_{tt}$, which yields $p_{tt} \leq 0.20$;
d) with p_{tt} in the range of 0.10 – 0.20, the corresponding Cu – H_α distance falls in the range of 0.23 – 0.26 nm; the respective range for p_{ct} is 0.10 – 0.25, which yields 0.22 – 0.25 nm for the Cu – H_α distance.

It was therefore concluded that the distance Cu – Pro^2-H_α does not differ much between the two structures. An evaluation of the probabilities of existence was therefore accomplished: The obtained values were $p_{tt} = 0.13 \pm 0.02$, $p_{tc} = 0.36 \pm 0.05$, $p_{ct} = 0.20 \pm 0.03$, and $p_{cc} = 0.31 \pm 0.05$. With this set of values all copper – proton distances were calculated (Table 2) and used as constraints in the molecular dynamics calculations. As in the previous case, the four copper complexes were subjected to 25 ps of restrained molecular dynamics at 300 K with the MM+ force field. Five structures per picosecond were sampled over the MD run. Three of the low-energy structures were superimposed (Figure 6). Metal binding of the ionized amide nitrogen following the "breaking" proline is a feature limited to the *trans* conformation of the X-Pro bond, whereas the *cis* conformation leads to the formation of a macrocyclic complex.

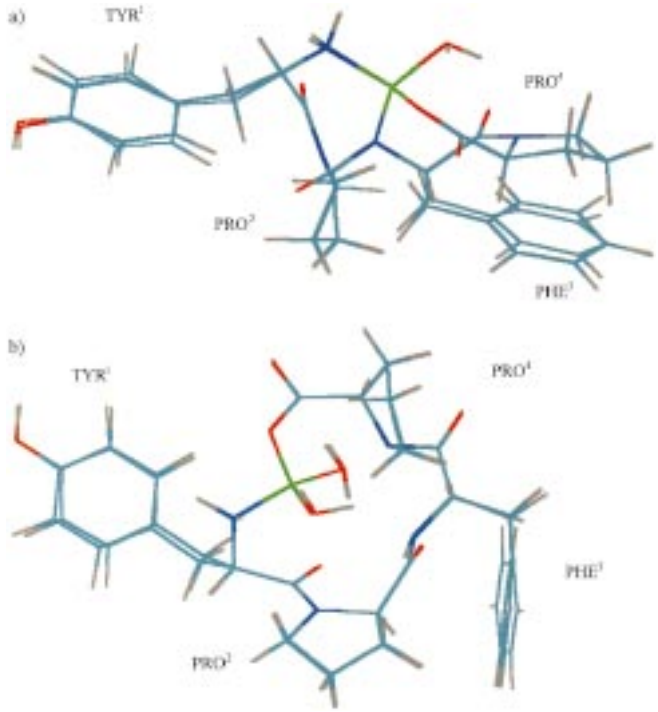

Figure 6. *Superposition of three low-energy stick models of the copper complexes of a)* tt *or* tc *and b)* ct *or* cc *isomers of BCM4. The copper atom is drawn in green and the coordination sphere has been completed by water molecules. All other atoms are in the CPK colors.*

It is worth noticing that the distribution of isomeric populations is changed by copper ions that apparently stabilize the least favored isomer. This may be relevant to all biological processes requiring recognition of any specific isomer, which can be aided or inhibited by the presence of copper or, most probably, other metal ions in the same environment. The biological significance of the phenomena we observed is not diminished by the choice of the solvent, although extension of the data obtained in DMSO to water is not straightforward. A *cis – trans* equilibrium is also present in water, where solute – solvent interactions may succeed in stabilizing definite isomers and, from this point of view, metal effects observed in DMSO are very likely to also play a role in an aqueous environment. Moreover, DMSO is thought to be a good solvent for mimicking the environment of living cells, in which many critical processes take place away from the bulk water (see, for example, ref. [34]).

Experimental Section

General: BCM4 was purchased from Bachem and used without further purification. Water solutions of the peptide had a pH of 6.7; 10 mM solutions were made in 100 % [$^2H_6$]DMSO (Merck) and carefully deoxygenated by a freezing – sealing – thawing cycle. A stock solution of $Cu(ClO_4)_2$ (Alpha Inorganics) (0.2 mM in [D_6]DMSO) was used for obtaining the desired copper concentration in the samples used for NMR spectroscopy. UV/Vis experiments were carried out with the Hewlett – Packard HP-8453 spectroscopy system at room temperature.

NMR spectroscopy: NMR experiments were carried out at 14.1 T (Bruker Avance 600), 11.7 T (Bruker DRX-500), and 4.7 T (Varian VXR-200) at controlled temperatures (± 0.2 K). A triple-resonance inverse $^1H - ^{13}C$ broad-band observe (BBO) probe was used at 600 MHz, direct detection probes were used in the other cases. Chemical shifts were referenced to tetramethylsilane (TMS) as internal standard. COSY and NOESY 2D NMR spectra were obtained with standard pulse sequences. TOCSY experiments were acquired with total spin-locking times in the range of 50 – 100 ms by using an MLEV-17 mixing sequence. ROESY spectra were obtained with mixing times in the range of 50 – 100 ms and the radiofrequency strength for the spin lock field at values lower than 3.5 kHz. Spin – lattice relaxation rates were measured with inversion recovery pulse sequences and calculated by exponential regression analysis of the recovery curves of longitudinal magnetization components. Selective spin – lattice relaxation rates were measured with DANTE-z pulse trains,[21] and calculated in the initial rate approximation by linear regression analysis of the initial part of the recovery curve of the involved longitudinal magnetization component.[22]

Molecular dynamics calculations: 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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