# *cis–trans* Isomerization of β-casomorphin peptides bound to copper(II): integration of EPR and NMR studies †

**Riccardo Basosi, Nicola D'Amelio, Elena Gaggelli, Rebecca Pogni and Gianni Valensin**

*Department of Chemistry, University of Siena, Via A. Moro, Siena 53100, Italy*

***Received (in Cambridge, UK) 21st June 2000, Accepted 17th January 2001***
***First published as an Advance Article on the web 16th February 2001***

Copper complexes of β-casomorphin peptides (BCM-7, BCM-5, BCM-4) were investigated by EPR and NMR in DMSO-$d_6$ solutions. Speciation of copper among many of the possible isomers was apparent. Computer simulations of low and room temperature EPR allowed the number of co-ordinated nitrogens in the major species (2 for BCM-4 and BCM-5, 4 for BCM-7) to be inferred and a rotational correlation time of 0.18 ns at 298 K to be evaluated for all complexes. All isomers of BCM-4 and BCM-5 were shown to bind copper, but the resulting structures were strictly determined by the conformational state of $^2$Pro. The *trans*, rather than the *cis*, conformation was shown to allow binding of the deprotonated $^3$Phe-NH; the terminal amino and carboxylate groups provided the other binding groups in all cases. Structures were obtained by constrained molecular dynamics using copper–proton distances obtained from paramagnetic nuclear relaxation rates. In the case of BCM-7, only the *cis-cis-trans* and/or the *cis-cis-cis* isomers were not binding copper. The conformational state of each Pro was shown to drive formation of the copper–nitrogen bond within the immediately adjacent residue, leading to the complex having four co-ordinated nitrogens in the case of the *trans-trans-trans* isomer.

β-Casomorphins (BCM) are opioid peptides originally identified in enzymatic digests of the bovine milk protein β-casein (hence the name β-casein exorphins).[1] They share with other μ-receptor agonists the sequence Tyr-X-Phe at the N-terminus as opposed to the Tyr-X-X-Phe sequence typical of δ selectivity (*e.g.*, enkephalins).

The occurrence of proline in the sequence imposes structural constraints that may determine secondary structure, even in the case of short chain peptides[2] such as Tyr-Pro-Phe-Pro-$NH_2$.[3] Another important feature is that peptidyl-proline bonds yield both *cis* and *trans* conformations that are relatively stable at temperatures above 0 °C.[4] Sequences containing *n* prolines therefore exist as $2^n$ stable isomers, with the energy barrier being large enough (>20 kcal $mol^{-1}$)[5] to lead to relatively slow *cis–trans* isomerization rates. A kinetic constant $k = 6.6 \times 10^{-5}$ $s^{-1}$ has, for example, been calculated for Phe-Pro at 283 K and pH 8.4.[6] Whilst the four isomers of BCM-5 (Tyr-Pro-Phe-Pro-Gly) and BCM-4 (Tyr-Pro-Phe-Pro) have been isolated and characterized,[7] no report has appeared so far about the eight isomers of BCM-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile).

The interaction of copper with opioid peptides, as well as neuro-transmitters and neuro-modulators, has also been thoroughly investigated since the metal is unevenly distributed in the body; it is found at relatively high concentrations in the brain, although its function is still rather obscure. Interaction with BCM-5 and BCM-7 has been previously considered,[8] but this did not take the isomeric equilibrium into account.

The present study was undertaken with a view to exploiting all of the advantages of EPR and NMR experiments, carried out in the same sample, for determining the ability of Cu(II) to co-ordinate all of the isomeric species of BCM-7 and the smaller BCM-5 and BCM-4 fragments.

## Experimental

Bovine β-casomorphin peptides were purchased from Bachem and used without further purification. Solutions were made in [$^2H_6$]-DMSO 100% (DMSO-$d_6$, Merck) and carefully deoxygenated by a freezing–sealing–thawing cycle. A stock solution of $Cu(ClO_4)_2$ (Alpha Inorganics) in DMSO-$d_6$ was used for obtaining the desired copper concentration in the NMR and EPR samples.

The X-band EPR spectra were obtained with a Bruker 200D SRC X-band spectrometer. Microwave frequencies were measured with a XL Microwave Model 3120 counter (Jagmar, Krakow, Poland). The spectrometer was interfaced with a PS/2 Technical Instruments Hardware computer and the data acquired using the EPR data system CS-EPR produced by Stelar Inc., Mede, Italy. The spectra were preliminarily corrected for baseline drift using a simple procedure based on a cubic spline function.[9]

The EPR simulation program COSMOS was written by us and is described in detail elsewhere.[10] It can cover virtually all of the dynamical conditions of copper complexes, from "fast tumbling" to "incipient slow motion". The program used for simulation of frozen solution spectra was a modified version of that described by Pilbrow and co-workers.[11] Both programs allow the simultaneous occurrence of the two isotopes of copper to be considered.

The best fit of the experimental spectra was found using the Simplex and Levenberg–Marquardt optimization procedures. The tolerances in the magnetic parameters during minimization were consistent with estimated experimental errors. Once a good simulation was obtained, input data was used to optimize magnetic parameters until the quality of the fit was invariant to further changes.

NMR experiments were carried out at 14.1 T on a Bruker Avance DRX 600 spectrometer at controlled temperatures (±1 K). Chemical shifts were referenced to internal TMS. TOCSY experiments were acquired with total spin-locking times in the range 50–100 ms using an MLEV-17 mixing sequence. ROESY spectra were obtained at mixing times in the range 50–100 ms with the *rf* 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 the longitudinal

† Magnified regions of the $^1$H NMR spectrum of β-casomorphin are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b004964f/

 DOI: 10.1039/b004964f

**Table 1** Magnetic parameters obtained by computer simulation of the low temperature EPR spectra of copper complexes[a]

| | BCM-7 | BCM-5, BCM-4 |
|---|---|---|
| $g_{\parallel}$ | 2.250 | 2.278 |
| $g_{\perp}$ | 2.053 | 2.066 |
| $10^4\ A^{Cu}_{\parallel}/cm^{-1}$ | 178 | 169 |
| $10^4\ A^{Cu}_{\perp}/cm^{-1}$ | 8.62 | 6.75 |
| $10^4\ A^{N}/cm^{-1}$ | 12.5 | 13.3 |
| Number of nitrogens | 4 | 2 |
| $(g_{\parallel}/A_{\parallel})$/cm | 126 | 135 |

[a] Estimated errors are ±0.001 for $g$ and ±0.0001 $cm^{-1}$ for $A^{Cu}$.

magnetization components. Spin–spin relaxation rates were measured with Carr–Purcell–Meiboom–Gill pulse sequences and calculated by exponential regression analysis of the decay curves of the transverse magnetization components.

Molecular structures were generated using the HYPERCHEM software package,[12] as implemented on a Pentium-120 MHz PC, by means of the ZINDO-1 semi-empirical method (for charge calculations) and the MM+ force field (for molecular mechanics and dynamics calculations).

## Results and discussion

Peptides have several possible candidates for binding groups for copper. In the absence of donor-containing side chains, the metal ion usually binds to the deprotonated terminal amino group and to the deprotonated amide nitrogens of the second, third and possibly fourth residues.[13] The occurrence of other complexes and even of macrocycles has, however, been reported, and the speciation of copper as a function of pH, solvent, and concentration has been widely investigated. The presence of proline in position 2 determines a break point to metal.[13,14] Many possibilities have again been demonstrated[8,14,15] but no attention has so far been paid to the possible role of proline conformation.

Low temperature EPR spectra were typical of square planar copper complexes with all BCM derivatives. In the $g_{\parallel}$ region three of the four expected transitions were observed, with the fourth being masked in the $g_{\perp}$ region. Contributions from the different isotopes of copper were not resolved as it usually occurs in the case of the relatively broad bandwidth of nitrogen-bound copper. The best-fit magnetic parameters are summarized in Table 1.

All the low temperature EPR spectra showed additional peaks in the $g_{\parallel}$ region that were interpreted in terms of the speciation of copper among complexes having different magnetic parameters. However, the absence of the typical midfield transition[16] and the large excess of the ligand ruled out the presence of dimeric species and of solvated copper.

The low temperature EPR spectra of BCM-7 and BCM-4 are shown in Fig. 1 together with the computer simulations obtained from the best fit parameters reported in Table 1. The number of co-ordinated nitrogens of BCM-7 was verified by the comparison of the sensible portions of the room temperature fourier transformed experimental and simulated EPR spectra.[17] Theoretical fitting of EPR spectra at 298 K (Fig. 2) allowed the rotational correlation time to be evaluated at $0.18 \pm 0.01_5$ ns.

The EPR spectra of the copper complexes of BCM-5 and BCM-4 were virtually identical and the best fitting parameters obtained from computer simulation were consistent with a major species having two co-ordinated nitrogens.

The EPR data therefore suggests that (i) the co-ordination mode in the major copper complex of BCM-7 is different from that of BCM-5 and BCM-4; (ii) speciation of copper occurs and (iii) 1:2 or 2:2 species have negligible existential probabilities.

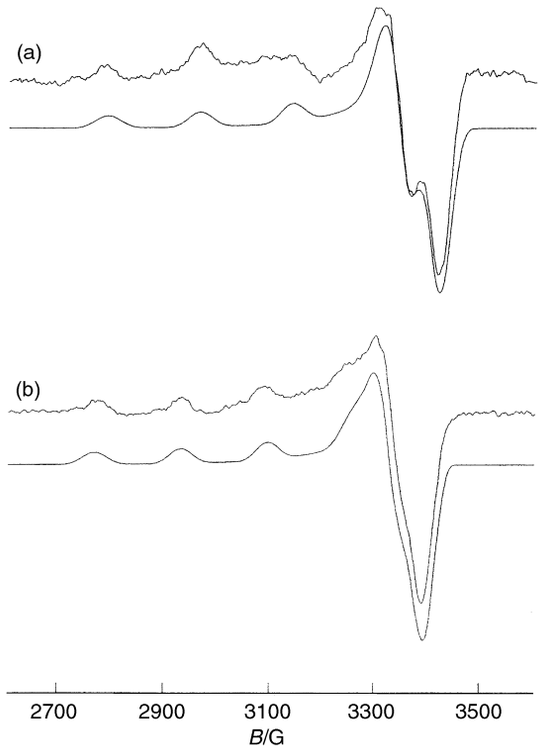


**Fig. 1** Experimental EPR spectra of copper complexes of (a) BCM-7 and (b) BCM-4 paired with the simulations giving the best fit. $T = 120$ K; $\nu = 9.627$ GHz.

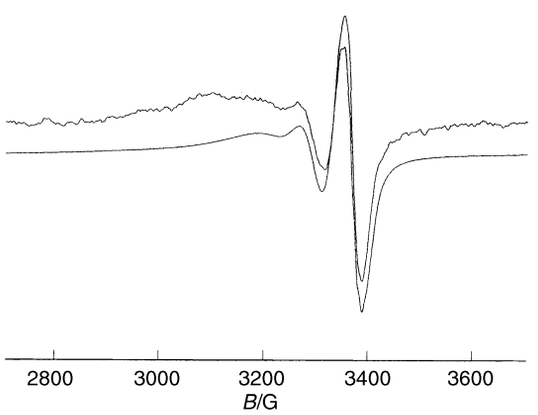


**Fig. 2** Room temperature EPR spectrum of the copper complex of BCM-7 paired with its best fit simulation. $T = 298$ K, $\nu = 9.619$ GHz.

The 600 MHz $^1$H-NMR spectrum of BCM-7 shows contributions from eight stable isomers, denoted *ttt*, *ttc*, *tct*, *ctt*, *tcc*, *ctc*, *cct*, *ccc,* where the letters *t* and *c* refer to the *trans* and *cis* conformations respectively. Even the TOCSY spectrum (Fig. 3) is not sufficient to resolve all the spin systems, especially those that belong to the 24 proline rings (3 prolines × 8 isomers). The final assignments were based on the following: (i) Tyr and Phe could be differentiated in ROESY spectra where aromatic protons are correlated to $H_{\beta\beta'}$; (ii) Pro $H_{\beta\beta'}$ and $H_{\gamma\gamma'}$ could be assigned in COSY spectra; (iii) ROESY exchange cross-peaks were connecting signals that differed for the *trans* or *cis* conformation of the following proline; (iv) all sets of signals could be subdivided into two groups according to the *trans* or *cis* state of the following proline; (v) *trans* or *cis* conformers could be distinguished by sequential NOEs. The *tt* isomer was identified by the occurrence of Tyr $H_{\alpha}$–$^2$Pro $H_{\delta\delta'}$ and Phe $H_{\alpha}$–$^4$Pro $H_{\delta\delta'}$ NOEs, *tc* by Tyr $H_{\alpha}$–$^2$Pro $H_{\delta\delta'}$ and Phe $H_{\alpha}$–$^4$Pro $H_{\alpha}$ NOEs, *ct* by Tyr $H_{\alpha}$–$^2$Pro $H_{\alpha}$ and Phe $H_{\alpha}$–$^4$Pro $H_{\delta\delta'}$ NOEs and *cc* by Tyr $H_{\alpha}$–$^2$Pro $H_{\alpha}$ and Phe $H_{\alpha}$–$^4$Pro $H_{\alpha}$ NOEs.

**Table 2** $^1$H-NMR chemical shifts (ppm) measured for β-casomorphin-7 (10 mmol dm$^{-3}$ in [$^2H_6$]-DMSO at $T$ = 298 K)

| | Conformation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *ttt* | *ttc* | *tct* | *tcc* | *ctt* | *ctc* | *cct* | *ccc* |
| $^1$Tyr $H_\alpha$ | 4.26 | | 3.75 | 3.68 | 3.34 | 3.31 | 3.07 | |
| $H_\beta$ | 2.93 | | 2.83–2.54 | 2.81–2.43 | 2.86–2.61 | 3.03–2.72 | 2.56 | |
| $H_{ortho}$ | 7.02 | | — | 7.02 | 6.90 | — | 6.86 | |
| $H_{meta}$ | 6.63 | | — | 6.63 | 6.63 | — | 6.62 | |
| $^3$Phe NH | 8.38 | 8.38 | 8.34 | 8.34 | 8.02 | 7.88 | | 7.88 |
| $H_\alpha$ | 4.57 | 4.51 | 4.71 | 4.73 | 4.51 | 4.70 | | 4.66 |
| $H_\beta$ | 2.96–2.87 | 2.96–2.87 | 3.04–2.86 | 3.00–2.88 | 2.87 | 3.04–2.81 | | 3.08–2.81 |
| $H_{ortho}$ | 7.15 | 7.15 | 7.23 | 7.23 | 7.15 | 7.23 | | 7.23 |
| $H_{meta, para}$ | | | | 7.07–7.03 | | | | |
| $^5$Gly NH | 8.15 | 8.13 | 7.84 | 7.88 | 7.86 | 7.72 | | |
| $H_\alpha$ | 3.87–3.76 | 4.44–3.61 | 4.01–3.79 | 4.01–3.79 | 3.87–3.48 | 3.97–3.42 | | |
| $^7$Ile NH | 8.16 | | 8.04 | | 7.95 | 7.90 | | |
| $H_\alpha$ | 4.14 | | 4.14 | | 4.09 | 4.09 | | |
| $H_\beta$ | 1.83 | | 1.83 | | 1.76 | 1.76 | | |
| $H_\gamma$ | 1.42 | | 1.42 | | 1.42 | 1.42 | | |
| $\gamma CH_3$ | 0.78 | | 0.78 | | 0.78 | 0.78 | | |
| $H_\delta$ | 0.88 | | 0.88 | | 0.83 | 0.83 | | |
| | *Trans* | | | | *Cis* | | | |
| $^{2,4,6}$Pro $H_\alpha$ | 4.58 | 4.44 | 4.34 | 4.23 | 3.70 | 3.63 | 3.58 | 3.45 |
| $H_\beta$ | 2.21 | 2.01 | 2.00 | 2.18 | 1.96 | 1.88 | 1.88 | — |
| $H_\gamma$ | 1.94–1.79 | 1.89 | 1.88–1.72 | 1.88–1.73 | 1.76–1.61 | 1.63–1.48 | 1.75 | — |
| $H_\delta$ | 3.43 | 3.63 | 3.59–3.34 | 3.37 | 3.18 | 3.52–3.44 | 3.33–3.25 | 3.73–3.17 |

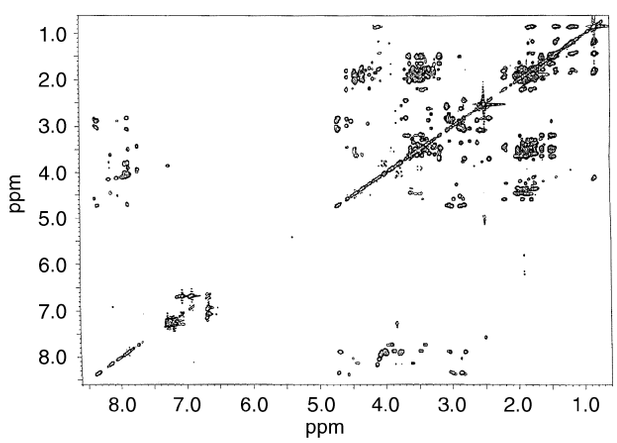


**Fig. 3** TOCSY spectrum of BCM-7 10 mM in DMSO-$d_6$; $T$ = 298 K.

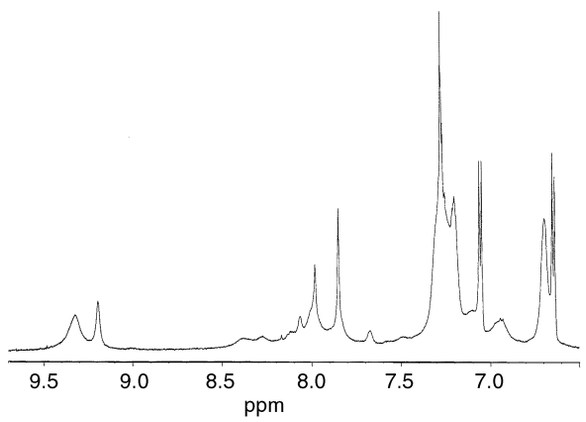


**Fig. 4** Low field region of the 600 MHz $^1$H-NMR spectrum of BCM-7 10 mM in DMSO-$d_6$ in the presence of 15 μM $Cu^{2+}$; $T$ = 298 K.

The assignments are summarized in Table 2. Addition of copper(II) determined extensive broadening of most NMR signals, especially at relatively high concentrations of the metal ion (Fig. 4). Interestingly, the hydroxy proton of tyrosine was split into at least two diversely broadened signals.

The NMR spectra of the smaller β-casomorphin fragments were completely assigned, as summarized in Table 3, by using the same delineated approach. In both cases it was possible to detect and integrate at least one set of resolved signals for each isomer. The approximate ratios of fractional populations were therefore evaluated at *tt*/*tc*/*ct*/*cc* : 53/12/26/9 for BCM-5 and 28/34/29/9 for BCM-4, which are in reasonable agreement with previous assessments.[7]

The addition of Cu(II) caused selective longitudinal relaxation rate enhancements, as shown in Table 4. It is apparent that all four isomers bind copper and that the *cis* or *trans* conformation of proline in position 2 determines the mode of binding. Transverse relaxation rate enhancements were also measured, yielding $R_{2p} \gg R_{1p}$ in all cases. The exchange process of peptide molecules between the metal-bound environment and the bulk solution was delineated by the temperature dependence of $R_{1p}$[18] and the data was consistent with fast exchange conditions for all the affected nuclear resonances, as expected in cases where $R_{2p}/R_{1p} \gg 1$ holds.[19]

The NMR data would appear to suggest that (i) all four isomers of BCM-4 and BCM-5 bind copper, as also do all but one of the eight isomers of BCM-7; and (ii) the conformation of $^2$Pro in BCM-4 and BCM-5 determines the structure of the copper complex. *Trans-trans* and *trans-cis* peptides in fact experience paramagnetic effects different from those of *cis-trans* and *cis-cis* isomers.

Structures of all copper complexes of BCM-4 and BCM-5 were solved by interpreting the nuclear relaxation rate enhancements in terms of the existing Solomon–Bloembergen–Morgan (SBM) theory.[20] The enhancement results from time averaging of nuclear relaxation rates in the metal co-ordination sphere and in the bulk [eqn. (1)], where $p_M$ is the fraction of

$$R_{ip} = \frac{p_M}{T_{iM} + \tau_M} \quad (i = 1,2) \qquad (1)$$

**Table 3** $^1$H-NMR chemical shifts (ppm) measured for β-casomorphin-5 (10 mM in [$^2H_6$]-DMSO) and β-casomorphin-4 (10 mM in [$^2H_6$]-DMSO) at $T = 298$ K

| | β-Casomorphin-5 conformation | | | | β-Casomorphin-4 conformation | | | |
|---|---|---|---|---|---|---|---|---|
| | *tt* | *tc* | *ct* | *cc* | *tt* | *tc* | *ct* | *cc* |
| $^1$Tyr $H_\alpha$ | 4.18 | 4.13 | 3.36 | 3.55 | 3.80 | 4.26 | 3.08 | 4.08 |
| $H_\beta$ | 2.96–2.81 | 2.97–2.73 | 2.76 | 2.89–2.84 | 2.83–2.51 | 2.93 | 2.58–2.55 | 2.95–2.68 |
| $H_{ortho}$ | 7.11 | 7.16 | 6.93 | 6.99 | 7.07 | 6.92 | 6.92 | 6.92 |
| $H_{meta}$ | 6.71 | 6.71 | 6.71 | 6.71 | 6.70 | 6.70 | 6.70 | 6.70 |
| OH | 9.46 | 9.46 | 9.46 | 9.46 | 9.26 | 9.26 | 9.26 | 9.26 |
| $^2$Pro $H_\alpha$ | 4.38 | 4.51 | 3.61 | 3.92 | 4.37 | 3.77 | 3.08 | 3.51 |
| $H_\beta$ | 2.00 | 2.00–1.79 | 1.65 | 1.70 | 1.95 | 1.86–1.80 | 1.64 | 1.46–1.39 |
| $H_\gamma$ | 1.77 | 1.78–1.69 | 1.50 | 1.75–1.61 | 1.75 | 1.63 | 1.51 | 1.17 |
| $H_\delta$ | 3.61–3.17 | 3.66–3.17 | 3.43–3.24 | 3.46–3.23 | 3.58–3.29 | 3.36–3.22 | 3.44–3.17 | 3.44–3.24 |
| $^3$Phe NH | 8.02 | 8.10 | 8.42 | 8.44 | 7.90 | 7.35 | 8.32 | 7.82 |
| $H_\alpha$ | 4.71 | 4.51 | 4.70 | 4.57 | 4.70 | 4.64 | 4.74 | 4.60 |
| $H_\beta$ | 3.08–2.84 | 3.08–2.84 | 3.09–2.83 | 2.91 | 3.03–2.82 | 2.91–2.84 | 2.97–2.87 | 2.89–2.84 |
| $H_{ortho}$ | — | — | — | — | 7.29 | 7.13 | 7.28 | — |
| Aromatic | 7.25–7.40 | | | | 7.16–7.38 | | | |
| $^4$Pro $H_\alpha$ | 4.36 | 3.79 | 4.36 | 3.90 | 4.26 | 4.06 | 4.26 | 4.50 |
| $H_\beta$ | 2.06–2.04 | 1.84 | 2.06–2.04 | 1.74 | 2.01 | 2.01 | 2.14 | 1.94 |
| $H_\gamma$ | 1.88 | 1.60–1.56 | 1.88 | 1.59 | 1.89–1.86 | 1.73–1.41 | 1.89–1.86 | 1.81 |
| $H_\delta$ | 3.61–3.52 | 3.43–3.25 | 3.61–3.52 | 3.44–3.24 | 3.64–3.43 | 3.41–3.27 | 3.64–3.43 | 3.61–3.24 |
| $^5$Gly NH | 8.01 | 8.32 | 8.06 | 8.29 | | | | |
| $H_\alpha$ | 3.76–3.74 | 3.66 | 3.79–3.71 | 3.68 | | | | |

**Table 4** $^1$H-NMR longitudinal relaxation rate enhancements ($R_{1p}$/s$^{-1}$, ±5%) measured for β-casomorphin-5 (10 mM in [$^2H_6$]-DMSO) and β-casomorphin-4 (10 mM in [$^2H_6$]-DMSO) upon addition of 1.3 μM $Cu^{2+}$ at $T = 298$ K

| | β-Casomorphin-5 conformation | | | | β-Casomorphin-4 conformation | | | |
|---|---|---|---|---|---|---|---|---|
| | *tt* | *tc* | *ct* | *cc* | *tt* | *tc* | *ct* | *cc* |
| $^1$Tyr $H_\alpha$ | 1.15 | | 6.09 | 1.67 | 0.67 | 1.85 | 5.91 | 1.53 |
| $H_\beta$ | 4.23 | 1.08 | 2.11 | 0.87 | 2.01 | 4.12 | 1.83 | 0.70 |
| $H_{\beta'}$ | | | 2.97 | 1.25 | | | 4.12 | 1.21 |
| $^2$Pro $H_\alpha$ | 1.77 | 0.57 | 3.64 | 1.53 | 1.15 | 1.76 | 3.12 | 1.48 |
| $H_\delta$ | 1.62 | | | | 0.81 | 1.99 | | |
| $H_{\delta'}$ | 2.31 | 0.62 | | | 1.26 | 1.60 | | |
| $^3$Phe NH | | | 7.49 | 2.19 | | | 4.82 | 1.62 |
| $H_\alpha$ | 1.33 | | 1.30 | 0.64 | 1.00 | 1.33 | 1.20 | 0.49 |
| $H_\beta$ | 1.65 | 0.55 | 3.67 | 1.67 | | | 6.65 | 1.78 |
| $H_{\beta'}$ | 3.77 | 0.97 | | | | | | |
| $H_{ortho}$ | | | | | 1.13 | 0.99 | 1.06 | 0.51 |
| $^4$Pro $H_\alpha$ | 1.16 | | 1.08 | | 1.06 | 1.71 | 3.12 | 0.87 |
| $H_\delta$ | 2.67 | 0.85 | 2.67 | 1.11 | 1.64 | 2.57 | | |
| $H_{\delta'}$ | 1.91 | 0.49 | 1.91 | | 1.12 | 1.98 | | |
| $^5$Gly NH | 1.20 | | 4.62 | 1.88 | | | | |
| $H_\alpha$ | 1.69 | 0.50 | 1.70 | 0.78 | | | | |
| $H_{\alpha'}$ | 1.90 | 0.59 | 1.93 | 0.80 | | | | |

metal-bound ligand, $T_{iM}$ is the nuclear relaxation time in the coordination sphere and $\tau_M = 1/k_{off}$. The temperature dependence of $R_{1p}$ and the occurrence of $R_{2p} \gg R_{1p}$ demonstrate that fast exchange conditions prevail at least for longitudinal relaxation rates ($\tau_M \ll T_{1M}$). This being the case, SBM theory provides eqn. (2) as the simplified expression for $R_{1p}$:[21]

$$R_{1p} = p_m \frac{\gamma_H^2 g_e^2 \beta_e^2}{10 r^6} \left\{ \frac{3\tau}{1 + \omega_H^2 \tau^2} \right\} \qquad (2)$$

In this equation $r$ is the proton–copper distance; $\omega_H = 3.77 \times 10^9$ rad s$^{-1}$ is the proton Larmor frequency; $\tau$ is the correlation time modulating the electron–nucleus dipole–dipole interaction; $\gamma_H = 2.6753 \times 10^4$ rad s$^{-1}$ G$^{-1}$ is the proton magnetogyric ratio; $g_e = 2.002322$ is the electronic $g$ factor; $\beta_e = 0.92731 \times 10^{-20}$ erg G$^{-1}$‡ is the Bohr magneton. The simplified equation usually holds for relatively small copper complexes where electron spin relaxation times yield negligible scalar contributions to $R_{1p}$.[19]

When distances are sought the other two unknowns, $p_M$ and $\tau$, must be independently obtained. The rotational correlation time is directly obtained in the simulation procedure of room temperature EPR spectra ($\tau = 0.18 \pm 0.01_5$ ns at 298 K in all cases). As for $p_M$, predominance of 1:1 complexes allowed the value, given by $C_{metal}/C_{peptide}$ weighted over the fractional population measured in the free state, to be assumed for each isomer. This assumption is justified by the small errors introduced in calculating distances. It can in fact be seen that a

‡ 1 erg = $10^{-7}$ J.

**Table 5** Copper–proton distances (nm, ±8%) calculated from $R_{1p}$ values measured at 298 K ($\tau = 0.18 \pm 0.01_5$ ns)

| | β-Casomorphin-5 conformation | | | | β-Casomorphin-4 conformation | | | |
|---|---|---|---|---|---|---|---|---|
| | *tt* | *tc* | *ct* | *cc* | *tt* | *tc* | *ct* | *cc* |
| $^1$Tyr $H_\alpha$ | 0.286 | | 0.192 | 0.201 | 0.281 | 0.245 | 0.196 | 0.203 |
| $H_\beta$ | 0.230 | 0.225 | 0.230 | 0.225 | 0.234 | 0.214 | 0.239 | 0.231 |
| $H_{\beta'}$ | | | 0.217 | 0.211 | | | 0.209 | 0.211 |
| $^2$Pro $H_\alpha$ | 0.266 | 0.250 | 0.210 | 0.204 | 0.257 | 0.247 | 0.219 | 0.204 |
| $H_\delta$ | 0.270 | | | | 0.272 | 0.242 | | |
| $H_{\delta'}$ | 0.255 | 0.247 | | | 0.253 | 0.251 | | |
| $^3$Phe NH | | | 0.186 | 0.192 | | | 0.203 | 0.201 |
| $H_\alpha$ | 0.279 | | 0.249 | 0.236 | 0.263 | 0.259 | 0.256 | 0.245 |
| $H_\beta$ | 0.269 | 0.252 | 0.209 | 0.201 | | | 0.193 | 0.198 |
| $H_{\beta'}$ | 0.235 | 0.229 | | | | | | |
| $H_{ortho}$ | | | | | 0.258 | 0.272 | | |
| $^4$Pro $H_\alpha$ | 0.285 | | 0.257 | | 0.260 | 0.248 | 0.262 | 0.244 |
| $H_\delta$ | 0.248 | 0.234 | 0.221 | 0.215 | 0.242 | 0.232 | 0.219 | 0.223 |
| $H_{\delta'}$ | 0.263 | 0.257 | 0.234 | | 0.258 | 0.242 | | |
| | | | | 0.197 | | | | |
| $^5$Gly NH | 0.284 | | 0.202 | | | | | |
| $H_\alpha$ | 0.268 | 0.256 | 0.238 | 0.228 | | | | |
| $H_{\alpha'}$ | 0.263 | 0.249 | 0.233 | 0.227 | | | | |

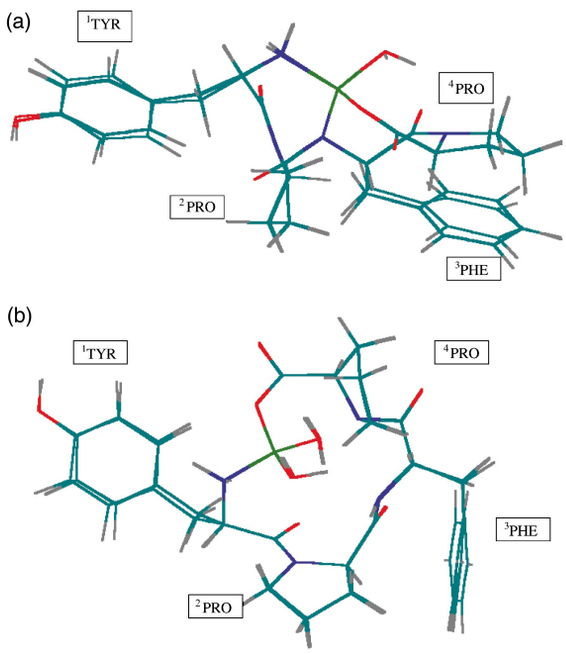


**Fig. 5** Superimposed stick models of some low-energy structures of the copper complexes of (a) *cis-cis* BCM-4 and (b) *trans-trans* BCM-4.

change in $p_M$ from 0.5 to 0.1 would modify the calculated $r$ by a factor of 1.3.

All proton–copper distances calculated by $R_{1p}$ values are summarized in Table 5. The two β-casomorphin fragments behave similarly, as also ratified by the absence of substantial differences in the EPR spectra. Consideration of the relative values of distances allows a subdivision of the four isomers of both peptides. The *trans* or *cis* conformation of $^2$Pro determines the binding mode; whereas the structure does not apparently depend upon the state of $^4$Pro.

The main observable difference is the absence of paramagnetic effects on $^3$Phe-NH in *trans-trans* or *trans-cis* isomers (Table 4). This suggests that this amide nitrogen is deprotonated and bound to copper in these cases only.

Molecular models of the complexes were obtained in the following way. A hydrated copper ion was added to an energy-minimized molecular model of each of the four isomers of either BCM-4 or BCM-5 and linked to the deprotonated terminal amino group. The eight complexes were then subjected to 25 ps restrained molecular dynamics at 300 K with the MM+ force field. Five structures per ps were sampled over the MD run. It emerged that the *trans-trans* and *trans-cis* isomers only allowed a favourable co-ordinating location of the amide nitrogen; whereas a copper–carboxylate interaction was evident in all cases, in agreement with the EPR evidence of a binding mode independent of the availability of the amide nitrogen of glycine. Copper was therefore bound to the carboxylate of all isomers and to the deprotonated nitrogen of *tt* and *tc* isomers, and the restrained molecular dynamics procedure was repeated. Some of the resulting structures are shown in Fig. 5. The RMSD value, calculated over the whole backbone, was 0.08 nm and the maximum violations lay in the range 0.02–0.04 nm. The average copper–proton distances, measured in the five lowest energy structures in the molecular model, are compared to those obtained from NMR relaxation analysis in Table 6 for two isomers of BCM-4. The agreement is rather good, especially if it is remembered that distances determined from NMR data are averaged over all the possible low- and high-energy conformational states in solution.

**Table 6** Copper–proton distances within the metal complexes of the *tt* and *cc* isomers of BCM-4 as measured (i) from paramagnetic relaxation rates ($r_{exp}$/nm) and (ii) in the five lowest energy structures of the minimized molecular model ($\langle r_{mod} \rangle$/nm)

| | *tt* | | *cc* | |
|---|---|---|---|---|
| | $r_{exp}$ | $\langle r_{mod} \rangle$ | $r_{exp}$ | $\langle r_{mod} \rangle$ |
| $^1$Tyr $H_\alpha$ | 0.281 | 0.291 | 0.203 | 0.210 |
| $^1$Tyr $H_\beta$ | 0.234 | 0.239 | 0.231 | 0.286 |
| $^1$Tyr $H_{\beta'}$ | | | 0.211 | 0.242 |
| $^2$Pro $H_\alpha$ | 0.257 | 0.265 | 0.204 | 0.230 |
| $^2$Pro $H_\delta$ | 0.272 | 0.278 | | |
| $^2$Pro $H_{\delta'}$ | 0.253 | 0.268 | | |
| $^3$Phe NH | | | 0.201 | 0.210 |
| $^3$Phe $H_\alpha$ | 0.263 | 0.287 | 0.245 | 0.234 |
| $^3$Phe $H_\beta$ | | | 0.198 | 0.224 |
| $^3$Phe $H_{ortho}$ | 0.258 | 0.326 | | |
| $^4$Pro $H_\alpha$ | 0.260 | 0.260 | 0.244 | 0.268 |
| $^4$Pro $H_\delta$ | 0.242 | 0.246 | 0.223 | 0.247 |
| $^4$Pro $H_{\delta'}$ | 0.258 | 0.269 | | |

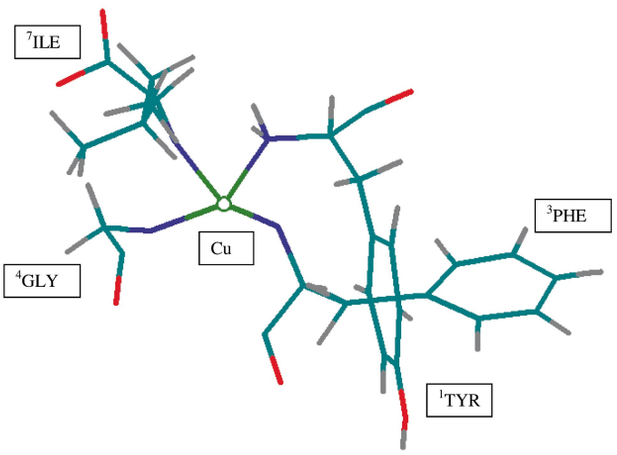


**Fig. 6** Molecular model of one of the low energy structures of the copper complex of *trans-trans-trans* BCM-7. Only residues bound to copper are shown for clarity.

As for BCM-7, the NMR data does not allow the paramagnetic contributions from isomeric complexes to be separated. Almost all isomers apparently bind copper. However, at higher copper concentrations, when several signals are greatly broadened (Fig. 4), Phe aromatic and Phe NH of *ccc* and/or *cct* isomers were virtually unaffected. It is therefore reasonable to conclude that these isomers either do not bind copper or else bind in a completely different fashion. Interestingly, the Tyr-OH was split into two components, with the low-field component being much more broadened than the other. A change in conformation is therefore apparent, with the folded tyrosine ring being brought into proximity with one of the three Pro residues such that it may be affected by the *cis* or *trans* conformation. Moreover, the two Tyr-OH protons interact differently with copper or, more probably, are variably broadened by exchange with residual water molecules.

Some inferences of the structure of the copper–BCM-7 complex were made with the aid of EPR data. The main species was in fact shown to be a copper complex having four co-ordinated nitrogens. The same procedure was therefore followed as in the case of BCM-4 and BCM-5. A hydrated copper ion was added and linked to the terminal amino group of the energy-minimized structure of each isomer. The unique restraints of the molecular dynamics run were the torsion angles indicative of proline and peptide-bond conformations. Again the state of $^{2}$Pro determined the proximity of the copper to $^{3}$Phe-NH; the copper–nitrogen bond was then formed and the molecular dynamics procedure was repeated. It transpired that the copper–nitrogen bond within any residue following Pro can be formed only when the involved proline is in its *trans* conformation. The molecular model of one of the energy-minimized structures of the copper–*ttt*-BCM-7 complex is shown in Fig. 6. It is interesting to note that what occurs for Ile at the C-terminus of BCM-7 does not hold for Gly in BCM-5. This can be reasonably ascribed to the p*K* of the amide nitrogen involved, which is expected to be diminished by the relatively long hydrocarbon substituent.

The fact that the conformational state of proline drives the binding mode of peptides to copper and, probably, other metal ions may be of relevance to protein folding,[22] channel operation by integral membrane proteins,[23] mitosis regulation,[24] enzymatic processes[7] and several other biological phenomena where the *cis*/*trans* isomerization of proline may act as a determinant step.

## References


1 E. Schlimme, H. Meisel and H. Frister, *Milk Proteins*, Steinkopff Verlag Darmstadt, Springer, New York, 1989.
2 A. L. Morris, M. W. MacArthur, A. G. Hutchinson and J. M Thornton, *Proteins*, 1992, **12**, 345.
3 G. Loew, C. Keys, B. Luke, W. Polgar and L. Toll, *Mol. Pharmacol.*, 1986, **29**, 546.
4 R. O. Fox, P. A. Evans and C. M. Dobson, *Nature*, 1986, **320**, 192.
5 C. Grathwohl and K. Wüthrich, *Biopolymers*, 1981, **20**, 2623.
6 F. Thunecke, A. Kálmán, F. Kálmán, S. Ma, A. S. Rathore and C. Horváth, *J. Chromatogr.*, *A*, 1996, **744**, 259.
7 (*a*) N. G. J. Delaet, P. M. F. Verheyden, D. Tourwé and G. Van Binst, *Biopolymers*, 1991, **31**, 1409; (*b*) A. Kálmán, F. Thunecke, R. Schmidt, P. W. Schiller and C. Horváth, *J. Chromatogr.*, *A*, 1996, **729**, 155.
8 (*a*) G. Formicka-Kozlowska, L. D. Pettit, I. Steel, B. Hartrodt, K. Neubert, P. Rekowski and G. Kupryszewski, *J. Inorg. Biochem.*, 1984, **22**, 155; (*b*) E. Chruscinska, M. Dyba, G. Micera, W. Ambroziak, J. Olczak, J. Zabrocki and H. Kozlowski, *J. Inorg. Biochem.*, 1998, **66**, 19; (*c*) E. Chruscinska, J. Olczak, J. Zabrocki, M. Dyba, G. Micera, D. Sanna and H. Kozlowski, *J. Inorg. Biochem.*, 1998, **69**, 91.
9 G. Della Lunga and R. Basosi, *J. Magn. Reson.*, *Ser. A*, 1995, **112**, 102.
10 G. Della Lunga, R. Pogni and R. Basosi, *J. Phys. Chem.*, 1994, **98**, 3937.
11 G. Rakhit, W. E. Antholine, W. Froncisz, J. S. Hyde, J. R. Pilbrow, J. R. Sinclair and B. Sarkar, *J. Inorg. Biochem.*, 1985, **25**, 217.
12 HYPERCHEM, Hypercube release 5.0, Hypercube Inc., Waterloo, Canada, 1997.
13 H. Sigel and R. B. Martin, *Chem. Rev.*, 1982, **82**, 385.
14 (*a*) H. Kozlowski, M. Bezer, L. D. Pettitt, M. Bataille and B. Hecquet, *J. Inorg. Biochem.*, 1983, **18**, 231; (*b*) M. Bataille, G. Formicka-Kozlowska, H. Kozlowski, L. D. Pettitt and I. Steel, *J. Chem. Soc.*, *Chem. Commun.*, 1984, 231; (*c*) M. Bezer, L. D. Pettitt, I. Steel, M. Bataille, S. Djemil and H. Kozlowski, *J. Inorg. Biochem.*, 1984, **20**, 13; (*d*) L. D. Pettitt, I. Steel, G. Formicka-Kozlowska, T. Tatarowski and M. Bataille, *J. Chem. Soc.*, *Dalton Trans.*, 1985, 535.
15 (*a*) G. Formicka-Kozlowska, H. Kozlowski, I. Z. Siemion, K. Sobczyk and E. Nawrocka, *J. Inorg. Biochem.*, 1981, **15**, 201; (*b*) I. Z. Siemion, A. Kubik, M. Jezowska-Bojczuk and H. Kozlowski, *J. Inorg. Biochem.*, 1984, **22**, 137; (*c*) M. Bataille, L. D. Pettitt, I. Steel, H. Kozlowski and T. Tatarowski, *J. Inorg. Biochem.*, 1985, **24**, 211; (*d*) C. E. Livera, L. D. Pettitt, M. Bataille, B. Perly, H. Kozlowski and B. Radomska, *J. Chem. Soc.*, *Dalton Trans.*, 1987, 661.
16 F. Boas, J. R. Pilbrow, C. R. Hartzell and T. D. Smith, *J. Chem. Soc. A*, 1969, 572.
17 G. Della Lunga, R. Pogni and R. Basosi, *J. Magn. Reson.*, *Ser. A*, 1995, **114**, 174.
18 T. J. Swift and R. E. Connick, *J. Chem. Phys.*, 1962, **37**, 307.
19 (*a*) G. Navon and G. Valensin, *Metal Ions in Bological Systems*, ed. H. Sigel, Marcel Dekker, New York, 1987, Vol. 21, pp. 1–45; (*b*) I. Bertini and C. Luchinat, *Coord. Chem. Rev.*, 1996, **150**, 1, and references therein.
20 (*a*) I. Solomon, *Phys. Rev.*, 1955, **99**, 559; (*b*) N. Bloembergen, *J. Chem. Phys.*, 1957, **27**, 572; (*c*) N. Bloembergen and L. O. Morgan, *J. Chem. Phys.*, 1961, **34**, 841.
21 (*a*) G. Valensin, R. Basosi, W. E. Antholine and E. Gaggelli, *J. Inorg. Biochem.*, 1985, **23**, 125; (*b*) E. Gaggelli, R. Basosi, R. Pogni and G. Valensin, *J. Inorg. Biochem.*, 1988, **32**, 7.
22 F. L. Texter, D. B. Spencer, R. Rosenstein and C. R. Matthews, *Biochemistry*, 1992, **31**, 5687.
23 C. M. Deber, M. Glibowicka and G. A. Woolley, *Biopolymers*, 1990, **29**, 149.
24 M. B. Yaffe, M. Schutkowski, M. Shen, X. Z. Zhou, P. T. Stukenberg, J.-U. Rahfeld, J. Xu, J. Kuang, M. W. Kirschner, G. Fischer, L. C. Cantley and K. P. Lu, *Science*, 1997, **278**, 1957.