Fine tuning the structure of the Cu^{2+} complex with the prion protein chicken repeat by proline isomerization†

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The interaction between the single hexarepeat unit of chicken prion protein [ChPrP(54–59)] and Cu(II) was investigated by NMR, finding different coordination modes for the *trans/trans* and *cis/trans* isomers.

The prion protein (PrP) is associated with lethal neurodegenerative disorders grouped as transmissible spongiform encephalopathies.[1,2] PrP can be found as either a pathogenic (PrPSc) or a normal cellular (PrPc) isoform. The biological function of PrPc, though widely debated, is still unclear.[3,4] Once it was discovered that PrP is a copper binding protein, strong evidence has been collected that PrPc has a role in normal brain copper metabolism,[5–7] either shuttling the metal from synaptic space to the cell interior[8] or acting as a carrier or a stress sensor for copper.[9] Since conversion into a protein aggregate may alter or abolish the function of PrPc, prion diseases may involve disturbance to brain copper homeostasis.[10,11] Solution structures have been obtained for recombinant PrP from many diverse species.[12–15] In particular chicken PrP shows *ca.* 30% identity with mammalian prion proteins and forms the same molecular architecture. Biological implications of Cu(II) interaction with avian PrP are not well established[16–18] although the 3D structures of avian and mammalian PrP are very close to each other.[13] Recently copper binding to the chicken prion protein was investigated, reporting an inter-repeat Cu(II) coordination.[19] When looking at the copper binding site within the monomeric unit of the mammalian octapeptide repeat (PHGGGWGQ), it became evident that the metal ion is first anchored to the imidazole nitrogen, then attaches two adjacent amide nitrogens of two Gly residues and the Trp side-chain is brought close to Cu^{2+} through a metal ion bound water molecule.[20,21] Since the avian PrP contains, in the same tandem region, hexapeptide repeats [ChPrP(53–58): PHNPGY] having Tyr instead of Trp as a presumably redox competent residue, delineation of the copper complex with such a unit was thought to yield valuable information on a generalized role for copper as an oxidation catalyst. The present results demonstrate not only that binding involves, after anchoring at the His site, also the Tyr residue either directly or through a metal-bound water molecule, but also that the binding mode can be fine tuned by *cis/trans* isomerization of proline which determines the structure of the complex, as also shown in other cases.[22]

† Electronic supplementary information (ESI) available: peptide synthesis, NMR measurements and ^{1}H-^{13}C HSQC spectra. See http://www.rsc.org/suppdata/cc/b5/b504986e/index.sht

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Potentiometric, EPR, CD and UV-vis measurements[23] provided evidence of a major single deprotonated species ($CuH_{-1}L$) dominating in the pH range 7–9 with copper bound to two nitrogens (one imidazole nitrogen and one ionized amide nitrogen, d–d band at 650–670 nm)[23–25] and the phenolate oxygen of tyrosine (d–d transition in the range 386–415 nm).[23,25] The relatively low intensity of the latter band suggested only a partial involvement of the phenolate ring in binding.

600 MHz ^{1}H NMR spectroscopy could easily distinguish two of the four isomers of ChPrP(53–58) and ChPrP(54–59) in D_2O at pD 7.8 and $T = 298$ K, identified by ROESY as *trans/trans* (73%) and *trans/cis* [ChPrP(53–58)] or *cis/trans* [ChPrP(54–59)] (both 27%). The *trans* and *cis* isomers were distinguished by the presence of a sequential NOE between Pro-Hδ or Pro-Hα with Hα of the preceding residue.[22] The two peptides were identically interacting with Cu(II), as manifest in paramagnetic effects on NMR parameters and potentiometric curves (data not shown). For the purposes of the present work, the possibility of detecting the Tyr aromatic protons in the two main isomers led us to prefer ChPrP(54–59) (see ESI†).

Addition of copper(II) was selectively affecting line widths and spin–lattice relaxation rates, as shown in Fig. 1 and summarized in Table 1. Anchoring of copper to the imidazole ring of histidine was demonstrated by the extensive line broadening of the imidazole aromatic protons and by the disappearance of imidazole connectivities in ^{1}H-^{13}C HSQC spectra (not shown), as usually observed in His-containing peptides interacting with copper(II).[26,27] The disappearance of the Hα signal in the 1D spectra (Fig. 1) and of the corresponding Hα–Cα cross-peak in the ^{1}H-^{13}C HSQC spectra of the *trans/trans* (*tt*) and not of the *cis/trans* (*ct*) isomer is direct evidence of the involvement of the His amide nitrogen in copper binding in one isomer and not in the other. Moreover the

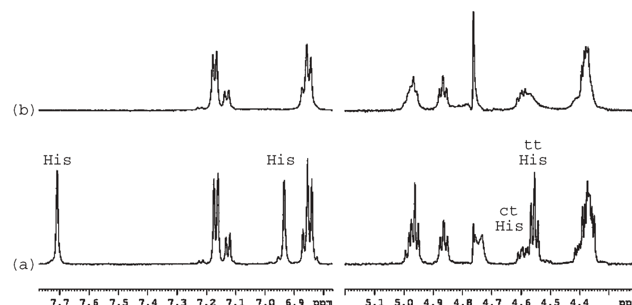

Fig. 1 ^{1}H NMR spectrum of free (a), and after the addition of 0.01 copper equivalents (b), ChPrP(54–59), 5 mM, pD = 7.8.

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Table 1 Paramagnetic relaxation enhancements and Cu(II)–H distances of ChPrP(54–59), 5.0 mM, in the presence of 0.05 Cu(II) equivalents in H_2O/D_2O at T = 298 K

	tt Isomer		*ct* Isomer	
	R_{1p} (s^{-1})	r (nm)	R_{1p} (s^{-1})	r (nm)
His-54 H_α	Broad	—	1.75	0.69
His-54 H_ε	Broad	0.31	Broad	0.31
His-54 H_δ	Broad	0.38[a]	Broad	0.38[a]
Asn-55 H_α	9.58	0.57	6.89	0.46
Asn-55 H_β	7.58–8.54	0.61–0.58		
Pro-56 H_β	12.67	0.52	1.81–3.88	0.69–0.57
Pro-56 H_δ	7.63–7.94	0.61–0.59		
Gly-57 H_α	2.07	0.79	2.03	0.67
Tyr-58 H_α	2.30	0.78		
Tyr-58 H_β	4.50–4.73	0.68–0.63		
Tyr-58 H_δ	4.07	0.69	4.26	0.56
Tyr-58 H_ε	7.62	0.61	8.82	0.35
Pro-59 H_α	2.88	0.75		
Pro-59 H_β	2.24	0.78	1.75	0.69
Pro-59 H_δ	1.73	0.82	2.39	0.64

[a] Average value calculated from R_{1p} values at [peptide]:[copper] ratios of 250:1 by using τ_M = 0.38 ms (see text for details).

still detectable Asn signals exclude its involvement in copper coordination. These findings, together with the relatively low intensity of the d–d transition in the 386–415 nm range, allow us to anticipate that the *tt* isomer binds the ionized His amide nitrogen, while the *ct* isomer binds the phenolate oxygen of the Tyr residue.

Interpretation of paramagnetic relaxation rates facilitated the elucidation of the structures of the complexes. It is known that in copper complexes with His-containing peptides structural delineation is possible only provided the exchange rate from the metal coordination sphere can be evaluated such that the contribution of R_{1b}^{-+} to R_{1p} can be obtained:[28,29]

$$R_{1p} = R_{1obs} - p_f R_{1f} = \frac{p_b}{R_{1b}^{-1} + \tau_M} \quad (1)$$

where $\tau_M = k_{off}^{-1}$ is the residence time of peptide molecules in the metal coordination sphere, p_f and p_b are the fractions of free and bound peptide, R_{1obs} and R_{1f} are the spin–lattice relaxation rates measured, respectively, after the addition of copper and in the metal-free solution and R_{1b} is the rate of ligand nuclei in the metal coordination sphere, given by the Solomon equation.[28–30]

Copper binding is expected to occur as a multi-step process starting with the entrance of the anchoring site into the metal coordination sphere.[25,31] In fact two different exchange times were measured: one shorter for the imidazole protons (τ_M^{im}) and the second longer for the other side-chain and backbone protons (τ_M^{bb}).[27] A separate estimate of τ_M^{bb} and τ_M^{im} was therefore obtained. The calculated R_{1p} values of the His Hε [Hε–Cu(II) distance 0.31 nm] allowed the determination of τ_M^{im} at 0.30 ± 0.10 ms,[26] by using τ_c = 0.20 ± 0.10 ns (calculated from the ratio between non-selective and selective spin–lattice relaxation rates).[32] In the same way, consideration of the fixed distance between copper and the Hε (0.35 nm) or Hδ (0.56 nm) protons of tyrosine when the phenolate oxygen is bound in the *ct* isomer permitted the calculation of τ_M^{bb} at 2.5 ± 0.5 ms. The obtained exchange times were then used to calculate all copper–proton distances (Table 1) from the R_{1p} values measured in the presence of 0.05 equivalents of Cu(II).

In contrast with Hε, the Hδ–Cu(II) distance depends on which nitrogen is bound to copper(II), being either 0.31 nm (Nε bound) or 0.51 nm (Nδ bound). The calculated 0.38 nm distance suggests an average between the isomeric complexes with involvement of Nδ for the main *tt* conformer and Nε for the secondary one. In fact, only binding to Nδ sterically allows further binding to the deprotonated amide nitrogen of the His residue.

Copper–proton distances were used in restrained molecular modelling of the Cu(II) complexes through a simulated annealing protocol. To take into account the observed coordination behaviour of copper, distance constraints were also imposed allowing copper binding to (i) the His-1 imidazolic Nδ and amide N^- for the *trans*/*trans* complex and (ii) His-1 imidazole Nε and Tyr-5 O^- for the *cis*/*trans* complex. Fig. 2 shows the best five structures thus obtained for the *tt* (Fig. 2a) and the *ct* (Fig. 2b) isomers. A value of backbone RMSD of 0.02 nm was obtained in both cases.

It can be concluded that NMR can take advantage from the slow *cis*/*trans* interchange rates in determining whether and how Cu(II), or any other metal ion, interacts with each single isomeric species. The diverse metal binding behaviour of the *tt* and *ct* isomers of the reported peptide is in fact unequivocally demonstrated by different paramagnetic contributions to selected proton resonances. The two obtained structures, together with inferences from UV-vis spectra, strongly support the involvement of the Tyr

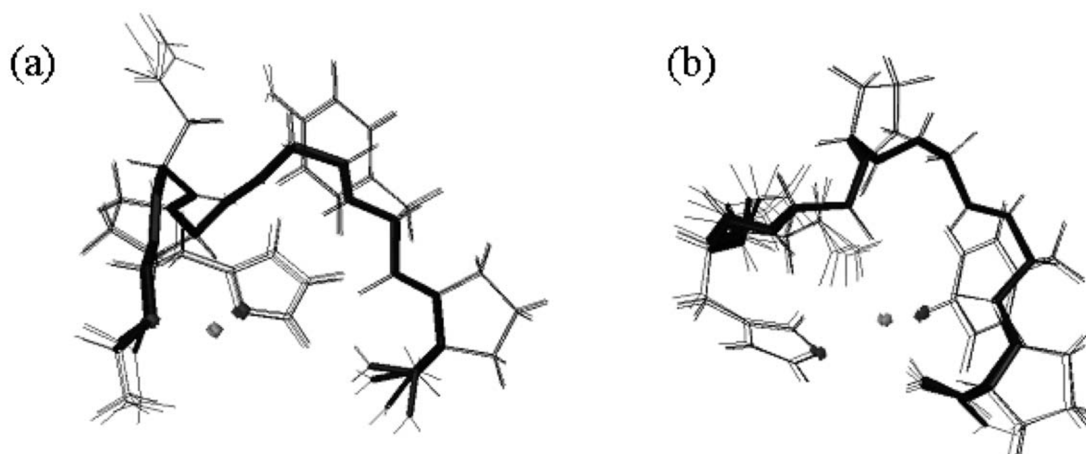

Fig. 2 Best five structures of *tt* (a) and *ct* (b) conformers of Cu(II)–ChPrP hexapeptide. The figure has been created with the program MolMol2 K.[33]

phenolato oxygen in Cu(II) binding of the *ct* isomer, even in the absence of any direct proof.

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