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Novel model peptide for Atx1-like metallochaperones†

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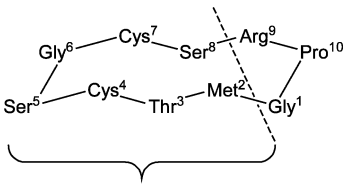
Received (in Cambridge, UK) 28th November 2003, Accepted 8th January 2004
First published as an Advance Article on the web 6th February 2004

The novel cyclodecapeptide c(GMTCSGCSRP) is able to bind soft metals with a selectivity for Hg^{2+} and Cu^{+} over Pb^{2+}, Cd^{2+} and Zn^{2+}, and is demonstrated to be an excellent structural model of the binding loop of the copper metallochaperone Atx1 in its apo and mercury loaded forms.

The CXXC binding motif is often encountered in metallo-proteins for the complexation of various ions (Fe^{2+}, Ni^{2+}, Cu^{+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}) which can be either essential or toxic for living organisms. Metallochaperones are a class of metal-binding proteins involved in the transport of metal ions and among them, the yeast protein Atx1 (73 amino acids) delivers copper in the +I oxidation state to a transporting ATPase (Ccc2) located in the Golgi membrane.[1,2] Atx1 binds Cu^{+} by means of a MXCXXC motif which is conserved[3,4] in many soft-metal transporters. Atx1 adopts a βαββαβ fold with the binding sequence located in a solvent-exposed loop anchored to the first β sheet and α helix.[5,6] Proteins containing the MXCXXC motif seem highly selective *in vivo*, it is therefore important to understand which factors govern this selectivity. This could lead to the conception of new selective complexing agents derived from Atx1-like chaperones for decorporation, depollution or design of biosensors. Few models of metallochaperones have been proposed, so far.[7,8] Here we report on a preliminary study of the complexation of heavy-metals with a cyclodecapeptide that mimics the Atx1 binding loop and presents a selectivity for Hg^{2+} and Cu^{+} over Pb^{2+}, Zn^{2+} and Cd^{2+}.

The 10-mer cyclopeptide c(GMTCSGCSRP) (**1**·H_2, Scheme 1) was chosen to mimic Atx1.‡ It provides i) the binding sequence MTCSGCS of the copper-chaperone; ii) a charged amino acid (R) to mimic the proximal Lys65 side-chain standing next to the metal binding site of Atx1 and to increase the solubility in water; iii) a XPGX motif able to form a β-turn[9] which could rigidify the cyclopeptide and act as an anchor for the metal binding site. The cyclic nature of the peptide and the presence of a rigid β-turn should pre-organize the MTCSGCS sequence into a loop and thus limit unfavourable entropic contribution associated with the coordination-induced structuration of the peptide compared to what happens with a linear peptide.



Scheme 1 Peptide **1**·H_2.

The solution structure of **1**·H_2 was investigated by 1D and 2D ^{1}H NMR experiments in H_2O/D_2O 9:1 (2.5 mM, pH 5.5, 298 K, 500 MHz). The ^{1}H NMR spectrum of **1**·H_2 displays a unique set of peaks that do not broaden when temperature is decreased to 278 K. Characteristic elements of a type II β-turn structure for the RPGM sequence were identified: a *trans* peptide bond for proline (*d*(Arg9 Hα, Pro10 Hδ$_1$) = 2.1 Å and *d*(Arg9 Hα, Pro10 Hδ$_2$) = 2.5 Å), a *cis* relationship between Gly1 HN and Pro10 Hα (2.1 Å), a relatively strong ROE cross-peak between Met2 HN and Pro10 Hα (3.6 Å) and a temperature coefficient ($\Delta\delta/\Delta T$ = −3.3 ppb) for Met2 HN indicative of a weak Arg9 CO···HN Met2 hydrogen bond.[10] However, only few medium range NOEs are observed except those between Met2, Cys7 and Arg9 side-chains. Together with the small dispersion of $^3J_{HN,H\alpha}$ values (all between 6.0 Hz and 8.0 Hz), this accounts for a rather unstructured peptide. A superimposition of low energy calculated structures§ (Fig. 1) clearly shows that the RPGM motif is well structured whereas the binding loop is more mobile.

Introduction of $HgCl_2$ (1.1 eq) in the water solution of **1**·H_2 leads to the formation of a 1:1 peptide/metal complex (**1**·Hg, ES/MS: *m*/*z* = 1180 [**1**·Hg+H]) without addition of base (final pH = 2.2). Its ^{1}H NMR spectrum displays sharp signals. Hα and Hβ protons of both cysteines are strongly downfield shifted (~0.5 and ~0.6 ppm, respectively), whereas those of other amino acids are not. This suggests that mercury is bound to the peptide by the two cysteine sulfur atoms only. This was confirmed by the ^{199}Hg NMR chemical shift (−937 ppm)[11,7] of **1**·Hg which is typical of a linear bicoordinate Hg^{2+} with thiolate ligands. A large number of medium range NOEs and multiple $^3J_{HN,H\alpha}$ values < 5.5 Hz or > 8.0 Hz

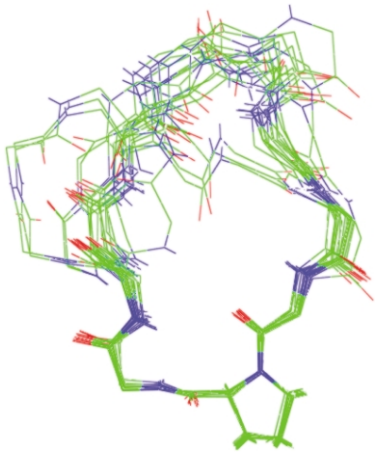


Fig. 1 Backbone superimposition of 20 low energy structures of the apo-peptide **1**·H_2 calculated with NMR derived constraints using X-PLOR[13] and drawn using MPV3D.[14] The side chain of Pro10 is displayed.

† Electronic supplementary information (ESI) available: ^{199}Hg NMR spectrum, ^{1}H-NMR chemical shifts, $^3J_{HN,H\alpha}$ coupling constants, distance restraints derived from ROESY spectra and calculated distances for **1**·H_2 and **1**·Hg. See http://www.rsc.org/suppdata/cc/b3/b315489k/

DOI: 10.1039/b315489k

support a well defined conformation for **1**·Hg. Structures calculated using X-PLOR§ (Fig. 2) reveal a unique conformation with a backbone RMSD of 0.19 Å. Comparison with the crystal structure of Atx1 in its Hg^{2+}-loaded form[5] shows that the structure of the CSGC turn coordinated to mercury is well reproduced (the RMSD for the (CSGC)-Hg motif between Atx1–Hg and **1**·Hg is 0.85 Å for the backbone and 1.24 Å for all atoms). The cyclodecapeptide also mimics the second coordination sphere interactions around the metal: in both structures, each coordinated S atom is hydrogen-bonded to an amide NH donor and an oxygen atom lies near Hg^{2+} (Thr3 CO in the model compound (*d*(Hg,O) = 3.0 Å) and Thr14 Oγ in Atx1–Hg (3.07 Å)). Such interactions are thought to play an important role in the selectivity of metal trafficking proteins.[12] Thus, the cyclodecapeptide **1**·H_2 appears to be a good structural model of the binding loop of the metallochaperone Atx1.

Binding of other soft metal ions (Pb^{2+}, Cd^{2+}, Zn^{2+} and Cu^{+}) was investigated by ES/MS and ^{1}H NMR spectroscopy after addition of excess (1.1–2 eq.) of metal ion into a water solution of **1**·H_2 (2.5 mM for NMR and 0.2 mM for ES/MS, initial pH ~ 5.5). Efficient binding of Cu^{+}, although weaker than Hg^{2+}, was observed for pH > 2.5. For Pb^{2+}, 2 equivalents of base were needed (pH > 5.5) to observe complete complexation, whereas **1** poorly binds Zn^{2+} and Cd^{2+} in the same conditions. In each case, the 1:1 peptide/metal stoichiometry was inferred by ES/MS. The binding affinities of **1**·H_2 for these ions, corresponding to equilibrium (1), were evaluated by ^{1}H NMR spectroscopy. The log β values (Table 1) show that **1**·H_2 presents a high selectivity toward Hg^{2+} and Cu^{+} over Pb^{2+}, Zn^{2+} and Cd^{2+}. The affinity for Cu^{+} is at least 5 orders of magnitude higher than for the three later, and especially, 7 orders of magnitude higher than for Zn^{2+}, the other biologically relevant ion.

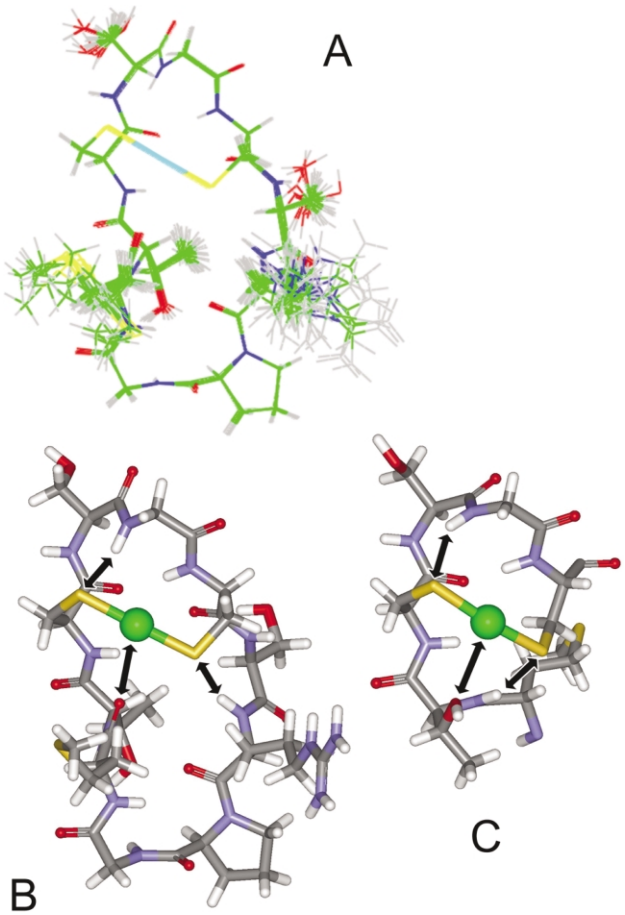


Fig. 2 NMR solution structure of **1**·Hg calculated using X-PLOR. (A) Superimposition of the 20 lowest energy structures. (B) Lowest energy structure of **1**·Hg showing side-chains, S···HN hydrogen bonds and Hg···O interaction. (C) (MTCSGC)-Hg motif of the crystal structure of Atx1-Hg (1.02 Å resolution).[5]

Table 1 Complex formation constants determined by ^{1}H NMR spectroscopy (298 K)

Ion	Hg^{2+}	Cu^{+}	Pb^{2+}	Cd^{2+}	Zn^{2+}
Log β	> 0	−2	−7	−9	−9

$$\mathbf{1}\cdot H_2 + M^{n+} \rightleftharpoons \mathbf{1}\cdot M + 2H^{+} \quad (1)$$

$$\beta = ([\mathbf{1}\cdot M] \times [H^{+}]^2)/([\mathbf{1}\cdot H_2] \times [M^{n+}])$$

In conclusion, the apo-cyclodecapeptide **1**·H_2 presents a MTCSGCS binding loop which is rather flexible, like its counterpart in apo-Atx1.[6] It forms stable complexes with Hg^{2+} and Cu^{+} which are selectively bound over Pb^{2+}, Cd^{2+} and Zn^{2+}. The solution structure of **1**·Hg shows that the cyclodecapeptide reproduces the first and second coordination sphere interactions found in Atx1. Thus, **1**·H_2 is a promising model to understand the selectivity of Atx1-like metallochaperones.

We are currently investigating the structure of complexes with other metals (coordination sphere and peptide conformation) as well as the effect of amino acid mutations on the binding affinities and selectivity.

The authors thank the Nuclear Toxicology program of the Commissariat à l'Energie Atomique (CEA) for financial support.

Notes and references

‡ The 10-mer protected linear precursor H_2NCys(Trt)-Ser(*t*Bu)-Arg(Pbf)-Pro-Gly-Met-Thr(*t*Bu)-Cys(Trt)-Ser(*t*Bu)-GlyOH was assembled on 2-chlorotrityl chloride resin using Fmoc chemistry, cleaved from the resin and cyclized in DMF. After deprotection of the side-chains, the cyclodecapeptide c(GMTCSGCSRP) (**1**·H_2) was purified by reversed-phase HPLC (52 mg, 17% overall yield, ES/MS: *m*/*z* 980.3 [**1**·H_2+H]).

§ Solution structures were calculated using the program X-PLOR 3.1 following standard refinement protocols.[13] Upper and lower limits for distance constraints were set to ±10% of the H–H distances obtained by integration of ROESY spectra (500 MHz, 200 ms). Φ dihedral constraints were derived from $^3J_{HN,H\alpha}$ coupling constants measured on ^{1}H NMR spectra or by soft-COSY experiments. Pseudo-atom corrections were applied to non-stereospecifically assigned methylenes. The S–Hg bond lengths were set to 2.33 Å, which is typical of linear bicoordinate RS–Hg–SR complexes.[7,8] No NOE violations greater than 0.5 Å and no dihedral angles violations greater than 5° were found.

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