Short oligopeptides with three cysteine residues as models of sulphur-rich Cu(I)- and Hg(II)-binding sites in proteins†

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The essential Cu(I) and the toxic Hg(II) ions possess similar coordination properties, and therefore, similar cysteine rich proteins participate in the control of their intracellular concentration. In this work we present the metal binding properties of linear and cyclic model peptides incorporating the three-cysteine motifs, CxCxxC or CxCxC, found in metallothioneins. Cu(I) binding to the series of peptides at physiological pH revealed to be rather complicated, with the formation of mixtures of polymetallic species. In contrast, the Hg(II) complexes display well-defined structures with spectroscopic features characteristic for a HgS2 and HgS3 coordination mode at pH = 2.0 and 7.4, respectively. Stability data reflect a ca. 20 orders of magnitude larger affinity of the peptides for Hg(II) \(\log{K_{\text{HgP}_{\text{pH7.4}}}}\) ≈ 41 than for \(\text{CuP}_{\text{pH7.4}}\) \(\log{K_{\text{CuP}_{\text{pH7.4}}}}\) ≈ 18. The different behaviour with the two metal ions demonstrates that the use of Hg(II) as a probe for Cu(I), coordinated by thiolate ligands in water, may not always be fully appropriate.

**Significance to metallomics**

Mercury(II) and copper(I) detoxification in cells involves similar sulphur-rich proteins, including metallothioneins. Short model peptides are used here to study the molecular interactions of these two soft cations with sequences containing three cysteine moieties found in these proteins. We demonstrate that the binding of Hg(II) and Cu(I) with these representative cysteine-rich sequences involves different molecular mechanisms, with the formation of various coordination complexes. Hence, the use of Hg(II) as a probe for Cu(I) coordination with sulphur-rich peptides or proteins in physiological conditions is demonstrated here to be not fully appropriate.

**Introduction**

Many metal ions, including Fe(II/III), Cu(I/II) or Zn(II), are essential micronutrients. Since both their deficiency and excess are harmful for the cells, the optimal concentration of these ions is controlled by regulation systems involving lots of proteins. Other metal ions, like Hg(II) and Cd(II), are toxic without any advantageous effects. The high affinity of these soft metal ions for soft sulphur bases, found in proteins and small ligands, such as glutathione, is one of the main factors leading to their toxicity. Indeed, interactions between these toxic metal ions and thiol-containing molecules induce misfolded protein structures or perturbation in the redox balance of the cells.

As a consequence of the similar coordination properties of the essential Cu(I) and the toxic Hg(II) ions, the cellular regulation and protection systems for controlling or reducing the concentration of these ions involve proteins with sulphur-rich metal coordination sites, usually dominated by Cys-thiolate donor groups. A varying number of Cys residues may allow the binding of Cu(I) or Hg(II) in isolated monometallic coordination sites or in polymetallic clusters. The strong preference of Hg(II) for bis-thiolate coordination environment is reflected by the published structures of several elements of the bacterial mercury resistance system, MerP,1 MerT,2 the N-terminal domain of MerA3 or the Hg(II)-bound form of the copper chaperone Atx1.4 Nevertheless, there are also examples of structures with more Hg(II)-thiolate bonds, e.g. the distorted tetrahedral tetrathiolate Hg(II)-centre of rubredoxin,5 or the tri- or tetra-coordinated metal ion environments in Hg(II)-bridged protein dimers, like those observed in the MerR metalloregulatory protein6 or in the human
copper chaperone HAH1. Cu(i) can also accommodate a linear CuS₂-type coordination mode, as indicated by structural data on the copper efflux regulator CueR or the metal binding domains of the copper transporter protein ATP7B. Trigonal CuS₂ centres were proposed in the binuclear Cu(i)-thiolate core of the repressor protein CopY and in one of the suggested metal-bridged dimeric forms of the copper chaperone CopZ, whereas the Cu(i)-bridged HAH1 dimer was shown also to include a fourth, weakly bound, thiolate in a pseudotetrahedral environment.

Di-, tri- or tetrathiolate coordination modes are also typical in polymeric clusters. Metallothioneins (MTs) are small, cysteine-rich proteins, found in all kingdoms of life from prokaryotes to mammals. Several isoforms of MTs evolved with high diversity of amino acid sequences and, pursuant to this, with different metal ion preferences, 3D structures and proposed biological functions. A novel approach for the classification of such a big family of proteins was developed by the groups of Capdevila and Atrian based on the metal binding features of MTs, i.e. on the criterion whether the MT binds Zn(u) either in homo or in heterometallic form, or only Cu(i) under physiological conditions. Trigonally coordinated Cu(i) ions strongly dominate in Cu(i)-loaded MTs displaying also less abundant Cu(i)S₂ centres. The only published Cu(i)-loaded MT crystal structure, a truncated form of the mononuclear Cu(i)-thiolate core of the repressor Ctr1, was confirmed to accommodate a linear unstructured peptide was considered as a predisposed structure that could pre-orient the Cys-sidechains for an easy accommodation of metal ions in a tri-coordinate fashion. The results confirmed the formation of a mononuclear Cu(i) complex with properties indicative of an HgS₃ coordination.

An interesting strategy to better understand the individual contributions of specific short sequences to the overall affinity of proteins for metal ions is to design and study model peptides. This approach could eventually lead to the identification of relevant peptide-based motifs for heavy metal chelation. Some of the above described metal-binding sites have been probed by using oligopeptides encompassing the relevant metal binding sequences of the modelled proteins. The metal-binding features of the metalloregulator CueR and the metallochaperone Atx1, both encompassing 2 cysteines in their metal-binding domain, with different sequences between the Cys residues, were investigated via oligopeptide models of the relevant metal-binding loops. A general finding was that the linear unstructured ligands, while displaying high affinity and interesting metal ion selectivity for Hg(u) or Cu(i) chelation, could not fully reproduce the efficiency of the modelled proteins. Cyclisation of the linear Atx1 model, resulting in a more rigid skeleton with pre-oriented Cys-sidechains, was an important step forward leading to notably increased metal binding affinities. The more challenging imitation of the HgS₃ type metal coordination sites was successfully achieved by different approaches. Pecoraro et al. successfully applied single oligopeptide chain three-helix bundles and three-stranded coiled coils to settle three Cys residues into optimal position for accommodating the Hg(u) ion in a tri-coordinate fashion under slightly alkaline conditions. These tri-thiolate bound species were in a pH-dependent equilibrium with HgS₂ type complexes, characterized by apparent pKₐ values ≥7. Using a different strategy, triiodal pseudopeptide ligands were designed with cysteine or d-penicillamine (d-Pen) moieties grafted onto a nitrilotriacetic acid scaffold. These constructs were able to stabilize the HgS₃ coordination mode in a broad pH range, starting even at pH ~ 5.5 with one of the d-Pen ligands. Interestingly, the behaviour of the same ligands in binding Cu(i) was less straightforward. Depending on the bulkiness and hydrophobicity of the arms attached to the triiodal template, the formation of mononuclear and (Cu₂Lig)₃ type complexes with (Cu₂S₃)ₙ cores was observed with high stabilities. In a recently published article, the Hg(u) binding of the cyclic peptide Pₓₐ incorporating a three Cys variant of the metal binding loop of Atx1 was discussed. This cyclic solvent accessible peptide was considered as a predisposed structure that could pre-orient the Cys-sidechains for an easy accommodation of metal ions in a tri-coordinate fashion. The results confirmed the formation of a mononuclear Hg(u) complex with properties indicative of an HgS₃ coordination.

Peptide scaffolds similar to Pₓₐ and comprising two typical metal binding motifs found in metallothioneins, namely the CxCxxC and CxCxxC sequences, are investigated in the present paper. Their binding properties for the two soft metal ions Cu(i) and Hg(u), exhibiting the largest affinities for metallothioneins, are studied and discussed in relation to their binding by detoxification systems.

**Experimental section**

**Materials**

Materials and solvents were purchased from Sigma Aldrich, Fluka, Acros Organics and used without further purification. Amino acid building blocks, resins and coupling agents were purchased from NovaBiochem. For aqueous solutions, ultrapure laboratory grade MilliQ water was used (resistivity 18 MΩ cm).

**Abbreviations**

AcO₂: acetic anhydride; AcN: acetonitrile; BCS: bathocuproine-disulphonate; DCM: dichloromethane; DIEA: N,N-diisopropyl-ethylamine; DTMNB: 5,5’-thio-bis-(2-nitrobenzoic acid); EDT: ethanedithiol; Et₂O: diethyl ether; Fmoc: 9-fluorenylmethoxycarbonyl; HBTU: 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBT: hydroxybenzotriazole; MeOH: methanol; NMP: N-methyl-2-pyrrolidone; TFA: trifluoroacetic acid; TIS: triisopropylsilane.

**Peptide synthesis and purification**

**Linear peptides.** Ac-GTCTSCGSRP-NH₂ (1) and Ac-GTCTSCGCSRP-NH₂ (2) peptides with acetyl and amide

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protection at their two termini were synthesized by standard solid phase peptide synthesis following the Fmoc procedure. Rink Amide AM resin (200–400 mesh, 0.67 mmol g⁻¹) was used as solid support. The average coupling mixture consisted of 4 equiv. of protected amino acid building blocks, 4 equiv. of HOBt, 4 equiv. of HBTU and 8 equiv. of DIEA dissolved in NMP. The reaction time was 1 h. The noncomplete coupling was monitored by Kaiser’s test. To avoid the formation of deleted products, the acetylation of the unreacted amino groups was performed in a mixture of 10% Ac₂O and 10% DIEA in DCM after each coupling reaction. The Fmoc deprotection was achieved by 20% piperidine solution in NMP. The cleavage of the peptide from the resin and the removal of the sidechain protecting groups were performed in one step in the mixture of TFA/EDT/H₂O/phenol/TIS (92/2.5/2.5/2/1 V/V%) for 4 h. After evaporation, the residue was precipitated in ice cold Et₂O and centrifuged (6000 rpm, 15 min).

Cyclic peptides. For c(SRPGTCSCG) (1), c(CSRPGTSCG) (2) and c(CSRPGATSCG) (3), linear precursors were first assembled on preloaded H-Gly-2-Cl-trityl resin (0.54 mmol g⁻¹) following the same method as described above. The protected precursor was cleaved from the resin by treatment with 2% TFA in DCM for 2 × 5 min. The solution was flushed into 20% pyridine in MeOH. After concentration, the residue was precipitated in ice cold Et₂O. The protected linear precursor (0.5 mM in DCM) was cleaved from the resin by treatment with 2% TFA in DCM after each coupling reaction. The Fmoc deprotection was achieved by 20% piperidine solution in NMP. The cleavage of the peptide from the resin and the removal of the sidechain protecting groups were performed in one step in the mixture of TFA/EDT/H₂O/phenol/TIS (92/2.5/2.5/2/1 V/V%) for 4 h. After evaporation, the residue was precipitated in ice cold Et₂O and centrifuged (6000 rpm, 15 min).

Sample preparation for physicochemical studies
The cysteine residues of the peptides and Cu(i) are sensitive to oxidation; therefore all sample preparations and manipulations were performed in a glove box, under an argon atmosphere. Fresh solution was made for each experiment in the appropriate buffer. 10% acetonitrile in volume was added to the Cu(ii) containing samples to overcome Cu(i) disproportion in water.

The final peptide concentration was determined by Ellman’s procedure, where DTNB reacts with the free thiols forming TNB (2-nitro-5-thiobenzoate) with ε₄₁₂ = 14 150 M⁻¹ cm⁻¹. Cu(i) solution was made from Cu(CH₃CN)₄PF₆ salt in acetonitrile. The final concentration was determined by using BCS forming a stable Cu(BCS)₂ complex with ε₄₈₃nm = 13 300 M⁻¹ cm⁻¹. A precise weight of high purity HgCl₂ was used to prepare Hg(ii) stock solution in water (~3 mM).

UV-Vis absorption and circular dichroism (CD) measurements
UV-Vis spectra were recorded on a Varian Cary 50 spectrophotometer equipped with optical fibers connected to an external cell holder in the glovebox. CD spectra were acquired with an Applied Photophysics Chirascan photometer. (1S,1R)-Camphorsulfonic acid served as the calibration compound for the instrument. Spectra were recorded in the range of 320–190 nm wavelength with 1 nm bandwidth and 2 s dwell time per point. For each sample 3 parallel spectra were recorded and the average of these spectra was smoothed by the Savitzky–Golay method with a “window size” 7. CD spectra are reported in molar ellipticity ([θ] in units of deg cm² dmol⁻¹). θ obs is the observed ellipticity in millidegrees, I is the optical path length in cm and c is the peptide concentration in mol dm⁻³.

With Cu(i), titrations were performed in individually prepared samples because of the slow complex formation. 2.5 mL volumes of peptide solution (20–30 μM) in phosphate buffer (20 mM, pH = 7.4) were transferred into UV cells (1.0 cm path length) and then different (0.0–3.0) equivalents of Cu(i) were added to these samples. The samples were equilibrated for at least 2 hours. To ensure the thermodynamic equilibrium was reached, the absorbance was measured regularly. In the case of Hg(ii) titrations, 2.5 mL of peptide solution (20–30 μM) in phosphate buffer (20 mM, pH = 7.4) was transferred into a UV cell (1.0 cm path length) and then aliquots of the Hg(ii) solution (~3 mM) were gradually added from 0.0 to 3.0 equivalents. pH titrations of the Hg(ii) complexes were performed in pure water in the pH range of 2–11 by adding aliquots of 0.1 M KOH. The pH was measured using a Metrohm 702 SM Titritron equipped with a Mettler Toledo InLab® Micro electrode. pKₐ values for the observed deprotonation processes were obtained by fitting the data by using the SPECFIT computer program.

ESI-MS experiments
Mass spectra were acquired on a LXQ-linear ion trap (THERMO Scientific) instrument equipped with an electrospay ion source. Electrospray full scan spectra in the range of m/z = 50–2000 amu were obtained by infusion through a fused silica tubing at a flow rate of 2–10 μL min⁻¹. The solutions were analysed in negative and positive ion modes. The LXQ calibration (m/z = 50–2000) was achieved according to the standard calibration procedure from the manufacturer (mixture of caffeine, MRFA and Ultramark 1621). The temperature of the heated capillary for the LXQ was set in the range of 200–250 °C, the ion-spray voltage was in the range of 3–6 kV and the injection time was 5–200 ms. The ligand solution (100 μM) was prepared in ammonium acetate buffer (20 mM, pH = 6.9) and aliquots of the appropriate metal ion were then added.

Determination of apparent stabilities of the metal ion complexes
The apparent stability constants at a given pH were determined by UV-Vis titration in the presence of a competitor. The Cu(i)
complexes were prepared by adding 0.9 equivalents of Cu(i) to three samples of each peptide (50 μM) in phosphate buffer (20 mM, pH = 7.4)/acetoni troile (9/1 V/V). Then 4, 8 and 12 equivalents (with respect to Cu(i) concentration) of BCS were added, respectively. The samples were equilibrated until the absorbance stabilized. Stability constants were calculated considering the formation of the CuP mononuclear complexes at the beginning of the titration according to the following equation:

\[
\beta_{\text{CuP}}^{\text{H}^+} = \frac{[\text{CuP}]}{[\text{Cu}]^n[\text{P}]}
\]

where CuP and P represent the complexed and free peptide at the pH of the studies, independent of the protonation state of the ligand, and Cu stands for the free Cu(i) ions.

The stabilities of the Hg(n)–peptide complex were determined at pH = 2.0 by using iodide (I−) as a competitor. Stability constants of the HgLn (n = 1–4) complexes were taken from the literature and recalculated for the conditions of the experiments (pH = 2.0, I− = 0.1 M NaClO4) by applying the SIT model. The molar absorption spectra of the four Hg(n)− complexes were determined by titrating a Hg(n) solution by the continuous addition of aliquots of I− solutions (0.01, 0.1 and 0.5 M KI). The recorded spectra were fitted with SPECFIT52–55 by fixing the stability constants of the HgLn complexes. The peptide solutions were prepared in water (30 μM, I− = 0.1 M NaClO4) and the pH was adjusted by a 1.0 M HCl solution. Samples for the competition experiments were prepared by adding 1.0 equivalent of Hg(n) to the peptide solutions. They were then titrated by the I− solutions by the same procedure and under the same conditions as described above. The pH of samples was under control throughout the experiments. SPECFIT52–55 was used for the evaluation of spectra. The molar absorption spectra and the stabilities of the HgLn complexes were fixed in the fitting procedure, allowing one to calculate the apparent stability of the HgP binary and HgPI ternary complexes (see the equations below), as well as their molar absorption spectra.

In the equations above, HgP (or HgPI) and P represent the complexed and free peptide at the pH of the studies, independent of the protonation state of the ligand, and Hg and I stand for the free Hg(n) and I− ions, respectively.

**Determination of acid dissociation constants (pKₐ)**

Deprotonation processes of one of the linear peptide ligands, 1, were followed by pH-potentiometric titrations in aqueous solution (T = 298.0 ± 0.1 K, c_ligand = 1.0 × 10⁻³ M, I = 0.1 M NaClO₄) following a protocol described earlier. An automatic titration set, including a PC-controlled Dosimat 665 (Metrohm) autoburette and an Orion 710A precision digital pH-meter equipped with an Orion 8103BNWP Ross Ultra semi micro pH electrode, was used to carry out the experiments. Argon atmosphere in the titration cell was applied in order to prevent the oxidation of the ligand. The data obtained in two parallel titrations were fitted using the PSEQUAD computer program based on the following general equilibrium process with the related equilibrium formation constants allowing the calculation of the pKₐ values for the individual deprotonation steps:

\[
qH + rL \rightleftharpoons H_rL_q
\]

where L denotes the non-protonated peptide and H the protons (charges are omitted for simplicity). Consequently, the composition of the neutral fully protonated peptide is H₃L. These pKₐ values were used in the calculation of the thermodynamic stability constants for the HgL and HgHL complexes from the apparent stabilities obtained at pH = 2.0. Details of these calculations are found in the ESL. Note that ‘L’ for the ligands is used in the text where the actual protonation states of the ligands/complexes are taken into account in calculations or in the description of species, whereas ‘P’ denotes the peptides in general, independent of their protonation states, as in the calculation of the apparent stabilities.

**Modelling of the trithiolate mercury complexes**

The six peptides were modelled in the apo and Hg(n)-bound form with a HgS₃ trigonal coordination. In the absence of structures for all the peptides but P⁵ᶜ, initial coordinates were built with the CHARMM program from standard values for the internal bonds, angles and dihedrals of amino acids in proteins. For peptide P⁵ᶜ, initial coordinates were either generated as for other peptides or taken from the solution NMR structure of the HgP⁵ᶜ complex.

**Model parameters.** For the holo peptides, a bonded model of the mercury site was used. Molecular mechanics force field parameters for surface mercury, absent from the CHARMM force fields, were deduced from ab initio DFT/B3LYP¹⁻¹⁻¹ calculations on the model system Hg³⁺{(S–CH₂–CH₃)}₃⁻ of tri-coordinated mercury using the Gaussian program. A total charge of –1 in the singlet spin state and using the LanL2dZ basis set was used. Bonded parameters were obtained by harmonic potential fitting of ab initio scans of the Hg–S distance, C–S–Hg angle and Hg–S–S–S improper angle. In this trigonal model, the S–Hg distance is 2.794 Å, the C–S–Hg angle is 125° and the Hg–S–S–S improper angle (calculated with Hg above the 3 sulfur atom plane in the counter clockwise direction) is 7.8°. Charges were obtained from RESP calculations following DFT/B3LYP optimization. Calculated charges are: Hg: +0.968, S: −0.798, all C(bonded to 2 hydrogens): 0.173, all C(bonded to 3 hydrogens): 0.069, van der Waals parameters were taken from the work of Šebesta et al. for Hg: e = 1.953 kcal mol⁻¹, R = 2.8 Å.

**Molecular dynamics simulations.** The geometries of the peptides were relaxed in vacuum by 1000-step energy minimization using the conjugated gradient method until the root-mean-square
(RMS) energy gradient was lower than 0.1 kcal mol\(^{-1}\) Å\(^{-1}\). Then the peptides were simulated in implicit water using the implicit solvent method EEF1 and the adapted extended atom CHARMM19_EEF1 force field.\(^{66}\) The systems were subjected to 40 to 300 ns MD simulations (depending on the convergence speed) using Langevin dynamics with a time step of 2 fs and a damping factor \(f_0\) of 20 ps\(^{-1}\) applied on non-hydrogen atoms. All peptides were simulated in the apo (no Hg) and holo (with Hg bound) forms.

### Results and discussion

#### Peptide design and synthesis

The cyclic peptide \(\text{P}^{\text{SC}}\) with three cysteine sidechains preoriented for Hg\(\text{II}\) coordination was recently reported by our group.\(^{40}\) All spectroscopic data obtained for the Hg\(\text{P}^{\text{SC}}\) mononuclear complex point to a HgS\(_3\) coordination mode, which is stable over a large pH range (pH = 5–9).

Five new peptides (Scheme 1) were designed in this work to monitor the effects of introducing a CxGx\(\beta\) motif instead of the CxCxC sequence found in P\(\text{SC}\) on the Cu(I) and Hg\(\text{II}\) complexation features. The positions of the three cysteines in the sequence of the newly designed linear and cyclic peptides were varied. An xPGx \(\beta\) turn inducing motif was introduced in the sequences of the cyclic peptides to rigidify the scaffold containing the metal-binding fragment CxCxG next to the turn in 1\(\text{C}\) or one amino acid apart in 2\(\text{C}\), 3\(\text{C}\), and 4\(\text{C}\) linear analogues of the cyclic decapeptides 1\(\text{L}\) and 2\(\text{L}\) synthesised to determine the impact of the cyclisation and flexibility of the backbone on the coordination properties. Finally, an additional amino acid, namely alanine, was inserted in peptide 3\(\text{C}\) with the idea of promoting larger flexibility in the cysteine sidechain orientation.

#### Cu(I) complexes

One of the six investigated peptides, P\(\text{SC}\), was previously demonstrated to stabilise the HgS\(_3\) coordination in a mononuclear complex.\(^{40}\) This raised the question whether a CuS\(_3\) geometry could also be observed with the latter peptide and with the new 3-Cys containing peptides presented in this paper. Cu(I) complexation of the ligands was studied by UV-Vis and CD spectroscopy and ESI-MS. All the six peptides display a rather similar complexation profile to illustrate the Cu(I)-binding features of the whole series. The titration of the peptides with Cu(I) at pH 7.4 was followed by UV-Vis spectroscopy. The absorbance stabilises surprisingly slowly with the equilibrium reached only after ca. 2 hours. Such long equilibration times were not observed before in similar systems with thiolate-containing peptides or pseudopeptides, even when Cu(I) clusters were formed from the very beginning of the titration.\(^{28,38,39}\) Titrations using individual samples were therefore conducted with Cu(I) to peptide concentration ratios from 0.0 to 3.0 in 0.25–0.5 steps to allow for long equilibration times and ensure that the thermodynamic equilibrium is reached. Fig. 1 shows the results of the UV titration of peptide 1\(\text{C}\) with Cu(I) at equilibrium. An intense band, characteristic for the S\(^{-}\) to Cu(I) charge transfer transition (LMCT),\(^{70}\) emerges at \(\lambda = 263\) nm with increasing Cu(I) concentration. The absorbance increases linearly up to 2.0 equiv. of Cu(I). The breakpoint observed for 2 equiv. of Cu(I) per peptide is indicative of the formation of polymetallic species with (Cu\(_{2}\)P\(_n\)) overall stoichiometry as previously observed with 3-Cys-containing tripeptide-like ligands.\(^{38,39}\) As seen before, the intensity of the S\(^{-}\) to Cu(I) LMCT is not sensitive to the composition of the complex and provides information only about the number of Cu(I)-thiolate bonds.\(^{38,39,71,72}\)

#### Scheme 1

Sequences of the studied peptides. Bonds in the turn motif are indicated in bold and the coordinating cysteine residues are highlighted in red.

![Scheme 1](image-url)
relatively low ([θ] < 1 × 10^4 deg cm^2 dmol^-1) compared to those obtained with other species involving 3 coordinated Cys ([θ] = 4 × 10^4 - 8 × 10^4 deg cm^2 dmol^-1). Moreover, no clear tendency can be observed in the evolution of the spectra upon Cu(i) addition up to 2.0 equivalents, as shown in Fig. S2 (ESI†). The mostly affected region falls below 250 nm, where the negative CD band is attributed to π → π* and possibly overlapping n → π* transitions, both belonging to the amide bonds of the peptide backbone, which suggests a conformational rearrangement upon Cu(i) addition. Since CD spectroscopy is more sensitive to the structure around Cu(i) than UV, these results indicate that the free peptides transform into more than one Cu(i) species. This assumption is confirmed by ESI-MS measurements in ammonium acetate buffer at pH 6.9 (Fig. S3, ESI†). Spectra recorded for samples with the peptides and 0.9 equivalents of Cu(i) show the formation of mononuclear complexes, CuP, and several polynuclear species, like Cu_2P_2 and Cu_4P_3. With the increase of the Cu(i) concentration, further species of higher nuclearity (Cu_4P_4, Cu_5P_3, Cu_6P_3) are detected. Therefore, the CD and ESI-MS experiments demonstrate that the apparently simple evolution of the LMCT bands in the UV titration is in fact due to the formation of a mixture of many polynuclear thiolate complexes.

Copper binding affinities were determined in the presence of BCS as a competitor. BCS forms a well-characterised Cu(BCS)_2 complex with Cu(i) according to eqn (1).

\[
\text{Cu}^+ + 2\text{BCS}^2- + \text{Cu(BCS)}_2^{3-} \rightleftharpoons \log \beta = 19.8 (1)
\]

This complex has an intense orange colour and a maximal absorbance at λ = 483 nm with an extinction coefficient ε = 13 300 M^-1 cm^{-1}. Solutions of the Cu(i)-peptide complexes (Cu(i):P ratio = 0.9:1) were titrated with BCS and the amount of Cu(i) displaced from the peptide by BCS was quantified based on the known absorption of the Cu(BCS)_2 complex. Since several Cu(i) complexes are formed with the six peptides, the fit of the spectroscopic competition results could not be perfectly implemented with a given complex stoichiometry. Hence, the apparent stability constants were calculated considering the formation of a CuP complex. The log β_CuP values are presented in Table 2.

All peptides display high affinity towards Cu(i) in the range typical of Cu(i) chaperone proteins, like Atx1.22 Hence, Cu(i) binding to the investigated 3-Cys containing peptides is characterized by a rather complicated speciation at pH = 7.4. Indeed, although the UV profile appears simple with a single endpoint at 2 equivalents of Cu(i), ESI-MS and CD clearly evidence several clusters, even with low Cu(i) concentration. This complicated mixture of species observed for the whole series of peptides highly contrasts with previous results obtained with tripodal pseudopeptides also incorporating three cysteine moieties, which form Cu(i) complexes, resembling those formed in metallothioneins.39,71,76 Indeed, the pre-orientation of the three thiolate groups in the tripodal pseudopeptides induces a well-defined metal binding cavity, stabilized by a network of hydrogen bonds.39 This structure perfectly controls the speciation of the Cu(i) complexes with only two identified species, namely the mononuclear complex and the Cu_6S_9 cluster both with Cu(I) ions in trithiolate environments.39,76

More recently, the highly constrained tetrapeptide Ac-Cys-D-Pro-Pro-Cys-NH_2 with a strong turn was shown to form exclusively a Cu_4S_6 trithiolate complex.77 The larger flexibility of the peptides described here could be responsible for the lack of control of the speciation of the Cu(i) complexes and ultimately for the formation of a mixture of polymeric species, with quite large stability. The determined apparent stability constants are similar in the whole peptide series, within the range of experimental errors, indicating that the structural differences have only a minor effect, if any, on the stability of the Cu(i) complexes.

### Hg(u) complexes

Hg(u) is a metal ion with soft character according to Pearson's theory,78 and as such, an often used probe of the oxygen and water sensitive Cu(i). The cyclic peptide P^AC has been recently demonstrated to form a complex with Hg(u) in a HgS_3 coordination environment, which is stable over a large pH range. Besides, the protonation of the mononuclear complex HgP^AC happens at a relatively low pH (pK_a value of 4.3) to produce a species with HgS_2 geometry.40 Hg(u) binding of the new series of peptides was therefore studied to reveal whether the behaviour of P^AC with Hg(u) is specific to the structure of this cyclic complex.
peptide or common for peptide sequences encompassing three Cys residues in a CxCxxC or CxCxC arrangement.

**Hg(n) binding at physiological pH.** The S" to Hg(n) LMCT bands of mercury–thiolate complexes are indicative of the metal ion coordination number and geometry, in contrast to similar bands of Cu(II)–thiolates. The HgS₃ coordination mode is characterized by an LMCT band in the wavelength range of 240–320 nm,²⁸,³⁷,⁷⁹ while the di-coordinate HgS₂ structures leave their fingerprint only in the higher energy UV region.²⁸,⁴⁰,⁸¹ Accordingly, the Hg(n) binding properties of the peptides were studied by UV-Vis spectroscopy at pH = 7.4. Titrations were conducted by gradually adding 0.1 equivalent of Hg(n) to the peptide solutions. The absorbance values stabilized after Hg(II) additions much faster (in less than 5 minutes) compared to the complexation of Cu(II) by the same peptides, which might be a result of a simpler speciation in the Hg(n)–ligand systems. All peptides showed very similar behaviour to ions coordinated by only two cysteine thiolate residues.²⁸,⁸⁰,⁸¹

The absorbance at 280 nm, characteristic of the HgS₃ geometry in the Hg(n) peptide complexes, decreases significantly with decreasing pH, which indicates the protonation of one cysteine to afford a linear HgS₂ complex at low pH, as observed previously with Hg(n) complexes of triple coiled-coil peptides.¹⁴,³⁵ Contrary to the latter systems, which define a highly protected hydrophobic metal-binding pocket, the flexible structure of the cyclic and linear peptides makes the transformation of the trithiolate coordination possible in excess of Hg(n) in Hg₃P₂ complexes.

**Protonation of the HgL complex.** The absorbance at 280 nm, characteristic of the HgS₃ geometry in the Hg(n) peptide complexes, decreases significantly with decreasing pH, which indicates the protonation of one cysteine to afford a linear HgS₂ complex at low pH, as observed previously with Hg(n) complexes of triple coiled-coil peptides.¹⁴,³⁵ Fig. 4 shows the pH titration for Hg₁C as an example. The spectroscopic data were satisfactorily fitted with one pKₐ value according to the following equation:

$$\text{HgL} + \text{H}^+ \rightleftharpoons \text{HgHL}$$

$$K_{\text{HgL}} = \frac{[\text{HgL}][\text{H}^+]}{[\text{HgHL}]}$$

where [HgL] and [HgL] denote the equilibrium concentrations of the mononuclear complexes including the peptide in different protonation states.

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**Fig. 2** UV titration of 1C with Hg(n) in phosphate buffer (20 mM, pH = 7.4). The inset shows the increase of the absorbance at 240 and 280 nm as a function of $c_{\text{Hg(n)}}/c_{\text{peptide}}$ ratio ($c_{\text{peptide}} = 30 \mu\text{M}$).

**Fig. 3** (+)ESI-MS spectra registered for 1C with different equivalents of Hg(n) in ammonium acetate buffer (20 mM, pH = 6.9). The comparison of the experimental and calculated isotopic envelop of the detected species is also presented. Asterisks mark the sodium adduct of the corresponding species. The notation 1C refers here to the neutral free peptide.
The $pK_a$ values of the Hg(n)–peptide complexes (see Table 2) follow the order of $pK_a^C < 3C ≈ 2C < 1C ≈ 1^l$. However, the differences observed between the $pK_a$ values in the series of peptides (maximum difference = 0.8) are quite small, which reflects the weak influence of the pattern of the three Cys residues (CxCxC or CxCxxC) and the separation of the metal binding fragment from the turn motif. $pK_a^C$ incorporating the CxCxxC sequence forms the HgS$_3$ coordination mode at a slightly lower pH than the other cyclic peptides. A larger separation from the PG-turn also seems to be favourable for the formation of the tris-thiolate complex in 3C. The highest $pK_a$ values are seen for the two linear peptides, which may be a consequence of their larger flexibility and a more significant reorganization necessary for the coordination of the third Cys sidechain. It is rather interesting that even the latter data (Table 2) are 1.7–1.8 log units lower than the $pK_a$ observed for the Hg(n)-complex of the tris-cysteine functionalized tripodal pseudopeptide ligand with amidated carboxyl groups, or of the three-stranded coiled coils ($pK_a$ values of 8.6 and 7.6 for sites d and a, respectively). This might be related to the presumably very different water-accessibility of the thiol groups, as hinted recently.

**Stabilities of the Hg(n) complexes.** Due to the high thiolphilicity of Hg(n), the determination of the stability of the complexes is rather challenging. Competition titrations with iodide ions were performed to measure the apparent stability constants at pH = 2.0. To the best of our knowledge, this is the first time that the well-characterized Hg(n)-complexing ability of iodide has been utilized for the determination of the stability of Hg(n)–thiolate complexes, which is probably due to the presence of several iodo complexes. According to the observed pH-dependent transformation of the Hg(n)–peptide complexes, the apparent stabilities determined at pH = 2.0 mostly correspond to the species with a HgS$_2$ structure. Hg(n) has been shown to form iodo complexes in four consecutive steps characterized by large formation constants. In spite of these large iodo complex stabilities, a high concentration of I$^-$ (1500–2000 equiv.) was necessary to withdraw Hg(n) from the peptide complexes which required the use of a background electrolyte ($I_0 = 0.1$ M NaClO$_4$) to lessen the change in the ionic strength during the titrations.

Therefore, the published stabilities of the iodo complexes were recalculate by applying the SIT model to the conditions of the experiments leading to the following formation constants: $\log \beta_{[HgI]^{2}} = 13.05$, $\log \beta_{[HgI]^{3}} = 24.09$, $\log \beta_{[HgI]^{4}} = 27.84$, $\log \beta_{[HgI]^{5}} = 29.91$. The result of the titration of Hg(n) with KI and the obtained molar spectra of the forming Hg(n)–I$^-$ complexes can be seen in Fig. 5A and Fig. S4 (ESI†), respectively.

Samples of the peptides containing 1.0 equivalent of Hg(n) were titrated with I$^-$ and the recorded spectra are presented in Fig. 5B. The first phase of the titrations, i.e. up to the presence of 10 equivalents of I$^-$, does not reflect considerable changes in the recorded spectra. Further addition of I$^-$ ions results in the appearance of new bands characteristic for the [HgI]$_3$ and [HgI]$_2$ species (compare to the spectra in Fig. S4, ESI†). A complete displacement of Hg(n) from the peptide complexes is achieved at ca. 2000 equiv. of I$^-$. The obtained spectra were fitted by SPECFIT by fixing the $log \beta$ values and the molar spectra of the Hg(n)-I$^-$ complexes. The best fits were obtained when the formation of a mixed ligand complex, HgPI, was also included in the models, besides the HgP species (inset in Fig. 5B). The appearance of such species is probably a consequence of the flexibility of the peptide structures. The HgPI complexes are present only in

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**Fig. 4** pH titration of 1C with 1.0 equivalent of Hg(i). The dashed line represents the spectrum of free 1C at pH = 2. The inset shows the evolution of the absorbance as a function of pH at 280 nm. Symbols represent the experimental points and the line is the fitted absorbance obtained by SPECFIT.

**Fig. 5** UV spectra recorded in the titration of (A) Hg(i) and (B) Hg1C with I$^-$, pH = 2.0, c$_{Hg(i)}$ = 30 μM (A), c$_{Hg(i)}$ = c$_{peptide}$ = 30 μM (B). The insets show the evolution of the absorbance at 322 nm (●) and 280 nm (○). Symbols represent the experimental data, and solid lines represent the absorbances calculated by SPECFIT.
the beginning part of the titrations in a ca. 20% relative proportion, except for HgP3C where a somewhat larger fraction of HgPI could be observed. The formation of the mixed ligand complexes, according to the HgP + I ⇄ HgPI equation, is characterized by stabilities falling in the range of log K \sim 1.5–2.5.

The apparent stability constants determined for the HgL complexes (Table 2) indicate rather similar affinities of Hg(n) to all peptides. Considering that at pH = 2.0 Hg(II) is coordinated only by two thiolate units, it is a plausible assumption that the preorientation of the donor groups has only a modest influence on the stabilities and the high thiophilicity of Hg(n) easily governs the formation of the favoured HgS2 structures. Formation constants for the different forms of the HgP species, i.e. for HgHL and HgL (where L denotes the fully deprotonated peptide), and apparent stabilities for pH = 7.4, were also estimated from the relevant conditional stability constants (log b_{HgL}^n) by applying the pKₐ values of the HgHL ⇄ HgL + H processes (Table 2) and the pKₐ values obtained for one of the free ligands, pKₐ^{HgL} = 9.26(1); pKₐ^{HgL}_{HL} = 8.56(1); pKₐ^{HgL}_{H2L} = 7.67(1) (see the Experimental part and the ESI†). The stability constants estimated for the Hg(n)-trithiolate HgL complexes span over a small range (maximum difference, Δlog b_{HgL} = 1) demonstrating a weak influence of the Cys-sidechain orientations in the chosen sequences (Table 2). Nevertheless, the two linear peptides display a slightly weaker affinity suggesting the need for a more pronounced rearrangement of the Cys sidechains. It is noteworthy to compare these data to the stability constants of Hg(n)-bisthiole complexes of highly constrained bis-thiol ligands. Our peptides, indeed, display very similar Hg(n)-binding affinities to that of the well-known soft metal ion chelator 2,3-dimercaptopropan-1-ol (BAL) (log b_{HgL} = 44.8),82 which forms a highly stable 5-membered chelate ring around the metal ion. Comparison of our data to the stability of the HgL species of the tetrapeptide CoPPC (log b_{HgL} = 40.0)81 clearly indicates that the structure of our peptides is prone to easily rearrange to a suitable form for the tridentate coordination of Hg(n) and thus the larger number of Hg(n)-thiolate bonds is revealed by higher affinities.

Modelling of the trithiolate HgL complexes

The six peptides were modelled in the apo and Hg(n)-bound form with a HgS3 trigonal coordination (see Experimental section for methods). Several independent simulations were run: first, for the Hg(n)-bound peptides three different simulations were run varying the orders of the cysteine sulfur atoms defining the Hg–S–S–S improper angle; then, for each best energy conformation in each system, new simulations for the related systems were run starting from there. The energy values reported in Table S2 (ESI†) refer to the simulation leading to the minimum average total energy for each peptide (over all simulations). Binding of Hg(n) to linear or cyclic peptides always results in stabilization meaning that the peptide structure organizes the 3 cysteine residues in an environment appropriate for HgS3 coordination. The stabilization expressed as ΔE = E(HgP) − E(P) is remarkably similar for all four cyclic peptides (between −11 and −12.8 kcal mol⁻¹ from Table S2, ESI†) with a relative error (ΔE/E) less than 1%. This stabilization varies from −13.1 to −9.5 kcal mol⁻¹ for the two linear peptides; however, this slightly larger difference may be due to an incomplete conformational search of the highly flexible linear apo peptides. Hence, the calculated stabilization energies, ΔE, are very similar in the series of peptides, which is in agreement with the comparable values of the stability constants of the HgL complexes determined experimentally (Table 2). Models of energy minimized structures of the Hg(n)-bound peptides, starting from the frame with the lowest potential energy during the dynamics simulation leading to minimum average total energy, are shown in Fig. 6 and Fig. S5 (ESI†) for cyclic and linear peptides, respectively. The three cysteine binding chains are well-disposed to afford the trithiolate coordination of the mercury ion in the six structures. In all the complexes, the positively charged sidechain of arginine (charge +1) is capping the HgS3, negatively charged binding site (charge −1), providing stabilizing electrostatic interactions.

Conclusions

Model peptides containing cysteine-rich sequences found in metallothioneins were studied for their metal-binding properties in relation to metal detoxification mechanisms. The two soft ions Cu(I) and Hg(n) were selected since they exhibit the largest affinities for these small detoxification proteins among endogenous and toxic metal ions, respectively. Three cysteine residues were introduced in CxCxC and CxCxC motifs in different positions within the sequence, in linear and cyclic derivatives. Overall, the six peptides display rather similar behaviour, which evidences minor contributions of the position of the three cysteine residues or cyclisation to the formation and stability of the Hg(n) and Cu(i) complexes.

Cu(i) binding to the series of peptides at physiological pH revealed to be rather complicated, with the formation of a mixture of polymetalic species. In contrast, cysteine-rich highly structured peptides77 or peptide-like ligands19,76 are able to
control the formation of well-defined Cu(i) complexes. Consequently, the complicated Cu(i)-complex speciation of the series of peptides, reported in this paper, has been assigned to their significantly larger flexibility. However, despite the formation of many polymetallic species, large affinity is achieved for the soft Cu(i) cation at physiological pH (10^{17.9}–10^{18}).

The binding of Hg(II), another soft metal ion often used as a probe for the oxygen and water sensitive Cu(i), demonstrates that the complexity of the Cu(i) speciation is due to the peculiar behaviour of Cu(i)-thiolate complexes in water and not to the cysteine-rich sequences chosen for the peptides. Interestingly, the HgS_2 coordination mode is stable over a large pH-range for all studied peptide complexes. Indeed, the protonation of the complex to give the HgS_2 linear coordination is observed with pK_a values ranging from 4.3 to 5.1, making the trithiolate coordination the major binding mode at physiological pH whatever the peptide sequence. The stabilities of the Hg(II) complexes (10^{10.6}–10^{11} at pH 7.4) are of the same order of magnitude as those reported for high affinity sulphur chelating agents such as BAL. 82 The large stability constants together with the low pK_a values and simulated structures clearly indicate that all the peptide sequences studied in this paper are adapted for an efficient trithiolate coordination of the thiophilic cation Hg(II).

Importantly, the striking differences observed in the coordination of Hg(II) and Cu(i) with the series of peptides indicate different molecular mechanisms involved in their binding to detoxification proteins. The sulphur-rich peptides studied here show more than 20 orders of magnitude larger affinity at pH 7.4 for Hg(II) (log K_{Hg}^{pH} ≈ 41) than for Cu(i) (log K_{Cu}^{pH} ≈ 18), due to the significantly softer character of Hg(II). Most importantly, Hg(II) forms well-defined complexes, whereas Cu(i)-coordination leads to mixtures of polymetallic species. This demonstrates the peculiar behaviour of Cu(i) thiolate complexes in water. Only highly constrained peptide sequences are able to promote the formation of well-defined Cu(i) complexes. The peptides studied here are probably too flexible to achieve such a control for Cu(i). Hence, the use of Hg(II) as a probe for Cu(i) coordination with sulphur-rich peptides or proteins in physiological conditions is demonstrated here to be not fully appropriate.

Conflicts of interest

There are no conflicts to declare.

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