Optical spectroscopic and reverse-phase HPLC analyses of Hg(II) binding to phytochelatins

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Optical spectroscopy and reverse-phase HPLC were used to investigate the binding of Hg(II) to plant metal-binding peptides (phytochelatins) with the structure $(\gamma\text{Glu-Cys})_2\text{Gly}$, $(\gamma\text{Glu-Cys})_3\text{Gly}$ and $(\gamma\text{Glu-Cys})_4\text{Gly}$. Glutathione-mediated transfer of Hg(II) into phytochelatins and the transfer of the metal ion from one phytochelatin to another was also studied using reverse-phase HPLC. The saturation of Hg(II)-induced bands in the UV/visible and CD spectra of $(\gamma\text{Glu-Cys})_2\text{Gly}$ suggested the formation of a single Hg(II)-binding species of this peptide with a stoichiometry of one metal ion per peptide molecule. The separation of apo- $(\gamma\text{Glu-Cys})_2\text{Gly}$ from its Hg(II) derivative on a C_{18} reverse-phase column also indicated the same metal-binding stoichiometry. The UV/visible spectra of both $(\gamma\text{Glu-Cys})_3\text{Gly}$ and $(\gamma\text{Glu-Cys})_4\text{Gly}$ at pH 7.4 showed distinct shoulders in the ligand-to-metal charge-transfer region at 280–290 nm. Two distinct Hg(II)-binding species, occurring at metal-binding stoichiometries of around 1.25 and 2.0 Hg(II) ions per

peptide molecule, were observed for $(\gamma\text{Glu-Cys})_3\text{Gly}$. These species exhibited specific spectral features in the charge-transfer region and were separable by HPLC. Similarly, two main Hg(II)-binding species of $(\gamma\text{Glu-Cys})_4\text{Gly}$ were observed by UV/visible and CD spectroscopy at metal-binding stoichiometries of around 1.25 and 2.5 respectively. Only a single peak of Hg(II)- $(\gamma\text{Glu-Cys})_4\text{Gly}$ complexes was resolved under the conditions used for HPLC. The overall Hg(II)-binding stoichiometries of phytochelatins were similar at pH 2.0 and at pH 7.4, indicating that pH did not influence the final Hg(II)-binding capacity of these peptides. The reverse-phase HPLC assays indicated a rapid transfer of Hg(II) from glutathione to phytochelatins. These assays also demonstrated a facile transfer of the metal ion from shorter- to longer-chain phytochelatins. The strength of Hg(II) binding to glutathione and phytochelatins followed the order: $\gamma\text{Glu-Cys-Gly} < (\gamma\text{Glu-Cys})_2\text{Gly} < (\gamma\text{Glu-Cys})_3\text{Gly} < (\gamma\text{Glu-Cys})_4\text{Gly}$.

INTRODUCTION

The living organisms survive in heavy-metal-contaminated environments by limiting the intracellular concentrations of the reactive free metal ions. Prokaryotes resist metals by four basic mechanisms: (i) modulation of their transport; (ii) active efflux; (iii) redox changes; and (iv) sequestration into innocuous complexes [1]. Eukaryotes may use all these mechanisms, but the metal resistance is generally attributable to the sequestration of toxic ions in complexes and/or within intracellular organelles. The metal ion sequestration in eukaryotes occurs via three main molecules: (i) glutathione (GSH) [2]; (ii) GSH-related phytochelatins with the general structure $(\gamma\text{Glu-Cys})_n\text{Gly}$ [3–6]; and (iii) cysteine-rich metallothioneins [7]. GSH appears to be the primary line of defence against metal toxicity in most organisms [2,8]. Metal-induced syntheses of metallothioneins occur in animals [7], several fungi [4], some prokaryotes [9] and perhaps in plants [10]. Plants, however, appear mainly to synthesize phytochelatins in response to heavy metals [3–6]. The fission yeast *Schizosaccharomyces pombe* also produces phytochelatins in response to many metals [11]. Some other fungi such as *Candida glabrata* [12], *Neurospora crassa* [13] and *Saccharomyces cerevisiae* [13] synthesize phytochelatins only in response to Cd(II).

The notion that phytochelatins are synthesized from GSH is supported by the following independent observations: (i) the inhibition of GSH synthesis in plants and yeasts leads to the loss of phytochelatin production [3–6]; (ii) phytochelatin synthesis is

also substantially reduced in GSH-deficient mutants of *S. pombe* [14] and *Arabidopsis thaliana* [15,16]; and finally (iii) the plants that produce homoglutathione in place of GSH synthesize phytochelatins with β -alanine, rather than glycine, at the C-terminus [3–6]. Zenk and co-workers isolated an enzyme from *Silene cucubalus* that transferred a $\gamma\text{Glu-Cys}$ dipeptidyl unit from one GSH molecule to another [17,18]. The enzyme also transferred a $\gamma\text{Glu-Cys}$ dipeptidyl unit from one phytochelatin molecule to another. The activity of this enzyme, trivially known as phytochelatin synthase, appeared to be tightly regulated by metal ions [17,18]. The chelation of metal ions by EDTA or newly synthesized phytochelatins led to the termination of the phytochelatin synthase reaction [17,18]. The activation of *Silene cucubalus* phytochelatin synthase by metal ions followed the order: $\text{Cd(II)} > \text{Ag(I)} > \text{Bi(III)} > \text{Pb(II)} > \text{Cu(II)} > \text{Zn(II)} > \text{Hg(II)} > \text{Au(I)}$ [17]. A close correspondence between the *in vitro* and *in vivo* studies on metal-dependent synthesis of phytochelatins suggests that these metal ions are probably the primary activators of phytochelatin synthase [6].

Sequestration of various heavy-metal ions by phytochelatins *in vitro* and *in vivo* suggested that these peptides detoxified metal ions [3–6]. The role of phytochelatins in detoxification of selected metal ions was confirmed by the isolation of GSH-normal but phytochelatin-deficient mutants from both *S. pombe* [14] and *A. thaliana* [15]. Mutations in *A. thaliana* phytochelatin synthase significantly decreased the tolerance of this plant to Cd(II) and Hg(II), but resistance to Cu(II) and Zn(II) was reduced only slightly [15]. It is interesting to note that the inhibition of GSH

Abbreviations used: LMCT, ligand-to-metal charge-transfer; n_2 , $(\gamma\text{Glu-Cys})_2\text{Gly}$; n_3 , $(\gamma\text{Glu-Cys})_3\text{Gly}$; n_4 , $(\gamma\text{Glu-Cys})_4\text{Gly}$; XAFS, X-ray absorption fine-structure spectroscopy

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biosynthesis, and thus of phytochelatin, in yeasts [8] and plants [15,16] affected Cu(II) tolerance only slightly. Although Cu(II)- and Zn(II)-dependent phytochelatin synthesis occurs both *in vivo* and *in vitro* [3–6], other pathways may be involved in tolerance to these metals. The yeasts *C. glabrata*, *N. crassa* and *S. cerevisiae* resist Cu(II) by synthesizing metallothioneins [4], but the exposure of these yeasts to Cd(II) leads to phytochelatin biosynthesis [12,13]. *A. thaliana* cells also synthesize metallothionein in response to Cu(II) stress [19]. Isolation and characterization of phytochelatin-deficient mutants from other species will lead to a better understanding of the roles that phytochelatins play in detoxification of metals other than Cd(II) and Hg(II).

The metal-binding characteristics of metallothioneins have been studied extensively [7]. In contrast, only a few investigations have been carried out on the metal-binding properties of phytochelatins. A detailed knowledge of the metal-binding characteristics of phytochelatins is necessary to clearly understand the roles of these short peptides in heavy-metal detoxification. The most important questions relating to the metal-binding properties of phytochelatins are as follows; (i) how are metals presented to phytochelatins?; (ii) what is the relationship between the metal-binding stoichiometry and the chain length of a given phytochelatin?; and (iii) are metals transferred from shorter-chain phytochelatins to longer-chain phytochelatins during their biosynthesis? The studies on the metal-binding characteristics of phytochelatins are particularly important, as some recent studies suggest that phytochelatin concentrations in marine phytoplankton can be good predictors of heavy-metal concentrations in natural waters [20–22]. Some information has been obtained on the Cd(II)-, Cu(I)- and Ag(I)-binding characteristics of phytochelatins [23–30]. The Cd(II)-binding properties of phytochelatins are complex in that the binding stoichiometry increases with the amount of inorganic sulphide present in the complex [23,24]. The Cd(II)-binding stoichiometry of $(\gamma\text{Glu-Cys})_2\text{Gly}$ (n_2) can vary from 0.5 to 5.0 Cd(II) ions per peptide molecule depending on the sulphide content of the complex [12,23,24]. The Cd(II)/sulphide molar ratio in these complexes can approach 0.8 [26]. Copper-phytochelatins luminesce at room temperature, indicating the presence of Cu(I)-thiolate clusters [25,27,28]. Luminescence studies indicated the formation of two distinct types of species for Cu(I)- $(\gamma\text{Glu-Cys})_2\text{Gly}$ and Cu(I)- $(\gamma\text{Glu-Cys})_3\text{Gly}$ [27]. Cu(I)-phytochelatins appear to exist in oligomeric forms and may contain different chain-length phytochelatins in one oligomer [25]. Cu(I)- and Ag(I)-binding stoichiometries of phytochelatins appear to depend on pH [27,29]. The binding of Ag(I) to phytochelatins was similar to that observed for Cu(I) at pH 7.0, but at pH values of 5.0 and below Ag(I)/-SH ratios approached one, suggesting the formation of linear polymers of the type -Ag-SR-Ag-SR-Ag-. Ag(I)-thiolate clusters in phytochelatins phosphoresce at 77 K [29], as has been observed for metallothioneins [7].

In this paper, we have used UV/visible and CD spectroscopy and reverse-phase HPLC to probe the binding of Hg(II) to phytochelatins. These studies are relevant to our understanding of the roles that phytochelatins play in Hg(II) detoxification. As mentioned above, Hg(II) activates phytochelatin synthase *in vitro* and induces phytochelatin synthesis in several different species [6,21]. Furthermore, mutations in GSH or phytochelatin biosynthesis lead to Hg(II) sensitivity [15,16]. As has been reported for Cu(I) [27], GSH-bound Hg(II) was quickly transferred to phytochelatins, indicating that the tripeptide may be a general donor of metal ions to phytochelatins. Facile transfer of Hg(II) from shorter-chain phytochelatins to longer-chain phytochelatins is also demonstrated. This finding is relevant to our understanding of the chain-elongation of phytochelatins *in vivo*.

MATERIALS AND METHODS

Reagents

Routinely used biochemical reagents were purchased from Sigma Chemical Company or Fisher Scientific. *S. pombe* 972 h^- was obtained from the American Type Culture Collection. The isolation of *C. glabrata* 2001-Cd^{R111} has been described previously [26]. *C. glabrata* cells were cultured in a defined medium [28], whereas *S. pombe* cells were grown in a complex medium containing tryptone, yeast extract and dextrose [25]. All yeast cultures were grown at 30 °C with shaking (225–250 rev./min). Cd and Hg concentrations were determined using flame atomic absorption (Perkin-Elmer model 3100 spectrophotometer) and flameless atomic absorption (Bachrach) respectively. HPLC analyses were carried out on a HP 1050 liquid chromatograph (Hewlett-Packard) controlled by HP Chemstation software. The chromatograms were analysed using public-domain software compatible with a HP Chemstation. The reverse-phase C₁₈ columns were purchased from Vydac, Hewlett-Packard or Phase Separations Inc.

Purification of phytochelatins and rat liver metallothionein-II

Phytochelatins were isolated from either *C. glabrata* 2001-Cd^{R111} or *S. pombe* cells exposed to CdSO₄ as described previously [27,29]. The yeasts rather than plants were chosen for the purification of phytochelatins due to the ease of manipulation and higher peptide yields for yeasts. *S. pombe* cells were grown to late logarithmic phase before exposure to CdSO₄ (1 mM final concentration) for 48 h. Stationary-phase cultures of *C. glabrata* were diluted to 1% (v/v) in the synthetic complete medium containing 10 mM CdSO₄ and grown for 24 h prior to harvest. The cells were disrupted using the bead-beater (Biospec) according to the recommendations of the manufacturer. This procedure has proven highly efficient for the breakage of both *C. glabrata* and *S. pombe* cells. The phytochelatins $(\gamma\text{Glu-Cys})_2\text{Gly}$ (n_2), $(\gamma\text{Glu-Cys})_3\text{Gly}$ (n_3) and $(\gamma\text{Glu-Cys})_4\text{Gly}$ (n_4) were purified by a combination of ion-exchange, gel-filtration (if needed) and reverse-phase HPLC as described previously [27,29]. The purified phytochelatins were quantified by thiol analyses [31]. The peptide samples were stored anaerobically in 0.1% trifluoroacetic acid at –20 °C or –70 °C depending on the length of storage. Rat liver metallothionein-II was isolated from animals injected with ZnSO₄. Male Sprague–Dawley rats weighing 180–220 g were administered a sterile saline solution of ZnSO₄ (10 mg of Zn/kg body wt) intraperitoneally on three consecutive days. The animals were killed using CO₂. The livers were stored at –20 °C prior to the purification of metallothioneins. The purification procedures were similar to those described previously [32], except that the Zn-metallothioneins were substituted with Cd(II) before chromatography. The purity of metallothioneins was checked by electrophoresis on polyacrylamide gels under non-denaturing conditions [33]. Thionein-II was prepared by acidification of the Cd(II)-protein to pH 2.0, followed by the removal of the metal ion by gel filtration on Sephadex G-25 equilibrated with 0.01 M HCl [27]. The protein concentrations were determined as described previously [27]. Thionein-II samples were stored anaerobically in 0.01 M HCl at –20 °C.

UV/visible and CD spectroscopy

The peptides were diluted to 12.5–50 μM (final concentration) in 0.01 M HCl (pH 2.0) or 0.1 M Tris/acetate (pH 7.4) in an anaerobic chamber (Plaslabs) and placed in septum-sealed cuvettes (Spectrocell). HgCl₂ solution prepared in 0.1 M HCl

was similarly placed in another cuvette. The UV/visible absorption and CD spectra of apophytochelatin were recorded on a double-beam spectrophotometer (Perkin-Elmer Lambda 3B) and a spectropolarimeter (Jasco J-600) respectively. Increasing molar equivalents of Hg(II) were injected into the cuvettes using a Hamilton syringe. The samples were mixed and the spectra recorded 5–10 min after Hg(II) addition. The binding of Hg(II) to phytochelatin appeared to be complete within the mixing time. In some cases the UV/visible and CD spectra were recorded in the presence of a 10-fold molar excess (with respect to the peptide concentration) of 2-mercaptoethanol. It has been shown that the presence of 2-mercaptoethanol prevented excess binding of Hg(II) to Hg(II) sensor MerR protein [34,35]. The spectral data were analysed using graphics software on a personal computer.

Reverse-phase HPLC analysis of Hg(II)-binding to phytochelatin

Preliminary experiments showed that Hg(II) binding leads to changes in the retention times of phytochelatin on a C₁₈ column (ODS2, Phase Separations Inc.). Run conditions were then optimized for individual phytochelatin to separate apophytochelatin from their Hg(II) derivatives. Hg(II)-binding stoichiometry was determined by quantifying apo and Hg(II) forms for each run. Aliquots, 20 nmol of n_2 or n_3 and 10 nmol of n_4 , were mixed with HgCl₂ solution or Hg(II)–GSH (see below) to achieve the specified Hg(II)/peptide molar ratios. The samples were diluted with 0.01 M HCl to a 1.0 ml final volume. Portions (0.3–0.5 ml) of the reconstituted samples were separated on the C₁₈ column (250 mm × 4 mm) using a gradient of acetonitrile in 0.1 % trifluoroacetic acid. The gradient conditions suitable for the separation of n_2 from Hg(II)– n_2 were as follows: 0–6 % acetonitrile (in 0.1 % trifluoroacetic acid) for 1 min, 6–18 % acetonitrile for 14 min and 18–60 % acetonitrile for 2 min. The free and Hg(II)-bound n_2 fractions were eluted at 9.6 and 9.9 min respectively. The separation of n_3 from its Hg(II) derivatives was achieved using the following gradient conditions: 0–10.8 % acetonitrile for 1 min and then 10.8–18 % acetonitrile for 15 min. The retention time of free n_3 was 8.8 min. Two species of Hg(II)– n_3 were resolved. The first species predominated at lower metal-binding stoichiometries and was eluted from the reverse-phase column at 8.6 min (see Figure 5). The principal Hg(II)– n_3 species at the higher metal-binding stoichiometries was eluted from the column at about 11.9 min (see Figure 5). The gradient conditions suitable for the separation of n_4 from its Hg(II) derivatives were: 0–8.4 % acetonitrile for 1 min and then 8.4–21 % acetonitrile for 24 min. The retention times for the free n_4 and Hg(II)– n_4 were 15.2 and 18.1 min respectively.

Transfer of Hg(II) from GSH to phytochelatin

The initial experiments on Hg(II) binding to n_3 and n_4 showed that these peptides were precipitated at higher metal loadings under acidic conditions. This problem was solved by replacing HgCl₂ with Hg(II)–GSH complex for reconstitution assays. These assays simultaneously demonstrated the transfer of Hg(II) from GSH to phytochelatin. Hg(II)–GSH solution was prepared by mixing HgCl₂ with a 10-fold molar excess of GSH in 0.01 M HCl. In agreement with the previous polarimetric studies [36], HPLC analyses of this solution showed the formation of Hg(II)–(GSH)₂ complexes (results not shown). Thus, 80 % of GSH in the Hg(II)–GSH solution was in the metal-free form. Hg(II)–phytochelatin complexes prepared with Hg(II)–GSH were stable even at very high metal loads, as indicated by the absence of aggregation or precipitation. GSH was clearly separated from its

Hg(II) derivative under the separation conditions used for n_2 , n_3 or n_4 (see Figures 2, 5 and 8).

Transfer of Hg(II) from shorter- to longer-chain phytochelatin

Two types of experiments were conducted to study the relative affinity of phytochelatin for Hg(II). In the first experiment, increasing equivalents of Hg(II)–GSH complex were added to a mixture of phytochelatin containing 10 nmol each of n_2 , n_3 and n_4 . The resulting mixtures were then separated on the Phase Separations C₁₈ column to determine the order of saturation of phytochelatin with Hg(II). In the second set of experiments, Hg(II)– n_2 was added to n_3 or n_4 to study the transfer of the metal ion from n_2 to n_3 or n_4 . Similarly, Hg(II)– n_3 complex was added to n_4 to examine the transfer of the metal ion from the shorter- to the longer-chain phytochelatin. The HPLC gradient conditions for all of these experiments were the same as those used for n_4 described above.

RESULTS AND DISCUSSION

Apophytochelatin, like thioneins, exhibit featureless absorption spectra in the 220–320 nm range [7,25,27,29]. However, the binding of metals to cysteinyl thiolates in phytochelatin or thioneins induces ligand-to-metal charge-transfer transitions (LMCTs) in the optical spectra [7,25,27,29]. Metal-induced qualitative and quantitative changes in the UV/visible absorption spectra of phytochelatin allow the determination of the metal-binding stoichiometries [25,27,29]. CD spectroscopy has also been extensively used to probe the binding of metals to thioneins [7]. The formation of three distinct Hg(II)-binding species of rabbit liver metallothionein-II was inferred from the unique CD spectral characteristics exhibited by the protein at specific metal loadings [37,38]. In this study, we have used both UV/visible and CD spectroscopy to elucidate the Hg(II)-binding properties of phytochelatin. The peptides chosen for this study, n_2 , n_3 and n_4 are the predominant phytochelatin produced in response to metal stress [3–6]. We have also developed and used reverse-phase HPLC assays to confirm the Hg(II)-binding stoichiometries of phytochelatin. As the best separations of phytochelatin from their Hg(II) derivatives (in HPLC assays) were achieved using a trifluoroacetic acid/acetonitrile system at pH 2.0, spectroscopic studies were also performed at acidic pH to determine whether the formation of a particular Hg(II)-phytochelatin species was a pH-dependent process. Studies on the binding of Ag(I) to phytochelatin have shown that the stoichiometry of binding was pH-dependent [29]. Additionally, studies on rabbit liver metallothionein-II showed that the formation of the Hg(II)₁₈ species of the protein is strongly pH-dependent [37,38]. The spectroscopic and HPLC data on individual phytochelatin are presented below.

Hg(II)-binding characteristics of n_2

The titration of Hg(II) into n_2 at pH 2.0 caused the appearance of an absorption shoulder at around 225 nm (Figure 1a). Another, much weaker, shoulder was present at ~265 nm (Figure 1a). This peptide appeared to bind 1–1.25 Hg(II) ions per molecule at acidic pH values, as indicated by the lack of any qualitative or quantitative changes in the spectrum upon titration of more than 1.25 Hg(II) equivalents (Figure 1a). The A_{225} increased linearly up to one Hg(II) equivalent and then flattened out (Figure 1a, inset). Raising the pH of titration to 7.4 did not appear to have any effect on the maximal Hg(II)-binding stoichiometry of n_2 as the peptide still bound 1–1.25 metal ion equivalents (Figure 1b, inset). The notable differences between the UV/visible spectra at

Figure 1 UV/visible absorption and CD spectroscopy of Hg(II)- n_2 complexes at pH 2.0 and 7.4

The metal-free peptide solution (50 μ M) in 0.01 M HCl (**a,c**) or in 0.1 M Tris/acetate, pH 7.4 (**b,d**) was titrated with increasing equivalents of Hg(II) as HgCl_2 . The titrations were performed in septum-sealed anaerobic cuvettes. The samples were scanned for UV/visible absorption (**a,b**) or CD spectroscopy (**c,d**) 5 min after the Hg(II) addition. The numbers on the curves indicate the Hg(II) equivalents added to the peptide. The insets (**a,b**) show plots of absorbance (at 225 nm) versus molar ratio of Hg(II)/peptide. Each point on the curve represents mean $\pm$ S.E.M. of five independent reconstitutions.

pH 2 and 7.4 were: (i) the presence of an absorption band at around 218 nm, due to the thiolate anion at pH 7.4; and (ii) a stronger shoulder at 265 nm at pH 7.4. The changes in the CD spectra of n_2 that occurred upon the titration of Hg(II) into the peptide at pH 2.0 (Figure 1c) or pH 7.4 (Figure 1d) confirmed the metal-binding stoichiometry derived from UV/visible spectroscopy. At pH 2.0, two positive bands at 226 and 253 nm increased in intensity with the added Hg(II) equivalents reaching a titration end point when one metal equivalent was titrated (Figures 1c and 1d). Only one band at 254 nm was seen upon the titration of Hg(II) into n_2 at pH 7.4. The CD spectra of metallothionein at acidic pH values are also different from those recorded at neutral pH [7]. It appears that only one type of Hg(II)- n_2 species is formed, as no qualitative changes were seen in the UV/visible or CD spectra upon titration of increasing equivalents of Hg(II) into the peptide. As described later, the Hg(II)-binding characteristics of n_2 are different from the longer-chain phytochelatin which appear to form two types of metal-binding species.

Reverse-phase HPLC analyses were developed (i) to study the transfer of the metal ion from GSH to phytochelatin, and (ii) to follow the conversion of metal-free peptides into their Hg(II) derivative(s). Identical results were obtained whether HgCl_2 or Hg(II)-GSH was used as the metal ion donor. Only the data obtained with Hg(II)-GSH are shown in Figure 2, to demonstrate the transfer of Hg(II) from GSH into n_2 . The binding of Hg(II) to n_2 increased the retention time slightly (Figure 2a). The titration of graded equivalents of Hg(II)-GSH to the peptide led to the reduction in the amount of apo- n_2 and concomitant

Figure 2 Reverse-phase HPLC analyses of the transfer of the metal ion from Hg(II)-GSH to n_2

(a) Graded equivalents of Hg(II)-GSH were added to n_2 diluted in 0.01 M HCl to achieve metal/ n_2 ratios of up to 1.5. Each titration mixture was then separated on the reverse-phase column using gradient conditions described in the text. The solid line shows the profile obtained for the peptide in the absence of any added Hg(II)-GSH. The broken line represents a typical chromatogram obtained following the addition of 1.5 equivalents of Hg(II) as GSH complex. Hg(II)-GSH peak appeared only when more than the saturating amounts of Hg(II) were added. (b) Peptide concentrations [free or the Hg(II)-bound form] were calculated from the respective peak areas and are plotted against the molar ratio of Hg(II)/peptide. Each point on the curve represents the mean $\pm$ S.E.M. from four independent HPLC runs.

increase in the quantity of Hg(II)- n_2 (Figures 2a and 2b). The peak area of the Hg(II)- n_2 complex increased linearly with the molar equivalents of Hg(II) added, reaching a titration end point at one Hg(II) ion per peptide molecule (Figure 2b). The peak area of the free n_2 decreased with increasing equivalents of Hg(II), reaching undetectable levels when saturating amounts of Hg(II) were added (Figure 2b). GSH was clearly separated from its Hg(II) derivative under the HPLC gradient conditions (Figure 2a). It is noteworthy that the Hg(II)-GSH peak appeared only when n_2 was completely saturated with the metal ion. Thus, the transfer of Hg(II) from GSH to the peptide may also be followed by monitoring the appearance of the Hg(II) derivative of the tripeptide. The formation of a stable GSH- n_2 -Hg(II) complex was not observed under the conditions of these experiments. The present studies extend our previous observations on the transfer of Cu(I) from GSH to phytochelatin [27]. Additional studies are required to determine whether GSH is a general donor of the metal ions to phytochelatin. The data presented in Figure 2 also suggest that Hg(II) binding to n_2 probably occurred in an 'all-or-nothing' fashion, in the sense that a given peptide molecule was either free or fully bound to the metal ion. Based upon the HPLC and optical spectroscopic studies, it can be concluded that n_2 binds one Hg(II) ion per peptide molecule. As discussed later, it appears likely that the Hg(II)-binding geometry in n_2 is two-coordinate, as is the case in GSH (results not shown).

Figure 3 UV/visible spectroscopy of Hg(II)- n_3 complexes at pH 2.0 (a) or at pH 7.4 (b,c)

Hg(II) titrations into n_3 were performed anaerobically in septum-sealed cuvettes. The final concentration of the peptide was 50 μ M for each titration. Inset (a) shows the changes in absorbance (at 225 nm) with the increasing equivalents of Hg(II) titrated at pH 2.0. Two Figures are used to show the spectra of Hg(II)- n_3 complexes at pH 7.4 (b and c) to clearly show the qualitative changes in the spectra upon titration of increasing equivalents of Hg(II). The changes in absorbance at 225 nm (b, inset) show two breakpoints: the first one occurring at 1.25 Hg(II) equivalents and the second occurring upon the titration of 2 equivalents of the metal ion. Hg(II)-induced changes in the absorbance at 290 nm also show similar breakpoints (c, inset). Each data point in the insets represents the mean $\pm$ S.E.M. from four independent titrations.

Hg(II)-binding characteristics of n_3

The titration of Hg(II) into n_3 at pH 2.0 led to the development of a shoulder at 225 nm in the UV/visible spectrum (Figure 3a). This shoulder was flattened upon the addition of more than 2.0 mol equivalents of Hg(II). The A_{225} of n_3 increased linearly up to the titration of 1.75–2.0 Hg(II) equivalents into the peptide (Figure 3a, inset). The n_3 samples showed precipitation at Hg(II) loadings above 1.75 equivalents. The sudden increase in absorbance at all wavelengths at Hg(II)/peptide ratios higher than 2.0 was probably due to aggregation/precipitation (Figure 3a). Precipitation was avoided in the presence of a 10-fold molar excess of GSH or 2-mercaptoethanol. Also, the precipitates could be dissolved by adding excess 2-mercaptoethanol or GSH. It is interesting to note that no precipitation occurred when Hg(II) was titrated into n_2 at pH 2.0. It is possible that the binding of excess Hg(II) to n_3 or n_4 (see below) at pH 2.0 results

Figure 4 CD spectroscopy of Hg(II)- n_3 complexes at pH 2.0 (a,b) and pH 7.4 (c,d)

Hg(II) titrations into n_3 were performed as in Figure 3. CD spectra obtained at each pH are shown in two Figures [0–1.0 and 1.0–2.0 Hg(II) equivalents] to show the qualitative changes that occur following the titration of more than 1 Hg(II) equivalent.

in the formation of large aggregates that eventually precipitate. It seems likely that GSH or 2-mercaptoethanol dissolved the precipitates due to chelation of the excess Hg(II) bound to the longer-chain phytochelatin. This interpretation will be consistent with the observation that the Hg(II)-GSH peak appeared in chromatograms only when the peptides were fully saturated with Hg(II) (see Figures 2, 5 and 8).

Although the data at pH 2.0 did not give a clear indication of the formation of more than one Hg(II)- n_3 species, the titration of the metal ion into n_3 at pH 7.4 clearly showed that two distinct Hg(II)-peptide species were formed (Figures 3b and 3c). Hg(II)-induced spectral changes were similar up to the addition of 1.25 mol equivalents of the metal ion (Figures 3b and 3c). The absorption shoulder at 290 nm decreased in intensity upon further titration of Hg(II) into the peptide. The spectrum of Hg(II)- n_3 stabilized upon the addition of more than 2.0 Hg(II) equivalents (Figure 3c). The Hg(II)-induced spectral features at pH 7.4 are better appreciated from the metal-ion-dependent changes in A_{225} and A_{290} (Figure insets to 3b and 3c). The A_{225} plot shows two break points: the first at 1.25 and the second at 2.0 mol equivalents of Hg(II) (Figure 3b, inset). The A_{290} plot shows that the absorbance increased up to the addition of 1.25 mol equivalents of Hg(II), whereafter it declined showing a breakpoint around 2.0 mol equivalents of the metal ion (Figure 3c, inset). The spectrum of Hg(II) $_2$ - n_3 is very similar to that of Hg(II)- n_2 , indicating that the metal-ion co-ordination may be similar in these two complexes. The CD spectral analyses of the titration of Hg(II) into n_3 supported the formation of two metal-

Figure 5 Reverse-phase HPLC analyses of Hg(II)-derivatives of n_3

(a) Hg(II) as Hg(II)–GSH complex was added to metal-free n_3 in increasing equivalents to obtain Hg(II)/ n_3 ratios of up to 2.0. The separation of metal-free n_3 from its Hg(II) derivatives was achieved by reverse-phase HPLC as described in the text. The solid line shows a typical chromatogram obtained without the addition of Hg(II) to the peptide. The dashed line shows the chromatogram obtained upon the titration of 0.75 Hg(II) equivalent into n_3 . The dotted line shows the chromatogram that was obtained upon the titration of 2.0 Hg(II) equivalents into n_3 . (b) The concentrations of metal-free n_3 and of the two species of Hg(II)– n_3 were calculated for each titration by HPLC analyses as in (a) and are plotted against the molar ratio of Hg(II)/peptide. Each data point represents the mean $\pm$ S.E.M. from four independent HPLC runs.

peptide species (Figures 4a, 4b, 4c and 4d). At pH 2.0, two positive bands centred at 230 and 260 nm were observed upon the addition of up to 0.75 Hg(II) equivalents (Figure 4a). These spectra are somewhat similar to those of the Hg(II)– n_2 complex. The 260 nm positive band was replaced with 265 and 290 nm negative bands upon titration of more than 0.75 Hg(II) equivalents (Figure 4b). These results suggest the formation of two chiral structures: the first one at 0.75–1.0 Hg(II) equivalents and the second at 1.75–2.0 Hg(II) equivalents. The CD spectra of Hg(II)– n_3 complexes at pH 7.4 showed a positive band at 233 nm and a negative band at 272 nm. The band intensities increased up to 0.75–1.0 equivalents of Hg(II), whereafter they declined (Figure 4c). A positive single band appears upon the binding of 1.5 Hg(II) equivalents to n_3 and stabilizes at 1.75 metal ions/peptide molecule (Figure 4d).

The reverse-phase HPLC analysis of Hg(II)– n_3 complexes was carried out using Hg(II)–GSH to prevent the precipitation that occurred at pH 2.0 (see above). The initial Hg(II)– n_3 species, with a retention time slightly shorter than the free peptide, predominated at Hg(II)/peptide molar ratios of up to 1 (Figures 5a and 5b). A second species with significantly increased retention time appeared at around 0.75 mol equivalent of Hg(II) and

Figure 6 UV/visible absorption spectroscopy of Hg(II)– n_4 complexes at pH 2.0 (a) and 7.4 (b,c)

The metal-free n_4 samples (12.5 μ M) were titrated with increasing equivalents of Hg(II) at pH 2.0 (0.01 M HCl) or at pH 7.4 (0.1 M Tris/acetate). The spectra obtained at pH 7.4 are shown in two Figures (b and c) to demonstrate the qualitative changes in the spectrum as more than one Hg(II) equivalent is titrated into the peptide. The inset (c) shows changes in the absorbances at selected wavelengths (234 and 280 nm) as increasing equivalents of Hg(II) are titrated into n_4 . Each data point represents the mean $\pm$ S.E.M. from four independent reconstitutions.

increased in concentration up to 1.75 equivalents, whereafter it stabilized. It is noteworthy that the peak due to free n_3 disappeared when more than 1 equivalent of Hg(II) was added. Thus, the formation of the second Hg(II)– n_3 species occurred upon the binding of additional Hg(II) to the first Hg(II)– n_3 species. As noted above for n_2 , the Hg(II)–GSH peak did not appear until n_3 was saturated with Hg(II). Thus, the HPLC results are consistent with the spectroscopic data which indicated the formation of two Hg(II)– n_3 species. For the sake of clarity, the possible co-ordination geometry of Hg(II) in these species is discussed following presentation of the data on n_4 .

Hg(II)-binding characteristics of n_4

The UV/visible spectra of Hg(II)– n_4 complexes at pH 2.0 (Figure 6a) were similar to those of Hg(II)– n_3 . The shoulder at $\sim$ 225 nm increased in intensity up to the titration of 2.0 Hg(II) equivalents (Figure 6a, inset). The loading of n_4 with more than 2.5 Hg(II) equivalents led to precipitation. The UV/visible spectra for Hg(II)– n_4 complexes at pH 7.4 showed a biphasic pattern

Figure 7 CD spectroscopy of Hg(II)- n_3 complexes at pH 2.0 (a,b) and pH 7.4 (c,d)

The metal-free n_4 samples (25 μ M) were titrated with increasing equivalents of Hg(II) at pH 2.0 or at pH 7.4 as in the legend to Figure 7. The spectra at each pH are shown in two sets to clearly demonstrate the qualitative changes that occur upon the titration of more than 1 Hg(II) equivalent.

(Figures 6b and 6c) which is described above for the Hg(II)- n_3 derivatives at the same pH. The initial Hg(II)- n_4 complex showed shoulders at ~ 240 nm and ~ 280 nm. These shoulders increased in intensity up to the titration of 1.25 Hg(II) equivalents (Figure 6b), whereafter the shape of the absorption spectrum changed (Figure 6c). The absorbances at wavelengths below 234 nm increased, whereas those above 234 nm decreased. The spectrum stabilized upon the addition of 2.5 or more Hg(II) equivalents to n_4 . These changes in the spectra of Hg(II)- n_4 are also presented as variations in the absorbances at 234 and 280 nm (Figure 6c, inset) to clearly demonstrate their significance. The A_{234} of Hg(II)- n_4 increased nearly linearly up to the addition of 1.25 Hg(II) equivalents. The titration of additional Hg(II) equivalents led to a slight decline in A_{234} (Figure 6c, inset). The absorbance at 280 nm of the Hg(II)- n_4 complex also increased up to the titration of 1.25 Hg(II) equivalents, whereafter a substantial decline occurred in A_{280} . The spectrum stabilized upon titration of more than 2.5 Hg(II) equivalents into n_4 . In contrast to the observations at acidic pH, no precipitation of Hg(II)- n_4 occurred, even at very high loadings of the metal ion, at pH 7.4. As noted above for n_3 , Hg(II)- n_4 complex containing maximal Hg(II) equivalents showed spectral features similar to those observed for Hg(II)- n_2 .

Although the CD spectra of Hg(II)- n_4 complexes at acidic pH exhibited noise (Figures 7a and 7b), some features of the titration are easily discernible. A positive CD envelope at 254 nm developed upon the titration of Hg(II) into n_4 and increased in

Figure 8 Reverse-phase HPLC analyses of Hg(II)-binding to n_4

(a) Hg(II) as Hg(II)-GSH complex was added to metal-free n_4 in increasing equivalents to obtain Hg(II)/ n_4 ratios of up to 4.0. The separation of metal-free n_4 from its Hg(II) derivative was achieved by reverse-phase HPLC as described in the text. The broken line shows the chromatogram obtained with metal-free n_4 sample. The chromatogram obtained upon the titration of 4.0 Hg(II) equivalents into n_4 is shown as a solid line. (b) The concentrations of metal-free n_4 and Hg(II)- n_4 complex were calculated for each Hg(II) titration using the HPLC assay as described in the text. Each data point represents the mean $\pm$ S.E.M. from four independent HPLC runs.

intensity up to the addition of 2 Hg(II) equivalents (Figures 7a and 7b). A sharp decrease in the 254 nm band intensity occurred upon the titration of 2.5 metal-ion equivalents. However, the CD envelope at 254 nm stabilized if an excess of 2-mercaptoethanol was included in the titration buffer (results not shown). A weak envelope appearing at 225 nm upon the titration of one or more Hg(II) equivalents indicated the formation of a second chiral structure (Figure 7b). The formation of two distinct Hg(II)- n_4 chiral species was clearly indicated by monitoring titration of the metal ion into the peptide at pH 7.4 (Figures 7c and 7d). A prominent negative CD envelope at ~ 275 nm, developing upon the binding of Hg(II) to n_4 , increased in intensity up to the titration of 1 metal-ion equivalent. This band intensity decreased upon the titration of additional Hg(II) equivalents into n_4 . A positive band at ~ 250 nm appeared upon the binding of 1.75 equivalents. This feature plateaued in the peptide complexes containing 2–2.25 Hg(II) equivalents.

The UV/visible and CD spectral data on the titration of Hg(II) into n_4 was confirmed by the reverse-phase HPLC analyses of the Hg(II)- n_4 complexes formed at various metal-binding stoichiometries. The HPLC analyses were performed using Hg(II)-GSH as the metal-ion donor. The binding of Hg(II) to n_4 induced a significant increase in the retention time of the peptide on the C₁₈ column (Figure 8a). Only a single Hg(II) derivative of n_4 was observed during the HPLC analyses. However, a plot of free peptide versus the molar ratio of Hg(II)/peptide showed a

Figure 9 The affinity of Hg(II)-binding to phytochelatins

Increasing amounts of Hg(II) as its GSH complex were added to a mixture of n_2 , n_3 and n_4 (10 nmol of each peptide) and the resulting mixture chromatographed on the C_{18} column using the acetonitrile gradient described in the text. The chromatogram obtained in the absence of any added Hg(II) is shown in (a). The chromatograms obtained following the addition of 15, 30 and 55 nmol of Hg(II) are shown in (b), (c) and (d) respectively. The peaks due to metal-free peptides and their Hg(II)-derivatives are indicated.

breakpoint at 1 Hg(II) equivalent (Figure 8b). The optical spectra described above did show the formation of a distinct species of n_4 containing one Hg(II) ion. It may be noted that the amount of Hg(II)- n_4 (based on HPLC peak area) did not increase linearly with the Hg(II) equivalents titrated into the peptide.

Co-ordination environment of Hg(II) in phytochelatins

Hg(II) can adopt a variety of co-ordination geometries with thiolate ligands, the most common geometries being linear digonal, trigonal and tetrahedral [34–38]. Hg(II) co-ordination observed with small organic compounds is generally linear digonal [34]. Polarimetric and proton NMR studies have shown that Hg(II) forms a two-co-ordinate complex with GSH, although transient complexes exhibiting higher co-ordination numbers do occur [36,39]. Our HPLC studies also suggest the formation of a Hg(II)-(GSH)₂ complex (results not shown). O'Halloran and co-workers [35] have reported the synthesis and characterization of a trigonal Hg(II) complex with (aliphatic) *t*-butyl thiolate [Et₄N][Hg(SBu^t)₃]. The UV/visible spectra of this compound was very similar to that of a Hg(II)-sensing protein MerR, leading to the suggestion that Hg(II) adopted a trigonal co-ordination in this protein [34]. Other studies, including genetic characterization, have shown that Hg(II) is indeed co-ordinated to three cysteinyl sulphurs [40]. The main distinguishing features of the UV/visible

spectrum of the above model compound and MerR consist of charge-transfer bands at 240, 260 and 290 nm [34]. Studies on Hg(II)₃-metallothionein from *N. crassa* [41] and Hg(II)₇-metallothionein from rabbit [37,38] or rat liver (this study; results not shown) also show similar characteristics. It was, therefore, suggested that the Hg(II) co-ordination in metallothioneins may be trigonal rather than pseudotetrahedral as has generally been believed [34].

There are strong parallels between the UV/visible spectra of Hg(II)- n_3 and Hg(II)- n_4 complexes and those of metallothioneins at pH 7.4. The 280–290 nm LMCT bands in the rat (this study; results not shown) or rabbit liver metallothionein-II [37,38] increase in intensity up to the titration of 7 Hg(II) equivalents. The band intensity declines upon further addition of Hg(II) into either metallothionein. The 290 nm band is completely flattened upon the titration of 10–11 metal ions per molecule. A third species forms upon the titration of 18 Hg(II) equivalents into the rabbit metallothionein [37,38]. A similar qualitative pattern is seen when Hg(II) is titrated into n_3 and n_4 (Figures 3 and 6). The Hg(II)-induced 280–290 nm LMCT bands increase in intensity up to the addition of 1–1.25 Hg(II) equivalents in both n_3 and n_4 . It appears likely that the metal co-ordination in Hg(II)- n_3 or Hg(II)- n_4 is very similar to that in Hg(II)₇-metallothionein from rat or rabbit liver. As mentioned above, a comparison with a model aliphatic compound suggests that Hg(II) is co-ordinated trigonally in Hg(II)₇-metallothioneins. Initial X-ray absorption fine structure spectroscopy (XAFS) analysis had also suggested a trigonal co-ordination for Hg(II) in metallothionein [42]. However, a recent XAFS study on Hg(II)₇-metallothionein [prepared from Zn(II)₇-metallothionein] suggested a co-ordination number of two for Hg(II), but with two unusually short bonds and two unusually long bonds that exhibited a much larger disorder [43]. It is unclear whether Hg(II)₇-metallothionein prepared from apometallothionein will exhibit the same Hg(II) co-ordination. It seems reasonable to assume that the metal-ion co-ordination in Hg(II)- n_3 is trigonal. Although, the spectral features of Hg(II)- n_4 at pH 7.4 are similar to those of Hg(II)- n_3 at the same pH, it is possible that the co-ordination is tetrahedral in Hg(II)- n_4 . Further structural characterization is required to resolve this issue. The metal-ion co-ordination appears to change when n_3 and n_4 are fully loaded with Hg(II) as the 280–290 nm LMCT bands are replaced by a shoulder at 265 nm (Figures 3 and 6). The UV/visible spectra, and to some extent CD spectra, of fully Hg(II)-substituted n_3 and n_4 at pH 7.4 are very similar to those of n_2 at the same pH. A comparison with a model compound exhibiting digonal co-ordination [34,35] suggests that the Hg(II) co-ordination in fully substituted phytochelatins is also two-co-ordinate. The phytochelatin with only two sulphurs (n_2) appears to form only the two-co-ordinate Hg(II) complex. Further structural characterization of Hg(II)-phytochelatins is required to confirm the present assignment of the metal-ion co-ordination environment.

Transfer of Hg(II) from shorter-chain to longer-chain phytochelatins

In vivo studies on a variety of plants and *S. pombe* have shown that the chain elongation of phytochelatins is a time-dependent process [11]. The longer the exposure of the yeast or plant cells to Cd(II), the longer is the chain length of phytochelatins [3–6]. A partially purified phytochelatin synthase also showed a time-dependent increase in the relative proportion of longer-chain phytochelatins [17,18]. The chain elongation may occur by transfer of γ Glu-Cys from GSH, or a phytochelatin, to another phytochelatin molecule. It is therefore important to determine if

Figure 10 The transfer of Hg(II) from shorter- to longer-chain phytochelatins

n_2 substituted with 1 Hg(II) equivalent was mixed with metal-free n_3 (a,b) or n_4 (c,d) and the mixture was separated by reverse-phase HPLC as described in the text. Similarly, n_3 containing 1.5 Hg(II) equivalents (in the presence of GSH) was mixed with n_4 to study the transfer of the metal ion (e,f,g). The chromatograms shown in (a) and (b) were obtained by adding 0.75 and 1.5 Hg(II) equivalents [as Hg(II)- n_2], respectively, to n_3 . The chromatograms resulting from mixing 1.0 and 2.25 Hg(II) equivalents (as n_2 complex) to n_4 are shown in (c) and (d) respectively. The transfer of Hg(II) from n_3 to n_4 was studied by mixing 1.0 (e), 2.0 (f) or 2.5 (g) Hg(II) equivalents as the shorter-chain phytochelatin with the longer-chain phytochelatin.

the metal ion bound to GSH or shorter-chain phytochelatins will transfer to longer-chain phytochelatins as these are synthesized. As shown above, Hg(II) bound to GSH is easily transferred to phytochelatins. Similarly, Cu(I) bound to GSH is also transferred to phytochelatins [27]. The transferability of Hg(II) from one phytochelatin to another and the relative strength of the binding of Hg(II) to phytochelatins of various chain lengths were determined using HPLC assays. In the first experiment, increasing equivalents of Hg(II) (as GSH complex) were titrated into a mixture of n_2 , n_3 and n_4 . The addition of 15 nmol of Hg(II) to the peptide mixture (containing 10 nmol of each peptide) resulted in the conversion of about 11 and 40 % of the free n_2 and n_4 into their Hg(II) derivatives (Figure 9a). The titration of 30 nmol of Hg(II) into the peptide mixture showed 85 % conversion of n_4 into its Hg(II) form, whereas only about 40 % of n_2 was converted into its Hg(II) form (Figure 9c). It appeared that the first Hg(II)- n_3 species was substantially formed upon titration of 30 nmol of Hg(II) into the peptide mixture. All three peptides were completely converted into their Hg(II) derivatives when 55 nmol of Hg(II) was titrated (Figure 9d). Only the second Hg(II)- n_3 species was seen upon complete titration of Hg(II) into the peptide mixture (Figure 9d). The Hg(II)-GSH peak occurred only when the Hg(II)-binding capacity (of all the peptides present

in the mixture) was exceeded. These results, therefore, show that the relative affinity of Hg(II) for GSH and phytochelatins followed the order $\text{GSH} < n_2 < n_3 < n_4$.

That Hg(II) could indeed be transferred from shorter-chain phytochelatins to longer-chain phytochelatins was shown by mixing Hg(II) derivatives of the shorter-chain phytochelatins with longer-chain apophytochelatins. The addition of increasing equivalents of Hg(II) as Hg(II)- n_2 to n_3 and n_4 resulted in the formation of Hg(II) derivatives of n_3 and n_4 with the concomitant release of apo- n_2 (Figures 10a, 10b, 10c and 10d). However, n_3 was not completely converted into its second Hg(II) species even when 1.5 Hg(II) equivalents as n_2 were added. n_4 was completely converted into its Hg(II) form when 2.25 Hg(II) equivalents as n_2 were added (Figure 10d). Similar experiments showed that the metal ion from Hg(II)- n_3 complexes was transferred to n_4 (Figures 10e, 10f and 10g). The addition of 2.5 Hg(II) equivalents as n_3 was required to completely convert n_4 into its Hg(II) derivative (Figure 10g). Although, the Hg(II)- n_3 complex added to n_4 was entirely in the form of the second Hg(II)- n_3 species, the chromatograms shown in Figures 10(e), 10(f) and 10(g) showed complete absence of the second Hg(II)- n_3 species, indicating that this species was converted into the first Hg(II)- n_3 species/apo- n_3 due to the transfer of the metal ion to n_4 . No noticeable transfer of

Hg(II) seemed to occur from longer-chain to shorter-chain phytochelatins (results not shown).

Conclusions

Both UV/visible and CD spectroscopy have proven excellent tools with which to study the metal-binding stoichiometries and obtain preliminary information on the formation of metal-thiolate clusters in the phytochelatins. Cu(I)-phytochelatins probably exist as oligomers [25,27], but initial gel-filtration studies do not suggest oligomerization of Hg(II)-phytochelatins (results not shown). Hg(II) may adopt digonal or higher co-ordination depending on the binding stoichiometry of phytochelatins. Reverse-phase HPLC has now been shown to provide invaluable information on the (i) metal-binding stoichiometry of phytochelatins, (ii) the transfer of metals from GSH to phytochelatins, and (iii) transfer of metal ions from shorter- to longer-chain phytochelatins. The binding of metals such as Cu(I) [28], Ag(I) [29], Hg(II) and Bi(III) (R. K. Mehra, V. R. Kodati and J. Miclat, unpublished work) alters the retention times of phytochelatins on reverse-phase HPLC. Thus, some of the analytical methods for phytochelatins that relied on retention times of apophytochelatins [20] need to be reassessed. However, the above-mentioned methods remain valid for Cd(II)- and Zn(II)-phytochelatins as these metals are released at the acidic pH used for HPLC.

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