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 (γGlu-Cys)Gly, (γGlu-Cys)2Gly and (γGlu-Cys)3Gly. 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 (γGlu-Cys)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-(γGlu-Cys)Gly from its Hg(II) derivative on a C18 reverse-phase column also indicated the same metal-binding stoichiometry. The UV/visible spectra of both (γGlu-Cys)Gly and (γGlu-Cys)2Gly 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 (γGlu-Cys)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 (γGlu-Cys)2Gly 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)–(γGlu-Cys)3Gly 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: γGlu-Cys–Gly < (γGlu-Cys)2Gly < (γGlu-Cys)3Gly.

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 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 (γGlu-Cys)nGly [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 γGlu-Cys dipeptidyl unit from one GSH molecule to another [17,18]. The enzyme also transferred a γGlu-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: Cd(II) > Ag(I) > Bi(III) > Pb(II) > Cu(II) > Zn(II) > Hg(II) > 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...
biosynthesis, and thus of phytochelatins, in yeasts [8] and plants [15,16] affected Cu(II) tolerance only slightly. Although Cu(II)- and Zn(II)-dependent phytochelatin synthesis occurs both \textit{in vivo} and \textit{in vitro} [3–6], other pathways may be involved in tolerance to these metals. The yeasts \textit{C. glabrata}, \textit{N. crassa} and \textit{S. cerevisiae} resist Cu(II) by synthesizing metallothioneins [4], but the exposure of these yeasts to Cd(II) leads to phytochelatin biosynthesis [12,13]. \textit{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 (γ-Glu-Cys),Gly (nγ) 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)-(γ-Glu-Cys),Gly and Cu(I)-(γ-Glu-Cys),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 appear to depend on pH [27,29,30]. 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 (γ-Glu-Cys),Gly (nγ) 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)-(γ-Glu-Cys),Gly and Cu(I)-(γ-Glu-Cys),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 \textit{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 \textit{in vivo}.

**MATERIALS AND METHODS**

**Reagents**

Routine use of biochemical reagents was purchased from Sigma Chemical Company and Fisher Scientific. \textit{S. pombe} 972 h−1 was obtained from the American Type Culture Collection. The isolation of \textit{C. glabrata} 2001-Cd\textsubscript{11111} has been described previously [26]. \textit{C. glabrata} cells were cultured in a defined medium [28], whereas \textit{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 C\textsubscript{18} columns were purchased from Vydac, Hewlett-Packard or Phase Separations Inc.

**Purification of phytochelatins and rat liver metallothionein-II**

Phytochelatins were isolated from either \textit{C. glabrata} 2001-Cd\textsubscript{11111} or \textit{S. pombe} cells exposed to Cd\textsubscript{SO\textsubscript{4}} 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. \textit{S. pombe} cells were grown to late logarithmic phase before exposure to Cd\textsubscript{SO\textsubscript{4}} (1 mM final concentration) for 48 h. Stationary-phase cultures of \textit{C. glabrata} were diluted to 1 % (v/v) in the synthetic complete medium containing 10 mM Cd\textsubscript{SO\textsubscript{4}} 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 \textit{C. glabrata} and \textit{S. pombe} cells. The phytochelatins (γ-Glu-Cys)\textsubscript{nγ}, Gly (nγ) and (γ-Glu-Cys)\textsubscript{nγ} (γ-Glu-Cys)\textsubscript{nγ} (γ-Glu-Cys) 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 %, trfluoroacetic acid at −20 °C or −70 °C depending on the length of storage. Rat liver metallothionein-II was isolated from animals injected with Zn\textsubscript{SO\textsubscript{4}}. Male Sprague–Dawley rats weighing 180–220 g were administered a sterile saline solution of Zn\textsubscript{SO\textsubscript{4}} (10 mg of Zn/kg body wt) intraperitoneally on three consecutive days. The animals were killed using CO\textsubscript{2}. 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). HgCl\textsubscript{2} solution prepared in 0.1 M HCl
was similarly placed in another cuvette. The UV/visible absorption and CD spectra of apophytochelatins 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 phytochelatins 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 phytochelatins**

Preliminary experiments showed that Hg(II) binding leads to changes in the retention times of phytochelatins on a C18 column (ODS2, Phase Separations Inc.). Run conditions were then optimized for individual phytochelatins to separate apophytochelatins 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 or n, and 10 nmol of n, 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 from Hg(II)–n 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 fractions were eluted at 9.6 and 9.9 min respectively. The separation of n 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 was 8.8 min. Two species of Hg(II)–n 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 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 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 and Hg(II)–n were 15.2 and 18.1 min respectively.

**Transfer of Hg(II) from GSH to phytochelatins**

The initial experiments on Hg(II) binding to n and n showed that these peptides were precipitated at higher metal loadings under acid 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 phytochelatins. 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)n 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, n, or n (see Figures 2, 5 and 8).

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

Two types of experiments were conducted to study the relative affinity of phytochelatins for Hg(II). In the first experiment, increasing equivalents of Hg(II)–GSH complex were added to a mixture of phytochelatins containing 10 nmol each of n, n, and n. The resulting mixtures were then separated on the Phase Separations C18 column to determine the order of saturation of phytochelatins with Hg(II). In the second set of experiments, Hg(II)–n, was added to n or n to study the transfer of the metal ion from n to n or n. Similarly, Hg(II)–n complex was added to n 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 described above.

**RESULTS AND DISCUSSION**

Apophytochelatins, 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 phytochelatins 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 phytochelatins 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 phytochelatins. The peptides chosen for this study, n, n, and n are the predominant phytochelatins 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 phytochelatins. As the best separations of phytochelatins 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 phytochelatins 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) species of the protein is strongly pH-dependent [37,38]. The spectroscopic and HPLC data on individual phytochelatins are presented below.

**Hg(II)-binding characteristics of n**

The titration of Hg(II) into n 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 A225 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 as the peptide still bound 1–1.25 metal ion equivalents (Figure 1b, inset). The notable differences between the UV/visible spectra at
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 transfer of Hg(II) from GSH to the peptide. As described later, the metal-binding stoichiometry derived from UV/visible spectroscopy (Figure 1c) or CD spectroscopy (Figure 1d) 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 ± S.E.M. of five independent recombinations.

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 µM) in 0.01 M HCl (a,b) 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 (e,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 ± S.E.M. of five independent recombinations.

Reverse-phase HPLC analyses were developed (i) to study the transfer of the metal ion from GSH to phytochelatins, 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 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 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 ± S.E.M. from four independent HPLC runs.

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 ± S.E.M. from four independent HPLC runs.
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 \( n_2 \) or \( n_3 \) (see below) at pH 2.0 results 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 phytochelatins. 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), whereas it declined showing a breakpoint around 2.0 mol equivalents of the metal ion (Figure 3c, inset). The spectrum of Hg(II)–\( n_3 \) 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-
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)–\(n_3\) 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 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 \(n_3\), 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.
Hg(II) binding to phytochelatins

( Figures 6b and 6c) which is described above for the Hg(II)–n₄ derivatives at the same pH. The initial Hg(II)–n₄ 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 n₄. These changes in the spectra of Hg(II)–n₄ are also presented as variations in the absorbances at 234 and 280 nm (Figure 6c, inset) to clearly demonstrate their significance. The A254 of Hg(II)–n₄ 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 A254 (Figure 6c, inset). The absorbance at 280 nm of the Hg(II)–n₄ complex also increased up to the titration of 1.25 Hg(II) equivalents, whereafter a substantial decline occurred in A254. The spectrum stabilized upon titration of more than 2.5 Hg(II) equivalents into n₄. In contrast to the observations at acidic pH, no precipitation of Hg(II)–n₄ occurred, even at very high loadings of the metal ion, at pH 7.4. As noted above for n₄, Hg(II)–n₄ complex containing maximal Hg(II) equivalents showed spectral features similar to those observed for Hg(II)–nₓ.

Although the CD spectra of Hg(II)–n₄ 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 n₄ and increased in 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 species was clearly indicated by monitoring titration of the metal ion into the peptide at pH 7.4 (Figure 7b). The formation of two distinct Hg(II)–n₄ chiral species was clearly indicated by monitoring titration of the metal ion into the peptide at pH 7.4 (Figures 7c and 7d).

Figure 7 CD spectroscopy of Hg(II)–n₄ complexes at pH 2.0 (a,b) and pH 7.4 (c,d)

The metal-free n₄ samples (25 µM) 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.

The UV/visible and CD spectral data on the titration of Hg(II) into n₄ was confirmed by the reverse-phase HPLC analyses of the Hg(II)–n₄ 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 n₄ induced a significant increase in the retention time of the peptide on the C₁₈ column (Figure 8a). Only a single Hg(II) derivative of n₄ was observed during the HPLC analyses. However, a plot of free peptide versus the molar ratio of Hg(II)/peptide showed a
Hg(II) is shown in the acetonitrile gradient described in the text. The chromatogram obtained in the absence of any sulphurs [40]. The main distinguishing features of the UV spectrum of the above model compound and MerR consist of charge-transfer bands at 240, 260 and 290 nm [34]. Studies on Hg(II)-metallothionein from N. crassa [41] and Hg(II)-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$_2$ and Hg(II)-n$_n$ 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$_2$ and n$_n$ (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$_2$ and n$_n$. It appears likely that the metal co-ordination in Hg(II)-n$_2$ or Hg(II)-n$_n$ is very similar to that in Hg(II)-n$_2$-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)-n$_2$-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)-n$_2$-metallothionein [prepared from Zn(II)-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)-n$_2$-metallothionein prepared from apometallothionein will exhibit the same Hg(II) co-ordination. It seems reasonable to assume that the metal co-ordination in Hg(II)-n$_n$ is trigonal. Although, the spectral features of Hg(II)-n$_n$ at pH 7.4 are similar to those of Hg(II)-n$_n$ at the same pH, it is possible that the co-ordination is tetrahedral in Hg(II)-n$_n$. Further structural characterization is required to resolve this issue. The metal-ion co-ordination appears to change when n$_2$ and n$_n$ are fully loaded with Hg(II) as the 280–290 nm LMCT bands are replaced by a shoulder at 265 nm [34]. The UV/visible spectra, and to some extent CD spectra, of fully Hg(II)-substituted n$_2$ and n$_n$ at pH 7.4 are very similar to those of n$_n$ 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 γGlu-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

$\text{n}_2$ substituted with 1 Hg(II) equivalent was mixed with metal-free $\text{n}_3$ (a, b) or $\text{n}_4$ (c, d) and the mixture was separated by reverse-phase HPLC as described in the text. Similarly, $\text{n}_3$ containing 1.5 Hg(II) equivalents (as Hg(II)$-\text{n}_3$), respectively, to $\text{n}_4$. The chromatograms resulting from mixing 1.0 and 2.25 Hg(II) equivalents (as $\text{n}_2$ complex) to $\text{n}_4$ are shown in (c) and (d) respectively. The transfer of Hg(II) from $\text{n}_3$ to $\text{n}_4$ 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.

Absorbance (214nm)

Time (min)

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 $\text{n}_3$, $\text{n}_4$ and $\text{n}_5$. 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 $\text{n}_3$ and $\text{n}_4$ into their Hg(II) derivatives (Figure 9a). The titration of 30 nmol of Hg(II) into the peptide mixture showed 85% conversion of $\text{n}_4$ into its Hg(II) form, whereas only about 40% of $\text{n}_3$ was converted into its Hg(II) form (Figure 9c). It appeared that the first Hg(II)$-\text{n}_2$ 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). The Hg(II)$-\text{GSH}$ 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 GSH $< \text{n}_4 < \text{n}_3 < \text{n}_5$.

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)$-\text{n}_2$ to $\text{n}_4$ resulted in the formation of Hg(II) derivatives of $\text{n}_4$ and $\text{n}_5$ with the concomitant release of apo-$\text{n}_3$ (Figures 10a, 10b, 10c and 10d). However, $\text{n}_3$ was not completely converted into its second Hg(II) species even when 1.5 Hg(II) equivalents as $\text{n}_3$ were added. $\text{n}_4$ was completely converted into its Hg(II) form when 2.25 Hg(II) equivalents as $\text{n}_4$ were added (Figure 10d). Similar experiments showed that the metal ion from Hg(II)$-\text{n}_4$ complexes was transferred to $\text{n}_5$ (Figures 10e, 10f and 10g). The addition of 2.5 Hg(II) equivalents as $\text{n}_5$ was required to completely convert $\text{n}_5$ into its Hg(II) derivative (Figure 10g). Although, the Hg(II)$-\text{n}_5$ complex added to $\text{n}_4$ was entirely in the form of the second Hg(II)$-\text{n}_5$ species, the chromatograms shown in Figures 10(e), 10(f) and 10(g) showed complete absence of the second Hg(II)$-\text{n}_5$ species, indicating that this species was converted into the first Hg(II)$-\text{n}_5$ species/apo-$\text{n}_5$ due to the transfer of the metal ion to $\text{n}_4$. 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 coordination 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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