Glutathione-mediated transfer of Cu(I) into phytochelatins

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Room temperature luminescence attributable to Cu(I)–thiolate clusters has been used to probe the transfer of Cu(I) from Cu(I)–glutathione complex to rabbit liver thionein-II and plant metal-binding peptides phytochelatins (γ -Glu-Cys)₂Gly, (γ -Glu-Cys)₃Gly and (γ -Glu-Cys)₄Gly. Reconstitutions were also performed using CuCl. The Cu(I)–binding stoichiometry of metallothionein or phytochelatins was generally independent of the Cu(I) donor. However, the luminescence of the reconstituted metallothionein or phytochelatins was higher when Cu(I)–GSH was the donor. This higher luminescence is presumably due to the stabilizing effect of GSH on Cu(I)–thiolate clusters. As expected, 12 Cu(I) ions were bound per molecule of metallothionein. The Cu(I) binding to phytochelatins depended on their chain length; the binding stoichiometries being 1.25, 2.0 and 2.5

for (γ -Glu-Cys)₂Gly, (γ -Glu-Cys)₃Gly and (γ -Glu-Cys)₄Gly respectively at neutral pH. A reduced stoichiometry for the longer phytochelatins was observed at alkaline pH. No GSH was found to associate with phytochelatins by a gel-filtration assay. The Cu(I) binding to (γ -Glu-Cys)₂Gly and (γ -Glu-Cys)₃Gly occurred in a biphasic manner in the sense that the relative luminescence increased approximately linearly with the amount of Cu(I) up to a certain molar ratio whereafter luminescence increased dramatically upon the binding of additional Cu(I). The luminescence intensity declined once the metal-binding sites were saturated. In analogy with the studies on metallothioneins, biphasic luminescence suggests the formation of two types of Cu(I) clusters in phytochelatins.

INTRODUCTION

The animal cells typically respond to metal stress by synthesizing cysteine-rich metallothioneins ([1] and references therein). These proteins buffer heavy metal ions in the intracellular environment and thus effectively decrease the concentrations of potentially toxic ions. Although plants harbour metallothionein-like genes [2], the exposure of plant cells to heavy metals generally results in the synthesis of glutathione-derived metal-chelating peptides variously called phytochelatins [3], cadystins [4] and γ -glutamyl peptides [5]. Some yeasts such as *Candida glabrata* [6] and *Schizosaccharomyces pombe* [4] and to a limited extent *Neurospora crassa* and *Saccharomyces cerevisiae* [7] also synthesize phytochelatins. These peptides have the general structure (γ -Glu-Cys)_nGly; the value of *n* varies from two to 11 depending on the species and conditions of exposure [8,9].

The synthesis of metallothioneins in animals occurs within a few hours of exposure to metal ions [10]. Similarly, there is some delay before phytochelatins are synthesized in response to metal ions [8,9]. Several lines of evidence suggest that glutathione plays a crucial role in defence against potentially toxic metal ions by sequestering them prior to the synthesis of metallothioneins or phytochelatins. *C. glabrata* cells exposed to Cd show predominant binding to glutathione up to 6 h and the binding species change to phytochelatins at later intervals [11]. Studies on *S. cerevisiae* showed the appearance of copper–GSH complex in cells exposed to ⁶⁴Cu [12]. A number of other investigators have provided evidence favouring an important role for GSH in Cu metabolism [13]. Cu or other metals associated with glutathione may be directly transferred to metallothionein. Ferreira et al. [14] have recently shown that Cu(I)–GSH complex was much more efficient than other Cu(I) compounds in transferring the metal to metallothionein. The importance of GSH in metal detoxification is highlighted by the fact that the inhibition of GSH biosynthesis sensitizes the cells to the toxic effects of metals [15,16].

Phytochelatins are presumably synthesized from glutathione by the action of an enzyme trivially known as phytochelatin synthase [17]. Certain other routes for the biosynthesis of phytochelatins have also been suggested [18]. The mechanisms by which metal ions are inserted into phytochelatins are yet to be defined, but it is likely that glutathione plays a crucial role in this process. Metal–glutathione complex may either be the actual substrate for the phytochelatin synthase reaction or metal ions may be transferred directly to the newly synthesized phytochelatins. Metal–glutathione complexes may also exist in association with phytochelatins. Cd-exposed *C. glabrata* cells appeared to contain such complexes prior to the total conversion of Cd–GSH complexes into phytochelatins [11]. The present studies show that Cu(I) is easily transferred from GSH to phytochelatins of various chain lengths. Gel filtration of phytochelatins reconstituted with Cu(I)–GSH did not show association of the tripeptide with any of the phytochelatins studied. However, the presence of glutathione appears to stabilize the Cu(I)–phytochelatins. This was indicated by higher luminescence of Cu(I)–phytochelatins reconstituted using Cu(I)–GSH than those reconstituted using CuCl.

MATERIALS AND METHODS

Reagents

GSH, glutathione reductase, NADPH, rabbit liver Cd,Zn-metallothionein-II and chromatography materials were purchased from Sigma Chemical Company. Cuprous chloride (99.995 + % pure) was obtained from Aldrich. All other routine reagents were of the highest purity available. The yeasts *C. glabrata* (A.T.C.C. 2001) and *S. pombe* (972 h[−]) were from the American Type Culture Collection. A Cd-resistant strain of *C. glabrata* used for the purification of some batches of (γ -Glu-Cys)₂Gly has been described [19]. All yeast strains were grown at 30 °C with shaking (225–250 rev./min) in a defined [6] or a complex yeast extract–

Abbreviations used: TFA, trifluoroacetic acid; n₂, (γ -Glu-Cys)₂Gly; n₃, (γ -Glu-Cys)₃Gly; n₄, (γ -Glu-Cys)₄Gly.

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tryptone–dextrose medium [20]. Metal concentrations were determined by atomic absorption spectroscopy using a Perkin-Elmer model 3100 spectrometer. HPLC purifications were performed on a Hewlett-Packard 1050 system controlled by HPCHEM software.

Purification of phytochelatins

Phytochelatins were purified from either *C. glabrata* or *S. pombe*. *C. glabrata* does not produce peptides longer than $(\gamma\text{-Glu-Cys})_2\text{Gly}$ [6], whereas *S. pombe* synthesizes longer phytochelatins [3,20]. For the induction of phytochelatins, *C. glabrata* was grown in the defined medium containing 1 mM CdSO_4 . The *S. pombe* cells were cultured in the complex medium for 24 h before the addition of CdSO_4 to a final concentration of 1 mM [20]. The cells were harvested by centrifugation at 5000 *g* for 15 min in a Sorvall GSA rotor. The purification procedures were similar to those described previously except that the gel-filtration purification step was generally not required prior to final purification by HPLC [5,6]. The Cd-binding peptides partially purified by ion-exchange chromatography [5] were acidified to pH 2.0 with concentrated HCl and then applied to a reverse-phase Vydac C_{18} column (25 cm $\times$ 1 cm) equilibrated with 0.1 % trifluoroacetic acid (TFA). The individual phytochelatins were eluted with a gradient of acetonitrile in 0.1 % TFA (0–18 % in 40 min) at a flow rate of 2.0 ml/min. The retention times for $(\gamma\text{-Glu-Cys})_2\text{Gly}$, $(\gamma\text{-Glu-Cys})_3\text{Gly}$ and $(\gamma\text{-Glu-Cys})_4\text{Gly}$ were 20.3, 27.1 and 32.3 min respectively. The peaks corresponding to individual phytochelatins were collected and pooled from a large number of runs. The peptides were concentrated by partial lyophilization and stored in 0.1 % TFA. Small amounts of acetonitrile (< 3 %) were present in the concentrated samples. The concentrations of phytochelatins in the stock solutions varied from 0.5 to 4 mM. The integrity of the concentrated samples was verified by analytical reverse-phase HPLC on a Hewlett-Packard ODS2 C_{18} (25 cm $\times$ 0.4 cm) column. After application of the sample (in 0.1 % TFA), the column was washed with 0.1 % TFA for 15 min. The peptides were then eluted with a linear gradient of acetonitrile in 0.1 % TFA (0–24 % in 20 min). The retention times for $(\gamma\text{-Glu-Cys})_2\text{Gly}$, $(\gamma\text{-Glu-Cys})_3\text{Gly}$ and $(\gamma\text{-Glu-Cys})_4\text{Gly}$ on this column were 22.6, 25.9 and 29.4 min respectively. The peptide concentrations were determined by -SH analysis using 2,2'-dithiodipyridine [21]. Degassed and nitrogen-saturated peptides were stored at -20°C or at -70°C depending on the length of storage.

Preparation of thionein

Rabbit liver Cd,Zn-metallothionein-II dissolved in distilled water was acidified to pH 2.0 with concentrated HCl to dissociate Cd and Zn from the protein. The thionein was separated from the released metals by gel filtration on a column (30 cm $\times$ 1.5 cm) of Sephadex G-25 equilibrated with 0.1 % TFA. The fractions were analysed for metals by atomic absorption spectrophotometry and the protein was located by measuring absorbance at 214 nm. The fractions free of metal and showing high protein content were pooled and stored under nitrogen at -20°C or at -70°C . The protein concentrations were determined by analysis of -SH groups [21] and absorbance at 220 nm [22].

Reconstitutions with Cu(I)

The reconstitutions were performed anaerobically with either CuCl or Cu(I)–GSH complex. CuCl was dissolved in degassed 0.1 M HCl containing 4 % NaCl in an anaerobic chamber (Plas Labs). Cu(I)–GSH was prepared by mixing 3 mol equivalents of

GSH (in 0.1 % TFA) with 1 mol equivalent of Cu(I) as CuCl solution. The pH was raised to 7.0 by rapid mixing with 1 M potassium phosphate buffer. Reconstitutions of individual phytochelatins or rabbit apothionein-II were performed using cuvettes equipped with screw caps lined with self-sealing septa. Appropriate amounts (generally 100 nmol for phytochelatins and 10 nmol for thionein) of the various peptides were diluted to a final volume of 2.0 ml that contained 200 μmol of potassium phosphate (pH 7.0). CuCl solution or Cu(I)–GSH were similarly placed in cuvettes. The screw caps containing septa were tightened and the samples used for spectroscopy analysis. Increasing equivalents of Cu(I) were added to the peptides by injecting 1–5 μl of the solution using a Hamilton syringe. The samples were mixed and scanned for spectral changes 8–10 min after Cu(I) addition. The Cu(I) binding to phytochelatins or metallothioneins was complete within the mixing time, as indicated by luminescence and absorption spectra recorded over a period of time after mixing. The luminescence was stable for at least a day provided the samples were kept anaerobic. At least two different isolates of phytochelatins were used for each analysis. Reconstitutions with individual peptides were carried out 6–8 times. The UV/visible and luminescence spectra were recorded on a Perkin-Elmer Lambda3 double-beam spectrophotometer and an LS50 spectrofluorimeter respectively. For luminescence measurements, the excitation wavelength was set at 290 nm. An emission filter (350 nm) was used to minimize interference from Raman scattering. Any remaining scattering was subtracted from the luminescence spectra. The fluorescence and UV/visible spectral data were manipulated by FLDM and PECSS softwares respectively. Additional manipulations were done using a graphics software on a personal computer.

Gel-filtration analysis of reconstituted samples

Cu(I)–GSH-reconstituted phytochelatins were fractionated on a column of Sephadex G-50 (30 cm $\times$ 1.5 cm) equilibrated with nitrogen-purged 0.1 M potassium phosphate (pH 7.0) to verify the transfer of Cu(I) to phytochelatins. The individual fractions were monitored for Cu by atomic absorption and total glutathione by a cyclic assay [23].

RESULTS AND DISCUSSION

Reconstitution of rabbit liver thionein-II with CuCl or Cu(I)–GSH

The addition of Cu(I) as GSH complex or as CuCl to thionein under anaerobic conditions led to the appearance of emission spectra characteristic of Cu(I)–thiolate clusters (Figures 1a–1f). We are presenting emission spectra of the reconstituted samples to show two important aspects of these results: (a) the relative luminescence increases slowly for the first six Cu(I) equivalents followed by a much greater rise for the next six Cu(I) equivalents, and (b) the nature of the spectrum changes after six equivalents have been added. The emission spectra are broad but the λ_{max} is closer to 600 nm for the first six equivalents but blue-shifts to about 550 nm for the next six Cu(I) equivalents (Figures 1a–1f). The emission intensity decreases with the addition of Cu(I) once the protein has accommodated 12 Cu(I) equivalents (Figures 1e and 1f). Several previous studies have shown that the room temperature luminescence from Cu(I)–thiolate clusters is dependent on the shielding of these clusters from the solvent to prevent solvent deactivation [24–26]. Consequently, Cu(I)–GSH complexes do not luminesce at room temperature [26]. However, strong luminescence is observed from Cu(I)–thiolate clusters in Cu(I)–GSH at liquid nitrogen-reduced temperatures due to significant reduction in the solvent deactivation of the excited

Figure 1 Cu(I) reconstitution of rabbit liver thionein-II

The metal-free protein ($5 \mu\text{M}$ in 0.1 M potassium phosphate, $\text{pH } 7.0$) was titrated with increasing equivalents of Cu(I) as CuCl (**a**, **c** and **e**) or Cu(I)-GSH (**b**, **d** and **f**). The Cu(I) solutions were injected into anaerobically sealed fluorescence cuvettes and the spectra recorded after 8–10 min of each addition. The maximal luminescence was observed at 12 ± 1 mol equivalent of Cu(I) (**c** and **d**). The numbers on the curves indicate the Cu(I) equivalents added to the protein solution. The fluorescence spectrum produced by thionein (without any added Cu) was subtracted from each spectrum to account for non-specific scattering.

states [26]. Thus, the reduction in luminescence intensity upon the addition of more than 12 mol equivalents of Cu(I) to mammalian metallothioneins has been interpreted to indicate the disruption of the native Cu(I)-thiolate clusters in the protein leading to increased solvent deactivation of the excited states [24,25]. It is possible that the decrease in luminescence of metallothioneins containing excess Cu(I) occurs due to other factors such as quenching of emission by these excess copper complexes.

The major differences between the emission spectra of the thionein reconstituted with Cu(I)-GSH and that reconstituted with CuCl are: (i) the higher emission intensity of thionein reconstituted with Cu(I)-GSH and (ii) less rapid decline of luminescence when more than 12 Cu(I) equivalents are added as GSH complex. Both of these effects may be attributed to the stabilization of Cu(I)-thiolate clusters by GSH. Similar effects are observed with several other Cu-chelating compounds [24]. Metallothionein samples reconstituted with Cu(I)-GSH and

Figure 2 Cu(I) reconstitutions of phytochelatin

The phytochelatin n_2 (a) and n_3 (b) were diluted to 2.0 ml to obtain a final concentration of 50 μ M for each peptide in 0.1 M potassium phosphate (pH 7.0). CuCl solution was injected and the spectra recorded after 8–10 min. The numbers on the curves indicate the Cu(I) equivalents added to each phytochelatin sample. The fluorescence spectrum produced by individual phytochelatin (without any added Cu) was subtracted from the spectra obtained following the addition of Cu(I). This was necessary to demonstrate the relatively weak luminescence emitted by both n_2 and n_3 at lower molar ratios of Cu(I)/peptide.

CuCl contained the same amount of Cl^- thus excluding the possibility that this ion was quenching luminescence. Furthermore, the addition of GSH to the metallothionein samples reconstituted with CuCl led to increased luminescence (data not shown, but see Figure 4), indicating that the higher luminescence of metallothionein samples reconstituted with Cu(I)–GSH was clearly due to the presence of GSH. The most significant point is

that CuCl and Cu(I)–GSH are equally efficient in donating the expected amounts of the metal ion to thionein. The notion that Cu(I) compounds other than Cu(I)–GSH cannot completely reconstitute metallothionein [14] is not supported by our studies. It may be noted that a large number of studies have shown that CuCl or cuprous acetonitrile [24–27] can fully reconstitute thioneins from various sources.

Metal ions in metallothionein are distributed in two clusters that are localized in the C-terminal α - and N-terminal β -domain [28,29]. In the case of the bivalent metal ions, four ions are localized in the α -domain and the remaining three are in the β -domain of the molecule [28,29]. However, six monovalent ions [Ag(I) or Cu(I)] are present in each domain to give a total stoichiometry of 12 monovalent metal ions per molecule [24]. The biphasic nature of luminescence observed upon the addition of Cu(I) to thionein (Figure 1, also see [25]) may be interpreted to indicate that luminescence recorded for the first six Cu(I) equivalents arises from the filling of the β -domain whereas that recorded for the next six equivalents results from the occupation of both the domains. Complete filling of both domains provides better solvent shielding than the occupation of only β -domain, leading to the higher luminescence upon the addition of the last six Cu(I) equivalents. The change in λ_{max} of emission also points to the formation of a new species. These results are relevant to the interpretation of the emission data of phytochelatin which also exhibit changes in λ_{max} with increasing stoichiometry (see below). We are not aware of any published data showing a change in the λ_{max} of emission as metallothionein is reconstituted with Cu(I). However, red-shifting of the λ_{max} of emission has been reported as different species of Ag(I)-metallothionein are formed [25].

Reconstitution of phytochelatin

Cu(I) as GSH complex or CuCl was added to $(\gamma\text{-Glu-Cys})_2\text{Gly}$ (referred to as n_2) in portions of 0.25 mol equivalents and the emission spectrum was recorded after each addition. The typical spectra of n_2 reconstituted with various mol equivalents of Cu(I) as CuCl are shown in Figure 2(a). These spectra were obtained by subtracting the baseline recorded for the apo-peptide. This step was necessary to demonstrate the weak luminescence emitted by n_2 or n_3 (Figure 2b) at lower molar ratios of Cu(I)/peptide. As shown later (Figure 3), only very small variations were observed in the luminescence emitted by Cu(I)-reconstituted peptides even at low Cu(I)/peptide molar ratios. To facilitate the interpretation of the luminescence data, the changes in the emission intensity at 600 nm as a function of molar ratio of Cu(I)/peptide are shown in Figures 3(a) and 3(b). The emission intensity rose slowly (and approximately linearly) as a function of the metal ion added up to 0.75 and 1.0 mol equivalents respectively, using Cu(I)–GSH and CuCl (Figures 3a and 3b). A dramatic increase occurred in the emission intensity upon the addition of Cu(I) up to 1.25 mol equivalent. The emission intensity declined when additional equivalents of the metal ion as CuCl were added (Figure 3a). However, the emission intensity stabilized after the addition of 1.25 mol equivalents when the source of Cu(I) was GSH complex (Figure 3b). An important qualitative difference between the emission spectra of n_2 reconstituted with 0.25–0.75 or 1.25 Cu(I) equivalents was significant red-shifting of λ_{max} at the higher stoichiometry (Figure 2a). A change in the λ_{max} and a significant increase in the emission intensity at higher stoichiometry suggests that the nature of Cu(I)–thiolate clusters is different at the two stoichiometries. A much tighter structure must exist at the stoichiometry of 1.25 Cu(I) equivalents/peptide resulting in decreased solvent deactivation and thus higher luminescence

Figure 3 Cu(I) reconstitutions of phytochelatins with CuCl and Cu(I)-GSH

The phytochelatins n_2 (a, b), n_3 (c, d) and n_4 (e, f) were diluted in potassium phosphate as described above in the legend to Figure 2 to obtain final concentrations of 50 μM for n_2 and n_3 and 25 μM for n_4 . Cu(I) as CuCl or Cu(I)-GSH was added in graded equivalents and the luminescence spectra were recorded. The titration curves shown in this Figure were then generated by plotting luminescence intensity at 575 nm (for n_3 and n_4) or 600 nm (for n_2) against molar ratio of Cu(I)/peptide. Each point on the curve represents mean $\pm$ S.E.M. from five independent determinations.

[25]. In analogy with the spectral data on thionein (Figure 1), it appears that n_2 can form two types of species; one predominating at about 0.75 Cu(I) equivalents and the other forming at 1.25 Cu(I) equivalents.

The reconstitution of $(\gamma\text{-Glu-Cys})_3\text{Gly}$ (referred to as n_3) with 0.5–1.5 mol equivalents of CuCl or Cu(I)-GSH caused an approximately linear increase in luminescence intensity (Figures 2b and 3c,d). The addition of the next 0.5 mol equivalent (a total of 2.0 equivalents) of Cu(I) dramatically increased luminescence (Figures 3c and 3d). This increase was much more pronounced in the case of Cu(I)-GSH (Figure 3d). The red-shifting of the λ_{max} of emission was readily apparent in samples reconstituted with CuCl (Figure 2b). A minimal but opposite effect of Cu(I)-GSH

on the λ_{max} of emission was noted (data not shown). The luminescence of n_3 declined rapidly when more than 2.0 mol equivalents of Cu(I) as CuCl were added (Figures 3c and 3d). The emission intensity of n_3 declined very slowly when Cu(I) in excess of saturating amounts was added as Cu(I)-GSH. The clear biphasic nature of luminescence and the red-shifting of λ_{max} of emission suggest that n_3 , like n_2 , forms two distinct species; one predominant at up to 1.5 mol equivalents of Cu(I) and the other at about 2.0 mol equivalents of Cu(I). As with n_2 , the species of n_3 at the higher stoichiometry of 2.0 Cu(I) equivalents enfolds a much tighter structure, as indicated by significantly higher luminescence due to probable protection from solvent deactivation.

Figure 4 Effect of GSH on luminescence of phytochelatin reconstituted with Cu(I)

The samples of n_2 , n_3 and n_4 reconstituted with 1.5, 3 and 4.0 mol equivalents respectively, of Cu(I) as CuCl were scanned for luminescence (— GSH curves). GSH was then added to achieve final concentrations identical to those used in experiments showed in Figure 3. The samples were scanned again (+ GSH curves). The increases in luminescence upon the addition of GSH occurred within the mixing time and were constant for at least 24 h.

The peptide (γ -Glu-Cys)₄Gly (referred to as n_4) exhibited very high levels of luminescence upon reconstitution with Cu(I) (Figures 3e and 3f). It may be noted that the molar concentration of n_4 used was half of that used for n_2 and n_3 reconstitutions shown in Figure 3. The addition of 0.5 mol equivalent of the metal ion as CuCl or GSH showed relatively low luminescence, but the intensity increased nearly linearly with the addition of up to 2.5 mol equivalent of CuCl (Figure 2e). The samples reconstituted with Cu(I)–GSH showed a higher luminescence increase between 1.5 and 2.5 mol equivalents, indicating the somewhat biphasic nature of the titration curve (Figure 3f). However, n_4 samples did not show the type of biphasic curves seen in n_2 and n_3 reconstitutions (Figure 3). Overall, GSH appeared to have only a marginal effect on the stability of luminescence of n_4 . It seems likely that Cu(I)–thiolate clusters formed in n_4 are much better protected from solvent deactivation, leading to signifi-

cantly higher luminescence in comparison with the clusters formed in n_2 or n_3 .

The higher luminescence intensities of samples reconstituted with Cu(I)–GSH were not due to any differences in the reconstitution assay buffer. The concentrations of phosphate (buffering ion) and of chloride ions (contributed by CuCl) were similar in the two assays. It may be noted that Cu(I)–GSH was prepared by mixing GSH with CuCl. To clearly demonstrate the stabilizing effects of GSH on Cu(I)–thiolate clusters, as indicated by emission measurements, the tripeptide was added to the samples of n_2 , n_3 and n_4 reconstituted with CuCl at concentrations equivalent to those in the Cu(I)–GSH titration studies. GSH addition led to increased luminescence in all cases (Figures 4a–4c). As expected from the data shown in Figure 3, the increase in luminescence was less pronounced in the case of n_4 . These results were highly reproducible as indicated by five independent determinations. The samples of n_2 , n_3 and n_4 used in this experiment were reconstituted with 1.5, 3 and 4 mol equivalents, respectively, of Cu(I) as CuCl prior to the addition of GSH. Similar results are obtained even when lower concentrations of Cu(I) are used (data not shown). The results presented in Figures 3 and 4 clearly show a stabilizing effect of GSH on the luminescence of Cu(I)–thiolate clusters in phytochelatin.

The Cu(I)–binding stoichiometries obtained from the luminescence data presented in Figure 3 are thus 1.25, 2.0 and 2.5 for n_2 , n_3 and n_4 respectively. These figures are higher than the previously reported stoichiometries of 0.5, 1.5 and 1.5 for n_2 , n_3 and n_4 respectively [20]. The biphasic nature of luminescence was also not observed previously. The possibility was considered that the discrepancy in the two studies may be related to the differences in the Cu(I) reconstitution protocols. The earlier study used individual reaction tubes for each Cu(I) concentration and CuCl was added under acidic pH to the peptide. The mixture was neutralized by adding 0.2 M dibasic potassium phosphate to bring the pH into the 7–8 range. Since pH of the individual peptides was not checked, some reactions might have occurred at higher pH. The present studies were carried out by adding increasing amounts of Cu(I) to the same peptide solution at pH 7.0. The present Cu(I) reconstitution protocol was prompted by two considerations: (i) the binding of Cu(I) to phytochelatin presumably occurs at neutral pH *in vivo*, and (ii) the same sample is used for the entire reconstitution assay, permitting several experiments with much lesser amounts of the peptides. A limited number of experiments on the reconstitution of n_3 with the two protocols indicated that the differences in binding stoichiometries are not related to the manner in which Cu(I) is added. The observed variations in the binding stoichiometries of at least n_3 and n_4 are probably due to the differences in the pH of the reconstitution assay, as indicated by reduced stoichiometry at higher pH. We carried out UV/visible absorption spectroscopy on the reconstituted peptides to corroborate the data obtained from luminescence measurements.

The titration of n_2 , n_3 and n_4 at neutral pH with CuCl showed that the luminescence data (Figure 3) is consistent with the UV/visible data (Figure 5). The 260 nm absorption shoulder increased in intensity as 0.25–1.25 mol equivalents of Cu(I) were added to n_2 (Figure 5a). This absorption shoulder disappeared upon the addition of higher Cu(I) equivalents (Figure 5a). The physical basis of this absorption shoulder is unknown, but its disappearance has been interpreted to indicate the disruption of cluster structure leading also to the loss of luminescence emitted by tightly enfolded Cu(I)–thiolate clusters [24]. Although structural evidence supporting this interpretation is yet to be obtained, this interpretation is consistent with the decreased Cu(I) binding to metallothioneins that were reconstituted with more than the

Figure 5 UV/visible spectroscopy of Cu(I)-reconstituted phytochelatins at pH 7.0

The phytochelatins were reconstituted with Cu(I) using CuCl as described in the legend to Figure 2. The samples were scanned for spectral changes 8–10 min after Cu(I) addition. The spectra obtained for n_2 (a), n_3 (b) and n_4 (c) are shown. The numbers on the spectra indicate the Cu(I) equivalents added.

saturation amounts of the metal ion [24]. Stillman [25] also reached similar conclusions based on the luminescence studies on metallothioneins. The Cu(I)-titration curves obtained for n_3 similarly show that the 260 nm absorption shoulder increased in intensity up to 2.0 mol equivalents and then disappeared (Figure 3b). The disappearance of the characteristic absorption shoulder

Figure 6 UV/visible spectroscopy of Cu(I)-reconstituted phytochelatins at pH 8.6

The phytochelatin samples were prepared anaerobically in degassed 0.1 M Tris/HCl, pH 8.6, and titrated with increasing equivalents of Cu(I) as CuCl. The spectra obtained for n_2 (a), n_3 (b) and n_4 (c) are shown. The numbers on the spectra indicate the Cu(I) equivalents added.

for n_4 occurred around 2.5–3.0. Cu(I) titration curves for the different phytochelatins at pH 8.6 showed that the stoichiometry for n_2 was similar to that observed at neutral pH (Figure 6a). However, Cu(I)-binding stoichiometries, based on the disappearance of the characteristic absorption shoulder, were lower at pH 8.5 (Figures 6b and 6c) and similar to those reported

Figure 7 Gel-filtration analysis of phytochelatin reconstitution with Cu(I)-GSH

Gel filtration of Cu(I)-GSH solution used for reconstitution (a), n_3 reconstituted with 2.0 mol equivalents of Cu(I)-GSH (b) and n_4 reconstituted with 2.5 mol equivalents of Cu(I)-GSH (c). The fraction size for the chromatograms shown in (a) and (b) was 1.2 ml, whereas the fraction size was 1.0 ml for the chromatogram in (c). The elution volumes for Cu(I)-GSH, Cu(I)- n_2 - Cu(I)- n_4 were similar under these conditions.

previously [20]. Some qualitative differences were also seen in the shapes (more pronounced shoulders at 230 and 300 nm) of the absorption spectra of n_3 at the two pH values. The luminescence intensities of the phytochelatin reconstituted with CuCl at higher pH were much lower than those at neutral pH (data not shown, [20]). This significant reduction in luminescence at higher pH could have obscured the formation of two types of Cu(I) clusters in the previous study.

These results suggest that the Cu(I)-binding stoichiometry of phytochelatin may vary with the pH of the reconstitution assay. However, the previous assignment of a Cu(I)-binding stoichiometry of 0.5 for n_2 [20] appears to be in error. Luminescence studies were not previously carried out on n_2 due to the limited availability of this peptide at that time. The correct Cu(I)-binding stoichiometry for n_2 based on both UV/visible and luminescence spectroscopy appears to be around 1.25. The effects of pH on the metal-binding stoichiometries of phytochelatin are not limited to Cu(I). The phytochelatin also bind higher amounts

of Ag(I) at lower pH (R. K. Mehra, unpublished work). The reduced stoichiometry at higher pH may result from the interactions of Cu(I) or Ag(I) with certain buffer ligands (such as $-\text{OH}$). This interpretation will be consistent with a significant reduction in the luminescence intensity of both Cu(I) phytochelatin ([20]; R. K. Mehra and P. Mulchandani, unpublished work) and metallothioneins at higher pH [24].

The binding stoichiometries reported here refer to Cu(I)-equivalents per peptide molecule. The non-integral stoichiometries suggest that Cu(I)-phytochelatin exist as oligomeric structures [5,20]. The gel-filtration studies under non-denaturing conditions (see below) indicated that there are not major differences between the elution volumes of different chain-length phytochelatin. Previous studies have suggested a molecular mass of 5–6 kDa for the native Cu-phytochelatin isolated from *S. pombe* [9]. Based on Cu(I)-stoichiometry/peptide and the estimated molecular size from gel filtration, up to four peptides of n_2 are likely to be present in the complex. Precise determination of the oligomer stoichiometry depends on estimation of the true molecular mass of these complexes. Electrospray mass spectroscopy can provide these estimates. Unfortunately, we have not yet been able to devise a matrix suitable for the ionization of metal-containing phytochelatin. The apophytochelatin do show peaks consistent with their molecular mass (data not shown).

GSH does not associate with Cu(I)-phytochelatin

Studies on metallothioneins have shown that GSH may complex with these proteins [30]. A putative GSH-binding site has also been identified in metallothionein [31]. Similarly, complexes consisting of Cd-GSH-phytochelatin occur in *C. glabrata* exposed to Cd [11]. It was, therefore, of interest to determine whether GSH-phytochelatin complexes were formed in the reconstitution assays using Cu(I)-GSH. The fractionation of Cu(I)-GSH on Sephadex G-25 showed that the Cu(I)-GSH was well separated from free GSH (Figure 7a). It is important to note a significant increase in the apparent molecular size of GSH upon the binding of Cu(I). The gel filtration of n_2 (data not shown), n_3 (Figure 7b) and n_4 (Figure 7c) reconstituted with Cu(I)-GSH showed the absence of GSH in the Cu peak (consisting of phytochelatin), indicating that: (i) Cu(I) was fully transferred to phytochelatin; and that (ii) GSH did not remain associated with the reconstituted phytochelatin. The molar ratio of Cu(I)/GSH was > 30 in the Cu peak (containing phytochelatin), indicating near absence of GSH in fractions containing phytochelatin. Based on the above data, the possibility of the formation of a transient Cu(I)-GSH-phytochelatin complex, however, cannot be excluded.

In conclusion, the present studies demonstrate that glutathione is an effective donor of Cu(I) to both metallothioneins and phytochelatin. Although similar binding stoichiometries are obtained whether CuCl or Cu(I)-GSH is used, the presence of GSH can have profound effects on the stability of Cu(I)-phytochelatin. Since GSH is the most important precursor for the phytochelatin biosynthesis [17], this tripeptide must be the *in vivo* donor of Cu(I) and perhaps other metals to phytochelatin. GSH did not appear to form a stable complex with Cu(I)-phytochelatin, but the formation of transient complexes cannot be ruled out. These studies also provide important new information regarding the formation of two different types of Cu(I)-thiolate clusters in n_2 and n_3 . The elucidation of the nature of these clusters requires further spectroscopic studies.

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