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Correlation of acid–base chemistry of phytochelatin PC2 with its coordination properties towards the toxic metal ion Cd(II)

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The acid–base properties and cadmium binding abilities of (γ-Glu-Cys)$_2$-Gly (PC2), a short phytochelatin, were studied in solution, using potentiometry, ^{1}H NMR and UV-vis spectroscopy. Macroscopic and microscopic constants established by potentiometry and NMR allowed the complete dissociation processes of the hexaprotic PC2 molecule to be explained. The stoichiometry of the complexes formed by phytochelatin with cadmium depends very strongly on reactant ratios. For different amounts of ligand with respect to the metal ion the CdH_2L and CdHL species exist in almost the same molar fractions. Above pH 6 complexes with one (CdL) or two ligand molecules bound (CdH_3L_2, CdH_2L_2, $CdHL_2$ and CdL_2, respectively) were found depending on the Cd(II) : peptide ratio. ^{1}H NMR and UV-vis spectroscopy show coordination of four sulfur atoms from two molecules of PC2 to one cadmium(II) ion (R_{Cd-S}: 2.52 Å). Amino groups, glutamic acid and glycine deprotonated carboxyls also participate in cadmium coordination, in contrast to thiolate groups, in monomeric complexes in acidic solutions. Quantitative comparison of metal ion binding strength by PC2 with low molecular weight peptide thiols (LMWT), glutathione and its fragments show that over a wide range of pH phytochelatin binds to cadmium(II) ions several times more strongly than LMWT.

Introduction

Phytochelatins (PCs) are the heavy metal inactivating peptides distributed widely in the plant kingdom. The inactivation or detoxification of heavy metals entering the cytoplasm and, thus, the protection of metal sensitive enzymes of life-supporting metabolic routes, is understood as the primary function of PCs in plant metal ion homeostasis.[1–3] However, there is currently no evidence that PCs have functions other than in metal detoxification.[4] Their molecules are structurally similar to glutathione and consist of repeating γ-glutamylcysteine dipeptide moiety such as (γ-Glu-Cys)$_n$-Gly, (PCn); n = 2–11. From some species aberrant PCs were isolated, in which the C-terminal amino acid glycine is replaced by β-alanine, serine, glutamic acid, glutamine named *iso*-PCs or without further amino acid – desglycine-PCs.[1–5] The presence of the γ-carboxyl in the peptide bonds of these molecules dictated a paradigm shift away from searches for genes defining the molecules to pathways of biosynthesis. It was found that these peptides are enzymatically synthesized from glutathione. A specific enzyme γ-glutamylcysteine dipeptidyl transpeptidase (PC synthase) catalyses the transfer of the γ-glutamylcysteine dipeptide moiety of glutathione to an acceptor glutathione molecule or a growing chain of PC. There is an absolute requirement of heavy metal ions for enzyme activity.[6] The cadmium(II) ion is the strongest activator of PC synthase and different species of its complexes with PCs based on the molecular weight could be recognized, from low through medium to high-molecular-weight complexes. They basically differ in the amount of accommodated sulfide ions that could be released in acidic environments.[2,4,7–9] The incorporation of sulfide ions increases both the amount of cadmium per molecule and the stability of the complex. Many other metals such as lead, zinc, silver, mercury, copper were found to be active in provoking PC synthesis when exposed to plant cells at non-toxic concentrations.[3,10] A number of comprehensive reviews concerning the metal tolerance in plants,[11] plant metallothioneins,[12] PCs and related peptides,[2] heavy metal detoxification in higher plants,[3] plant responses to metal toxicity,[13] response to cadmium in higher plants,[7] heavy metal-binding peptides and proteins in plants,[8] PCs and their role in heavy metal detoxification[4,14] have been published in the last decade.

PCs contain carboxylate, amino, and thiolate groups, which are able to associate with protons. These multidentate bioligands exist in a great number of protonated forms that can be characterized in terms of microconstants. Furthermore, the type and extent of metal–ligand interactions are also considerably influenced by the protonation stage of the ligand. Consequently, a knowledge of proton-binding characteristics is of primary importance for a thorough understanding of metal–ligand interactions.

In the present paper we deal with the acid–base chemistry of PC2 and its interaction with cadmium(II) ions. For the first time, full acid–base and chelating properties of phytochelatin are presented. To determine macroconstants, microconstants and stability constants of its cadmium(II) complexes, ^{1}H NMR and potentiometric experiments have been used.

Experimental

Materials

TSP (sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate), DTNB (5,5′-dithiobis(2-nitrobenzoic) acid), NaOD and DCl solutions in D_2O were purchased from Sigma Chemical Co. (St. Louis, MO), NaOH, HNO_3, nitrates of potassium and cadmium(II), sodium perchlorate were purchased from Merck (Darmstadt, Germany). D_2O (99.9%) was from Cambridge Isotope Laboratories.

PC2 synthesis

The peptide was prepared by the solid phase method of Merrifield,[15] using Boc-Bzl strategy. Deprotection and splitting from the resin was done by liquid hydrogen fluoride. Preparative runs for peptide purification were performed on a Knauer apparatus (Bad Hamburg, Germany) equipped with a steel column (250 × 10 internal diameter) filled with LiChrospher 100 RP-18 (12 μm) (Merck) using the same gradient system (1% per min). All runs used a UV detector wavelength of 220 nm. Purified peptides (purity over 92%) were freeze dried and kept under nitrogen in a freezer. The peptides were characterized by amino acid analysis and FAB MS spectroscopy. Moreover, the purity of the peptides was

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Table 1 Protonation and stability constants of PC2 and its cadmium(II) complexes ($I = 0.1$ M KNO_3, T = 25 °C) determined by potentiometry

Protonic species	logβ	pK_a	Cadmium species	logβ	p$K_a{}'$[a]
HL	10.25(2)	10.25	CdH_2L	27.50(2)	–
H_2L	19.94(1)	9.69	CdHL	22.82(1)	4.68
H_3L	28.53(2)	8.59	CdL	16.14(4)	6.68
H_4L	32.83(2)	4.30	CdH_3L_2	48.10(8)	–
H_5L	36.00(2)	3.17	CdH_2L_2	41.25(3)	6.85
H_6L	38.43(2)	2.43	$CdHL_2$	31.72(3)	9.53
			CdL_2	21.35(4)	10.37

[a] Constants calculated from log β values of cadmium species.

determined by analytical HPLC experiments performed on a LaChrom Merck–Hitachi system, equipped with a L-7450 model diode array detector and a steel column (250 × 4 internal diameter) filled with LiChrospher 100 RP-18 (Merck). The flow rate was 1 ml per min, and a 0–100% linear gradient (2% per min) of 80 : 20 (v/v) $CH_3OH + H_2O$ (both phases contain 0.1% of TFA (trifluoroactic acid)) was used.[16] The identity and purity of the peptide was confirmed by mass spectrometry, utilizing a Finnigan MAT TSQ 700 (Finnigan MAT, San Jose, CA, USA) mass spectrometer equipped with a Finnigan electrospray ionization source. The *m*/*z* values found/calculated were 540.0/539.6 $(M + H)^+$. The purity of phytochelatin PC2 was determined by potentiometric titrations to exceed 96%.

Potentiometry

Potentiometric titrations of PC2 and its cadmium complexes in the presence of 0.1 M KNO_3 were performed at 25 °C using pH-metric titrations over the pH range 2.5–10.5 (Molspin automatic titrator, Molspin Ltd., Newcastle-upon-Tyne, UK) with 0.1 M NaOH as titrant. Changes in pH were monitored with a combined glass-Ag/AgCl electrode (Mettler Toledo) calibrated daily for hydrogen ion concentration by HNO_3 titrations.[17] Sample volumes of 1.5 ml, PC2 concentrations of 2 mM and M : L molar ratios of 1 : 0 (ligand titrations), 1 : 1, 1 : 1.5, 1 : 2 were used. These data were analysed using the SUPERQUAD program.[18] Standard deviations computed by SUPERQUAD refer to random errors only.

NMR spectrometry

^1H NMR spectra of 2 mM PC2 metal free samples in D_2O and 3 mM PC2 containing varied amounts of Cd(II) (1 : 1 and 1 : 2) were recorded at 25 °C, on Bruker AMX-300 and AMX-500 spectrometers (Karlsruhe, Germany). TSP (sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate) was used as an internal standard. pH* (pH-meter reading in D_2O using a glass electrode calibrated with standard buffers in H_2O) was corrected for isotopic effects and transformed into pH.[19]

Electronic absorption spectroscopy

The electronic absorption spectra of PC2 and its cadmium(II) complexes were recorded at 25 °C on a Cary 50 Bio spectrophotometer (Varian Inc. Scientific Instruments, USA) over the spectral range of 190–300 nm in 1 cm cuvettes in 50 mM phosphate buffers. The exact concentration of PC2 was assayed spectrophotometrically at 412 nm with Ellman's reagent (DTNB).[20] 6.0×10^{-5} M samples of phytochelatin were used in stoichiometry analysis experiments at different concentrations of cadmium(II) ion, from 0 M to 8.0×10^{-5} M.

Theoretical calculations

Structural calculations based on potential energy minimizations were done for PC2 complexes with Cd(II), using semi-empirical MNDO/d methods implemented under HyperChem 7. The following optimisation criteria were used: RMS gradient 0.42 kJ mol^{-1}, convergence limit $< 10^{-8}$ and polarizability field strength $< 10^{-4}$ a.u.[21]

Definitions of constants

p$K_i = -\log K_i$; $K_i = [H_{n-1}L] \times [H]/[H_nL]$; dissociation macroconstant.

p$k_j = -\log k_j$; $k_j = [H_{n-1}L^*] \times [H]/[H_nL]$; dissociation microconstant of particular chemical group (*).

$\beta = [M_iH_jL_k]/([M]^i \times [H]^j \times [L]^k)$; overall complex stability constant or protonation constant (H_jL_k).

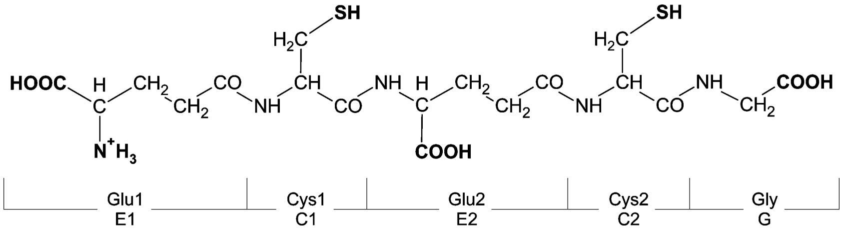
Results

Acid–base properties

The molecule of PC2 behaves as a hexaprotic acid (Scheme 1). A fully protonated PC2 molecule undergoes two reversible proton dissociation steps in fairly well separated pH ranges. The protons of the three carboxyl groups dissociate in the acidic pH range while the protons of ammonium and two sulfhydryl groups dissociate in the basic pH region.

If ionization occurs simultaneously at three groups, macroscopic constants are the composite of the microscopic constants for ionization from the individual groups.[22] In such cases, it is not possible to describe the acid–base chemistry at the molecular level in terms of macroscopic constants. The entire process of dissociation with macro- and micro-dissociation equilibria for the PC2 molecule is described in Scheme 2.

Using potentiometric titrations only macroconstants can be determined. The first part of Table 1 shows macrodissociation constants calculated from protonation constants determined directly from experiments. In such a way it was necessary to use a technique that allows monitoring of the protonation sites. ^1H NMR spectra at selected pH values in the region from 1.4 to 11.2 have been obtained and gave us helpful information about

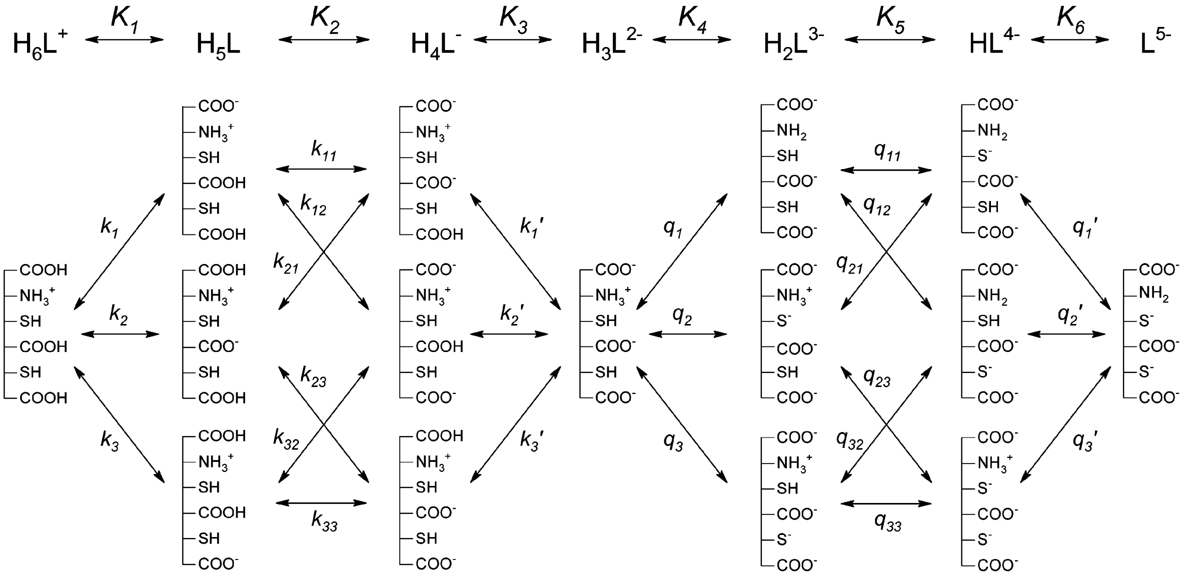


Scheme 1 The structure of a fully protonated molecule of phytochelatin PC2, (γ-Glu-Cys)$_2$-Gly.

Table 2 Micro- and macro-constants of the PC2 ionization processes calculated on the basis of ^{1}H NMR experiments

Microconstants[a]				Macroconstants[b]
$pk_1= 2.72(1)$	$pk_{23}= 3.79(7)$	$pq_1= 9.54(8)$	$pq_{23}= 9.18(2)$	$pK_1= 2.68(2)$
$pk_2= 3.92(2)$	$pk_{32}= 2.68(1)$	$pq_2= 9.13(1)$	$pq_{32}= 9.63(8)$	$pK_2= 3.60(3)$
$pk_3= 3.98(3)$	$pk_{33}= 3.74(7)$	$pq_3= 9.00(1)$	$pq_{33}= 9.31(2)$	$pK_3= 4.24(2)$
$pk_{11}= 3.79(1)$	$pk_1' = 4.01(7)$	$pq_{11}= 9.07(8)$	$pq_1' = 9.67(8)$	$pK_4= 8.73(5)$
$pk_{12}= 3.93(1)$	$pk_2' = 3.87(7)$	$pq_{12}= 9.09(8)$	$pq_2' = 9.65(8)$	$pK_5= 9.31(5)$
$pk_{21}= 2.59(1)$	$pk_3' = 2.81(7)$	$pq_{21}= 9.48(8)$	$pq_3' = 9.96(8)$	$pK_6= 10.23(4)$

[a] Microconstants fitting using eqn. (3). [b] Macroconstants calculated according to eqn. (2).



Scheme 2 Macroscopic and microscopic ionization scheme for the PC2 molecule.

the acid–base chemistry of PC2. Comparison of our ^{1}H NMR experimental data with those reported as reference data for PC2[23] showed almost identical chemical shifts for all protons.

Fig. 1 presents changes in chemical shifts of each proton of the PC2 molecule. COSY and TOCSY experiments for proton correlations have been obtained. In acid–base investigations the chemical shifts of the α-protons have been chosen. Conversion of the chemical shifts of the protons near to the protonation sites, using eqn. (1), to ionization fractions of particular groups (F) allows the direct determination of the group constants listed in Table 2.

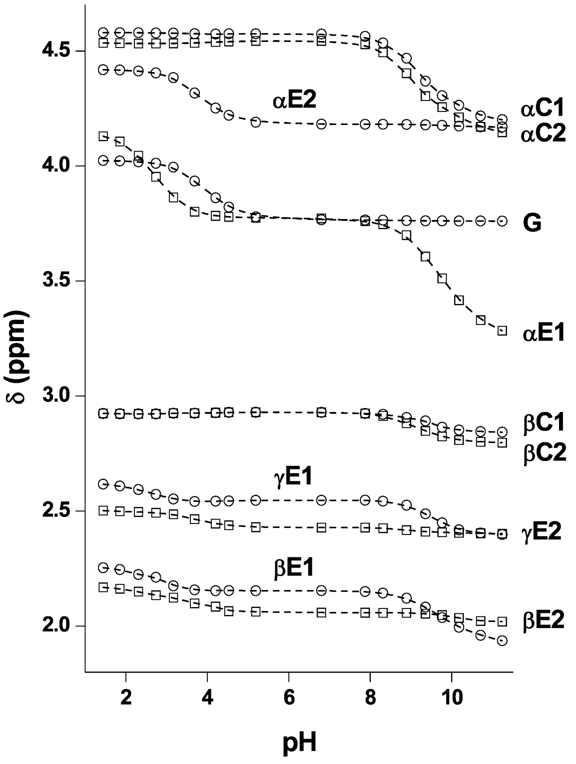


Fig. 1 The pH dependence of the chemical shifts of the PC2 protons.

$$F = (\delta - \delta_p)/(\delta_u - \delta_p) \tag{1}$$

δ represents the experimental average of the chemical shifts, while δ_p and δ_u are the values of the particular groups when they are fully protonated or deprotonated, respectively.[24]

Both macroconstants and microconstants, summarized in Table 2, were evaluated by non-linear least squares curve fitting of fractional ionization data as a function of pH at two separate regions as shown in Fig. 2.

The macroconstants are needed for the calculation of the microconstants described below. pK_1, pK_2 and pK_3 were determined from the sum of ionization fractions of carboxylic groups at two glutamic acid (F_{E1} and F_{E2}) and glycin (F_G) residues in the pH region from 1.4 to 6.8 following eqn. (2) (Fig. 2A).

$$F_{E1} + F_{E2} + F_G = \frac{10^{pH-pK_1} + 2\times10^{2\times pH-pK_1-pK_2} + 3\times10^{3\times pH-pK_1-pK_2-pK_3}}{1+10^{pH-pK_1} + 10^{2\times pH-pK_1-pK_2} + 10^{3\times pH-pK_1-pK_2-pK_3}} \tag{2}$$

The microconstants were determined using eqn. (3) where $\alpha = pk_1$ and $\beta = pk_2 + pk_{23} = pk_3 + pk_{33}$.

$$F_{E1} - F_{E2} - F_G = \frac{2\times10^{pH-\alpha} - 2\times10^{2\times pH-\beta} - 10\times^{3\times pH-pK_1-pK_2-pK_3}}{1+10^{pH-pK_1} + 10^{2\times pH-pK_1-pK_2} + 10^{3\times pH-pK_1-pK_2-pK_3}} \tag{3}$$

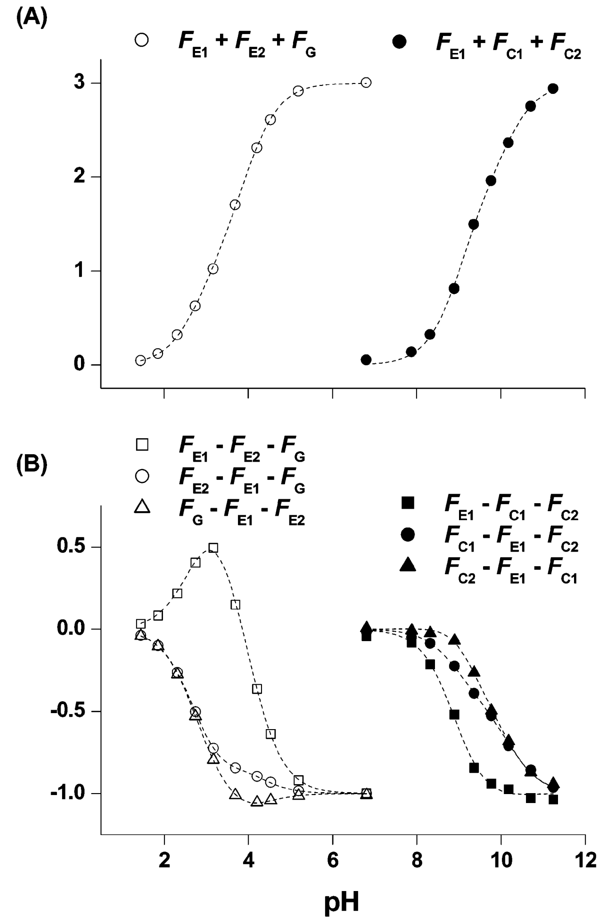


Fig. 2 Evaluation of macrodissociation and microdissociation constants. Non-linear least squares curve fitting of properly arranged fractional ionization data following eqn. (2) (A) and (3) (B).

Replacement of the left side in this equation by the expression $F_{E2} - F_{E1} - F_G$ and $F_G - F_{E1} - F_{E2}$ allows evaluation of $\alpha = pk_2, \beta = pk_1 + pk_{12} = pk_3 + pk_{32}$ and $\alpha = pk_3, \beta = pk_1 + pk_{11} = pk_2 + pk_{21}$ respectively (Fig. 2B). The rest of the microconstants were then calculated from relations such as $pk_{11} + pk_1' = pk_{12} + pk_2'$, $pk_{21} + pk_1' = pk_{23} + pk_3'$ and $pk_{32} + pk_2' = pk_{33} + pk_3'$.

Microconstants and macroconstants that characterize acid–base properties at basic pH were determined in the same way as described above but from ionization fractions of the amino group at the glutamyl (F_{E1}) residue and the thiolate groups at two cysteinyl (F_{C1} and F_{C2}) residues in the pH region 6.8 to 11.4. Fig. 3 represents the pH distribution diagram of fractionally ionized PC2 microforms calculated from evaluated microconstants.

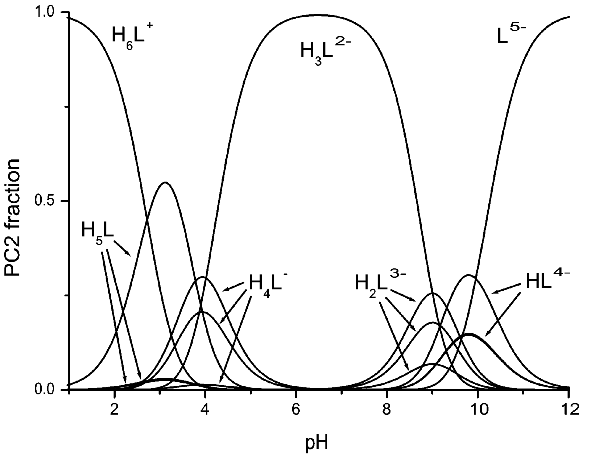


Fig. 3 pH distribution of the fractionally ionized forms of PC_2, calculated from the microconstants listed in Table 2.

Coordination mode of PC2 with cadmium

Potentiometric titrations indicate that PC2 forms very stable complexes with cadmium(II) ions over a wide pH range. The stoichiometry of these species strongly depends on the metal : ligand ratio. In the solutions of 1 : 1 molar ratio, three equimolar complexes are formed above pH 3 with stoichiometries CdH_2L, CdHL and CdL. In the presence of at least a two-fold excess of ligand over cadmium(II) ions additional bis-complexes are formed. CdH_3L_2, CdH_2L_2, $CdHL_2$ and CdL_2 appear in solution at pH 6 with two phytochelatin molecules coordinated to one cadmium(II) ion. Fig. 4 presents the species distribution diagrams for the complexes mentioned above, with metal : ligand ratios of 1 : 1 and 1 : 2 respectively. Concentration values of reactants were chosen in such a way as to compare with the ^1H NMR results. It is worth noticing that independently of the ratio the first two species possess the same stoichiometries and exist in almost identical fractions. Only above pH 5 do the processes in solution become dependent on the metal : ligand ratio.

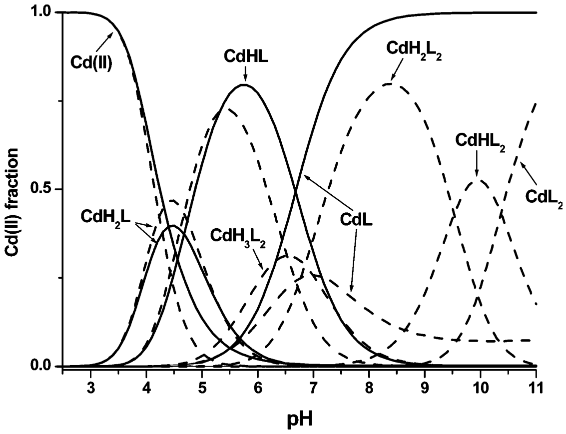


Fig. 4 Species distribution of Cd(II) : PC2 complexes. The line — represents ratio 2mM PC2 : 2 mM Cd(II) and line --- 1mM PC2 : 1 mM Cd(II).

Electronic spectra recorded over the UV region for the solutions having ligand excess show a distinct band at 245 nm and $\varepsilon = 6700\ M^{-1}\ cm^{-1}$ (pH 9.0), while the solutions at pH 5.5 do not exhibit this band at any metal to ligand molar ratio. Both pH values were chosen according to the presence of predominant species, the equimolar complex at pH 5.5 and the bis-complex in solutions above pH 7. Absorption at 245 nm in basic solution depends very strongly on the reactant ratio. Fig. 5

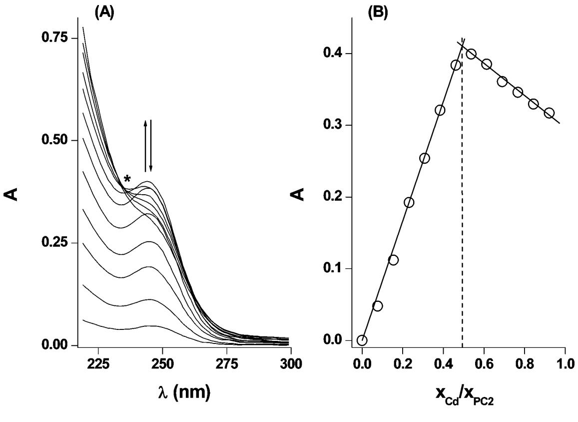


Fig. 5 Stoichiometrical analysis of the complexes formed at pH = 9.0. UV spectra of PC2 (1.0×10^{-5} M) at different concentrations of cadmium ions ($0–1.0 \times 10^{-5}$ M) (A) and absorption dependence on the molar ratio of reactants x_{Cd}/x_{PC2} (B). Arrows indicate systematic intensity increase or decrease, and star represents isosbestic point at 235 nm.

presents spectra of PC2 obtained at different concentrations of cadmium(II) ions (A). Increasing absorption at 245 nm (B) indicates stoichiometric dependence on the Cd : PC2 ratio. The highest absorption exists at a Cd(II) : PC2 ratio of 1 : 2. Further addition of cadmium(II) ions causes a decrease of the band intensity until a 1 : 1 ratio is reached, while an excess of the metal has no influence on the absorption (B).

^{1}H NMR spectra recorded both at a 1 : 1 ratio and with a double excess of phytochelatin show that cadmium(II) ions prefer to coordinate to peptide sulfur atoms over a wide range of pH. All spectra recorded at equimolar solutions show line broadening. Fig. 6 presents a comparison of the chemical shifts of selected PC2 protons both in the presence and absence of cadmium(II) ions. The α and β protons of Cys2 show sulfur participation in metal ion coordination from pH 3.5 in every ^{1}H NMR spectra, while chemical shifts of the protons indicate Cys1 thiolate binding above pH 4.0 independently of the reactant ratio. Changes in chemical shifts of N– and C– terminal protons suggest the partial coordination of the amino and carboxyl groups of Glu1 and glycine carboxyl to cadmium(II) in acidic solution.

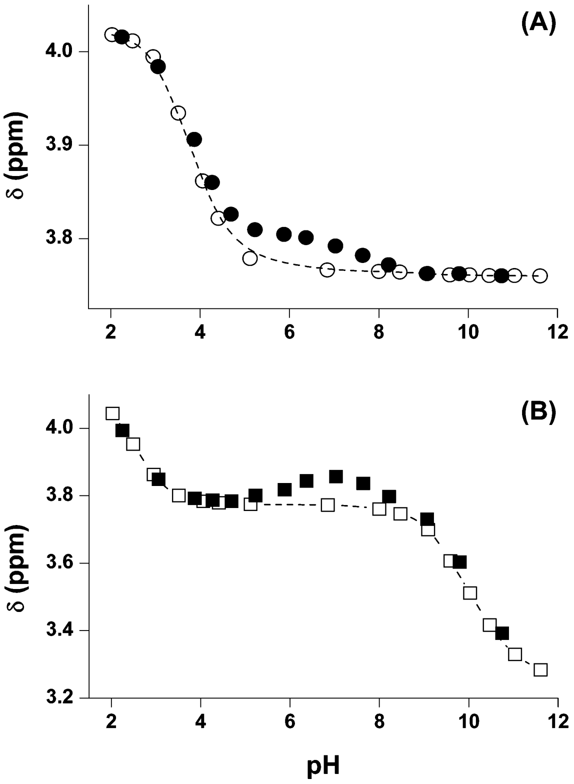


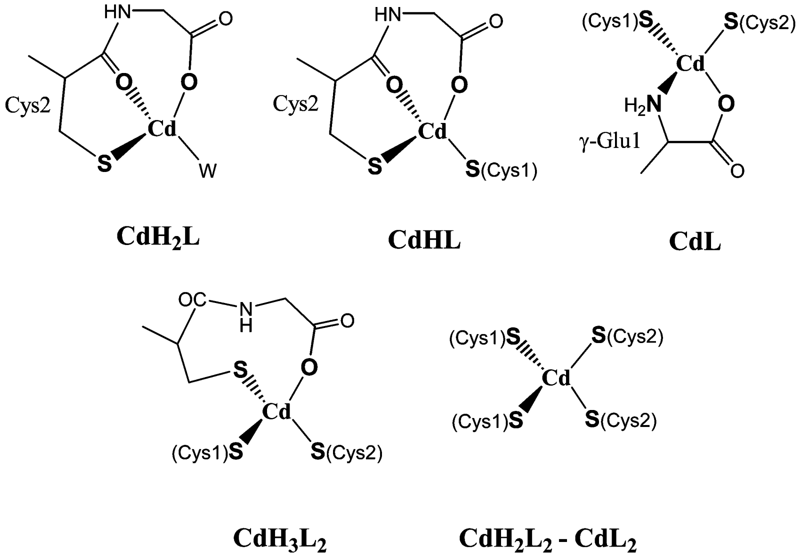
Fig. 6 Comparison of ^{1}H NMR glycine (A) and α-Glu (B) proton chemical shift of free 2 mM PC2 (○ and □) with: 2mM PC2 : 1 mM Cd(II) (● and ■).

Discussion and conclusions

The values of the protonation constants obtained from the pH-metric and ^{1}H NMR titrations fit in between the characteristic values for glutathione and its derivatives with γ-peptide bonds.[25–27] As noted in the Results section, the deprotonation of the amino and thiol groups proceeded almost independently of each other, but pH ranges for these three processes overlap. All three carboxyl groups also deprotonate over a similar pH range but independently. The macroscopic constants obtained from potentiometric measurements stay in good agreement with adequate macroconstants calculated on the basis of ^{1}H NMR data and differ slightly from the values obtained under the same temperature but in 1 M KNO_3 solution (pK_1= 2.39, pK_2= 3.18, pK_3 = 4.01, pK_4= 8.75, pK_5 = 9.03, pK_6 = 10.04).[28] These differences arise only from the ten-fold increased ionic strength.

A complete understanding of the deprotonation processes is possible after taking the microscopic ionization scheme into consideration (Scheme 2). The speciation presented in Fig. 3 clearly shows that individual LH_5, LH_4^-, LH_2^{3-} and LH^{4-} species exist in triplicate. The dominating LH_5 micro-species results from the deprotonation of the most acidic Glu1, while the two remaining ones originate from Glu2 and Gly carboxyl group deprotonations. Further spontaneous deprotonation leads to formation of three LH_4^- species. Two of them, which possess ionized Glu1 carboxyl, dominate what is the consequence of the previous deprotonation. Their formation is depicted in Scheme 2 as k_{11} and k_{12} while the third, the least represented one results from the sum of the k_{23} and k_{33} processes. The large difference in protonation constants of carboxyl groups and distinctly more basic amine and thiol groups (over 4 logarithmic units) causes the existence of only one species of stoichiometry LH_3^{2-} over a wide range of pH. Deprotonation of the remaining basic functions proceed *via* a very similar pattern. Both thiol groups deprotonate first and result in the formation of two, dominant LH_2^{3-} microspecies. Their further ionizations lead to the prevailing LH^{4-} microform with ionized thiol functions and a still protonated amine. All three LH^{4-} species result after dissociation in one L^{5-} species, fully deprotonated and prevailing in solution above pH 11.

Apart from the dissociating groups, the PC2 molecule also contains four potentially donating peptide bonds, thus offering various coordination sites for metal ions. The complexation pattern can be influenced mainly by metal preference towards a specific donor, its availability resulting from dissociation processes and steric hindrance. Moreover, the possible stability gain resulting from chelate ring formation is also important. Within the PC2 molecule, the most favorable binding sites are the sulfur atoms provided by the thiol groups, as in the case of the similar multicysteinyl peptides.[29] As presented above the dominant LH_3^{2-} species over a wide pH range possess three deprotonated carboxyl groups with both the thiols and the amine protonated. ^{1}H NMR chemical shifts without metal and with cadmium indicates that anchoring of the Cd(II) ion occurs at the Cys2 sulfur atom following the electrostatic attraction of the metal by the neighboring carboxylate of the glycine residue (Fig. 6A). Rabenstein *et al.* previously proposed a similar coordination mode for glutathione Cd(II) complexes.[30] Increasing concentration of microspecies with a glycine deprotonated function (Fig. 3) causes binding of the metal ions to the C-terminal tail of phytochelatin with the simultaneous deprotonation of the most acidic Cys2 thiol group. The species distribution diagram calculated on the basis of stability constants obtained from potentiometric titrations shows that the dominating species in solution above pH 4.0 is a CdH_2L complex with a $\{S_1O_3\}$ coordination sphere. Carbonyl oxygen from the peptide bond between Cys2 and Gly participates also in cadmium donation giving two chelate rings (Scheme 3). Deprotonation of the next sulfhydryl of Cys1 (pK_a': 4.68) gives a very stable monomeric CdHL complex $\{S_2O_2\}$. The precise analysis of the chemical shifts of PC2 in the presence of Cd(II) in comparison with phytochelatin alone shows that apart from the C-terminal carboxylate and cysteine donor, the N-terminal Glu1 amino and carboxyl groups complete the coordination resulting in the CdL complex (Fig. 6B). In the case of this species, the stabilization accompanying the strong chelate effect, following five-membered ring formation, surpasses the preference of the cadmium ion towards binding both Glu1 typical amino acid functions (pK_a': 6.68). With the rise of pH, the γ-Glu1 amine deprotonation increases and its participation in Cd(II) binding as CdL species can be observed in the ^{1}H NMR spectra above pH 5, at equimolar reagent ratio and with an excess of ligand. Fractional concentration of the form depends strongly on the PC2 : Cd(II) ratio. Molecular modelling and ^{1}H NMR data indicate that glycine carboxylate is removed from the cadmium coordination sphere in the CdL complex



Scheme 3 Proposed structures of the Cd(II)–PC2 complexes. W represents a water molecule.

$\{S_2N_1O_1\}$. This is a result of an N-terminal function domination over C-terminal ones towards binding of Cd(II) ions, chelated already by two sulfur donors. The latter complex exists in solution independently of the metal : ligand ratio but with the increase in phytochelatin concentration above a 1 : 1 ratio the CdL complex becomes a minor species at the expense of the bis-ligand species. The coordination sphere of the CdH_3L_2 complex posses three sulfur atoms, two of them come from one PC2 molecule, bound only through cysteine residues. The third comes from another ligand molecule and participates in Cd(II) chelation with glycine carboxyl respectively, as presented on Scheme 3 $\{S_3O\}$. The coordination modes of the CdH_2L_2, $CdHL_2$ and CdL_2 species are identical (pK_a' (log β CdH_3L_2 − log β CdH_2L_2): 6.85). The Cd(II) ion is coordinated by four sulfur atoms $\{S_4\}$ of two PC2 molecules and the differences between these complexes derive from the different protonation states of the two amino groups. pK_a' values: 9.53 (log β CdH_2L_2 − log β $CdHL_2$) and 10.37 (log β $CdHL_2$ − log β CdL_2) are close to that of spontaneous amine deprotonation in free PC2 molecules (Table 1). Finally, these complexes include two PC2 molecules bound symmetrically, only by sulfur donors, with large chelate rings. These systems present a similar cadmium binding fashion to the Cd–metallothioneins[12] and have a very strong susceptibility towards sulfur donation.

Previous potentiometric studies on the PC2 and cadmium(II) system[28] showed distinctly different coordination modes. Although similar protonation constants were obtained, the complex stoichiometries and stability constants presented therein were dissimilar. For example, the calculations based on those data at pH 5.0 and 7.3 with a PC2 : Cd(II) ratio of 75 µM : 50 µM, revealed that cadmium exists at 98.7 and 0.28% as a free metal, while our results, presented here, clearly indicate 45.5 and 9×10^{-5}%, respectively. A pH value of 7.3 was chosen as being typical for the cytoplasm of higher cell plants.[31]

With an excess of PC2, the NMR experiment performed in the pH range where bis-ligand complexes dominate, indicates the involvement of only four sulfur atoms in cadmium chelation. This is confirmed by the presence of the characteristic CT band at 245 nm (Fig. 5). This transition originates from four sulfur atom donations to the Cd(II) ion in the complex of tetrahedral geometry.[32–34] This band reaches the highest absorption by a Cd(II) : PC2 ratio of 1 : 2. This phenomenon seems to confirm the two-ligand involvement in the coordination by means of all four thiol groups. A supporting fact for the formation of the bis-complexes with PC2 could be the formation of the thermodynamically more favorable macrochelate 14-membered ring.

In the case of biologically important monothiol ligands like cysteine, its analogues and short peptides containing this amino acid residue Cd(II) ions are chelated in the formation of mononuclear species, where beside sulfur donors, other functions may be involved in the coordination process. With an increase in the Cd : ligand ratio polynuclear complexes appear with participating bridging sulfur atoms.[35,36] For reasons of strong affinity of cadmium towards sulfur donors, in the case of ligands containing two thiol functions, we observe the formation of complexes with domination of sulfur donations up to Cd–S_4 centers. This coordination mode is the most common also due to entropic reasons. Similarly to the monothiols, an excess of Cd(II) ions determines the increasing amount of bridging sulfur atoms.[32] Such bridge formation exists neither when steric hindrance is present nor when the ligand molecule participates in other types of interaction.[37] The presence of the larger amount of sulfur donors in one ligand molecule increases the metal-to-ligand ratio of the complexes. The M : L ratio equal to 3 : 4 appears to be typical for the species of cadmium with PC3.[3] This rule allows for the conclusion that the most dominant complex in the case of PC4 may have a 1 : 1 ratio and starting with PC5, similar clusters to the metallothioneins might be observed. Therefore, studying Cd(II) complexation by longer phytochelatins would require the application of *i.e.* ^{113}Cd-NMR which is sensitive to the differentiation between cadmium ion environments in polynuclear complexes.[38]

Semiempirical calculations for the CdH_2L_2 stoichiometry, which could be relevant to natural conditions, presented large conformational diversity. Molecular dynamics performed for this species resulted in many conformers of similar potential energy without distinguishing any particular "conformational family". Application of NMDO/d methods showed the almost perfect tetrahedral structure, with S–Cd–S angles: 103.95–119.60° (average: 109.3°), resultant from four sulfur atoms coordinated to a cadmium ion with typical Cd–S distances of 2.494–2.529 Å for phytochelatins, metallothioneins and their models.[39–41] The distances calculated are in very good agreement with a terminal type of sulfur binding to cadmium ions: 2.54–2.47 Å, while the distances of the μ_2 bridging type are a little longer: 2.62–2.56 Å.[42] Fig. 7 presents the stereo-view of the CdH_2L_2 conformer possessing the lowest potential energy.

The high conformational flexibility of the phytochelatin complexes may have significant importance in the detoxifiaction of plant cells from cadmium ions. The possibility of Cd(II) ion transfer from the low molecular weight thiols to PC2 (probably mediated by ternary complexes) and further to another PC type of ligand originates from the cadmium(II) complexes

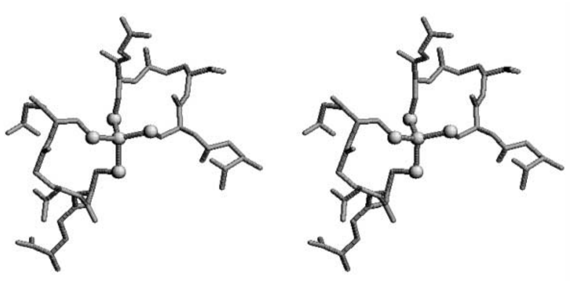


Fig. 7 Steroview of the calculated CdH_2L_2 complex structure. The cadmium(II) ion is coordinated by four sulfur atoms (4S complex) with characteristic tetrahedral geometry and bond lengths: 2.494–2.529 Å. Hydrogen atoms were neglected.

lability and their conformational disorder. With an excess of thiols *in vivo* (both phytochelatins and shorter thiol-containing peptides) the most likely scenario is that the Cd(II) ion is bound by sulfur donors only. The comparison of the stability constants of PC2 complexes with those of glutathione and other LMWT (low molecular weight thiols), presented in Fig. 8, clearly indicates the domination of PC2 cadmium species over the rest of the complexes considered.[43–47]

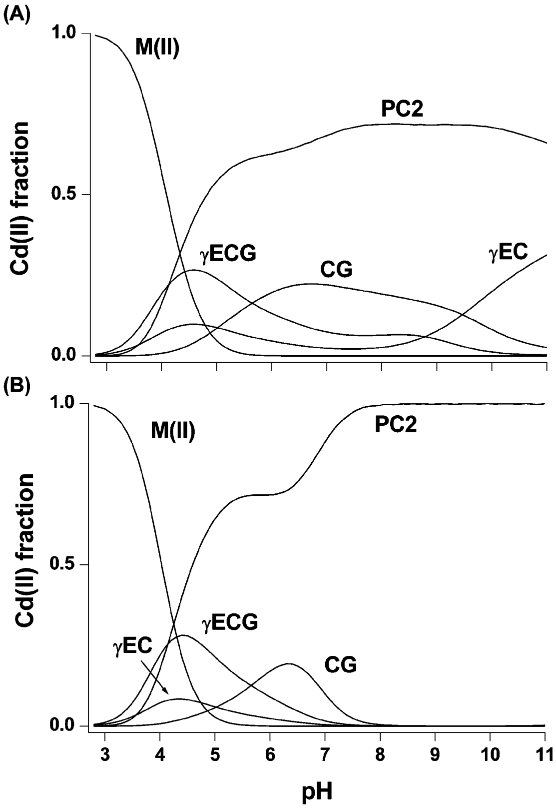


Fig. 8 Competition diagrams for Cd(II)–thiol peptide complexes. γECG (GSH), CG, PC2, γEC = 1mM : 1 mM Cd(II) (A) and 0.1 mM Cd(II) (B).

The complexation mode presented in this paper and the indication that terminal functions participate in Cd(II) ion binding is the first complete description of cadmium interactions with phytochelatin.

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