Properties of tobacco (*Nicotiana tabacum*) cadmium-binding peptide(s)

Unique non-metallothionein cadmium ligands

R. Neil REESE and George J. WAGNER*

Department of Agronomy, ASC-N, N212, University of Kentucky, Lexington, KY 40546-0091, U.S.A.

The chemical and physical characteristics of Cd-binding peptides isolated from tobacco (*Nicotiana tabacum*) leaves and suspension-cultured tobacco cells were determined and compared with properties of rat liver Cd,Zn-thionein. Some emphasis was placed on metal-binding and specificity properties. Cd-peptides of apparent M_r 6000 and 2000 were induced in tobacco leaves by growth of plants with 90 μM -Cd. Only the apparent- M_r -2000 Cd-peptide was induced in the leaves of tobacco plants grown in the presence of 3 μM -Cd. In cultured tobacco cells exposed to a wide range of Cd levels (3–180 μM), a peptide of apparent M_r 2000 was observed. Under denaturing conditions [6 M-guanidinium chloride (GdmCl) with or without 100 mM-2-mercaptoethanol], all of the above forms were shown to have an M_r of approx. 1300, compared with an M_r of 6000 for Cd,Zn-thionein. The apparent disaggregation of the M_r -6000 form by GdmCl to what appears to be the unit Cd-binding peptide was reversible. Tobacco-derived Cd-peptide contained approx. 40, 35 and 15 residues of glutamate/glutamine, cysteine and glycine respectively, with serine, lysine, and aromatic residues being absent. Tobacco Cd-peptide had an isoelectric point (pI) of 3.15, which is lower than the $\text{pI} \geq 4$ reported for metallothionein. A 50% dissociation of Cd occurred at pH 5 and 3.5 for the tobacco Cd-peptide and Cd,Zn-thionein respectively, and GdmCl was shown to cause Cd dissociation from tobacco peptide, but not from metallothionein. No evidence was obtained for Zn induction *in vivo* of, or Zn binding *in vitro* to, tobacco Cd-peptide. Copper induced a low- M_r metal-binding component in cultured tobacco cells which did not appear to be identical with the peptide induced by Cd. Properties of tobacco Cd-peptide and Cd,Zn-thionein, including metal affinity and selectivity, are greatly different, except for the common presence of 30 residues of cysteine/100 residues.

INTRODUCTION

In higher plants, cadmium causes the formation of low- M_r (apparent M_r 2000–10000; the higher apparent- M_r forms probably being aggregates) soluble peptides which are capable of binding this metal. Available data indicate that these Cd-peptides are highly acidic, are composed predominantly of Glu/Gln (30–50 residue %), Cys (30–40 residue %) and Gly (10–15 residue %) and are lacking in aromatic and basic amino acid residues (see Rauser, 1984; Wagner, 1984; Grill *et al.*, 1985). Although there is evidence to suggest the occurrence of Cd-mercaptide chromophores, the mode(s) of metal binding to plant Cd-peptides has not been established. In plant tissues containing high levels of Cd, these peptides are often the principal form of soluble Cd (Rauser, 1984; Wagner, 1984), but their chemical and physical characterization is still incomplete and their function(s) are unknown. Murasugi *et al.* (1981) demonstrated Cd-induction of a similar peptide in fission yeast, which contains Glu, Cys and Gly in a 3:3:1 (by residue) ratio. Grill *et al.* (1985) recently showed that a small ($M_r \sim 1000$ –1800) Cd-binding peptide from suspension-cultured *Rauwolfia serpentina* cells contains the primary sequence:

Inducibility of metallothionein (MT) by Zn, Hg, Au, Cu, as well as by Cd, and high stability of Cd binding, are features which relate to proposed roles for this protein in toxic-metal sequestration and, in at least multicellular organisms, essential-metal (Zn, Cu) homeostasis. In the present study, physical and chemical properties of Cd-peptides of tobacco leaf and suspension-cultured cells were compared with those of MT. In particular, stability of Cd binding and inducibility by Zn were examined in an attempt to understand the possible function(s) of plant Cd-peptide(s) *in vivo*.

MATERIALS AND METHODS

Growth of tissue

Tobacco (*Nicotiana tabacum* L., cv. KY 14) plants were grown in open-ended 50 ml plastic tubes containing vermiculite (two plants/tube). In all, 40 tubes were bathed in 18 litres of half-strength Hoagland's solution no. 1 (Hoagland & Arnon, 1950), with 18 μM -iron added as Fe-EDTA. Cd, as CdSO₄, was added to the nutrient medium 2 weeks after seeding and plants were harvested after a minimum of 3 weeks exposure to the metal. To maintain nutrient and Cd levels, solutions were changed weekly. For labelling with ³⁵S, plants were grown in

Abbreviations used: GdmCl, guanidinium chloride; residue %, residues/100 residues; MT, metallothionein.

* To whom correspondence and reprint requests should be sent.

microcentrifuge tubes (1.5 ml) containing vermiculite, from which the cap and bottom had been removed. Tubes were suspended in 2-litre plastic tubs and plants were grown as described above, except that 1 week after Cd addition plants were transferred into a modified Hoagland's solution containing 0.2 mCi of ^{35}S (aq. H_2SO_4 ; 40 mCi/ml; ICN Biomedicals). In this solution ZnSO_4 and CuSO_4 were replaced by their respective chloride salts. After 1 week, plants were transferred to half-strength Hoagland's solution no. 1 for the remainder of the growing period. At harvest, leaves were immediately frozen on solid CO_2 and freeze-dried. Plants grown with 90 μM -Cd were stunted and chlorotic, indicating toxicity at this level. Those grown with 3 μM -Cd were healthy and perhaps more vigorous than control plants.

Suspension-cultured tobacco cells (*N. tabacum* cv. Wisconsin 38) were grown in modified medium of Gamborg & Miller (B5) as described previously (Reese & Roberts, 1984). Cd, Cu and Zn (sulphate salts) and ^{35}S , where used, were added on day 0. Cells were harvested on day 5 by filtration on to Whatman no. 1 paper.

Purification of tobacco Cd-binding peptides

Freeze-dried leaf tissue was homogenized under a stream of N_2 in 25 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer, pH 7.4, containing 5 mM-2-mercaptoethanol (' KPO_4 buffer') at 4 °C with a Polytron tissue grinder (Brinkman Industries). Approx. 4 ml of N_2 -purged buffer was used per g of freeze-dried leaf tissue. The brei was filtered through three layers of cheesecloth, centrifuged at 10000 g for 10 min, and the supernatant removed and re-centrifuged at 100000 g for 1 h. The resulting supernatant was desalted on a column (7 cm $\times$ 100 cm) of Sephadex G-25 (coarse grade). Cd-containing void- and post-void-volume fractions were pooled and freeze-dried. This material was redissolved in KPO_4 buffer and chromatographed on a column (2.5 cm $\times$ 60 cm) of Sephadex G-50 equilibrated with the same buffer. Fractions containing Cd were pooled, exhaustively dialysed against deionized water (using Spectra/Por tubing; 1000- M_r cut-off; Spectrum Medical Industries) and freeze-dried. This material is referred to as 'G-50-prepared'. The Cd in column fractions was monitored directly by flame atomic-absorption spectrophotometry.

Suspension-cultured cells were processed as above, using 2 ml of KPO_4 buffer (10 mM-2-mercaptoethanol)/g fresh weight of cells, and the G-25 desalting step was omitted. The presence of ^{35}S -labelled protein was monitored by liquid-scintillation spectrometry.

Tobacco Cd-binding peptides were further purified by using an electrophoretic system described previously (Wagner, 1984; system C). Gels contained 20% (w/v) acrylamide/bisacrylamide (225:9, w/w), 0.15 M-Tris/HCl buffer, pH 7.6, 0.028% ammonium persulphate and 0.019% (v/v) *NNNN*-tetramethylethylenediamine. The running buffer contained 0.15 M-Tricine titrated to pH 6.95 with Tris. The gels (1.3 mm thick $\times$ 10 cm long) were electrophoresed at 30 mA, 10 °C, until the electrophoretic front (marked by dinitrophenylaspartate) was about 1 cm from the bottom (approx. 2.5 h). Gels were sectioned into 4 mm strips, and the strips placed in test tubes containing 2 ml of deionized water for 24 h at 10 °C to leach the peptides. Location of Cd-binding peptide in leaching solution was monitored by ^{112}Cd atomic-absorption analysis and as ^{109}Cd where

proteins were labelled by 'exchange binding' (Wagner, 1984) before electrophoresis. In some experiments ^{35}S -labelled peptides were monitored directly by liquid-scintillation spectrometry. Peptide purified by gel filtration on Sephadex G-50 and subsequent electrophoresis as described above is referred to as 'G-50-prepared-electrophoretically purified' peptide.

Preparation of MT

MT was induced in Sprague-Dawley rats by subcutaneous injection of CdSO_4 (3 mg/kg body wt.) as described by Winge *et al.* (1975). Livers from three rats were ground (Polytron) in 4 vol. of KPO_4 buffer. The homogenate was centrifuged at 27000 g for 15 min and the supernatant heated to 65 °C for 10 min to precipitate denaturable proteins. After centrifugation as described above, the supernatant was decanted and freeze-dried. Dried material was dissolved in deionized water and fractionated on a column (2.5 cm $\times$ 60 cm) of Sephadex G-50 (fine grade) equilibrated with KPO_4 buffer. Cd-containing fractions were pooled, dialysed in Spectra/Por tubing (3500- M_r cut-off) against deionized water and freeze-dried. Metallothionein was purified by ion-exchange chromatography on a column (1.5 cm $\times$ 20 cm) of DEAE-Sephadex A-25 essentially as described by Winge *et al.* (1975).

Amino acid analysis

Electrophoretically purified tobacco Cd-binding peptide and ion-exchange-purified MT were performic-acid-oxidized (Hirs, 1967) and hydrolysed for 22 h in 6 M-HCl at 110 °C. Amino acid content was determined with a Beckman 6300 automatic amino acid analyser.

Isoelectric focusing

Tube and slab acrylamide gels were prepared by following the procedures of Righetti & Drysdale as previously described (Wagner, 1984). After focusing, gels were sectioned into 3 mm pieces and placed in 1 ml of freshly boiled water at room temperature to determine the pH profile. The position of Cd-binding peptides was determined as described above for acrylamide gels. G-50-prepared and G-50-prepared-electrophoretically purified samples gave the same results (monitored as ^{112}Cd , ^{109}Cd or ^{35}S -labelled ligand).

Gel filtration under dissociating conditions

M_r determinations of tobacco Cd-binding peptides and rat liver MT were made by gel filtration on a Bio-Gel A 0.5 m column (2.5 cm $\times$ 60 cm) equilibrated with 6 M-GdmCl, with or without 100 mM-2-mercaptoethanol, as described previously (Wagner, 1984). To increase resolution of the M_r of the tobacco Cd-peptide, performic-acid-oxidized (Hirs, 1967) peptides were also analysed on a Bio-Gel P-4 column (0.8 cm $\times$ 60 cm) equilibrated with 6 M-GdmCl (constant flow rate of 0.18 ml/min; 0.7 ml fractions). The column was calibrated with Blue Dextran 2000, insulin A, cytochrome *c* CNBr fragments II and III, angiotensin I, enkephalinamide, and dinitrophenylaspartate (all obtained from Sigma). Fluorescamine labelling of low- M_r standards was useful in column calibration, as previously noted (Wagner, 1984).

Cd dissociation studies

Dissociation of Cd from tobacco Cd-binding peptides and Cd,Zn-thionein was measured by using 0.8 cm × 25 cm Sephadex G-15 desalting columns. Peptide and MT were labelled with ^{109}Cd by exchange binding and dissolved in 1 ml of buffer (50 mM-ammonium citrate, pH 2.5–4.0; 50 mM-sodium acetate, pH 4.0–6.0; 50 mM-Tris/HCl, pH 6.5–8.0). The same buffers were used for this purpose by Kagi & Vallee (1960). The protein concentration of samples was between 120 and 140 $\mu\text{g/ml}$. After being left for at least 1 h at room temperature, the samples were desalted on columns equilibrated with the corresponding buffer; ^{112}Cd and/or ^{109}Cd in the void volume was used to determine the percentage of Cd which remained bound to peptide. That considered bound to Cd-peptide was eluted from Sephadex G-15 before the standard (Cd-glutathione and $^{109}\text{CdCl}_2$). Elution of these components was unchanged by addition of 0.5 M-NaCl to the elution buffer, indicating a lack of ionic interaction of components with the resin. Electrophoresis of the void-volume fraction showed that essentially all Cd was associated with Cd-binding peptide.

The effects of GdmCl or dissociation of Cd from tobacco Cd-peptide and MT were measured by using various concentrations of GdmCl buffered with 50 mM- KPO_4 , pH 7.4. Samples were incubated for 24 h then chromatographed on Sephadex G-25 columns (0.8 cm × 25 cm) equilibrated with the corresponding concentration of buffered GdmCl. Although exchange-bound ^{109}Cd was used in these experiments, dissociation of endogenously bound ^{112}Cd was also checked and found to parallel that of ^{109}Cd . As observed previously for cabbage peptide (Wagner, 1984), exchange-bound ^{109}Cd served as a useful marker for tobacco Cd-binding peptides under all conditions. No 2-mercaptoethanol was used in these experiments because preliminary results showed its addition had no significant effect on Cd dissociation.

RESULTS AND DISCUSSION

The M_r values for rat liver MT and the tobacco Cd-binding peptide were determined by gel filtration. Under non-denaturing conditions, MT was observed to have an apparent M_r of 10000, a value which is in agreement with those reported in the literature (Kagi & Vallee, 1960). Tobacco Cd-binding peptides isolated from 90 μM -Cd-treated plants had apparent M_r values of about 6000 (usually 6000, but variable from 4000 to 8000) and 2000. Only about 10% of the Cd occurred as the M_r -2000 peptide. Extracts from leaves of 3 μM -Cd-treated plants contained only the apparent- M_r -2000 peptide. In cultured tobacco cells an apparent- M_r -2000 peptide was also observed; however, the apparent M_r of this peptide was occasionally seen to vary between 2000 and 4000. A similar variability in the apparent M_r of Cd peptides from *Pisum* (pea) has been reported (Grünhage *et al.*, 1985).

Under denaturing conditions on the Bio-Gel A 0.5m column, the M_r of rat liver MT was 6000, a value that is in agreement with that calculated from its amino acid composition (Winge *et al.*, 1984; Kagi *et al.*, 1984). Cd-peptide isolated from 90 μM -Cd-treated plants, 3 μM -Cd-treated plants and cultured cells had M_r values of about 1500 under these conditions. Consistent with this,

more highly resolving chromatography on Bio-Gel P4 revealed the M_r of tobacco peptide to be about 1300, although some variability between the M_r of peptides isolated from leaves and cultured cells was observed. Minor differences in M_r may result from interactions with phenolic compounds (Van Sumere *et al.*, 1975). Brown coloration of purified Cd-binding peptides was greatest in material recovered from 90 μM -Cd-treated plants (highly stressed) and least in that extracted from tissue-cultured cells.

The effects of denaturing and non-denaturing conditions on the molecular size of the leaf M_r -6000 Cd-peptide can be seen in Fig. 1. After denaturation with 6 M-GdmCl/100 mM-2-mercaptoethanol and subsequent removal of denaturant by dialysis, the peptide appeared to reaggregate or renature. The reaggregation/renaturation resulted, however, in a broadening of the Cd-containing peak (Fig. 1c), with much of the material having a somewhat lower apparent M_r . This shift was accompanied by a loss of about 60% of Cd and may indicate some involvement of the metal in the formation of the M_r -6000 form (presumed aggregate). In additional experiments utilizing ^{35}S -labelled peptide or ^{109}Cd exchange binding, similar results to those shown in Fig. 1 were obtained. Under the same conditions as in Fig. 1, the Cd-peptide from 3 μM -treated plants and cultured cells showed no comparable shift in size, and MT appeared to shift from M_r 10000 to M_r 6000 and back. The position of MT elution in this system is shown in Fig. 1. Omission of the 2-mercaptoethanol had no apparent effect on the denaturation of these peptides.

The reaggregation/renaturation of the M_r -6000 form appears to be unique to peptides isolated from plants grown in the presence of relatively high levels of Cd. Similar results were obtained with apparent- M_r -10000 Cd-peptide from cabbage grown with 180 μM -Cd, where denaturation also resulted in an M_r -2000 component (Wagner, 1984) which, after removal of denaturant, reaggregated/renatured to M_r 10000 (results not shown). Grill *et al.* (1985) concluded that the Cd-peptide of *Rauwolfia* cells grown in media containing 200 μM -Cd occurs as a trimer under non-denaturing conditions. Attempts to cause aggregation of the M_r -2000 peptide from 3 μM -Cd-grown tobacco plants were unsuccessful. Exposure to ≥ 100 mM-Cd *in vitro* caused the peptide to precipitate, but there was no evidence for the formation of a soluble M_r -6000 aggregate under this or any other experimental condition tested.

The difference between the apparent M_r of MT under native conditions and its true M_r may be due to a non-globular shape resulting from the protein's charged nature (Kagi *et al.*, 1974). It is unlikely that the extreme difference observed between the apparent M_r of the tobacco leaf M_r -6000 and cabbage M_r -10000 peptides under non-denaturing and denaturing conditions is the result of shape alone. The occurrence of only the M_r -2000 form in 3 μM -Cd-grown plants, but of both the M_r -2000 and M_r -6000 forms in the 90 μM -Cd-grown plants (all forms having the same amino acid composition; see below), suggests some form of aggregation phenomenon in the case of the M_r -6000 form. The basis of this phenomenon is not understood, but these results suggest that tobacco Cd-binding peptides are composed of the same M_r -1300 peptide.

Amino acid compositions of G-50 prepared-electrophoretically purified Cd-peptide from tobacco leaves

Fig. 1. Disaggregation-reaggregation of apparent- M_r -6000 tobacco Cd-peptide

(a) Non-denaturing gel-filtration on a column (2.5 cm $\times$ 60 cm) of Sephadex G-50 with 25 mM-potassium phosphate buffer (pH 7.4)/5 mM-mercaptoethanol as eluent; 5.5 ml fractions were collected. (b) Denaturing gel-filtration on a column (2.5 cm $\times$ 60 cm) of Bio-Gel A 0.5m with 25 mM-potassium phosphate buffer (pH 7.4)/6 M-GdmCl/100 mM-mercaptoethanol; 2.0 ml fractions were collected. (c) Same as (a). Fractions, marked by the bar in (a) (total amount of Cd: 53.6 μ g) were pooled, dialysed against deionized water and freeze-dried. The material was dissolved in 6 M-GdmCl for denaturing chromatography (b). Cd-containing fractions (marked by the bar) from the Bio-Gel column were pooled, dialysed against deionized water and freeze-dried. The recovery of Cd was 18.2 μ g. Elution of MT under similar conditions

and cultured cells were essentially identical (Table 1) and similar to that reported for cabbage (Wagner, 1984) *Agrostis* (Rauser, 1984) and *Rauwolfia* cultured cells (Grill *et al.*, 1985). These results suggest that production of unique Cd-peptides may be a common response of plants in the presence of Cd. The data of Table 1 show the actual amino acid compositions obtained with plant peptides on a residue % basis. Calculations based on a M_r -1300 unit peptide (see above) indicate that Cd-peptide has an average composition of Glu₄Cys₄Gly₁. Other amino acids occurring at very low levels may be the result of phenolic interaction between peptide and amino acids/proteins present during homogenization. Consistent with this suggestion is the observation that the amino acid profiles of cultured-cell peptides contained insignificant levels of amino acids other than Glu, Cys and Gly. Peptide from this source was virtually colourless, whereas that from leaves was tan-coloured. Oxidation products of proteins with plant phenolics appear tan/brown in colour (Van Sumere *et al.*, 1975). Gravimetric analysis of G-50-prepared-electrophoretically purified peptide from cultured cells indicated that virtually all the dry weight of this, the most pure of the tobacco peptides isolated (Table 1), was accounted for by Glu, Gly, Cys and Cd (results not shown).

Anti-(rat liver MT) antibody was prepared in rabbits by using essentially the procedure used by Winge *et al.* (1975). Reactivity to MT and cross-reactivity with tobacco Cd-peptide was assessed by using rocket immunoelectrophoresis. A concentration-dependent response was observed with MT, whereas no immunoprecipitation was detected with tobacco Cd-peptides (results not shown). These results are consistent with the observed differences in amino acid composition between MT and the tobacco Cd-peptide. Efforts to raise antibodies to the M_r -6000 tobacco Cd-peptide using the same approach were unsuccessful.

All of the tobacco Cd-peptide from leaf and cultured cells had pI 3.15, which supports the contention that they are all composed of the same unit peptide. Nordberg *et al.* (1972), using a preparative sucrose-gradient isoelectric-focusing system, reported that rabbit MT I and MT II have pI values of 4.5 and 3.9 respectively, and previously Kagi & Vallee (1960) showed equine MT to have a pI between 4.5 and 5.5. Our attempts to focus rat liver MT using the acrylamide system indicated a pI of 4.8 when protein was added to the cathodal end. Values of pI for rabbit MT, equine MT and that found here for rat liver MT are consistent with our observations of the migration of MT in the Tricine/gel system described in the Materials and methods section. In this system, which separates small acidic molecules, MT migrated behind Bromophenol Blue (pI $\sim$ 4.0), whereas tobacco Cd-protein migrated with the electrophoretic front (near the highly acidic dinitropyridylaspartate) and well ahead of Bromophenol Blue.

Differences between the pI of MT and those of tobacco Cd-peptides are presumably due to the high Glu content of plant Cd-peptides and the presence of basic amino acid residues (Ser and Lys) in MT. Basic residues

is indicated by the arrows. Calibration standards: 1, chymotrypsinogen (25.8 kDa); 2, cytochrome *c* (12.5 kDa); 3, insulin B (3.5 kDa); 4, cyanocobalamin (1.35 kDa).

Table 1. Comparison of amino acid compositions of tobacco Cd-binding peptides, rat liver MT, fission-yeast cadystin A, and the major Cd-peptide from *Rauvolfia serpentina*

Amino acid	Composition						
	Residue %*			Calculated number of residues/molecule			
	Tobacco leaf M_r -6000 Cd-peptide	Tobacco leaf M_r -2000 Cd-peptide	Cultured-tobacco-cell M_r 2000 Cd-peptide	Rat liver MT†	Cultured-tobacco-Cell Cd-peptide‡	Fission-yeast cadystin§	<i>Rauvolfia serpentina</i> Cd-peptide
Cys	27.0	28.0	35.1	20	4	3	4
Asx	2.7	3.4	0	4	0	0	0
Thr	0.9	1.2	0.5	4	0	0	0
Ser	4.1	5.1	1.1	11	0	0	0
Glx	40.6	36.5	47.9	1	4	3	4
Pro	0	0	0	2	0	0	0
Gly	15.2	16.5	13.0	6	1	1	1
Ala	2.3	2.6	1.2	3	0	0	0
Val	1.0	1.1	0	2	0	0	0
Met	0	0	0	1	0	0	0
Ile	0.8	1.2	0.3	0	0	0	0
Leu	1.3	1.3	0.5	0	0	0	0
Tyr	0.6	0.5	0	0	0	0	0
Phe	0.7	0.6	0	0	0	0	0
His	0.6	0.8	0.3	0	0	0	0
Trp	0.3	0.6	0	0	0	0	0
Lys	1.7	0.9	0.1	7	0	0	0
Arg	0.6	0.4	0	0	0	0	0

* Similar results obtained with at least four separate preparations of tobacco peptides.

† Winge *et al.* (1975) 'isoform I'.

‡ Calculated from amino acid analysis, assuming an M_r of 1300 and 85% efficiency of performic acid oxidation of Cys (Hirs, 1967).

§ From Murasugi *et al.* (1981).

|| From Grill *et al.* (1985).

are absent from plant Cd-peptides. Vasak *et al.* (1985) have suggested that Lys residues juxtaposed to Cys groups in MT act to neutralize the acidic nature of Cd-Cys bonds. That the difference between the pI of MT and that of tobacco Cd-peptide is not more than 1.7 pH units may be due to the larger number of possible acidic Cd-chromophores per molecule in the former. The Cys/Cd ratio of about 3:1 observed here for tobacco Cd-peptides is similar to that reported for cabbage (Wagner, 1984) and MT (Kagi & Vallee, 1960; Armitage *et al.*, 1982). Because tobacco Cd-peptide is smaller than MT, the Cd-binding capacity is expected to be about 1–2 mol of Cd per mol of M_r -1300 ligand compared with a possible 7 mol of Cd per mol of MT.

Stability of metal binding, measured as the pH at which 50% of metal is lost, is regarded to be a useful criterion for distinguishing MT and non-MT metal-binding proteins (Vasak & Armitage, 1986). Values for 50% Cd and Zn dissociation from MT are 3 and 4.5 respectively [the bar on Fig. 2 marks the range of values for pH of 50% dissociation reported by Kagi & Vallee (1960, 1961) for equine MT]. A value of 3 was obtained here for rat liver MT, whereas that for tobacco peptide was found to range from 5 to 5.8 (Fig. 2). Kagi & Vallee (1961) calculated conditional Cd and Zn binding constants for equine MT ($K_{3Cd} = 10^{25.5}$ and $K_{3Zn} = 10^{21}$ respectively) based upon the pH of 50% metal dissociation. By comparison, making the same basic assumptions, including co-ordination of each Cd ion with three Cys residues and assuming no ligand binding to the G-15 columns used here, K_{3Cd} for tobacco Cd-peptides is estimated to be about 10^{19} .

Fig. 2. Effect of pH on Cd dissociation from MT and tobacco Cd-peptides

MT and tobacco Cd-binding peptides (G-50-prepared) were equilibrated (≥ 1 h; room temperature) in buffers having specific pH (see the Materials and methods section) and subsequently desalted to determine the percentage of Cd remaining bound. The bar marked by the asterisk indicates the pH causing 50% Cd dissociation from equine MT reported by Kagi & Vallee (1960, 1961). ○, 3 μ M-Cd leaf peptide; ●, 90 μ M-Cd leaf peptide; ▲, cultured cell peptide; △, MT; ■, electrophoretically purified cultured-cell Cd-peptide.

Differences in these conditional binding constants provide further evidence that the MT has a higher affinity for Cd than does the tobacco peptide. Similar data were obtained with G-50-prepared and G-50-prepared-electrophoretically purified (■, Fig. 2) tobacco Cd-peptide. Exchange-bound ^{109}Cd and ^{112}Cd bound *in vivo* were used to monitor Cd-peptide in both cases. As noted above, electrophoresis experiments indicate that more than 95% of Cd associated with G-50-prepared Cd-peptide was associated with Cd-peptide after purification by electrophoresis. This result validates the use of G-50-prepared material and exchange binding with ^{109}Cd to study the pH-dependency of Cd binding (Fig. 2) and the influence of GdmCl on Cd-binding (Fig. 3). Whereas MT always contains bound Zn, no Zn was found to be associated with tobacco Cd-peptide. Attempts to exchange bind radiolabelled ^{65}Zn or 10, 100 and 1000 mM unlabelled Zn to tobacco peptide through mass action or by removal of Cd at low pH, followed by addition of Zn and titration to pH 8, failed to provide evidence for Zn binding. These results support the contention that tobacco Cd-peptide ligand is non-MT-like with regard to binding of Zn.

Denaturing chromatography using GdmCl (Fig. 1) showed that high concentrations of GdmCl caused the partial dissociation of Cd from tobacco Cd-peptide. Therefore a series of experiments were conducted in which tobacco Cd-peptide (G-50-prepared peptide isolated from tobacco leaves and G-50-prepared-electrophoretically purified peptide from cultured cells) was exposed to various concentrations of GdmCl at 22° and 35 °C. The data (Fig. 3) show that an increase in GdmCl concentration or temperature resulted in increased dissociation of Cd from the ligand. Although the reasons for the apparent break in the curves at ≤ 1 M-GdmCl and the quantitative differences between the apparent Cd affinities of leaf and cultured-cell peptide are not known,

Fig. 3. Effects of GdmCl concentration at 22 and 35 °C and at pH 7.4 on the dissociation of Cd from MT and the M_r -6000 tobacco Cd-peptide

Samples were equilibrated for 24 h before chromatography. Δ, MT, 22 °C; ■, electrophoretically purified cultured-cell peptide, 22 °C; □, electrophoretically purified cultured-cell peptide, 35 °C; ●, 90 μM-Cd-induced leaf peptide, 22 °C; ○, 90 μM-Cd-induced leaf peptide, 35 °C.

the data are consistent with observed heterogeneity of Cd binding by tobacco peptide and may also reflect differences involved in formation of the M_r -6000 aggregate. No comparable dissociation of Cd from MT was observed (Fig. 3), providing further evidence that the affinity of MT for Cd is higher than that of tobacco Cd-binding peptide for this metal. A linear decrease to 80% dissociation of Cd from G-50-prepared tobacco Cd-binding peptide over 24 h was observed in the presence of 4 M-GdmCl (results not shown). These results are consistent with the limited retention of Cd observed in the experiments described in Fig. 1.

MT is induced by Zn as well as by Cd and other metals (Kagi *et al.*, 1984). To examine the possibility that ligand of tobacco Cd-peptides is induced by Zn or Cu, tobacco suspension cultures were grown in the presence of ^{35}S to label any Cys-rich peptide. Control cultures contained only the low levels of Zn (7 μM) and Cu (0.1 μM) found in basal B5 medium. Cd, Cu and Zn cultures were grown in the presence of 90 μM-metal, added as the respective sulphate salt. Elution profiles (Figs. 4a–4d) of extracts from all the ^{35}S -labelled cultures contained substantial label in the elution region corresponding to components of < 1 kDa (free-salt region). An apparent- M_r -2000 ^{35}S -labelled peptide, containing typically > 70% of eluate Cd, was induced in the Cd-treated cultures (Fig. 4c). In Cu-treated cultures, Cu was coincident with ^{35}S under an apparent- M_r -2000 peak, and gel electrophoresis of this peak showed that the ^{35}S -labelled material migrated with the front in a manner similar to the Cd-binding peptides. However, Cu migrated independently of ^{35}S (results not shown). Exchange binding of ^{109}Cd demonstrated that the ^{35}S -enriched anionic Cu-induced material is capable of binding Cd, but preliminary analysis indicates that the amino acid composition of this material differs from that of tobacco Cd-peptide or MT (results not shown). Further characterization of components induced by Cu in this system is required. However, it appears that cultured tobacco cells exposed to Cd or Cu do not respond in an identical manner.

No M_r -2000 ^{35}S -labelled component was observed in control cultures or in those exposed to Zn (Figs. 4a and 4d). Amino acid analysis of the ≤ 2 kDa regions of profiles from Zn and control cultures gave no indication of the presence of a Cys+Glu-enriched component (result not shown). In addition, increasing the level of Zn to 180 μM failed to induce any ^{35}S -labelled peptide. These data, along with those noted above regarding the lack of Zn-binding *in vitro*, indicate that, under the conditions used, Zn is apparently not capable of causing significant formation of tobacco Cd-peptide, nor is it able to bind to its ligand. Grill *et al.* (1985) reported the absence of Cd-binding peptides in *Rauwolfia* control cultures, but stated in passing (no data was provided) that induction of the peptide by Zn was observed. We emphasize that, in our study, growth of tobacco cell cultures in the presence of 180 μM-Zn produced no observable anionic ^{35}S -enriched Cd-peptide-like component. Similarly, growth of tobacco plants in up to 500 μM-Zn produced no Cd-peptide-like component capable of binding ^{109}Cd (results not shown).

Zn induces MT and has been suggested to be its primary inducer in some animal organs (Onosaka & Cherian, 1982). The lack of a significant Zn induction of, and Zn binding to, tobacco Cd-peptide represents an

Fig. 4. Sephadex G-50 elution profiles (column size: 1.5 cm $\times$ 95 cm; 2.5 ml fractions) of aqueous extracts of cultured tobacco cells grown in the presence of 0.1 mCi of ^{35}S /150 ml of culture

(a) Control cultures, no metals added except those found in B5 medium; (b) cultures containing 90 μM -Cd; (c) cultures containing 90 μM -Cu; (d) cultures containing 90 μM -Zn. Calibration standards [1, chymotrypsinogen (25.8 kDa); 2, cytochrome *c* (12.5 kDa); 3, insulin B-chain (3.5 kDa)] are marked by arrows.

important difference between this peptide and MT. Kagi & Vallee (1960) demonstrated that Zn binding to MT is less stable than is Cd binding, with 50% dissociation of Zn occurring at a pH > 1 unit higher than that which causes an equivalent dissociation of Cd. If Zn binding to tobacco Cd-peptide does occur *in vivo*, with a relative stability like that of MT (Cd versus Zn), then, at physiological pH, Zn binding would be very weak and of little value in a Zn sequestration or homeostatic role. The inability of plant Cd-binding peptide to bind substantial amounts of Zn is not surprising, given that

metal specificity in heavy-metal tolerance is known to occur in plants (Woolhouse, 1983), and available evidence relates Cd tolerance in plants to the occurrence of low- M_r , Cys-rich peptides (Jackson *et al.*, 1984). Presumably, in plants tolerance to Zn occurs by a different mechanism.

Conclusion

Results presented here show that tobacco Cd-peptides and MT differ substantially in metal affinity and selectivity as well as in M_r , pI, and amino acid composition. Furthermore, results regarding the apparent lack of Zn induction of, or binding to, ligand of tobacco Cd-peptide are of particular significance in that numerous proposed roles of MT in animals involve Zn storage (Karin, 1985). Further studies of the effects of Cu on induction of metal-binding peptides in plants will be of particular interest with regard to understanding the function(s) of these peptides.

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