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Poly(γ-glutamylcysteinyl)glycine: Its role in cadmium resistance in plant cells

(heavy metal resistance/plant cell cultures/metallothionein)

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ABSTRACT **Angiosperms can be selected for the ability to grow in the presence of normally toxic concentrations of certain trace metal ions. Addition of Cd and Cu to Cd-resistant *Datura innoxia* cell cultures results in the rapid synthesis and accumulation of sulfur-rich, metal-binding polypeptides. The structure of these compounds was determined using amino acid analysis, ^{13}C NMR, and site-specific enzymic digestion. These compounds are poly(γ-glutamylcysteinyl)glycines. Greater than 80% of the cellular Cd is bound to the bis and tris forms in Cd-resistant cells. There is a direct correlation between the maximum accumulation of the metal-binding polypeptides and the concentration of toxic ions to which the cells are resistant. In the presence of metal ions, the polypeptides form multimeric aggregates that can be resolved by gel chromatography. Cd binds to both the high and low molecular weight aggregates, whereas Cu preferentially binds to the higher molecular weight forms. The presence of γ-carboxamide linkages between glutamyl and adjacent cysteinyl residues indicates that these polypeptides are products of biosynthetic pathways. Poly(γ-glutamylcysteinyl)glycines bind metals and, in this respect, appear to be functional analogs of the protein metallothionein. However, in the absence of supraoptimal concentrations of trace metal ions, the functions of metallothionein in animals and microorganisms and poly(γ-glutamylcysteinyl)glycines in plants may differ.**

Populations of angiosperms have rapidly colonized several environments containing high concentrations of certain metal ions (1). Metal-resistant cultivars of some common grasses are commercially available for the revegetation of contaminated sites (2). While much is known about the ecological genetics and evolution of metal tolerance in plants (1), the physiological, biochemical, and genetic basis of tolerance is not as well understood in the majority of resistant ecotypes. Production of metallothionein-like metal complexes correlates with resistance to Cu and Cd in some plants and cultured plant cells (3–8). However, the chemical structures of such compounds produced by tolerant plants have not been fully elucidated. Improved understanding of the chemical form of metals in plants is also of ecological and nutritional importance since the chemical form of metals affects their mobility through food chains (9).

Suspension cell cultures of *Datura innoxia* have been selected for resistance to various concentrations of $CdCl_2$ (7). Variant cell lines retain the ability to grow in normally toxic concentrations of Cd after growth in its absence for >700 generations. Resistance to Cd correlates with synthesis of large amounts of small, cysteine-rich, Cd complexes (7, 8). The structures of these complexes are described.

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MATERIALS AND METHODS

Maintenance of Plant Suspension Cultures. Suspension cultures of Cd-sensitive and -tolerant cell lines of *D. innoxia* were maintained in the dark in 50-ml batch suspension cultures as described by Jackson *et al.* (7). Under these conditions, a single signal, corresponding to unchelated Cd, was detected in the medium by ^{113}Cd NMR. Cells were diluted every 48 hr to maintain a concentration of cells in logarithmic phase.

Radioisotope Labeling of Cells. All labeled compounds and ^{109}Cd were purchased from duPont–New England Nuclear, and specific activities were those provided by the manufacturer. Carrier-free ^{67}Cu was a gift of the Isotope and Nuclear Chemistry Division, Los Alamos National Laboratory. Polypeptides were labeled *in vivo* by growing the cells in medium containing 300 μM Cd plus carrier-free ^{109}Cd at 0.1 μCi/ml (1 Ci = 37 GBq). Alternatively, polypeptides were labeled by growth of the cells in medium containing 300 μM unlabeled Cd plus [^3H]glutamate (10 μCi/ml), [^3H]glycine (10 μCi/ml), [^3H]glutathione (10 μCi/ml), or [^{35}S]cysteine (2.5 μCi/ml) added to the culture medium in the absence of additional unlabeled amino acids. Polypeptides were labeled *in vitro* by carboxymethylation using iodo[^3H]acetic acid according to the methods of Cole *et al.* (10).

^{13}C Labeling of Cells and Polypeptides. L-[1,5-$^{13}C_2$]Glutamic acid and ^{13}C-labeled diglutathione were obtained from the National Stable Isotope Resource (Los Alamos National Laboratory, Los Alamos, NM). Glutamate, differentially labeled at the 1 and 5 carbons, was produced biosynthetically by incubating *Brevibacterium flavum* in culture medium that contained [1-^{13}C]acetic acid (11). Cd-resistant *D. innoxia* cells were grown for 48 hr in medium containing [^{13}C]glutamate at 100 mg/liter of medium and 250 μM $CdCl_2$ to produce ^{13}C-labeled polypeptide. Polypeptides were isolated from these cells as described below.

Cell Extraction and Isolation of Polypeptides. Cells were extracted as described by Jackson *et al.* (7). The supernatant was subjected to analytical gel filtration on 2.2 × 100 cm columns of Sephadex G-50 (fine) (Pharmacia) equilibrated at 4°C with 50 mM Tris·HCl, pH 7.8. Preparative amounts of polypeptide were obtained by subjecting larger amounts of supernatant to gel filtration through 5 × 150 cm columns of Sephadex G-50 (fine). Samples from these preparative columns were pooled and further purified by covalent chromatography on thiopropyl-Sepharose 6B (12). Resulting isolates were desalted and concentrated by ultrafiltration utilizing an Amicon ultrafiltration cell containing a YC05 filter, which excludes molecules of molecular weight >500. Polypeptide samples isolated in this way were pure as determined by denaturing gel filtration analysis, amino acid content analysis, ^{13}C NMR analysis, and enzyme digestion followed by paper electrophoresis.

Abbreviation: (γ-Glu-Cys)$_n$Gly, poly(γ-glutamylcysteinyl)glycine.

Denatured molecular weights of the polypeptides were determined by passage of carboxymethylated polypeptides through denaturing guanidine hydrochloride gel filtration columns packed with either Sephadex G-50 (fine) or Sephadex G-10. Columns were calibrated with denatured molecular weight markers prior to analysis of the denatured polypeptides.

Enzymic Digestion of Polypeptides and Analysis of Digestion Products. Purified polypeptides were digested for different periods with carboxypeptidase Y (Sigma) at 25°C in 50 mM phosphate buffer, pH 6.25, or carboxypeptidase A (Sigma) at 25°C in 450 mM KCl/50 mM Tris·HCl, pH 7.5. Digested samples were loaded onto filter paper saturated with formic acid/acetic acid/distilled water, 26:120:1000 (vol/vol), and electrophoresed at 3 kV for 45 min in a high-voltage paper electrophoresis cell (Chemei Erzeugnisse Und, Muttenz, Switzerland) as described by Antener *et al.* (13). The paper was removed from the apparatus, immediately air dried at 50°C, and stained with 3% (wt/vol) ninhydrin (Bio-Rad) in ethylene glycol monomethyl ether. The stain was allowed to develop overnight prior to analysis of the digestion products. The positions of the different amino acids were determined by comparison to the positions of glutamate, glycine, cysteine, and glutathione samples analyzed on the same paper. Amounts of each amino acid were determined by elution of spots containing the different amino acids from the paper with absolute methanol followed by determination of the absorbance at 500 nm for each sample.

Amino Acid Analysis. Lyophilized extracts were oxidized to convert cysteine to cysteic acid and then hydrolyzed in 6 M HCl at 110°C for 24 hr. Samples were resuspended in citrate buffer and analyzed for amino acid composition using an ion-exchange automated amino acid analyzer.

Amino Acid Sequence Determination. Extracts were treated with trifluoroacetic acid for 90 min at 55°C to remove any acetyl groups that might have been present, then directly subjected to Edman degradation and amino acid analysis using an automated amino acid sequencer. A portion of the extracts was treated with *Staphylococcus aureus* V8 prior to Edman degradation.

Acetylation of Peptides. *N*-Acetyl derivatives of glutamate, α-L-glutamyl-L-alanine, and diglutathione were prepared by treatment with the mixed anhydride of pivalic acid and acetic acid, prepared by the method of Horton *et al.* (14). A 2-fold molar excess of the acetic pivalic anhydride was added to an aqueous solution of the peptide (50 mg/ml; pH 10.2). The reaction mixture was stirred vigorously for 1 hr then was extracted with chloroform to remove excess anhydride. Unreacted polypeptides were removed by chromatography on Dowex AG-50 (Bio-Rad) in the H^+ form. The fraction eluted from the column (0.5 × 10 cm) with water was lyophilized and prepared for analysis by ^{13}C NMR spectroscopy.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were obtained at 75.47 MHz on a Bruker WM-300 wide-bore NMR spectrometer using the following acquisition parameters: sweep width, 14.7 KHz; digital resolution, 1.8 Hz; pulse, 45°; recycle time, 10.8 s; and 25°C. Protons were decoupled using 1 W of forward power and the WALTZ 16 composite pulse sequence (15). Apopolypeptide samples (50 mg or less) were dissolved in 3.5 ml of $^2H_2O/H_2O$, 50:50 (vol/vol). Dioxane (25 μl) was added as an internal chemical shift reference (67.2 ppm). Dithiothreitol was added in a 2-fold molar excess to reduce the cysteine thiol groups, and the pH was adjusted with 2 M HCl or NaOH. The samples were sealed in 10-ml NMR tubes under an Ar atmosphere.

RESULTS

Cadmium-Induced Trace Metal-Binding Complexes in *D. innoxia*. Most of the soluble Cd (>80%) in Cd-resistant *D. innoxia* cells is bound to a collection of small complexes of native molecular weights between 1500 and 4000 (peaks 1, 2, 3, and 4; Fig. 1). These complexes differ in their metal binding *in vivo*. Larger complexes bind Cu and Cd, whereas only Cd was detected bound to the smaller complexes. Addition of Cu to cells growing in the presence of Cd causes the displacement of Cd from the larger complexes suggesting that these compounds have a higher affinity for Cu. Cells selected for resistance to Cd are also resistant to normally toxic concentrations of Cu.

Gel filtration chromatography of extracts from cells grown in [^3H]leucine and [^{35}S]cysteine reveals an increase in the ratio of $^{35}S/^3H$ corresponding to the elution position of peaks 1 to 4 in Fig. 1. This increase is not found in chromatographs of extracts from Cd-sensitive cells or from Cd-resistant cells grown in the absence of Cd.

Cell lines grown in the maximum tolerable concentration of Cd show a correlation between the amount of ^{109}Cd incorporated into peaks 1 to 4 (Fig. 1) and the degree of Cd tolerance (Table 1). There is also a direct correlation between the amount of Cd and the amount of [^{35}S]cysteine in peaks 1 to 4 suggesting a direct correlation between the maximum amount of metal-binding polypeptide synthesized and the level of resistance to different concentrations of metal ion. Such a correlation also exists if all cell lines are grown in 300 μM Cd, a concentration toxic to all but the most resistant line. However, exposure of less-resistant lines to this concentration of Cd leads to rapid cell death. Therefore, reduced polypeptide synthesis in these lines may not be the result of the inability to synthesize adequate amounts of polypeptide, but may result from a significant decrease in viability. These data, therefore, suggest that these polypeptides are involved in the mechanism of Cd resistance. However, it is not known whether the metal-resistance genes are directly responsible for the overproduction of poly(γ-glutamylcysteinyl)glycine [(γ-Glu-Cys)$_n$Gly] in resistant cells.

Covalent chromatography on thiopropyl-Sepharose 6B of pooled fractions corresponding to peaks 1 to 4 (Fig. 1) of an extract from cells grown in the presence of Cd and [^{35}S]cysteine results in the collection of fractions containing >90% of the [^{35}S]cysteine loaded onto the column upon elution with 2-mercaptoethanol. Fractions containing this cysteine-rich material were pooled, subjected to ultrafiltration, and further analyzed.

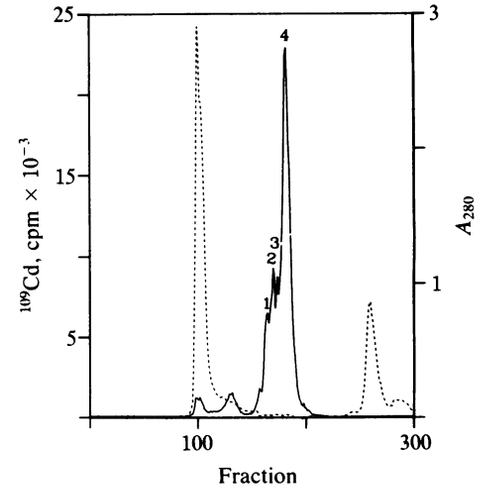

FIG. 1. Native gel chromatography of trace metal-binding complexes. Cd-resistant *D. innoxia* cells were grown for four generations in 300 μM Cd. Cells were exposed to 0.15 μCi of ^{109}Cd per ml for 24 hr prior to extraction. Extracts of cells were centrifuged, and the soluble portion of the extract was analyzed by passage through a 150 × 5 cm Sephadex G-50 (fine) column (7). Solid line, ^{109}Cd; dashed line, A_{280}.

Table 1. Maximum accumulation of metal-binding complexes in *D. innoxia* cells resistant to various concentrations of Cd

Level of Cd resistance, μM Cd	^{109}Cd bound, cpm $\times 10^{-3}$
50	14.4
120	35.8
160	61.4
200	65.7
250	94.3

D. innoxia cells resistant to various concentrations of Cd were exposed for 24 hr to ^{109}Cd (0.15 μCi/μmol Cd added to the medium) at the maximum concentration of Cd in which they could grow rapidly. Cell extracts were analyzed by gel chromatography through columns of Sephadex G-50 (fine). The amount of ^{109}Cd bound to fractions containing the metal-binding polypeptides was used to determine the amount of metal-binding polypeptide present. Correlation coefficient, $r = 0.987$; significant at $P = 0.001$.

Molecular Weight Determination. The above material was analyzed by gel chromatography on a column of Sephadex G-50 (fine) in 6 M guanidine hydrochloride. A broad peak with an apparent molecular weight of 776 was observed (Fig. 2). However, chromatography of a similar extract on a column of Sephadex G-10, under reducing conditions in 50 mM sodium dithionite, resolved two components, one excluded from the gel and one partially included. This suggests at least two compounds, one with a molecular weight slightly below 700, and one or more with molecular weights above 700. Repeated ultrafiltration of a sample labeled with [^{35}S]cysteine under reducing conditions using a YM2 membrane, which excludes materials with molecular weights >1000, excluded only 4% of the ^{35}S-labeled material from passage through the membrane. This indicates that the major components all have molecular weights <1000.

Amino Acid Analysis. The amino acid composition of a typical polypeptide preparation purified from resistant cells by gel filtration and covalent chromatography is 39.5% cysteine, 41.9% glutamate, and 18.5% glycine (values in molar fraction). A preparation from 200 ml of cell culture yielded 25 mg of polypeptide. All samples isolated from cells resistant to different concentrations of Cd contained only cysteine, glutamate, and glycine in molar ratios between 2:2:1 and 3:3:1. Samples from most resistant cells had a molar ratio closer to 2:2:1. A stoichiometric compound containing these three amino acids with a ratio of 3:3:1 would have a molecular weight of 771, whereas a ratio of 2:2:1 results in a molecular weight of 539. The apparent molecular weights of the complexes are, therefore, consistent with the existence of two polypeptides containing five and seven amino acids in the above ratios.

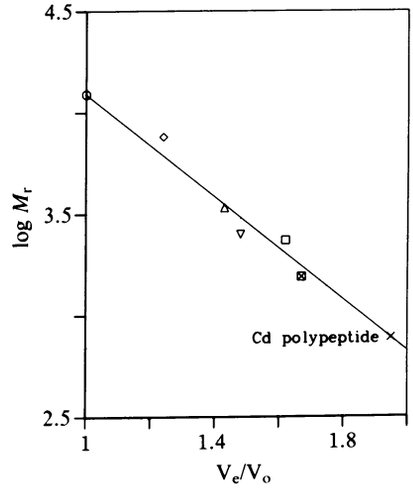

FIG. 2. Estimation of the denatured molecular weight of Cd-binding polypeptides from *D. innoxia*. Cysteine-rich, metal-binding polypeptides purified by covalent chromatography on thiopropyl-Sepharose 6B were analyzed by separation on a column containing Sephadex G-50 (fine) in 6 M guanidine hydrochloride. Molecular weights were determined by comparison of the migration of metal-binding polypeptides to the migration of the α and β fragments of insulin (□ and △, respectively), cytochrome *c* (○), and CNBr fragments I, II, and III of cytochrome *c* (◇, ▽, and ⊠, respectively) (16). Analysis of the results yields a molecular weight of 776 for the metal-binding polypeptide.

Polypeptide mixtures were not sensitive to Edman degradation (17), even after treatment with trifluoroacetic acid to remove any acetyl groups that may have been present. Treatment with *S. aureus* V8, known to cleave glutamyl α-carboxamide bonds (18), did not generate polypeptide fragments that were sensitive to Edman degradation. These results suggest that the N-terminal amino acid is glutamate and that all glutamate residues are linked to adjacent amino acids through γ-carboxamide linkages. Glutathione binds Cd with a stoichiometry of 1:1, 1:2, or 1:3 at pH 7.0. However, the apparent molecular weights of the denatured polypeptides are higher than glutathione, the observed ratio of the three amino acids is inconsistent with Cd–glutathione complexes, and oxidized apopolypeptides are not a substrate for glutathione reductase in a standard assay (19). These results suggest that the metal-binding polypeptides are not glutathione but may be synthesized from this compound.

Amino Acid Sequence. The presence of γ-carboxamide linkages between glutamate and adjacent amino acids, starting with the carboxamide linkage closest to the N-terminal end of the polypeptides, prevented the sequencing of the compounds by Edman degradation. Two different enzymes were, therefore, used to determine the amino acid sequences of the polypeptides. Incubation with carboxypeptidase Y, which cleaves the C-terminal amino acid from a polypeptide only if that amino acid is glycine (20), released this amino acid from the C terminus of the polypeptides. Results of digestion of the polypeptides with carboxypeptidase A, which releases amino acids sequentially from the C terminus (21), are shown in Fig. 3. These results demonstrate the sequence of the C terminus of the polypeptides to be -Glu-Cys-Gly-COOH. The slow release of cysteine and, consequently, glutamate is consistent with the preference of carboxypeptidase A for α- rather than γ-carboxamide linkages (21). The N-terminal amino acid is known to be glutamate. This leaves only one cysteine residue to be accounted for. Thus, for the five amino acid polypeptide, the amino acid sequence is H_2N-Glu-Cys-Glu-Cys-Gly-COOH. The partial amino acid sequence of the seven amino acid polypeptide is similarly shown to be H_2N-Glu-Xaa-Xaa-Xaa-Glu-Cys-Gly-COOH where Xaa represents an unknown amino acid. However, the ^{13}C NMR data (see below) indicate that the chemical environments of all of the α- and γ-carbons of glutamate are similar in the smaller and larger polypeptides. The larger polypeptide, therefore, differs from the smaller polypeptide only in the number of repeating subsets containing γ-carboxamide linkages. This implies that the amino acid sequence of the large molecule is H_2N-Glu-Cys-Glu-Cys-Glu-Cys-Gly-COOH.

NMR Spectroscopy. To confirm that the polypeptides contain the repeating unit Glu-Cys linked by γ-carboxamide bonds, the apopolypeptide isolated from cultures grown in L-[1,5-$^{13}C_2$]glutamate was examined by ^{13}C NMR spectroscopy. The ^{13}C NMR spectrum (Fig. 4*B*) has two resonances at 178.4 and 176.6 ppm, which arise from the enriched carbons, C-1 and C-5 of glutamate, respectively. The fact that only two resonances were resolved suggests that the two or three glutamyl residues in the apopolypeptide are in similar chemical environments, consistent with a repeating unit

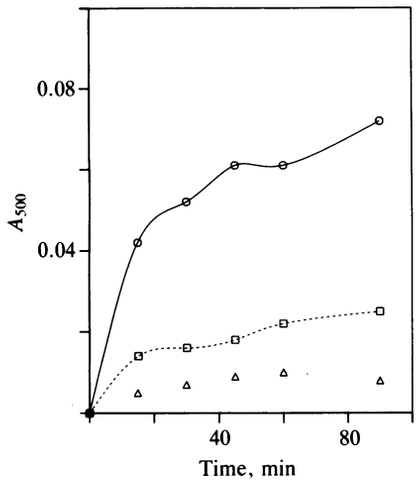

FIG. 3. Digestion of metal-binding polypeptides with carboxypeptidase A. Purified metal-binding apopolypeptides were subjected to digestion with carboxypeptidase A. Samples were analyzed at various time points by paper electrophoresis. The identity of the different amino acids released was determined by comparison of the electrophoretic profile to that obtained for the three amino acids known to be present in the polypeptides. Relative amounts of each amino acid released were determined by staining the paper with ninhydrin, then eluting the bound ninhydrin with methanol and immediately determining the absorbance at 500 nm. Values were compared to a standard curve generated by reaction with known amounts of each amino acid. ○, Glycine; □, cysteine; △, glutamate.

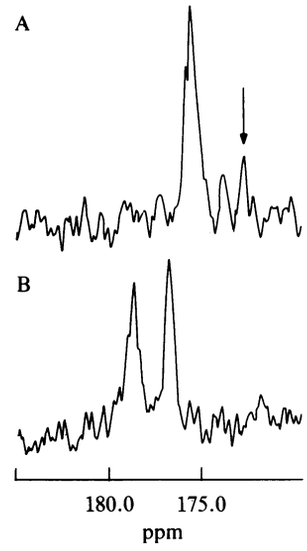

FIG. 4. NMR spectra of ^{13}C-labeled metal-binding apopolypeptides. Cd-resistant *D. innoxia* cells were grown in 300 μM Cd plus L-[1,5-^{13}C]glutamic acid at 10 mg/liter. Metal-binding polypeptides were isolated and subjected to ^{13}C NMR analysis. Spectra were determined at pH 1.5 (*A*) and pH 8.0 (*B*). The larger peak on the spectrum taken at pH 8.0 corresponds to the γ-carbon, whereas the smaller "up-field" peak corresponds to the α-carbon of glutamate residues. At pH 1.5 the peaks corresponding to the α- and γ-carbons overlap. The α-carbon undergoes a greater chemical shift with acid titration indicating that it is part of a free carboxyl group that can be titrated through its pKa. The arrow in *A* indicates the position of the peak representing C-1 of the N-terminal glutamyl residue.

structure. To confirm the existence of γ-carboxamide linkages, the chemical shift and pH titration behavior of these peaks were compared to those of tripeptides in which the internal glutamyl residue is either α-linked (*N*-acetyl-α-L-glutamyl-L-alanine) or γ-linked (*N*-acetyl-γ-L-glutamyl-L-cysteine). The results are presented in Table 2. ^{13}C-chemical-shift assignments of the carboxyl resonances in glutathione have been made by Jung *et al.* (22) and are confirmed here using glutathione that was prepared chemically from L-[1,5-$^{13}C_2$]glutamate and [1-^{13}C]glycine. Acetylation of this peptide allowed unequivocal assignment of the carbonyl resonances in *N*-acetylglutathione. Assignments of the carbonyl resonances in an α-linked tripeptide were made by comparing ^{13}C NMR spectra of glutamate (23), *N*-acetyl-L-glutamate, and L-α-glutamyl-L-alanine with that of *N*-acetyl-α-L-glutamyl-L-alanine. Assignment of carboxamide resonances was aided by obtaining spectra using $^2H_2O/H_2O$, 50:50 (vol/vol), mixture as a solvent. Since there is an isotope shift of the carboxamide resonance associated with deuterium substitution of the amide proton (24), the carboxamides appeared a double peaks, whereas the free carboxyls appeared as single peaks.

The chemical shifts and pH dependence of the glutamyl-carboxyl resonances of the reduced apopolypeptide are similar to those of the γ-linked *N*-acetylglutathione and quite distinct from those of the α-linked *N*-acetyl-α-L-glutamyl-L-alanine. The small shift differences between the glutamyl resonances in the polypeptide and *N*-acetylglutathione represent the fact that the α-amino group of the glutamyl residues are linked to cysteine in the polypeptide and an acetyl group in the model compound. These data demonstrate clearly the existence of γ-linked glutamyl residues in the polypeptide.

The small peak (173 ppm) in the pH 1.5 spectrum of the apopolypeptide (Fig. 4*A*, arrow) is similar in position to the glutamyl C-1 of glutathione (172.6) and probably represents the C-1 of the N-terminal glutamyl residue. The NMR results

Table 2. ^{13}C NMR analysis of compounds containing α- and γ-carboxyl groups

Compound	Chemical form			Chemical shift*			
				pH 1.5		pH 8.0	
	α-NH_2	C-1	C-5	C-1	C-5	C-1	C-5
Glutamic acid	Amino	Carboxyl	Carboxyl	173.1	177.3	175.3	182.1
N-Acetylglutamic acid	Amido	Carboxyl	Carboxyl	176.2	177.3	179.6	182.9
N-Acetyl-α-glutamylalanine	Amido	Amido	Carboxyl	174.0	177.9	173.8	182.4
Glutathione	Amino	Carboxyl	Amido	172.6	175.3	176.0	176.0
N-Acetylglutathione	Amido	Carboxyl	Amido	175.4	175.8	179.2	176.6
Cd-binding polypeptides	Amido	Carboxyl	Amido	175.4	175.4	178.6	176.6

Results of NMR analysis of ^{13}C-labeled metal-binding polypeptides were compared to results obtained for ^{13}C-labeled compounds of known chemical structure. Comparisons of the chemical shifts at pH 1.5 and 8.0 for the C-1 and C-5 carbons of the metal-binding polypeptides demonstrate the similarity of signals from the metal-binding polypeptides to those obtained from compounds containing amide linkages through C-5 of glutamate.

*Chemical shifts are in ppm downfield from tetramethylsilane using dioxane as an internal reference (67.2 ppm).

presented here are, therefore, consistent with the polypeptide structure H_2N-(γ-Glu-Cys)$_n$-Gly-COOH.

DISCUSSION

Poly(γ-glutamylcysteinyl)glycines bind the majority of the cellular Cd in Cd-resistant *D. innoxia* cells. Bis- and tris(γ-glutamylcysteinyl)glycine, (γ-Glu-Cys)$_2$Gly and (γ-Glu-Cys)$_3$Gly, account for 96% of the metal-binding polypeptides in these cells. Synthesis of these compounds is induced by Cd and Cu. These polypeptides form multimeric aggregates between 1.5 and 4 kDa in the presence of these ions.

Exposure of cells growing in the presence of sublethal concentrations of Cd to a normally nontoxic concentration of Cu leads to displacement of Cd by Cu from larger polypeptides and subsequent cell death (8). This suggests that production and accumulation of these polypeptides are related to resistance. Resistance to increasing concentrations of Cd correlates with both the initial rate of *de novo* synthesis upon exposure to the metal ions (8) and the maximum accumulation of (γ-Glu-Cys)$_n$Gly (Table 1). While these data strongly suggest that production of these compounds is involved in the mechanism of resistance to Cd and Cu in these plant cells, they do not confirm that overproduction of (γ-Glu-Cys)$_n$Gly is a direct product of enzymes encoded by resistance genes.

Results presented here demonstrate that metal-binding complexes of Cd-resistant *D. innoxia* consist of (γ-Glu-Cys)$_n$Gly, not metallothionein. Comparison of the properties of these metal-binding polypeptides to the metal-binding complexes isolated from other plants suggests that many of these latter compounds may be similar, or identical, to (γ-Glu-Cys)$_n$Gly (25). The metal-binding polypeptides (γ-Glu-Cys)$_n$Gly are not confined to angiosperms. These Cd-binding compounds were first identified in, and isolated from, the primitive fission yeast, *Schizosaccharomyces pombe*. Cadystin, from this yeast, is also composed of (γ-Glu-Cys)$_n$Gly (26, 27). Cadystin A is (γ-Glu-Cys)$_3$Gly, whereas cadystin B is (γ-Glu-Cys)$_2$Gly. Small amounts of (γ-Glu-Cys)$_n$Gly were also identified in suspension cultures of *Rauvolfia serpentina* (28).

The data presented here suggest that, in Cd-resistant *D. innoxia*, (γ-Glu-Cys)$_n$Gly is functionally analogous to metallothionein accumulated in metal-tolerant mammalian cells upon exposure to Cd. However, in the absence of supraoptimal concentrations of trace metals the function of (γ-Glu-Cys)$_n$Gly and metallothionein may differ. It has been suggested that (γ-Glu-Cys)$_n$Gly could be involved in donating reduced sulfur to *O*-acetylserine in the formation of cysteine (29).

Note Added in Proof. Following the submission of this manuscript, a paper was published by Grill, E., Winnacker, E.-L. & Zenk, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 439–443, detailing the synthesis of (γ-Glu-Cys)$_n$Gly in cultures of *Rauvolfia serpentina* and other plant cell suspension cultures.

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