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The Composition of Metals Bound to Class III Metallothionein (Phytochelatin and Its Desglycyl Peptide) Induced by Various Metals in Root Cultures of *Rubia tinctorum*[1]

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The induction of phytochelatins (PCs) and their desglycyl peptides (both are referred to as class III metallothionein [CIIIMT]) by exposure to various metals (Ag^{+}, As^{3+}, As^{5+}, Cd^{2+}, Cu^{2+}, Ga^{3+}, Hg^{2+}, In^{3+}, Ni^{2+}, Pb^{2+}, Pd^{2+}, Se^{4+}, and Zn^{2+}) and the metal composition in the CIIIMTs were investigated in root cultures of *Rubia tinctorum* L. All of these metal species induced PCs to various degrees when analyzed by the postcolumn derivatization high-performance liquid chromatography method. The desglycyl peptides of PCs often were also present. However, only Ag, Cd, and Cu were bound to the CIIIMTs that they induced when analyzed by the high-performance liquid chromatography-inductively coupled plasma-atomic emission spectrometry method. Cu was also bound to the CIIIMTs induced by Ag^{+}, As^{3+}, and Cd^{2+}. After Ag^{+} exposure, an Fe peak that may be of Fe-CIIIMT was also observed. However, most of the metal species studied were not bound to the CIIIMTs that they induced.

In response to excess heavy metals, plants induce SH-containing peptides called PCs (Rauser, 1990; Robinson et al., 1993), which are class III MTs (Kägi, 1993). The general structure of PCs is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, which means that they are Cys-rich (Grill et al., 1985). PCs with n from 2 to 11 have been described (Gekeler et al., 1989).

PC is successively synthesized by γ-glutamylcysteine dipeptidyl transpeptidase (PC synthase) with glutathione as a precursor (Grill et al., 1989; Hayashi et al., 1991). The enzyme is activated by heavy metals in vitro (Grill et al., 1989). Because the γ-carboxyamide bond is not synthesized by ribosomes, it is thought that PC synthesis is not controlled by a gene (Scheller et al., 1987; Robinson et al., 1988). Thus, the biosynthetic pathway has been clarified to a considerable extent.

Grill et al. (1987) studied PC induction by various metals and found that Ag^{+}, As^{5+}, Au^{+}, Bi^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, Se^{4+}, Sn^{2+}, Te^{4+}, W^{6+}, and Zn^{2+} induce PC. Among these, several are so-called soft metals (Pearson, 1963), which have high affinity for the SH group. Therefore, the soft metals that induce PC may be incorporated into the induced Cys-rich PC. In fact, some soft metals such as Cd reportedly bind to the PCs that they induce (Grill et al., 1985; Jackson et al., 1987). However, it has not been fully elucidated whether the respective PC-inducing metals are similarly incorporated. This may be due partly to the lack of a suitable analytical method.

With a high-performance liquid chromatograph connected directly with an inductively coupled plasma-atomic emission spectrometer in a simultaneous multielement analytical mode (Ebdon et al., 1987), the chemical state of various metals can be analyzed simultaneously (Maitani et al., 1991). Therefore, HPLC-ICP may be suitable for determining the metal composition bound to PCs.

Here, we investigated in *Rubia tinctorum* L. root cultures (a) whether various metals induce PCs (peptide) and if so (b) which binds to the induced PCs. The induction of PC and the metal composition were analyzed by means of postcolumn derivatization HPLC (Grill et al., 1987) and HPLC-ICP, respectively. Various soft metals (Pearson, 1963) and metals that have an electronic configuration similar to Cd^{2+} (d^{10}), such as Ga^{3+} and In^{3+}, were tested.

MATERIALS AND METHODS

Reagents

Silver nitrate, cadmium chloride hemipentahydrate, cupric chloride dihydrate, gallium chloride, indium sulfate, mercuric chloride, nickel chloride hexahydrate, lead nitrate, palladium chloride, zinc chloride, 5,5′-dithiobis(2-nitrobenzoic acid), and GSH were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium *m*-arsenite, sodium arsenate heptahydrate, BSO, and Tris were obtained from Sigma. Sodium selenite pentahydrate was purchased from Merck (Darmstadt, Germany). Other chemicals were of reagent grade or of the highest grade commercially available.

Tissue Culture in Medium Containing Test Metals

Root cultures established previously (Kubota et al., 1995) were subcultured every 4 weeks. After the last subculture, the root cultures (about 0.25 g fresh weight) were main-

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Abbreviations: BSO, L-buthionine-[*S*,*R*]-sulfoximine; CIIIMT, class III metallothionein; ICP, inductively coupled plasma-atomic emission spectrometry; MT, metallothionein; PC, phytochelatin; SH, thiol.

tained for 7 d in 10 mL of Murashige-Skoog liquid medium (Murashige and Skoog, 1962) in 50-mL Erlenmeyer flasks on a rotary shaker at 100 rpm at 25°C in the dark. Various metals (10, 100, or 1000 μM) were then added to the medium. The root cultures were maintained for 3 d, then washed with distilled water and stored at −80°C.

HPLC-ICP Analysis of Metal Complexes

Root cultures were homogenized with a Polytron tissue grinder (Kinematica, Littau, Switzerland) in 4 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM KCl and 1.5 mM $MgCl_2$ (Delhaize et al., 1989) under an N_2 atmosphere to prevent oxidation (Suzuki and Maitani, 1983). The homogenates were centrifuged at 100,000*g* for 60 min at 4°C. A 100-μL aliquot of the supernatant fraction was applied to a high-performance liquid chromatograph (LC-6A; Shimadzu, Kyoto, Japan) equipped with a gel-filtration column (Asahipak GS520HQ, 7.6 mm i.d. × 300 mm; Showa Denko, Tokyo, Japan). The sample was eluted with 10 mM Tris-HCl buffer (pH 7.4) containing 0.9% NaCl at a flow rate of 0.6 mL min^{-1}. The eluate was introduced continuously to the nebulizer tube of an ICP machine (ICAP-61; Thermo-Jarrell Ash, Waltham, MA). The atomic emission intensities of the respective elements were integrated for 3 s and stored every 3 s using a personal computer. HPLC-ICP chromatograms were obtained as described by Maitani et al. (1994).

Postcolumn Derivatization HPLC Analysis of PC

PCs were analyzed according to the method of Grill et al. (1987) with some modifications reported previously (Kubota et al., 1995). The assignments of the respective peaks were performed with an electrospray ionization-mass spectrometer after fractionation without postcolumn derivatization as reported by Kubota et al. (1995).

Metal Determination

About 0.1 g of the cultures and a 0.5-mL aliquot of the homogenates and the supernatant fractions were digested with mixed acid (5:1, v/v, HNO_3:$HClO_4$), and the solutions were diluted with distilled water to 5 and 3 mL, respectively. Metal concentrations were determined by means of ICP.

RESULTS

Root cultures of *R. tinctorum* were exposed to various metals for 3 d. Doses were applied that were based on the toxicity determined in a preliminary experiment and on published data (Grill et al., 1987) except for Pd^{2+}, for which solubility was the determining factor. The growth in the root cultures exposed to the various metals, the metal concentrations in the root cultures, and the recovery from the homogenate of the cultures into the supernatant fraction are shown in Table I. Se^{4+} (selenite) caused the greatest inhibition of growth under our experimental conditions. However, for each metal, the growth was at least half of that of the control. Pd^{2+} markedly enhanced growth, although the reason for this remains unclear.

Table I. *Relative root growth in culture, root metal concentrations, and the recovery of root-associated metal into the supernatant after exposure for 3 d*

After an incubation without test metals for 7 d after the subculture, root cultures were exposed to metals at the described concentrations for 3 d.

Metal	Dose	Growth[a]	Concentration[b]	Recovery[c]
	μM		μg g^{-1}	%
Ag^{+}	100	5.7	77	44
As^{3+}	100	6.1	25	82
As^{5+}	100	6.8	15	78
Cd^{2+}	100	8.4	42	46
Cu^{2+}	100	6.7	28	63
Ga^{3+}	1000	8.7	27	ND[d]
Hg^{2+}	10	5.7	–[e]	–
In^{3+}	1000	7.2	63	99
Ni^{2+}	100	8.0	12	73
Pb^{2+}	1000	5.4	1700	1
Pd^{2+}	100	12.0	3	ND
Se^{4+}	100	3.9	32	49
Zn^{2+}	1000	7.8	290	27
Control	–	6.7	–	–

[a] Growth is defined as the ratio of the wet weight after the exposure to that on the subculture. [b] Values are means of two samples. [c] Two samples were mixed and analyzed. [d] ND, Not detected. [e] –, Not determined.

Pb^{2+} was taken up at a remarkably high concentration (Table I). However, it was recovered at an extremely low level (1%) in the supernatant fraction. The concentration of Ag in the Ag^{+}-treated cultures (77 μg g^{-1}) was comparable with that in those exposed to Pb^{2+}, when differences in the dose (10-fold) and the atomic weight (Ag, 107.9; Pb, 207.2) were taken into account (calculated value was about 1500 μg g^{-1}). However, the recovery of Ag in the supernatant fraction (44%) was markedly higher than that of Pb. More than 50% of As^{3+}, As^{5+}, Cu^{2+}, In^{3+}, and Ni^{2+} was recovered in the supernatant fractions.

Figure 1 shows the postcolumn derivatization HPLC chromatogram from the root cultures exposed to Ag^{+} as an example of PC detection. Root cultures were extracted with an alkaline solution, and SH-containing substances were detected (Grill et al., 1987). PCs [(γ-Glu-Cys)$_n$-Gly] with *n* from 2 to 5 (peaks 1, 3, 5, and 7) and their desglycyl peptides (peaks 2, 4, 6, and 8, respectively [Kubota et al., 1995]) were detected from 12 to 21 min (hereafter both are referred to as CIIIMTs). Although the peaks located between peaks 4 and 5 might also be of CIIIMT analogs such as (γ-Glu-Cys)$_n$-Glu (Meuwly et al., 1995), they could not be identified. Peaks at 2.9 and 5.3 min were ascribed to Cys and GSH (and γ-glutamylcysteine), respectively, by co-chromatography with the standards. The peaks at 26 to 40 min also observed in the control were those of color constituents.

The peak at 8.2 min (Fig. 1) was ascribed to sulfide, since the peak height was increased when Na_2S was added and since, in our preliminary experiment, sulfide ions were detected in the supernatant fraction of the root cultures exposed to 1 mM $CdCl_2$ with 2 mM GSH for 5 d, which

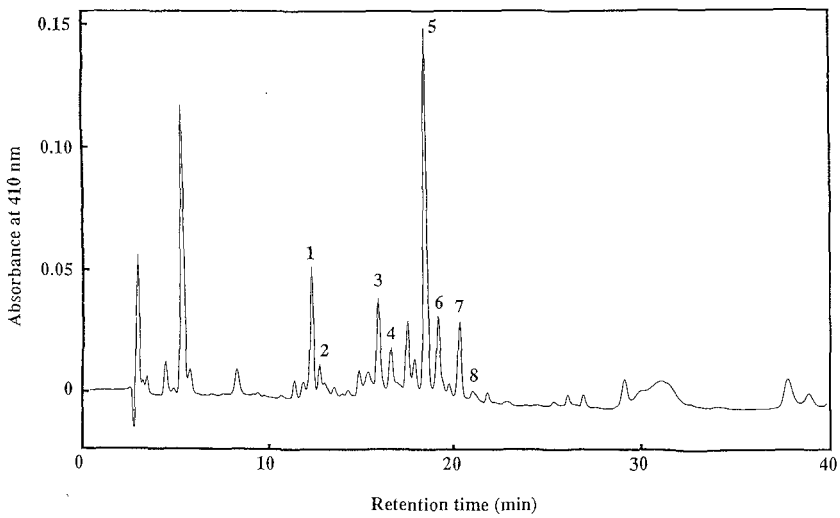

Figure 1. HPLC chromatogram generated by postcolumn derivatization. Root cultures were exposed to $AgNO_3$ (100 μM) for 3 d and the induced PCs and their desglycyl peptides were analyzed as SH-containing peptides as described in "Materials and Methods." Peaks 1, 3, 5, and 7 are of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ and peaks 2, 4, 6, and 8 are of $(\gamma\text{-Glu-Cys})_n$ with $n = 2, 3, 4$, and 5, respectively.

contained a large amount of CIIIMT. However, the sulfide ion may not have been detected quantitatively, because a faint odor of H_2S was evident upon acidification for HPLC analysis in such a case.

The levels of PCs and their desglycyl peptides induced by exposing root cultures to the respective metals for 3 d are shown in Table II. The amounts were calculated from the postcolumn derivatization HPLC chromatograms and are expressed for the individual species and the total γ-Glu-Cys. All of the metals investigated induced PCs to various degrees and many of them also induced their desglycyl peptides. PC_5 [$(\gamma\text{-Glu-Cys})_5\text{-Gly}$] and d-$PC_5$ [$(\gamma\text{-Glu-Cys})_5$] were detected only in root cultures exposed to Ag^+. The total γ-Glu-Cys contents in root cultures exposed to Ag^+, As^{3+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} were more than 30 μmol g^{-1} under our experimental conditions.

Figure 2 shows the HPLC-ICP chromatograms of the root cultures exposed to Ag^+. The void volume of the column was 8.0 min. An Ag peak that was absent in the control was detected at 13.8 min. The addition of $AgNO_3$ to the control supernatant fraction did not generate a peak at this retention time. Furthermore, the induction of the peak was dramatically inhibited by exposure of roots to 2 mM BSO, an inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979), which is characteristic of CIIIMT induction (Grill et al., 1987; Kubota et al., 1995). Therefore, the Ag peak was ascribed to Ag ions bound to the CIIIMTs detected by means of postcolumn derivatization HPLC (Table II). Although CIIIMT molecules contain S derived from Cys, the corresponding S peak of Ag-CIIIMT was not detected in the chromatogram for S (Fig. 2), because the sensitivity of S in the spectrometry was about one-sixth of that of Ag.

Thus, a peak of Ag-CIIIMT was detected. However, Ag-CIIIMTs could not be separated into the individual Ag peptides, such as Ag-PC_4. That the single peak contains various

Table II. *Levels of PCs and their desglycyl peptides induced by various metal ions*

Root cultures were exposed to metals for 3 d.

Metal	Dose	CIIIMT[a]								Total γ-Glu-Cys
		PC_2[b]	d-PC_2[b]	PC_3	d-PC_3	PC_4	d-PC_4	PC_5	d-PC_5	
	μM					*nmol* g^{-1}				*nmol* g^{-1}[c]
Ag^+	100	15.2	3.8	9.0	4.6	22.4	7.8	4.2	0.8	224.4
As^{3+}	100	21.4	4.0	1.0	0.4	0.2	0.0	0.0	0.0	55.6
As^{5+}	100	6.2	2.0	0.4	0.2	0.0	0.0	0.0	0.0	18.2
Cd^{2+}	100	7.0	0.4	9.4	2.2	14.8	1.4	0.0	0.0	114.0
Cu^{2+}	100	1.2	0.2	3.0	1.0	2.4	0.8	0.0	0.0	27.2
Ga^{3+}	1000	1.2	0.2	0.4	0.2	0.2	0.2	0.0	0.0	5.4
Hg^{2+}	10	20.4	2.8	4.0	0.8	1.4	0.4	0.0	0.0	67.2
In^{3+}	1000	1.6	0.4	0.6	0.4	0.2	0.0	0.0	0.0	7.2
Ni^{2+}	100	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2
Pb^{2+}	1000	6.0	2.6	11.2	4.2	2.4	0.4	0.0	0.0	74.4
Pd^{2+}	100	3.6	0.0	0.2	0.0	0.0	0.0	0.0	0.0	8.2
Se^{4+}	100	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.6
Zn^{2+}	1000	7.0	0.0	0.8	0.2	0.0	0.0	0.0	0.0	17.2
Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] Mol of each species per g fresh weight of cultures. [b] PC_n designates $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ and d-PC_n $(\gamma\text{-Gly-Cys})_n$ [c] Total mol of γ-Glu-Cys units per fresh weight of cultures.

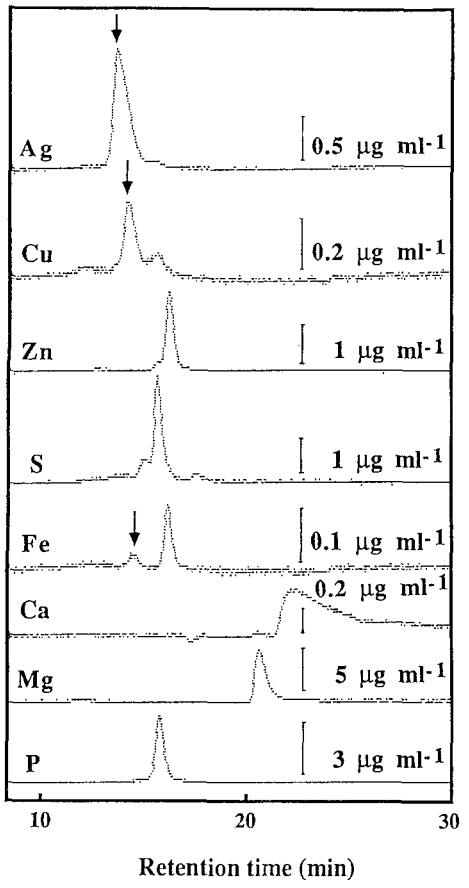

Figure 2. HPLC-ICP chromatograms of the supernatant fraction obtained from the root cultures exposed to Ag^+. Root cultures were exposed to $AgNO_3$ for 3 d. The supernatant fraction was applied to the HPLC-ICP system and the levels of the elements in the eluate were monitored continuously. The vertical bars indicate detection levels for the elements in the spectrometer. Arrows indicate peaks of metals bound to CIIIMT.

PCs and their desglycyl peptides has been demonstrated in root cultures exposed to Cd^{2+} (Kubota et al., 1995).

A Cu peak (the largest one in Fig. 2), which was absent in the control group, also appeared after exposure to Ag^+. The Cu peak was almost completely inhibited by BSO. Consequently, the peak was ascribed to Cu ions bound to the CIIIMT. An Fe peak with a shorter retention time (Fig. 2) may also be of Fe-CIIIMT, because it was absent in the control and inhibited by BSO. The difference in the retention times of CIIIMT bound to Ag, Cu, and Fe may be explained by the difference in bulk and/or charge of the metal-CIIIMT complexes or by the selectivity of metals toward the respective CIIIMTs.

Major unresolved peaks were evident in the chromatograms for Zn and S, whereas only one peak was detected for Ca, Mg, and P. These were also found in the control. Since the gel-filtration column also exhibits ionic interaction (Asahipak technical data sheet No. 14, Asahi Chemical Industry, Kawasaki, Japan), the retention times of free Ca and Mg were very long. When the standard solutions for Ag^+, Cu^{2+}, Fe^{2+}, and Zn^{2+} were passed through the column, metal peaks were not detected, probably because of adsorption via ionic interaction.

Figure 3 shows the HPLC-ICP chromatograms of the root cultures exposed to As^{3+}, Cd^{2+}, and Cu^{2+}, along with that of the control. Cu is sequestered in the induced PC (Reese et al., 1988). After exposure to Cu^{2+}, Cu bound to CIIIMT was detected at 14.1 min; the native Cu peak at 16.2 min was also observed in the control.

When the cultures were exposed to Cd^{2+}, two Cd peaks appeared (Fig. 3), which were absent in the control and inhibited by BSO. The larger peak with the shorter retention time (14.0 min) was Cd-CIIIMT as shown previously (Kubota et al., 1995), and the smaller peak (15.6 min) may have been a smaller Cd complex such as Cd-GSH. In addition, the Cu-CIIIMT peak, which was absent in the control and inhibited by BSO, was also detected after Cd^{2+} exposure.

After exposure to As^{3+}, two As peaks were detected at retention times later than those for several metal-CIIIMT peaks. The peaks at 16.9 and 19.8 min were ascribed to free As^{5+} (arsenate) and As^{3+} (arsenite), respectively. Although a peak of As-CIIIMT was not detected, a Cu peak appeared near the retention time of Cu-CIIIMT induced by Cu^{2+}. The peak was ascribed to Cu-CIIIMT based on the BSO experiment. The finding that Cu-CIIIMT detected after As^{3+} exposure eluted more slowly than those after exposure to Cu^{2+} and Cd^{2+} may be explained by the fact that a major-

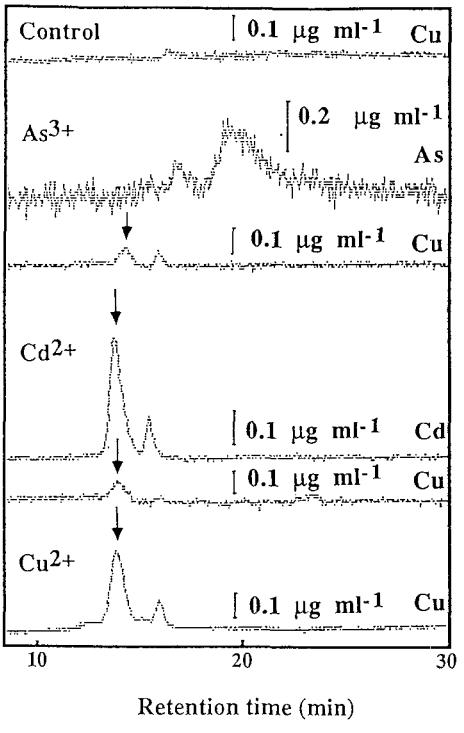

Figure 3. HPLC-ICP chromatograms of control root cultures and those exposed to As^{3+}, Cd^{2+}, and Cu^{2+}. Root cultures were exposed to the metals for 3 d. The vertical bars indicate detection levels for elements in the spectrometer. Arrows indicate that the peak is (or may be) of CIIIMT. See the legend to Figure 2.

ity of the CIIIMTs induced by As^{3+} was PC_2, whereas PC_3 and PC_4 were major constituents after Cu^{2+} and Cd^{2+} exposure (Table II).

Although a large amount of CIIIMT was induced after exposure to Pb^{2+} (Table II), Pb was not incorporated into the induced CIIIMT (data not shown). Other metals, including Zn^{2+}, were not detected as bound to the induced CIIIMTs either. This may partly be due to the low level of induced CIIIMT. Hg could not be studied with HPLC-ICP, because the emission line of Hg was used to calibrate the wavelength in our ICP machine.

DISCUSSION

Grill et al. (1987) reported the PC induction by Ag^{+}, As^{5+}, Au^{+}, Bi^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, Se^{4+}, Sn^{2+}, Te^{4+}, W^{6+}, and Zn^{2+} in cell suspensions of *Rauvolfia serpentina*. We reconfirmed their results for Ag^{+}, As^{5+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Se^{4+}, and Zn^{2+} and, moreover, identified PC induction by Ga^{3+}, In^{3+}, and Pd^{2+} in root cultures of *R. tinctorum*. Furthermore, we found that most metals and metalloids studied also induced the desglycyl peptides of PCs.

The desglycyl peptides of PCs have been described in maize (Rauser, 1993), rice (Klapkeck et al., 1994), and yeast (Mehra and Winge, 1988; Mehra et al., 1988). Therefore, the desglycyl derivatives are not specific in *R. tinctorum* and might have been ignored in many other plants as minor ingredients. Although the comparative ability of PC and its desglycyl peptide to sequester metals has not yet been elucidated, the amounts of both types of CIIIMTs induced by the various metals (Table II) do not seem to suggest selectivity toward various metals. Rather, the induced amounts seem to indicate that the C-terminal Gly is not essential for sequestration (Mehra and Winge, 1988).

When the root cultures were exposed to Ag^{+}, As^{3+}, or Cd^{2+}, Cu was bound to the CIIIMT as in the case of Cu exposure. Since Ag^{+}, As^{3+}, and Cd^{2+} induced a large amount of CIIIMT, Cu may be incorporated into the metal-free CIIIMT. Although the valency of Cu was not determined in this study, it may be present as Cu^{+} (Reese et al., 1988) as it is in class I and II MTs (Prinz and Weser, 1975; Suzuki and Maitani, 1981; Bordas et al., 1982).

An Fe peak was induced when a large amount of CIIIMT was induced by Ag^{+} exposure (smaller one in Fig. 2). An Fe-PC has been identified in *Datura innoxia* (Jackson et al., 1992). Therefore, the Fe peak may be of Fe-CIIIMT. If so, the retention time longer than those of Ag-CIIIMT and Cu-CIIIMT on the gel-filtration column may suggest that the carboxyl groups in CIIIMT also participate in the coordination with Fe. However, the peak could not be identified, because this peak was undetectable after exposure to 1 mM $FeCl_2$ or $FeCl_3$. Studies to identify Fe-CIIIMT in *R. tinctorum* are now in progress in our laboratory.

Induced class I MT always contains Zn ions (Kägi, 1993), regardless of the nature of the inducer or whether or not the inducer itself is incorporated, when a sufficient amount of class I MT is induced. In this study, however, the level of CIIIMT induction by Zn^{2+} was low (Table II), and Zn was not detected as Zn-CIIIMT even after Zn^{2+} exposure. In this study, the root cultures were maintained in normal Murashige-Skoog medium, which contains 30 μM Zn^{2+} (Murashige and Skoog, 1962). The concentration is about one-thirtieth of the exposed dose of Zn^{2+}. Therefore, the root cultures already had tolerance to Zn^{2+} stress, and hence they may have not responded to Zn ions sensitively even during exposure at 1 mM. Further experiments using culture medium without Zn^{2+} are needed to elucidate the CIIIMT inducibility of Zn ions and to detect Zn-CIIIMT in *R. tinctorum* cultures.

One of the roles of PC (and probably of CIIIMT) is to protect plants against toxic metals (Steffens et al., 1986). Hg^{2+} has a linear configuration in coordination compounds. Therefore, one molecule of small PC_2 can effectively protect plants against the Hg^{2+} toxicity. The most abundant CIIIMT in Hg^{2+} exposure was PC_2 in this study. This result answers the purpose of the induction, if Hg is indeed bound to the induced CIIIMT.

2,3-Dimercaptopropanol, an antidote for As^{3+} (arsenite), has two SH groups (Goyer, 1986). Therefore, PC_2 might be suitable for protection against As^{3+} exposure. However, As was apparently not bound to the CIIIMT. Why CIIIMT is induced by various metals and metalloids that are not sequestered into it remains to be determined.

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