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Detoxification of Arsenic by Phytochelatins in Plants[1]

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As is a ubiquitous element present in the atmosphere as well as in the aquatic and terrestrial environments. Arsenite and arsenate are the major forms of As intoxication, and these anions are readily taken up by plants. Both anions efficiently induce the biosynthesis of phytochelatins (PCs) ([γ-glutamate-cysteine]$_n$-glycine) in vivo and in vitro. The rapid induction of the metal-binding PCs has been observed in cell suspension cultures of *Rauvolfia serpentina*, in seedlings of Arabidopsis, and in enzyme preparations of *Silene vulgaris* upon challenge to arsenicals. The rate of PC formation in enzyme preparations was lower compared with Cd-induced biosynthesis, but was accompanied by a prolonged induction phase that resulted finally in higher peptide levels. An approximately 3:1 ratio of the sulfhydryl groups from PCs to As is compatible with reported As-glutathione complexes. The identity of the As-induced PCs and of reconstituted metal-peptide complexes has unequivocally been demonstrated by electrospray ionization mass spectroscopy. Gel filtration experiments and inhibitor studies also indicate a complexation and detoxification of As by the induced PCs.

As is a toxic element ubiquitously encountered in the environment and in organisms (Cullen and Reimer, 1989). Substantial amounts of As are released by geological activities and by anthropogenic impacts such as smelting operations and fossil fuel combustion, accounting for 1.2×10^4 to 2.6×10^4 tons of emission into the atmosphere (Nriagu and Pacyna, 1988; Ochiai, 1995). For instance, widespread groundwater pollution by As compounds has drawn considerable attention and raised serious concern in Bangladesh and other locations (Dhar et al., 1997; Kaiser, 1998).

As poisoning is known to interfere with the cell's sulfhydryl groups. In humans, the toxicity of trivalent As is mainly due to its binding to the sulfhydryl groups of lipoic acid (Webb, 1966; Kalef and Gitler, 1994). Therefore, As intoxication can be ameliorated by the administration of dithiols such as 2,3-dimercaptopropanol or 1,2-ethanedithiol (Webb and van Heyningen, 1947; Whittaker, 1947), which compete for As binding.

In mammals (Lakso and Peoples, 1975; Aposhian, 1997), fungi, and algae (Edmonds and Francesconi, 1981; Cullen and Reimer, 1989), detoxification of As usually involves methylation and other biotransformations such as incorporation of As into organic molecules by the formation of e.g. arsenocholine, arsenobetaine, or arsenosugars. In bacteria, a widespread tolerance mechanism for arsenate, $As^{V}O_4^{3-}$, is based on an efflux system that exports arsenic specifically and ATP-dependently as $As^{III}O_2^{-}$ from the cell generated by cytosolic reduction (Silver, 1996). At least in terrestrial higher plants, these processes seem to form no major routes of detoxification (Nissen and Benson, 1982).

Plants face arsenical compounds mainly in the form of the anions arsenite and arsenate; the latter competes with phosphate and is readily taken up (Warren et al., 1964). Both anions have been reported to trigger the formation of phytochelatins (PCs) in plants (Grill et al., 1987; Maitani et al., 1996).

PCs are heavy-metal-binding peptides derived from glutathione (GSH) and have the general structure (γ-Glu-Cys)$_n$-Gly (n = 2–11) (Grill et al., 1985; Zenk, 1996). Their biosynthesis is due to the transpeptidation of γ-glutamylcysteinyl dipeptides from GSH by the action of a constitutively present PC synthase (Grill et al., 1989; Chen et al., 1997). Recently, the gene encoding PC synthase has been cloned by several laboratories (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). PC synthase is activated by heavy metal ions such as Cd^{2+}, Cu^{2+}, Ag^{+}, Hg^{2+}, and Pb^{2+}, which are characterized as class B and borderline elements (Nieboer and Richardson, 1980). Subsequently, these ions are complexed by the induced PCs via thiolate coordination (Grill et al., 1985; Grill, 1989; Strasdeit et al., 1991; Mehra et al., 1995, 1996a, 1996b; Mehra and Mulchandani, 1995; Salt et al., 1995; Pickering et al., 1999).

Since the immobilized metals are less toxic than the free ions, PCs are considered to be part of the detoxifying mechanism of higher plants (Grill et al., 1985; Zenk, 1996), algae (Gekeler et al., 1988), and some fungi (Kondo et al., 1985; Kneer et al., 1992). This concept is supported by inhibitor studies (Grill et al., 1987; Reese and Wagner, 1987; Gussarsson et al., 1996), biochemical studies (Kneer and Zenk, 1992), and mutant analyses (Mutoh and Hayashi, 1988; Howden and Cobbett, 1992; Howden et al., 1995, Ha et al., 1999). The analysis of a PC-deficient Arabidopsis mutant showed a detoxifying role for PCs, at least for Cd^{2+} and Hg^{2+} (Howden and Cobbett, 1992). Furthermore, PCs appear to be involved in the homeostasis of Zn^{2+} and Cu^{+}/Cu^{2+} by providing a transient storage form for the ions (Grill et al., 1988; Thumann et al., 1991).

Under the premises of a detoxifying role of the metal-binding peptides a sequestration of As compounds by induced PCs has been implied (Grill et al., 1987). This as-

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sumption is supported by in vitro experiments that documented the formation of complexes between GSH and As compounds (Jocelyn, 1972; Scott et al., 1993; Gailer and Lindner, 1998). In addition, Arabidopsis and *Schizosaccharomyces pombe* mutants lacking PC synthase activity display an enhanced sensitivity toward arsenate (Ha et al., 1999).

More surprising were data by Maitani et al. (1996) that failed to demonstrate an As-PC complex. The result could indicate that PCs do not fulfill a detoxifying function during As poisoning. This challenging finding prompted us to examine the cellular role of PCs during As poisoning in plants. We report the unequivocal induction of PCs by As in vivo and in vitro and provide clear evidence for the formation of As-PC complexes, in accordance with a detoxifying role for the peptides.

MATERIALS AND METHODS

Plant Material

Rauvolfia serpentina Benth. ex Kurz and *Silene vulgaris* (Moench) Garcke cell suspension cultures were cultivated as reported previously (Grill et al., 1987). For experiments, 6 g (fresh weight) of cells were diluted into 30 mL of fresh medium (Linsmaier and Skoog, 1965). Three days after transfer, cells were treated with different concentrations of As or Cd (three replicates each). In the inhibitor studies, buthionine sulfoximine (BSO) (Griffith and Meister, 1979) was added 3 h before heavy metal administration to the cell suspension cultures. After 4 d of exposure, cells were harvested and frozen in liquid nitrogen for PC analysis by HPLC (Grill et al., 1987).

Seeds of Arabidopsis (L.) Heynh. (ecotype RLD) were surface-sterilized with ethanol and NaOCl (Estelle and Somerville, 1987) and placed on solidified Murashige and Skoog medium (Murashige and Skoog, 1962). Plates were stored vertically at 22°C with an illumination of 100 μmol m^{-2} s^{-1} and a light/dark cycle of 16/8 h. One-week-old seedlings were transferred onto solidified Murashige and Skoog medium containing different concentrations of As or Cd ions. Growth of seedlings was determined by a root growth assay (Estelle and Somerville, 1987). Two weeks after transfer the plants were harvested and frozen in liquid nitrogen for PC and protein (Bradford, 1976) analyses.

Chemicals Used

All chemicals were of the highest grade available from Fluka (Buchs, Switzerland). Metal salts used in the experiments were $Cd(NO_3)_2$, $NaAsO_2$, and Na_2HAsO_4.

In Vitro PC Synthase Assay

The purification procedure for PC synthase from *S. vulgaris* cells was according to the method of Friederich et al. (1998). Cells were filtered and treated with liquid nitrogen, extracted in 10 mM Tris-Cl, pH 8.0, containing 10 mM β-mercaptoethanol (standard buffer), centrifuged for 30 min (10,000*g*, 4°C), and filtered. After the 15% (w/v) ammonium sulfate precipitation, the cell-free extract was clarified by centrifugation (30 min, 10,000*g*), and chromatographed on a phenyl sepharose column (CL-4B, Pharmacia, Uppsala). Column-bound proteins were washed and eluted with standard buffer containing 10% (w/v) ethylene glycol and fractionized. Fractions with enzymatic activity were detected by analysis for PCs synthesized (Grill et al., 1989), pooled, and subsequently dialyzed against standard buffer prior to the in vitro experiments.

All in vitro experiments were carried out in a reaction volume of 2 mL in a tightly closed reaction tube (Eppendorf Scientific, Hamburg, Germany). The assay contained approximately 0.5 nkat of PC synthase and variable concentrations of GSH and As from 1 to 50 mM and 0.1 to 1 mM, respectively, buffered at pH 8.0. EDTA (5 mM) was included in the enzyme assay to complex bivalent cations such as Cu^{2+} and Zn^{2+}, which are present in the extract and can otherwise provide a residual PC synthase activity.

Reconstitution of As-GSH and As-PC Complexes

Complexes of GSH and As were generated by incubating an aqueous solution of the tripeptide with arsenite under nitrogen to prevent oxidation, as described in Scott et al. (1993). PC_2-arsenite complexes were obtained in vitro by the procedure replacing GSH for the pentapeptide. The concentrations of peptide (10 mM) and metalloid resulted in a stoichiometry of one As to three thiol groups.

As Determination

The As content was quantified by atomic absorption spectroscopy of the samples in the graphite furnace mode at 193.7 nm (model 1100B spectrometer, Perkin-Elmer/Applied Biosystems, Foster City, CA).

PC Analysis

PC determination was by HPLC analysis and post-column derivatization essentially as previously described (Kneer and Zenk, 1997; Friederich et al., 1998). However, we used freshly prepared $NaBH_4$ solution (1 mg/mL) in double-distilled water instead of sodium hydroxide solution and a prolonged incubation time for reduction (10 min).

Gel Filtration Analysis

Gel filtration analyses were performed on a fast-protein liquid chromatograph (BioLogic, Bio-Rad Laboratories, Hercules, CA) equipped with a column (1.6 × 64 cm) containing Sephadex G-25 (Pharmacia). The chromatography was carried out in the presence of either 10 mM Tris-Cl (pH 8.0) or 10 mM sodium acetate (pH 4.0) in 100 mM NaCl solution. Cell-free extracts were prepared by thawing cells in a one-half volume of buffer (10 mM Tris-Cl, pH 8.0, or 10 mM sodium acetate, pH 4.0) and subsequent centrifugation of the slurry at 10,000*g* for 30 min at 4°C. Eight milliliters of the supernatant was applied to the column and eluted at a flow rate of 0.8 mL/min. Fractions of the eluate (2-mL fraction size) were tested for As and PC levels.

Purification of PCs for Mass Spectroscopic Analysis

Cell-free extracts of the *R. serpentina* cells were prepared and chromatographed on a Sephadex G-25 column as mentioned above. Fractions containing PCs were concentrated by partial lyophilization and isolated by HPLC with UV detection at 220 nm (Grill et al., 1991). PC fractions were lyophilized and just before electrospray ionization mass spectroscopy (ESI-MS) analysis diluted to an approximate concentration of 0.1 mg/mL with 10% (v/v) acetonitrile in water. The analysis was performed on a MAT LCQ instrument (Finnigan, San Jose, CA).

RESULTS

PC Formation in Vivo

In general, PC biosynthesis is triggered by heavy metal cations such as Cd^{2+} and Zn^{2+}. The induction of PCs by the anion arsenate has been observed in a survey for peptide-inducing metal ions (Grill et al., 1987), and suggests a unique mode of PC synthase activation. To substantiate the previous finding, the arsenate- and arsenite-triggered induction characteristics of PC peptides were investigated in detail with *R. serpentina* suspension cells. As outlined in Figure 1A, arsenite treatment (100 μM) resulted in the pronounced formation of PC_2, PC_3, and trace amounts of PC_4 and PC_5, as concluded from HPLC analysis for sulfhydryl groups. A comparable pattern of PC formation was observed in arsenate-treated cells (data not shown) and is characteristic of many other PC-inducing metal ions (Grill et al., 1987). The retention times of the PC peaks in the analysis perfectly correlated with peaks of PC standards and of extracts of cells treated with 100 μM Cd^{2+} (Fig. 1B), while in extracts of cells cultivated in metal-free medium, these fractions were not detected (Fig. 1C).

A difference between As and Cd-challenged cells was the abundance of individual PC peptides: the dominant species was PC_2 in the presence of arsenite, whereas PC_3 prevailed in the presence of Cd ions. The predominance of the PC_2 peptides was also observed with arsenate-treated cells and occurred over the whole concentration range of arsenite and arsenate tested (data not shown). In contrast to Cd, arsenite or arsenate treatment did not lead to a significant depletion of the GSH pool. The toxic action of both As species is indicated by the growth reduction of *R. serpentina* compared with untreated cell suspension cultures, and was evident above 30 and 10 μM arsenite and arsenate concentrations, respectively (Fig. 2). Optimal PC induction levels were observed at growth-inhibitory and sublethal concentrations of inducers, as previously reported for Cd (Grill et al., 1986, 1987).

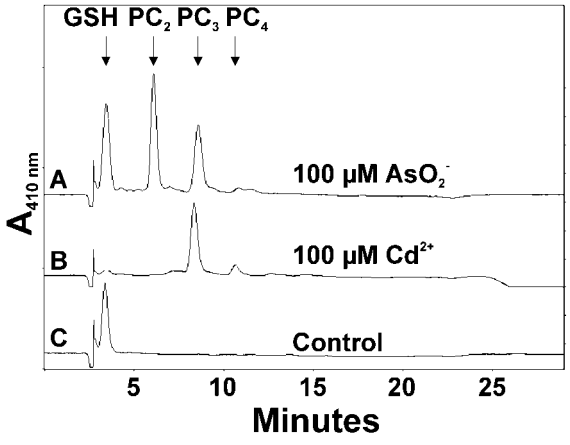

Figure 1. PC analysis of extracts of *R. serpentina*. The elution profiles of cell extracts treated for 4 d with 100 μM AsO_2^- (A), 100 μM Cd^{2+} (B), or without metal (control, C) with sulfhydryl group-specific detection. The peaks indicated by arrows are: glutathione γECG (GSH), $(\gamma EC)_2G$ (PC_2), $(\gamma EC)_3G$ (PC_3), and $(\gamma EC)_4G$ (PC_4).

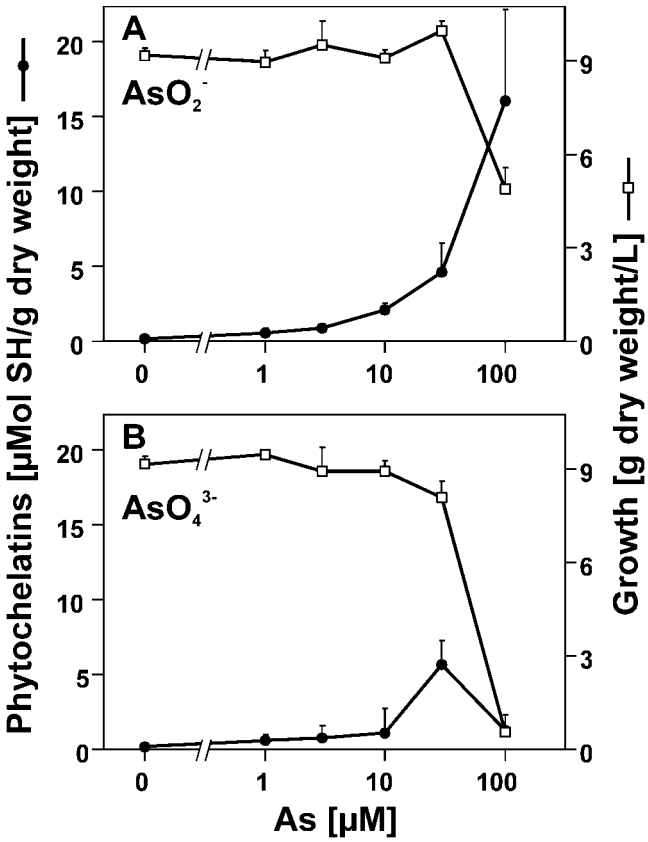

Figure 2. Dependence of growth and PC production on arsenite and arsenate. *R. serpentina* cell cultures were cultivated in the presence of varying concentrations of arsenite (AsO_2^-) (A) or arsenate (AsO_4^{3-}) (B) for 4 d. Growth is expressed as the gain of biomass of the inoculum (10 g dry weight/L). The analysis was performed in triplicate.

The maximal yield of PCs was observed at 100 μM arsenite and 30 μM arsenate concentrations, which gave rise to 16 and 6 μmol GluCys units (in PCs per gram dry weight), while growth was inhibited by approximately 50% and 90%, respectively. In comparison, cells treated in parallel without metal contained 0.2 μmol GluCys unit, and treatment with 100 μM Cd^{2+} produced about 7 μmol GluCys units (in PCs per gram dry weight; data not shown). The induction rates were especially dramatic with arsenite as the inducer, reaching factors of approximately 90-fold at optimal metalloid concentrations. Even at the

non-inhibitory level of 30 μM arsenite, an increase in PC levels by a factor of about 25 compared with the untreated control occurred.

Cell-free extracts of 40 μM and 100 μM arsenite-treated *Silene cucubalus* suspension cultures (4 d) contained substantial levels of PCs and As (0.37 and 0.92 μmol GluCys units in PCs and 0.11 and 0.32 μmol of As per gram fresh weight, respectively), corresponding to an approximate ratio of three thiol groups to 1 As.

A similar toxicity of As was observed in Arabidopsis seedlings (Fig. 3) by using inhibition of root growth and a well-established marker for metal toxicity (Howden and Cobbett, 1992). Root growth was analyzed after transfer of 1-week-old seedlings onto solidified medium containing various concentrations of arsenite for 6 d. A negative effect of arsenite ions on root elongation was visible at a concentration of 10 μM. In Arabidopsis treated with 100 μM arsenite, the roots stopped growing and the seedlings produced more PCs (63 μmol GluCys units in PC/g protein) than after challenge with the same concentration of Cd^{2+}, which is considered to be an optimal PC inducer (Grill et al., 1987) (36 μmol GluCys units in PC/g protein). Again, the strongest PC induction occurred at toxic levels of metalloid reflected by the exertion of growth inhibition. However, the non-inhibiting concentration of 3 μM arsenite resulted in an approximately 100-fold increase of the PC level in unchallenged Arabidopsis seedlings below 0.1 μmol GluCys unit (in PC/g protein).

To unequivocally confirm the identity of As-induced PC peptides, PC_2 and PC_3 were isolated from de-proteinated extracts of arsenite-challenged *R. serpentina* cells by preparative reversed phase-HPLC. Subsequently, the material was subjected to ESI-MS and compared with authentic PC species purified from Cd-treated cells. In both cases, the peptides isolated from As-exposed plant cells generated peak patterns characteristic for penta- and nonameric PC peptides with mass peaks for PC_2 ($m+H^+$: 540) (Fig. 4) and for PC_3 ($m+H^+$: 772; data not shown). In addition, major signals at molecular masses of 538 and 770, corresponding to the intramolecular oxidation products of PC_2 and PC_3, respectively, were detected. These signals were shifted to the expected masses of the PC species upon prolonged reduction of the isolated material by borohydride. Thus, the production of PCs represents unequivocally a reaction of plants toward exposure to As ions.

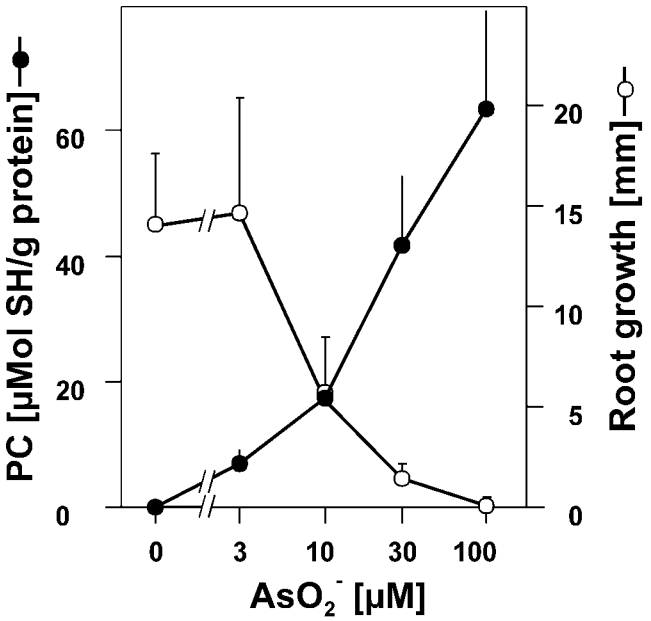

Figure 3. Root growth and PC content of arsenite-exposed Arabidopsis. One-week-old seedlings were transferred to heavy-metal-containing solidified medium under sterile conditions. Root growth (n = 20) within 6 d after transfer was determined and, after another 8 d, pools of 10 seedlings each (n = 4) were analyzed for protein and PC levels.

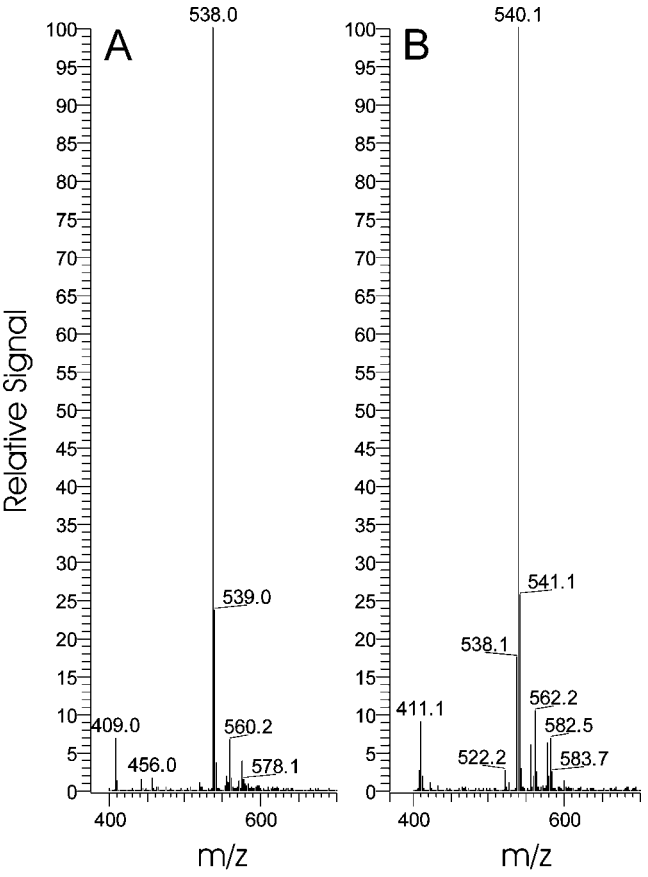

Figure 4. ESI-MS spectra of arsenite-induced PCs. PC_2 purified from AsO_2^--treated *R. serpentina* cells (A) was compared with authentic PC_2 (B). The major mass signal in A corresponds to the oxidized pentapeptide ($m+H^+$, 538). The intramolecular oxidation of the sulfhydryl groups has been frequently observed in those preparations. After prolonged reduction of the As-induced PCs, the mass peak of 540, which is identical to the signal of the sulfhydryl-reduced standard (B; $m+H^+$, 540), was generated (not shown).

As-Induced in Vitro Biosynthesis of PCs

The observed induction of PC peptides by As anions could reflect a direct activation of the PC synthase by As compounds, or it could be indirectly triggered by the As-mediated liberation of cellularly complexed heavy metal

ions such as Zn^{2+} and Cu^{2+}, which subsequently activate the synthase. An indirect pathway operates, for example, during the induction of metallothioneins by Mn^{2+} or Pb^{2+} (Bracken and Klaassen, 1987). To examine the mechanism of induction, cell-free extracts of *S. vulgaris* (Grill et al., 1989) have been assayed for PC synthase activity in the presence of EDTA. The rationale of the experiment was to inactivate Zn^{2+} and Cu^{2+} present in the enzyme preparation with EDTA, which would not interfere with the anions arsenite and arsenate. Metal analyses of the enzyme preparation revealed levels of Zn^{2+} and Cu^{2+} corresponding to 14.4 and 3.1 μM, respectively. Therefore, a millimolar concentration of EDTA would be more than sufficient to efficiently scavenge those ions. The presence of 5 mM EDTA completely excluded any residual activity of PC synthase, while the addition of As anions (up to 1 mM) strongly activated the enzyme (Fig. 5A). PC biosynthesis was detectable in the presence of as little as 10 μM arsenite, however, PC formation was optimal in the concentration range of 1 mM arsenite and 10 mM GSH, yielding 0.75 μmol GluCys unit in PC per minute per gram of protein. The PC formation leveled off after about 24 h, yielding 2.7 mM GluCys moieties incorporated into PCs, which corresponds to a ratio of metal/GluCys of 1 to 2.7 (Fig. 5A).

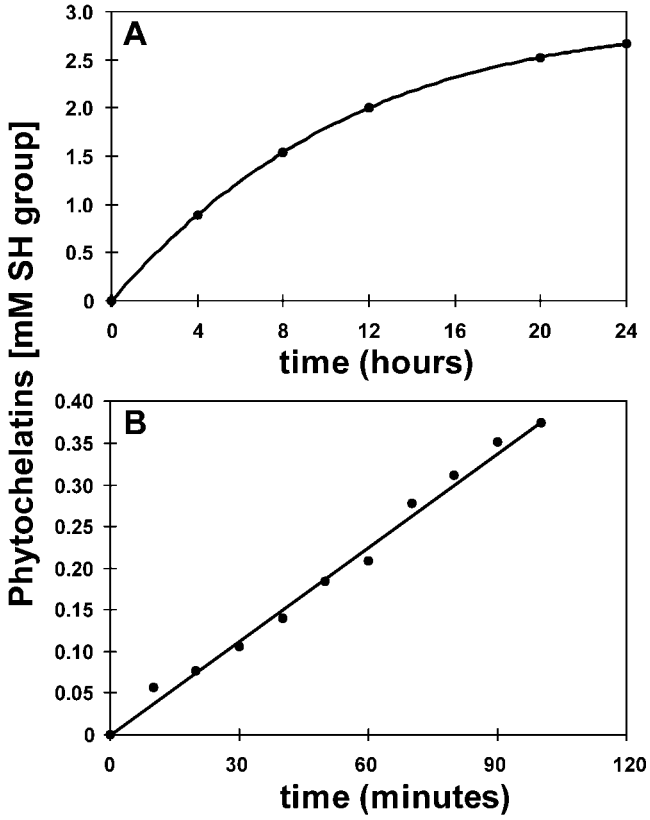

Figure 5. Kinetics of in vitro PC biosynthesis. PC formation catalyzed by PC synthase of *S. vulgaris* (0.5 nkat) in the presence of 1 mM arsenite and 10 mM GSH reaching equilibrium (A) and the initial phase of enzymatic activation (B). Inclusion of 5 mM EDTA in the assay prevented any residual activity of the enzyme (approximately 1% of the level achieved by arsenite activation).

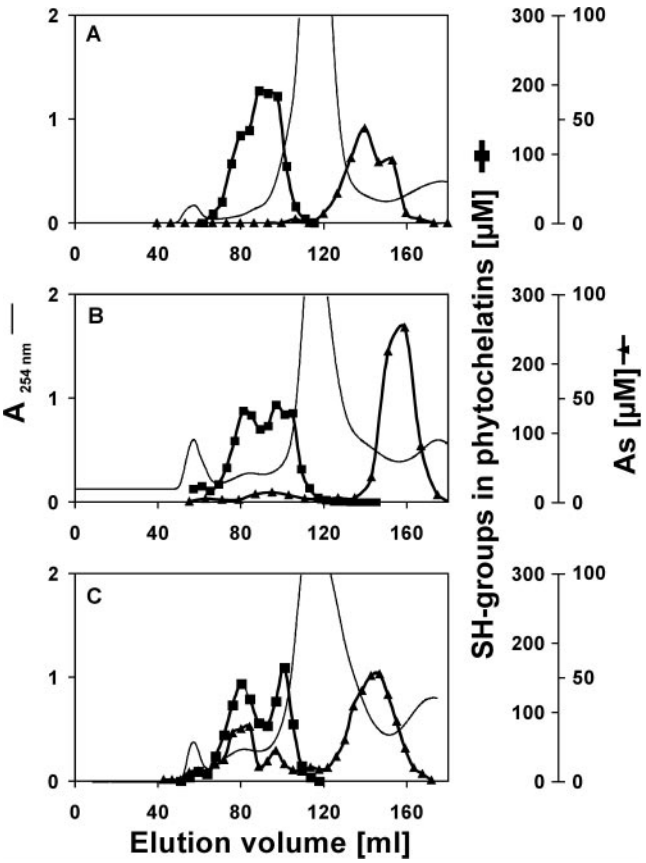

Figure 6. Gel filtration experiments of arsenite-treated *R. serpentina*. Cell-free extracts of suspension cells were chromatographed on a Sephadex G25 size exclusion column. The plant material was extracted and analyzed at pH 8.0 (A), pH 4.0 (B), or pH 4.0, and chromatographed in the presence of 5 μM arsenite (C). In the data shown in C, 35% of the applied metalloid coeluted with the PC fraction.

The activation of PC synthase occurred immediately upon administration of the inducer in the initial phase (Fig. 5B), and PCs accumulated linearly over time (for at least 4 h). The induced peptides were isolated and subjected to MS analysis. Signals of mass peaks identical to the data presented in Figure 4A were recorded.

A parallel experiment conducted with 1 mM Cd^{2+} as an inducing ion yielded a 1.5-fold increased initial rate of PC formation (1.10 μmol GluCys units in PC per minute per gram of protein) compared with the enzyme activation with 1 mM arsenite, although net PC production was finally lower (2 mM GluCys moieties in PC instead of 2.7 mM GluCys moieties in the presence of As).

These results clearly support PC induction by the direct mode of enzymatic activation in vitro.

Evidence for Complexation of As by PC

To determine the existence of an As-PC complex, gel filtration experiments were performed with both in vivo-induced peptides and in vitro-reconstituted complexes. Surprisingly, initial attempts to demonstrate co-chromato-

graphy of PCs and As failed under standard pH conditions (pH 8.0; Grill et al., 1985) using arsenite-induced (80 μM, 4 d) extracts of *R. serpentina* cells (Fig. 6A). Since As is known to undergo oxidation/reduction reactions easily and to form less stable interactions with sulfhydryl groups compared with Cd^{2+}, the chromatographic analysis was carried out with weakly acidic buffers recommended to prevent oxidation and to stabilize As complexes (Jocelyn, 1972). The gel filtration analysis carried out at pH 4.0 revealed co-chromatography of a small fraction of the metalloid with PCs (10%), while the majority of As applied to the column (90%) co-eluted with uncomplexed arsenite (Fig. 6B).

In an attempt to further stabilize the association of As and the induced peptides, a low concentration of arsenite (5 μM) was included in the running buffer during size exclusion chromatography. Indeed, in contrast to the initial gel filtration experiments, 35% of the As level applied by the sample co-eluted with the PC fraction (Fig. 6C). The double-peaked PC elution profile correlated with two peaks of As abundance, which is indicative of a complex formation. The approximately 1:5 ratio of metal to sulfhydryl groups in those fractions was unexpectedly low. Attempts to gain further evidence for a specific binding of As to PCs by purifying the presumed complex were unsuccessful.

Inhibitor studies, however, clearly supported the existence of this complex. In the presence of a specific inhibitor of GSH biosynthesis, 1 mM BSO (Griffith and Meister, 1979), PC induction by 30 μM arsenite was reduced by 75% in *R. serpentina* cells (Fig. 7) and the GSH concentration was reduced by 50%. Subsequent gel filtration analysis of the cell-free extract performed as above revealed no As co-eluting with the PC fraction (detection level at 0.15 μM metal, which corresponds to 6% of the sample concentration). In addition, the analysis documented an increased toxicity of arsenite in the presence of BSO (Fig. 7). The presence of BSO resulted in complete growth inhibition of 30 μM arsenite-challenged cells, while in the absence of the inhibitor the same metalloid concentration did not negatively affect the growth of the cell culture.

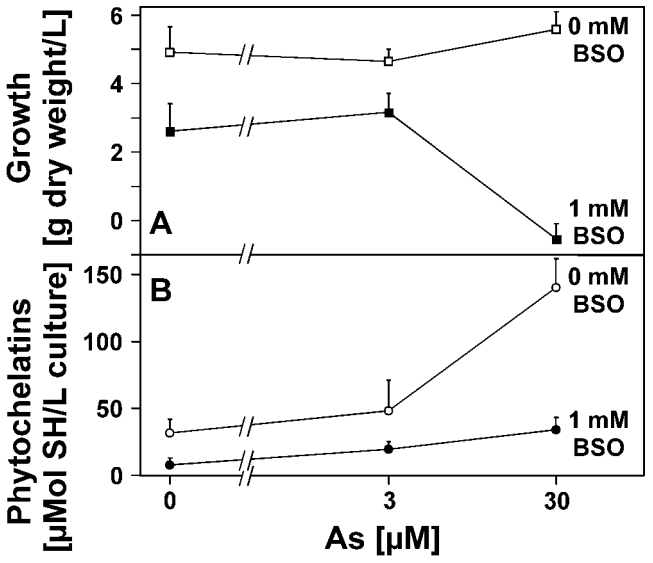

Figure 7. Growth (A) and PC formation (B) of cell cultures from *R. serpentina* in the absence and presence of the GSH biosynthesis inhibitor BSO. Prior administration of varying concentrations of arsenite to the plant material accounted for 7.4 g dry weight/L, which contained 38.1 μM GSH and 17.7 μM SH-groups in PCs. Growth is expressed as gain of biomass within 4 d. All values are the means of three replicates.

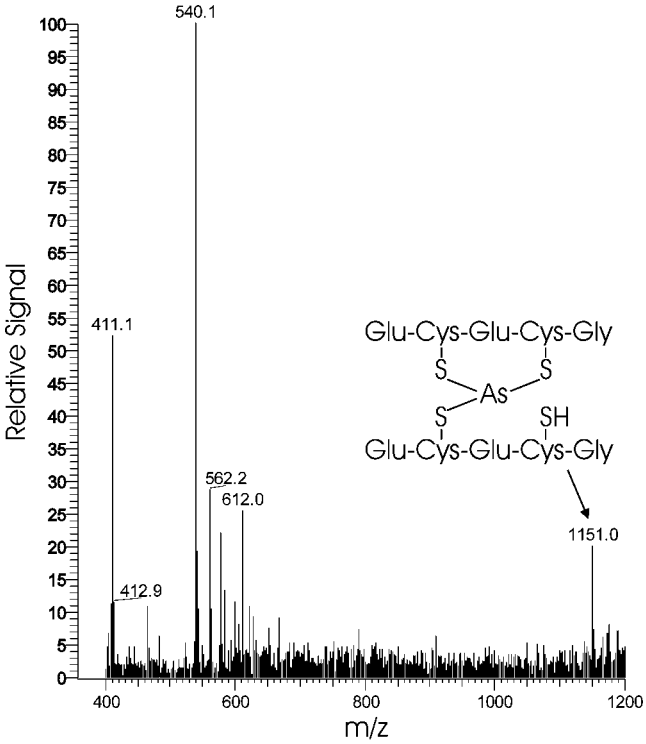

Figure 8. ESI-MS spectrum of the reconstituted As-PC_2 complex. The analysis reveals the characteristic signal of the free PC_2 ($m+H^+$ 540) and also of a presumed As-PC_2 complex ($m+H^+$ 1,151). The signal at 1,151 corresponds to a protonated complex ($m+H^+$) formed by two molecules of PC_2 (molecular mass: 539) ligating one As molecule (atomic mass: 75) via three thiolate coordinations (deprotonated sulfhydryl groups). The structural model $(\gamma\text{-Glu-Cys-}\gamma\text{-Glu-Cys-Gly})_2$As tentatively proposed would be in agreement with this finding.

The surprising destabilization of the As-PC complex in alkaline buffer prompted us to reconstitute complexes of As and PC_2 or GSH by incubating the peptides in a molar ratio of 3 to 1 with arsenite ions under nitrogen. The samples were analyzed by gel filtration experiments at alkaline or weakly acidic pH values. Fractionation analysis of the As-GSH sample documented a co-elution of the metal with the tripeptide to an extent of 95% and of 43% at pH 4.0 and 8.0, respectively. In agreement with the gel filtration data obtained from in vivo-induced material, a corresponding As-PC_2 co-elution was only observed in the weakly acidic buffer (38% metal co-eluted).

The existence of an As-PC_2 complex was further supported by MS analysis of the reconstituted complex via ESI-MS. As shown in Figure 8, a mass peak at 1,151 was detected in the reconstituted material that was absent in As-free PC_2 samples. This mass peak corresponds exactly

to a predicted complex of two PC_2 molecules coordinating one As ion via three thiol groups ($[\gamma$-Glu-Cys-γ-Glu-Cys-Gly$]_2$As). The additional signals observed at masses of 540 and 612 are generated by metal-free PC_2 (compare Fig. 4) and, presumably, by a cationic complex of one PC_2 molecule coordinating one As ($[\gamma$-Glu-Cys-γ-Glu-Cys-Gly$]As^+$), respectively.

DISCUSSION

Structural analyses of PC-metal complexes by EXAFS studies (Strasdeit et al., 1991; Pickering et al., 1999) and optical spectroscopy (Mehra et al., 1995, 1996a, 1996b) have documented a ligandation of Cd^{2+}, Ag^+, Hg^{2+}, and Pb^{2+} by thiolate coordination, as is known for corresponding metallothionein-metal complexes (Kägi, 1991). In the case of the PC-inducing anions arsenate and arsenite, the question of metal coordination is intriguing. The present study unequivocally established the formation of PCs in cell cultures of *R. serpentina* and in Arabidopsis plants. In addition, enzyme preparations of the PC synthase from *S. vulgaris* were capable of producing the metal-binding peptides in the presence of the cation chelator EDTA upon challenge with arsenate and arsenite. The in vivo and in vitro biosynthesis of PCs resulted in a stoichiometry of metal to Cys residues provided by PCs of approximately 1 to 3.

The findings imply a direct mode of enzymatic activation and a subsequent sequestration of the As by the formed PCs. Evidence for the complexation of As by PCs stems from MS analysis and from gel filtration studies of cell-free extracts. The PC fraction also contained substantial amounts of As, while extracts of BSO-treated cells revealed both a strongly reduced level of PCs and no detectable co-elution of PCs and metal in the chromatographic analysis.

As-PC complexes were found to be unstable in alkaline buffer solutions but could be stabilized by weak acids, as has been reported for As-GSH complexes (Jocelyn, 1972). NMR structural analyses of As-GSH complexes generated by the incubation of the tripeptide with arsenite revealed a coordination of As^{3+} by three peptide molecules (Scott et al., 1993). In the presence of arsenate instead of arsenite, the same coordination pattern was observed in vitro due to a reduction of the metalloid ion to As^{3+} by GSH (Jocelyn, 1972). Therefore, it is conceivable that PCs coordinate the As in the same mode. The formation of a reconstituted As-PC_2 complex and the corresponding mass signal identified by ESI-MS analyses is in perfect accordance with the structural model of three thiol groups provided by two PC_2 molecules that coordinate one As molecule.

In vivo, compounds with vicinal sulfhydryl groups such as dimercaptopropanol (Webb and van Heyningen, 1947) or peptides with a Cys-Cys-X-X-Cys-Cys motif (Griffin et al., 1998) interact specifically and with high affinity with arsenicals. PCs are multidental sulfhydryl-containing molecules, which is good evidence for a corresponding interaction of As and PCs. In contrast to Cd^{2+} and Zn^{2+}, As easily undergoes redox reactions. The instability of As-PC complexes observed in vitro possibly reflects the facile oxidation of PCs catalyzed by As^{III}/As^{V} transitions that are favored in alkaline versus acidic conditions and lead to a disruption of the oxidized PC complex. Indeed, a large increase in oxidized sulfhydryl groups was evident in PCs purified from As-treated cells compared with those from Cd-treated cells. The reducing power of PC molecules is much stronger than of GSH and could provide a basis to explain the more stable interaction of As-GSH complexes than As-PC complexes at alkaline conditions. In this context the previously reported failure to identify an As-PC complex by gel filtration in alkaline buffer is conceivable (Maitani et al., 1996).

The reducing environment within the cell, however, where the reduced form of GSH predominates (Hwang et al., 1992), is expected to prevent the oxidational destabilization of the PC-metal complex. The acidic milieu of the vacuole, the presumed final storage compartment for PC-metal complexes (Vögeli-Lange and Wagner, 1990), would also counteract a redox-based destabilization of the complex. Although the direct interaction of As and PCs has not been proven in vivo, the identification of As-PC complexes in cell-free extracts of challenged plant cells, the induction of PCs by As in vivo and in vitro, and reconstitution experiments in vitro strongly argue for the existence of such an complex in planta. The elucidation of the cellular localization and the actual structure of As-PC complexes requires further investigations, e.g. by employing electron probe microscopy (Van Steveninck et al., 1990; Lichtenberger and Neumann, 1997) or whole-cell NMR analysis (Cullen and Hettipathirana, 1994).

Both the documented binding of the metalloid to PCs and the inhibitor study presented here indicate a detoxifying role for PCs. The PCs share with dimercaptopropanol (the antidote of As intoxication) the feature of having vicinal sulfhydryl groups, which are known to provide a more avid complexation of As than monodental sulfhydryl compounds such as GSH (Jocelyn, 1972). Therefore, the inhibition of PC biosynthesis via BSO resulted in a pronounced increase of As toxicity. Furthermore, mutants of Arabidopsis and *Schizosaccharomyces pombe* deficient in PC synthase activity revealed an enhanced sensitivity toward arsenate (Ha et al., 1999). Conversely, the overexpression of a plant PC synthase in yeast resulted in enhanced resistance to arsenite and arsenate (Vatamaniuk et al., 1999). In light of this evidence, we favor the concept of metal complexation and detoxification by the PC peptides during As poisoning.

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