Mechanism of Heavy Metal Ion Activation of Phytochelatin (PC) Synthase

BLOCKED THIOLS ARE SUFFICIENT FOR PC SYNTHASE-CATALYZED TRANSPEPTIDATION OF GLUTATHIONE AND RELATED THIO PEPTIDES*

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The dependence of phytochelatin synthase (γ-glutamylcysteine dipeptidyltranspeptidase (PCS), EC 2.3.2.15) on heavy metals for activity has invariably been interpreted in terms of direct metal binding to the enzyme. Here we show, through analyses of immunopurified, recombinant PCS1 from Arabidopsis thaliana (AtPCS1), that free metal ions are not essential for catalysis. Although AtPCS1 appears to be primarily activated posttranslationally in the intact plant and purified AtPCS1 is able to bind heavy metals directly, metal binding per se is not responsible for catalytic activation. As exemplified by Cd2+- and Zn2+-dependent AtPCS1-mediated catalysis, the kinetics of PC synthesis approximate a substituted enzyme mechanism in which micromolar heavy metal glutathione thiolate (e.g. Cd-GSγ or Zn-GSγ) and free glutathione act as γ-Glu-Cys acceptor and donor. Further, as demonstrated by the facility of AtPCS1 for the net synthesis of S-alkyl-PCS from S-alkyl-glutathiones with biphasic kinetics, consistent with the sufficiency of S-alkylglutathiones as both γ-Glu-Cys donors and acceptors in media devoid of metals, even heavy metal thiolates are dispensable. It is plausible that the dependence of AtPCS1 on the provision of heavy metal ions for activity in media containing glutathione and other thiol peptides is a reflection of this enzyme’s requirement for glutathione-like peptides containing blocked thiol groups for activity.

Phytochelatins (PCSs)1 are (γ-Glu-Cys)n-Xaa polymers whose synthesis from glutathione (GSH) is promoted by heavy metals (1, 2). First identified in the fission yeast Schizosaccharomyces pombe and termed cadystins (3), PCs have since been found in some fungi, some marine diatoms, and all plant species investigated (4). PCs contain 2–11 γ-Glu-Cys repeats, act as high affinity metal chelators, and facilitate the vacuolar sequestration of heavy metals, most notably Cd2+ (2). PC-deficient Arabidopsis cad1 mutants are hypersensitive to Cd2+ salts (5), Cd-Pc complexes localize preferentially to the vacuole of intact plant cells (6), and in plant cell lines capable of tolerating high levels of Cd2+ at least 90% of this metal is accumulated as Cd-PC complexes (2). In the organism for which the molecular basis of PC-dependent metal detoxification is best understood, S. pombe, vacuolar Cd2+ sequestration is mediated by a 90.5-kDa vacuolar ATP-binding cassette transporter, heavy metal tolerance factor 1 (HMT1), that catalyzes the MgATP-energized uptake of Cd-PCS and apoPCS into the vacuoles of wild type but not hmt1 cells (7, 8). HMT1 homologs have not yet been isolated from plants, but an MgATP-energized transport pathway for PCs and Cd-PCS, analogous to that identified in S. pombe, has been characterized in vacuolar membrane vesicles isolated from oat roots (9).

Although it is more than a decade since the first report of the partial purification of heavy metal-, primarily Cd2+-, activated enzymes (PC syntheses; γ-glutamylcysteine dipeptidyltranspeptidases, EC 2.3.2.15) competent in the synthesis of PCs from GSH and related thiol tripeptides, by the net transfer of a γ-Glu-Cys unit from one thiol peptide to another or to a previously synthesized PC molecule (10), it is only in the last year that three groups have simultaneously and independently cloned and characterized genes encoding this enzyme. Isolated from Arabidopsis, S. pombe, and wheat, these genes, designated AtPCS1, SpPCS, and TaPCS1, respectively, encode 40–50% sequence-similar 50–55-kDa polypeptides active in the synthesis of PCs from GSH (11–13). All known cad1 mutants are mutated in AtPCS1 (12), SpPCS disruptants are hypersensitive to heavy metals and deficient in cellular PCs (11), and heterologous expression of AtPCS1 in Saccharomyces cerevisiae, an organism that lacks PCS homologs and does not otherwise synthesize appreciable amounts of PCs, confers increased heavy metal tolerance and elicits Cd2+-dependent intracellular PC accumulation (13). As established by the capacity of cell-free extracts from AtPCS1- or SpPCS-transformed cells of Escherichia coli (12) and of purified FLAG epitope-tagged AtPCS1 (AtPCS1-FLAG) for the Cd2+-activated synthesis of short chain PCs from GSH in vitro (13), each of these gene products is not only necessary but also sufficient for the elaboration of PCs.

A physiologically crucial and biochemically intriguing property of PC synthase is its susceptibility to activation by heavy metals. It is by virtue of the activation of PC synthase-catalyzed PC biosynthesis by agents, heavy metal ions, that poison most enzymes that plants and fungi are able to mount a PC-based response to heavy metals.

Few investigators have considered explicitly how heavy metals activate PC synthase but those that have considered it have assumed that activation is consequent on the direct binding of metal ions to the enzyme (2, 4). Indeed, in the most recent model

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1 The abbreviations used are: PC, phytochelatin containing n γ-Glu-Cys repeats; PCS, PC synthase; AtPCS1, Arabidopsis thaliana PC synthase 1; Cd-GSγ, bis(glutathionato)cadmium; DTFN, 5,5′-dithiobis(2-nitrobenzoic acid); MT, metallothionein; RP-HPLC, reverse-phase high pressure liquid chromatography; HMT1, heavy metal tolerance factor 1; Zn-GSγ, bis(glutathionato)zinc; kb, kilobase pair(s).
for PC synthase action, it has been proposed that the strongly conserved N-terminal half of the enzyme is responsible for catalysis and that activation arises from the binding of metal ions to residues, possibly cysteine residues, within this domain (4). The presence of five conserved cysteine residues, two of which are vicinal, in the N-terminal halves of AtPCS1, SpPCS, and TaPCS1 is at least consistent with this notion, as is the observation that the three most extreme Arabidopsis cad1 alleles have amino acid substitutions in this region (12). An extension of this model, proposed to ascribe a role to the more sequence-divergent C-terminal half of the molecule and to account for the properties of the least extreme cad1 allele, cad1-5, a nonsense mutation causing premature termination and deletion of the C-terminal segment, is the concept of a C-terminal “metal-sensing domain” whose multiple cysteine residues bind heavy metals and bring them into contact with the putative “activation” site within the N-terminal, catalytic half of the molecule.

In the experiments described here we exploit the ease with which AtPCS1-FLAG can be purified to near homogeneity from AtPCS1:FLAG-transformed *S. cerevisiae* to yield high activity PC synthase preparations (13) to examine the mechanism by which this class of transpeptidase is activated by heavy metals. In so doing, we establish that although AtPCS1-FLAG confers tolerance to and is subject to posttranslational activation by a broad range of heavy metals, direct interaction of the enzyme with free metal ions is not the primary mode of activation. Instead, heavy metal ions are required for the formation of heavy metal peptide, GSH or PC, thiolates that serve as cosubstrates for catalysis via a substituted enzyme mechanism. On the basis of these findings and the efficacy of S-alkylglutathiones as substrates for the synthesis of S-alkyl-PCs in the complete absence of metal ions, it is inferred that AtPCS1 catalyzes the polymerization of GSH-derived thiol peptides containing blocked thiol groups, regardless of whether the substrate-active species is a heavy metal thiolate or thioether. If heavy metals do directly bind AtPCS1 in vivo it is to a limited extent and associated with only minor augmentation of synthetic activity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plant Materials—**The ycf1Δ mutant *S. cerevisiae* strain DTY167 (MATa ura3–52 leu2–3,112 his–Δ200 trp1–901 lys2–801 suc2–50 yef1::hisG), deficient in vacuolar Cd2+ sequestration (14), was employed for the studies of heterologously expressed AtPCS1:FLAG. *Arabidopsis thaliana* cv Columbia was the source of the RNA used for the Northern analyses.

**Heterologous Expression of FLAG-tagged AtPCS1—**For constitutive expression of immunoreactive protein in *S. cerevisiae* strain DTY167, yeast-*E. coli* shuttle vector pYES3 (15), containing *AtPCS1* cDNA insert engineered to encode an AtPCS1 C-terminal FLAG (DYKDDDDK) epitope tag fusion (pYES3-AtPCS1::FLAG), was used as described (13).

**Purification of AtPCS1-FLAG—**The soluble fraction from pYES3-AtPCS1:FLAG-transformed DTY167 (DTY167/pYES3-AtPCS1::FLAG) cells was prepared by the disruption of spheroplasts as described (13).

**Equilibrium Dialysis of AtPCS1-FLAG—**Binding of Cd2+ was determined by equilibrium dialysis of 400–800 μM (160 μg) samples of purified AtPCS1-FLAG against 80-ml volumes of 10 mM Tris-HCl buffer, pH 7.8, containing 0.5–20 μM 109CdCl2 (specific activity 22 Ci/mmol) for 12 h at 4 °C in 4 ml mini-collodion membrane tubes (molecular weight cutoff of 25,000, Schleicher & Schuell). Protein-bound 109Cd was estimated by measuring the radioactivity of the bulk medium outside the dialysis tube and that of the solution within the dialysis tube and determining the increase in 109Cd radioactivity consequent on AtPCS1-FLAG binding of Cd2+ to AtPCS1-FLAG was estimated by measuring the decrease in equilibrium binding of a half-saturating (Kd) concentration of 109Cd2+ as the result of inclusion of a range of concentrations of Cu2+ or Zn2+ in the equilibrium dialysis buffer.

**Northern Analyses—**To assess the effects of treatment with heavy metal salts on the steady state levels of AtPCS1 transcripts, 21-day-old seedlings grown in Gamborg’s B-5 medium were transferred into fresh medium containing 25 or 100 μM CdSO4, CuSO4, or ZnSO4 and incubated with shaking at 22 °C for an additional 6 or 24 h before RNA extraction. Control seedlings were treated in an identical manner except that CdCl2, CuSO4, and ZnSO4 were not added to the culture medium.

Total RNA was extracted from roots and shoots in Trizol Reagent (Life Technologies, Inc.), resolved on formaldehyde-agarose gels, blotted, and hybridized with 32P-labeled, random-primed 1.5-kb NotI/SmaI restriction fragment corresponding to the coding sequence of *AtPCS1* as described (13). The filters were washed twice in 2 × SSC, 0.1% (w/v) SDS (5 min at room temperature), twice in 0.2 × SSC, 0.1% SDS (15 min at 42 °C), and twice in 0.1 × SSC, 0.1% SDS (15 min at 65 °C). 32P-Labeled bands were visualized and quantitated with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

**Amino Acid Analyses—**The chain lengths of the PCs and S-alkyl-PCs synthesized from GSH or S-alkylglutathionates, respectively, were determined by estimating their Glu/Gly or Glu/Ala ratios (ratio = n = number of Glu-Cys or Glu-(alkyl-Cys) repeats per Gly) after acid hydrolysis of the appropriate HPLC fractions. Aliquots of the fractions were taken to dryness in pyrolyzed glass tubes, hydrolyzed in gas-phase 6 N HCl for 20 h at 110 °C before ion-exchange chromatography, postcolumn derivatization with O-phthalaldehyde, and fluorescence detection (17).

**Calculation of Concentrations of Free Heavy Metal Ions and Their Complexes—**The concentrations of free heavy metal ions and their complexes with GSH and other ligands in the reaction media were determined by estimating their Glu/Gly or Glu/Ala ratios (ratio = n = number of Glu-Cys or Glu-(alkyl-Cys) repeats per Gly) after acid hydrolysis of the appropriate HPLC fractions. Aliquots of the fractions were taken to dryness in pyrolyzed glass tubes, hydrolyzed in gas-phase 6 N HCl for 20 h at 110 °C before ion-exchange chromatography, postcolumn derivatization with O-phthalaldehyde, and fluorescence detection (17).
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10^{-3} M^{-1}; [Cd{\text{GS}_{2}}]/[Cd^{2+}]/[GS] = 5.13 \times 10^{-3} M^{-1}; [Cd{\text{GS}_{2}}]/[Cd^{2+}]/[GS] = 2.24 \times 10^{-3} M^{-1}; [Cd{\text{GS}_{2}}]/[Cd^{2+}]/[H]^{2+}/[GS] = 2.75 \times 10^{-3} M^{-1}; [Cd{\text{H-GS}_{2}}]/[Cd^{2+}]/[H]^{2+}/[GS] = 5.50 \times 10^{-2} M^{-1}; [Cd{\text{H-GS}_{2}}]/[Cd^{2+}]/[H]^{2+}/[GS] = 2.51 \times 10^{-2} M^{-1}; [Zn{\text{GS}_{2}}]/[Zn^{2+}]/[GS] = 3.24 \times 10^{-2} M^{-1}; [Zn{\text{GS}_{2}}]/[Zn^{2+}]/[GS] = 3.16 \times 10^{-2} M^{-1}; [Zn{\text{H-GS}}]/[Zn^{2+}]/[H]^{2+}/[GS] = 1.02 \times 10^{-2} M^{-1}; [Zn{\text{H-GS}}]/[Zn^{2+}]/[H]^{2+}/[GS] = 1.20 \times 10^{-2} M^{-1}.  

Other Computations—Kinetic parameters. AtPCS1-FLAG-catalyzed heavy metal-binding constants, and stoichiometries of binding were estimated by nonlinear least squares analysis (20) using the Ultrafit nonlinear curve-fitting package from BioSoft (Fergusom, MO).

Protein Estimations—Protein was estimated by the dye-binding method (21).

Chemicals—S-Methylglutathione, S-ethylglutathione, S-propylglutathione, S-butyrylglutathione, and S-hexylglutathione were purchased from Sigma. 100\text{mg} of CdSO_{4} (78.4 Ci/mmol) was from Amersham Pharmacia Biotech. All of the other, general, reagents were obtained from Fisher Scientific, Research Organics Inc., or Sigma.

RESULTS

AtPCS1 Is Constitutively Expressed in the Intact Plant—High stringency Northern analyses revealed a single 1.7-kb band of similar intensity after hybridization of random-primed 32P-labeled AtPCS1 cDNA with total RNA extracted from roots and shoots of 21-day-old Arabidopsis seedlings, regardless of whether the seedlings had been exposed to 25 or 100 \mu M Cd^{2+} (CdSO_{4}), the most potent activator of AtPCS1-catalyzed PC synthesis (below), Cu^{2+} (CuSO_{4}), an activator of intermediate potency, or Zn^{2+} (ZnSO_{4}), an activator of weak to moderate potency, for 6 h (data not shown) or for 24 h before RNA extraction (Fig. 1). From these results and those from earlier biochemical investigations, demonstrating that extractable PC synthase activity is not enhanced by the pretreatment of plant tissues or cell suspension cultures with heavy metal salts (8), modulation of AtPCS1 by heavy metals was inferred to be exerted at the enzyme level. All subsequent experiments were therefore directed at determining how metal ions interact with the enzyme to elicit PC synthetic activity and were performed on recombiant AtPCS1 (AtPCS1-FLAG). For this purpose, heterologously expressed AtPCS1-FLAG was 30- to 50-fold immunopurified from the soluble fraction of yeast-AtPCS1::FLAG-transformed S. cerevisiae strain DTY167 to yield a single anti-FLAG antibody-reactive, Mr = 58,000 polypeptide species (13) whose activity consistently exceeded 35 \mu mol/mg/min when assayed in standard reaction media containing 25 \mu M CdCl_{2}, 3.3 mM GSH, and 200 mM Hepes-BTP buffer (pH 8.0).

AtPCS1-FLAG Is Activated by a Broad Range of Heavy Metals and Is Sufficient for the Synthesis of PC2–6—AtPCS1-FLAG retained all of the known characteristics of the PC synthetic activities of plant extracts. It was subject to activation by a broad range of heavy metal cations and oxyanions and was competent in the synthesis of both short chain and long chain PCs. AtPCS1-FLAG-catalyzed PC synthesis from GSH was obligatorily dependent on the provision of heavy metals. No activity was detectable when metals were omitted from the reaction medium, but the addition of Cd^{2+}, Hg^{2+}, As^{3+}, AsO_{2}^{3-}, Cu^{2+}, Zn^{2+}, Pb^{2+}, AsO_{2}^{3-}, Mg^{2+}, and Ni^{2+} at total concentrations of 50 \mu M increased the capacity of AtPCS1-FLAG for PC synthesis from GSH by 47.7, 40.3, 27.7, 27.2, 10.6, 8.3, 4.8, 3.9, and 3.6-fold, respectively, versus Co^{2+}, the least stimulatory metal ion examined (Fig. 2). Because in no case, with the exception of Cu^{2+}, did pretreatment of the terminated reaction media with sodium borohydride before RP-HPLC markedly change the estimated reduced thiol contents of the PCs synthesized or the apparent rank order with which the metal cations or oxyanions promoted PC synthesis (Fig. 2), it was concluded that the effects of most of the metal cations and oxyanions examined were exerted at the enzyme level, not at the level of the oxidation state and amenability of the thiol peptide reaction products to detection with DTNB. Cu^{2+} was an exception in that prior reduction of the reaction products with sodium borohydride doubled DTNB reactivity (Fig. 2), suggesting an approximately 1:1 ratio of oxidized:reduced thiols in the PCs synthesized in media containing this metal ion.

As exemplified by the results obtained with standard reaction medium containing Cd^{2+}, AtPCS1-FLAG was competent in the sequential synthesis of PC2–6 from GSH (Fig. 3). For shorter term (0–60 min) incubations, net synthesis of PC2–4 was evident within 2, 5, and 20 min, respectively, of the addition of enzyme (Fig. 3, inset), indicating that whereas GSH, alone, was sufficient for the synthesis of PC2, the net synthesis of PC3 and PC4 required not only GSH but also PC2 and PC3, respectively. For longer term incubations, the net synthesis of not only PC3, PC5, and PC6 but also PC4 and PC5 was evident (Fig. 3). Qualitatively similar time dependences and ranges of chain length were observed when PC synthase activity was activated by Cu^{2+} or Zn^{2+} instead of Cd^{2+} (data not shown). As would be predicted when GSH is the prevalent thiol peptide in the reaction medium and PC_{n-1} is derived from PC_{n} + GSH, the final amounts of thiol equivalents (= \gamma-Glu-Cys units) incorporated into PC_{n} decreased exponentially with increase in chain length (Fig. 3).

AtPCS1-FLAG Is Active in Media Depleted of Free Metal Ions—All of the metals employed for the experiments summarized in Figs. 2 and 3 elicited net PC synthesis despite the presence of a 66- to 132-fold molar excess of GSH- and/or...
PC-associated thiols in the reaction media. Given that the complexes formed between heavy metals and thiol compounds are among the most stable known, it was decided to determine the likely concentrations of free metal ions and their complexes under conditions in which AtPCS1-FLAG-catalyzed PC synthesis was sustained.

The concentrations of free metal ions and their complexes were estimated by substitution of the stability constants of the complexes formed between the metal ions concerned and GSH into the SOLCON computer program. In the first instance, Cd$^{2+}$ and Zn$^{2+}$ were chosen as model metal ions, because of the ready availability of comprehensive compilations of the appropriate stability constants for these and their ligands, and the calculations were based on the composition of the standard reaction medium containing 25 μM metal chloride, 3.3 mM GSH, and 200 mM HEPES-BTP buffer (pH 8.0).

Two crucial insights were gained from these analyses. The first was that under the conditions in which AtPCS1-FLAG catalyzed high rates of PC synthesis from GSH, the concentrations of free Cd$^{2+}$ and free Zn$^{2+}$ ([Cd$^{2+}$]$_{free}$ and [Zn$^{2+}$]$_{free}$) were only of the order of 10$^{-10}$ and 10$^{-9}$ M, respectively (Table I). The second was that more than 98% of the total Cd$^{2+}$ and more than 80% of the total Zn$^{2+}$ added to the reaction medium were associated with GSH as their corresponding bidentate thiol peptides. Although AtPCS1-FLAG bound$^{[10]}$Cd$^{2+}$ to the putative activation site of AtPCS1-FLAG, (ii) The active substrates, or one of the active substrates, for AtPCS1-FLAG-catalyzed PC synthesis are heavy metal thiolates. Although direct interaction of the enzyme with heavy metals may not be a requirement for catalysis, there is a requirement for substrate containing thiol-associated heavy metal.

**AtPCS1-FLAG Binds Heavy Metals at Only Low to Moderate Affinity**—Of these three explanations, the first, direct binding, seemed the least capable of accounting for the activations measured in media containing millimolar concentrations of thiold peptides. Although AtPCS1-FLAG bound$^{[10]}$Cd$^{2+}$ at high capacity ($B_{max}$ = 7.09 ± 0.94) as determined by equilibrium dialysis, the ligand-binding constant ($K_L$ = 0.54 ± 0.21 μM) was 6 orders of magnitude greater than the value of [Cd$^{2+}$]$_{free}$ calculated for the standard reaction medium (Fig. 4A). A similar pattern was inferred for Cu$^{2+}$ and Zn$^{2+}$. Inclusion of 1–20 μM Cu$^{2+}$ in dialysis buffer containing a concentration of 10$^{-5}$Cd$^{2+}$ approximating its $K_L$ for binding to AtPCS1-FLAG (0.5 μM) decreased equilibrium binding of 10$^{-5}$Cd$^{2+}$ to approximately 50% of the control ($-Cu^{2+}$ level) in a manner consistent with a $K_L$ for Cu$^{2+}$ binding of 5.6 ± 1.5 μM (Fig. 4B). Inclusion of the same concentrations of Zn$^{2+}$ in the dialysis buffer exerted little or no effect on the equilibrium binding of 0.5 μM 10$^{-5}$Cd$^{2+}$ (Fig. 4B).

**Heavy Metal Thiolates and Free GSH as Candidate Substrates for AtPCS1-FLAG**—In agreement with the conclusions drawn from the equilibrium binding measurements, and as would be predicted from explanations (ii) and (iii), analyses of the steady state kinetics of AtPCS1-FLAG-catalyzed PC synthesis demonstrated that activity was strictly dependent on thiolate and free GSH concentration, not free metal ion concentration. Providing that the incubations were of sufficiently short duration (180 s) as to enable precise initial rate measurements and ensure exclusive synthesis of PCs, no precluding complications attending the synthesis of longer chain PCs, the kinetics of Cd$^{2+}$-activated PC synthesis were uniform. When free GSH concentration was adjusted to values of 0.6–6.6 mM, and the concentrations of Cd-GS$_2$ were enumerated using the SOLCON program, the initial rates of AtPCS1-FLAG-catalyzed PC synthesis ($V$) approximated a series of Michaelis-Menten functions (Fig. 5A). In all cases, and in support of the notion that free metal ions are not essential for catalysis, other than through their interaction with substrate thiols, free GSH concentrations in excess of those required to complex Cd$^{2+}$ increased, rather than decreased, PC synthesis.

In strict agreement with the possibility that the reaction catalyzed by AtPCS1-FLAG proceeds via a substituted enzyme.

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**TABLE I**

Concentrations of heavy metal ions and their complexes with GSH in standard AtPCS1-FLAG reaction medium.

| Species | Concentration | Species | Concentration |
|---------|---------------|---------|---------------|
| Cd$^{2+}$ | $6.638 \times 10^{-20}$ | Zn$^{2+}$ | $4.585 \times 10^{-6}$ |
| GSH$_{free}$ | 3.2399 | GSH$_{free}$ | 3.2444 |
| Cd · GS | $10^{-4}$ | Zn · GS | 0.0045 |
| Cd · GS$_2$ | 0.0249 | Zn · GS$_2$ | 0.0205 |

Cd-GS$_2$ and Zn-GS$_2$ complexes before transfer of the metal ion to the putative activation site of AtPCS1-FLAG. (iii) The active substrates, or one of the active substrates, for AtPCS1-FLAG-catalyzed PC synthesis are heavy metal thiolates. Although direct interaction of the enzyme with heavy metals may not be a requirement for catalysis, there is a requirement for substrate containing thiol-associated heavy metal.
Methyl derivatives. Not only peptides containing blocked thiols might serve as substrates regardless of whether blocking is a consequence of heavy metal thiolate formation or some other thiol-specific modification.

The results summarized in Figs. 6 and 7 indeed demonstrate that explanation (ii) cannot be generally applicable in that substrate thiol-specific modifications other than those associated with heavy metal thiolate formation render thiol peptides amenable to transpeptidation by AtPCS1-FLAG.

When assayed in media devoid of metal salts, AtPCS1-FLAG catalyzed the net synthesis of S-methyl-PCs from S-methylglutathione with a time dependence (Fig. 6) similar to that for the synthesis of unsubstituted PCs from the equivalent concentration of GSH in media containing heavy metals (Fig. 3). The sequence of appearance of S-methyl-PC2, S-methyl-PC3, and S-methyl-PC4 in the reaction medium during shorter term (0–60 min) incubations (Fig. 6, inset) was consistent with a precursor-product relationship analogous to that inferred for the synthesis of unsubstituted PCs from GSH (Fig. 3, inset), and longer term (6 h) incubations resulted in the net formation of S-methyl-PC3, in addition to S-methyl-PC2,4 (Fig. 6).

The facility of AtPCS1-FLAG for catalyzing the synthesis of S-alkyl-PCs from S-alkylglutathiones was not restricted to S-methyl derivatives. Not only S-methylglutathione but also S-ethyl-, S-propyl-, S-butylyl-, and S-hexylglutathiones were subject to transpeptidation by AtPCS1-FLAG (Table II). The initial rates of metal ion-independent S-alkyl-PC synthesis were similar for all of the S-alkylglutathione derivatives examined.

**FIG. 4.** Concentration dependence of equilibrium binding of Cd$^{2+}$ (A) and concentration dependence of competition between Cu$^{2+}$ or Zn$^{2+}$ and Cd$^{2+}$ for equilibrium binding to purified AtPCS1-FLAG (B). In A, aliquots of purified AtPCS1-FLAG (160 μg) were dialyzed against Tris-HCl buffer (pH 7.8) containing the indicated concentrations of $^{109}$CdCl$_2$ at 4 °C for 12 h. In B, aliquots of purified AtPCS1-FLAG (160 μg) were dialyzed against buffer containing 0.5 μM $^{109}$CdCl$_2$ and the concentrations of CuCl$_2$ (●) and ZnCl$_2$ (□) indicated. Protein-bound radioactivity ($^{109}$Cd) was estimated as the increment in which partial reactions catalyzed by AtPCS1 necessitates formation of a substituted enzyme intermediate. Nominally, AtPCS1-FLAG catalyzes a reaction of the form:

$$\text{AtPCS1} + \gamma\text{-Glu-Cys-Gly} \rightarrow \text{AtPCS1-γ-Glu-Cys + Gly} \quad (\text{Eq. 1})$$

$$\text{AtPCS1-γ-Glu-Cys} + X\cdot(\gamma\text{-Glu-Cys-Gly})_2 \rightarrow \text{AtPCS1}$$

$$+ X\cdot(\gamma\text{-Glu-Cys)-Gly + γ-Glu-Cys-Gly} \quad (\text{Eq. 2})$$

in which X is heavy metal and GSH (or XGSH$_2$) and XGSH$_2$ (or GSH) are γ-Glu-Cys donor and acceptor, respectively.

AtPCS1-FLAG Is Not Obligatorily Dependent on Heavy Metal Ions—Although the reaction scheme depicted in Equations 1 and 2 does not automatically preclude explanation (ii), the possibility that thiolates act to shuttle activatory metal ion to the enzyme, it does raise the question of whether thiol...
(26–31 μmol of S-alkyl-PC₁₂₅/min = 52–62 μmol of γ-Glu-S-alkyl-Cys units incorporated/mg/min) and comparable with the rates of metal ion-dependent PC synthesis from unsubstituted GSH (Figs. 2 and 3).

A notable feature of AtPCS1-FLAG-catalyzed S-methyl-PC₂ synthesis from S-methylglutathione was the biphasic nature of the substrate saturation curve. Plots of the initial velocity of S-methyl-PC₂ synthesis (v) versus S-methylglutathione concentration ([S-Ch₃-GS]) revealed an inflection at 3 mM S-methylglutathione (Fig. 7A) and Hanes-Woolf plots of [S-Ch₃-GS]/v versus [S-Ch₃-GS] clearly resolved the saturation curve into two strictly linear (Michaelian) components (Fig. 7B): a high affinity, low capacity component (Kₘ₁ = 1.0 ± 0.2 mM; Vₘₐₓ₁ = 38.1 ± 2.2 μmol/mg/min) evident at S-methylglutathione concentrations of 2 mM and less and a low affinity, high capacity component (Kₘ₂ = 9.8 ± 1.6 mM; Vₘₐₓ₂ = 115.6 ± 18.9 μmol/mg/min) evident at S-methylglutathione concentrations of 3 mM and greater. Behavior of this type would be expected if, as implied by the kinetics of Cd²⁺-dependent PC synthesis from GSH, S-methylglutathione must be capable of substituting for both the high affinity and low affinity substrates, Cd-GS₂ and free GSH, respectively. The near coincidence of the Kₘ for S-methylglutathione-dependent S-methyl-PC₂ synthesis (Fig. 7B) with Kₘ(GSH) for Cd²⁺-dependent PC₂ synthesis from Cd-GS₂ and GSH (13.6 ± 3.3 mM, Fig. 5) but the approximately 110-fold greater value of Kₘ for S-methyl-PC₂ synthesis (Fig. 7B) versus Kₘ(Cd-GS₂) for PC₂ synthesis (Fig. 5) indicates that S-methylglutathione is a markedly more effective stereochemo-

cal analog of GSH than of Cd-GS₂.

S-Alkyl-PC Synthesis Is Promoted by but Not Obligatory Dependent on Heavy Metal Ions—On the one hand, the capacity of S-alkylglutathiones to serve as substrates for S-alkyl-PC synthesis in the complete absence of heavy metals established that blocked thiols on the substrate are sufficient for core catalysis. On the other hand, the sufficiency of S-alkylglutathiones as substrates despite their inability to form thiolates provided a unique opportunity to assess the influence of free heavy metal ions on AtPCS1-FLAG activity under conditions in which heavy metal-substrate interactions are minimized.

The effects of heavy metal ions on activity were examined by measuring the initial rates of AtPCS1-FLAG-catalyzed S-methyl-PC₂ synthesis from S-methylglutathione in reaction media containing different concentrations of Cd²⁺ and by determining the effects of maximally activating concentrations of Cd²⁺ on the S-methylglutathione concentration dependence of S-methyl-PC₂ synthesis.
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The results of these experiments were extremely informative in three respects. With respect to core catalysis it was evident that although Cd$^{2+}$ promoted the synthesis of S-methyl-PC$_2$, the promotions were moderate in that approximately 50% of synthetic activity was sustained in the complete absence of metal ions (Figs. 7 and 8). With respect to the kinetics of S-methyl-PC$_2$ synthesis from S-methylglutathione, the effects of stimulatory concentrations of Cd$^{2+}$ were exerted primarily at the $V_{\text{max}}$ level. The biphasic substrate concentration dependence of S-methyl-PC$_2$ synthesis was retained in reaction media containing a maximally activating concentration of Cd$^{2+}$ (0.5 mM) and both $V_{\text{max}}$ and $V_{\text{max}}$ were increased by 1.7- and 1.9-fold versus control media lacking metal ions (Fig. 7). By contrast, $K_{\text{m}}$ was unaffected (10.2 ± 0.1 mM with and 9.8 ± 1.6 mM without Cd$^{2+}$, Fig. 7), and $K_{\text{m}}$ was increased from 1.0 ± 0.2 mM to 1.4 ± 0.1 mM (Fig. 7). With respect to the facility of AtPCS1-FLAG for binding heavy metals, the concentrations of Cd$^{2+}$ required for activation of S-methyl-PC$_2$ synthesis (0.025–1.00 mM, Fig. 8), although commensurate with the concentrations required for direct binding to the enzyme as determined by equilibrium dialysis (Fig. 4), were more than 5 orders of magnitude greater than those prevailing in reaction media containing unsubstituted GSH (Table I).

The effects of Cd$^{2+}$ were not attributable to activation consequent on the removal of endogenous heavy metal from AtPCS1-FLAG during extraction and/or purification. Pretreatment of purified AtPCS1-FLAG with 1 mM EGTA and subsequent dialytic removal of the chelator before the measurement of S-methyl-PC$_2$ synthesis neither decreased the activity of the enzyme versus control samples pretreated in an identical manner in media lacking chelator nor influenced the concentration dependence of or degree to which the enzyme was activated by the direct addition of Cd$^{2+}$ to the reaction medium (Fig. 8). The concentrations of Cd$^{2+}$ required for half-maximal and maximal activation of AtPCS1-FLAG, 0.025 and 0.50 mM, respectively, were the same regardless of whether the enzyme had or had not been pretreated with EGTA (Fig. 8). Both EGTA-pretreated and control enzyme were less stimulated by Cd$^{2+}$ concentrations in excess of 1 mM and inhibited by concentrations in excess of 5 mM (Fig. 8).

The results of these investigations reveal that AtPCS1, and by implication other PC synthases, are almost exclusively regulated by heavy metals at the posttranslational level and catalyze a bisubstrate transpeptidation reaction in which both free GSH and its corresponding heavy metal thiolate are substrates. Further, it is shown that although both free GSH and its heavy metal thiolate are ordinarily required for maximal activity, other compounds, for instance S-substituted glutathione derivatives, can substitute for both in such a way as to overcome the enzyme’s otherwise obligatory requirement for heavy metals for activity.

The facility with which S-alkyl-PCs can be synthesized from S-alkylglutathionnes in the complete absence of added heavy metal ions is significant in two respects. (i) In the context of the finding that in reaction media containing concentrations of GSH optimal for heavy metal-dependent PC synthesis, most of the heavy metal present is complexed with GSH, the high activity of S-alkylglutathionnes as substrates in the absence of heavy metals implies that heavy metal ions do not activate catalysis in media containing free thiols through direct interaction with the enzyme but instead do so through interaction with the substrate. As would be expected if this were the case, the activity of AtPCS1-FLAG at a given concentration of free GSH increases as a simple Michaelian function of Cd-GS$_2$ or Zn-GS$_2$ concentration, and AtPCS1-FLAG although able to bind heavy metal ions directly does so at too low an affinity for direct binding to be appreciable in media containing thiol peptides. That the capacity of AtPCS1-FLAG for S-methyl-PC$_2$ synthesis from S-methylglutathione in media lacking added metal ions is retained despite exhaustive pretreatment with metal chelator excludes the possibility of very high affinity substoichiometric heavy metal binding and/or retention of bound metal throughout the extraction and purification procedures used for preparation of the enzyme used in these experiments. (ii) It demonstrates that at least some glutathione derivatives containing blocked thiol groups are sufficient for recognition by and transpeptidation by AtPCS1-FLAG. With specific regard to S-alkylglutathionnes it suggests that this class of compounds bears sufficient resemblance to free GSH and its heavy metal thiolates to serve as both substrate and cosubstrate. S-Alkylglutathionnes can act as both donor and acceptor in the transpeptidation reaction in so far as neither thiol-associated heavy metal on the substrate (or cosubstrate) or a free thiol on the cosubstrate (or substrate) are absolute prerequisites for the transpeptidation reaction. The biphasic substrate concentration dependence of S-methyl-PC$_2$ synthesis from S-methylglutathione, the fact that a high affinity component can be clearly resolved from a low affinity component, is consistent with this explanation if it is assumed that the former corresponds to “metal thiolate-like” binding and the latter to “free GSH-like” binding. The 100-fold lower affinity of AtPCS1-FLAG for S-methylglutathione versus metal thiolates but its approximately equivalent affinity for S-methylglutathione and GSH is explicable in terms of the closer stereochemical resemblance of S-methylglutathione to GSH than to, for example, Cd-GS$_2$ or Zn-GS$_2$.

Previous investigations of partially purified preparations of PC synthase from Silene cubulatus cell suspension cultures have shown that another S-substituted glutathione, S-bimane-glutathione, can serve as a substrate (10). However, in the studies of the enzyme from this source neither incorporation of S-bimane-glutathione into PCs nor alleviation of the dependence of activity on heavy metals was determined. Substrate
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Fig. 9. Model for heavy metal-activated PC synthesis and heavy metal-independent S-alkyl-PC synthesis by AtPCS1. Step 1 is the formation of a γEC acyl-enzyme intermediate concomitant with the cleavage of Gly from the first substrate. Step 2 is transfer of the γEC unit from the substituted enzyme intermediate to the second substrate to generate a product containing one additional γEC repeat. Step 3 is transport of the product from the cytosol into the vacuole. Solid arrows denote the core catalytic pathway. Dashed arrows denote an auxiliary catalytic pathway in which heavy metals, such as Cd²⁺, accelerate catalysis by binding to the enzyme at a site distinct from but coupled with the substrate-binding site(s). The sequence-conserved N-terminal and sequence-divergent C-terminal halves of AtPCS1 are depicted in black and white, respectively. Steps 1 and 2 are inferred to have different substrate requirements in that R is H or CH₃ through C₆H₁₁ and R' is a heavy metal or CH₃ through C₆H₁₁. The R- and R'-substituted forms of glutathione are considered to participate in Steps 1 and 2, respectively (or vice versa) but not both.

activity was assessed by monitoring a partial reaction, Gly release, not S-bimane-PC formation, and all of the assays were performed in media containing 100 μM Cd²⁺ (10).

The sufficiency of blocked thiol groups on at least one of the two substrate molecules required for core catalysis by AtPCS1-FLAG does not necessarily preclude the augmentation of activity by direct metal ion binding to the enzyme. Indeed, when the reaction conditions are designed so as to be compatible with the availability of not only sufficient substrate but also adequate concentrations of free metal ions, by exploiting the capacity of S-alkylglutathiones to act as substrates despite their inability to form heavy metal thiolates, promotion of S-alkyl-PC synthesis up and above that conferred by the provision of substrate containing blocked thiol groups is readily detectable. However, while undoubtedly of mechanistic interest and consistent with direct modulation of catalytic turnover by heavy metal binding, this effect is unlikely to be appreciable in vivo or in vitro when the dominant thiol peptide is unsubstituted GSH. The free Cd²⁺ concentrations required for half-maximal stimulation of S-methyl-PC₂ synthesis are more than 5 orders of magnitude greater than those that prevail when the rates of synthesis of PCs from unsubstituted GSH are maximal.

Implicit in the finding that the steady state kinetics of AtPCS1-FLAG-catalyzed PC₂ synthesis from GSH in media containing heavy metal ions approximate a scheme in which heavy metal thiolate, as exemplified by Cd-GS₂ or Zn-GS₂, and free GSH interact via a substituted enzyme intermediate, not via a ternary complex, to form PC₂, is the concept of formation of an enzyme covalent intermediate during catalysis. Specifically, given that PC synthase is a dipeptidyltranspeptidase (1, 2, 10), the kinetics of heavy metal-dependent PC₂ synthesis from GSH implicate the formation, coincident with cleavage of the Cys-Gly peptide bond of the first substrate (GSH or Cd-GS₂), of an enzyme γ-Glu-Cys acyl intermediate, which in turn plays the role of activated donor for transeptidation of the second substrate (Cd-GS₂ or GSH). If correct, an important corollary follows from this interpretation: the likelihood that the initial nucleophilic attack on the scissile bond of the first substrate is by an enzyme hydroxyl-derived oxygen or thiol-derived thiolate anion and results in the formation of a γ-Glu-Cys-enzyme oxyster or thioester, respectively. A mechanism analogous to that of serine proteases (23), cysteine proteases (24), and cysteine hydrolases (25–27) may therefore be invoked, in which case at least some of the energy required for condensation of the γ-Glu-Cys unit from the first substrate with the α-amino group of the second substrate during PC synthesis is derived from an enzyme oxyster of intermediate energy or an enzyme thioester of high energy formed during the first phase of the catalytic cycle.

A scheme summarizing these conclusions is shown in Fig. 9. According to this scheme, a substantially modified version of that proposed by Cobbett (4, see “Introduction”), AtPCS1 is considered to catalyze a dipeptidyltranspeptidation reaction in which the γ-Glu-Cys donor acylates the enzyme, concomitant with the release of Gly. The activated γ-Glu-Cys-AtPCS1 acyl intermediate so formed then transfers the γ-Glu-Cys unit to the second substrate to generate a product extended by the condensation of one new γ-Glu-Cys repeat with the N terminus of the acceptor. The minimum condition that must be satisfied for this reaction to proceed is that at least one of the thiol groups on one of the substrate molecules is blocked either through heavy metal thiolate formation or S-alkylation. Although heavy metals are not crucial for core catalysis, which is presumably mediated by the conserved N-terminal half of the enzyme, other than through substrate thiolate formation, they are capable of augmenting activity in the presence of substrate-active S-alkyl derivatives. However, unlike heavy metal-mediated catalytic activation in media containing unsubstituted thiols, heavy metal-mediated augmentation requires relatively high concentrations of free metal ions and appears to derive from the direct binding of metal ions to AtPCS1. In light of the dispensability of this binding reaction for core catalysis it is inferred to be at a site distinct from the active site, possibly via the multiple Cys residues found in the more sequence-divergent C-terminal half of the molecule.

In addition to these enzymological insights, two informative physiological implications follow from the results presented. The first is that, contrary to the prevailing model (2, 28), termination of the reactions catalyzed by PC synthases cannot be solely contingent on the chelation of heavy metals because GSH and PC complexes containing heavy metal are active substrate species. Instead, termination more likely results from exhaustion of the heavy metal pool such that free thiols (GSH and apo-PCs) compete with thiolates for the high affinity site of the synthase. Diminution of the substrate-active thiolate pool, whether it be by the incorporation of heavy metals into higher order, substrate-inactive metal-PC complexes or by the removal of metal-PC complexes from the cytosolic pool into the
The vacuole is probably the determining factor for ensuring that PC synthesis meets but does not exceed demand.

The second physiological implication of the utilization by AtPCS1-FLAG of heavy metal thiolates as substrates is that the cytosolic concentration of free heavy metal ions need not increase even transiently for net PC synthesis. Given the high values of the stability constants of heavy metal-GSH complexes and the fact that the prevailing concentration of GSH, the most abundant intracellular thiol, is between 1 and 10 mM (29), any metal that gains access to the cytosol would be expected to be rapidly converted to its corresponding thiolate. The GSH thiolates so formed, because of the moderately high and constitutive expression of PCS genes would, in turn, be incorporated into derivatives, PCs, that also bind heavy metals but at higher affinity (2).

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