Phytochelin Synthase, a Dipeptidyltransferase That Undergoes Multisite Acylation with \(\gamma\)-Glutamylcysteine during Catalysis

STOICHIOMETRIC AND SITE-DIRECTED MUTAGENIC ANALYSIS OF ARABIDOPSIS THALIANA

PCS1-CATALYZED PHYTOCHELATIN SYNTHESIS*

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Phytochelin (PC) synthase has been assumed to be a \(\gamma\)-glutamylcysteine dipeptidyl transpeptidase (EC 2.3.2.15) and, more recently, as exemplified by analyses of the immunopurified recombinant enzyme from Arabidopsis thaliana (AtPCS1-FLAG), has been shown to catalyze a PC synthetic reaction with kinetics that approximate a bisubstrate-substituted enzyme mechanism in which millimolar concentrations of free GSH and micromolar concentrations of heavy metal-GSH thiolates (e.g. cadmium-GS\(_2\)) or millimolar concentrations of \(S\)-alkylglutathiones serve as cosubstrates. Here, we show, by direct analyses of the stoichiometry of AtPCS1-FLAG-catalyzed PC synthesis, the kinetics and stoichiometry of acylation of the enzyme and release of free glycine from \(\gamma\)-Gly-Cys donors, and the effects of the Cys-to-Ser or -Ala and Ser-to-Ala substitution of conserved residues in the catalytic N-terminal half of the enzyme, that PC synthesis is indeed a dipeptidyltransferase that undergoes \(\gamma\)-Glu-Cys acylation at two sites during catalysis, one of which, in accord with a cysteine protease model, likely corresponds to or is at least tightly coupled with \(\gamma\)-Glu-Cys acylation by free GSH, because its acylation not only depends on the provision of \(\text{Cd}^{2+}\) or GSH with a blocked, \(S\)-alkylated thiol group, but is also necessary for net PC synthesis. We conclude that des-Gly-PCs are not generated as an immediate by-product, but rather that the enzyme catalyzes a dipeptidyl transfer reaction in which some of the energy liberated upon cleavage of the Cys-Gly bonds of the \(\gamma\)-Glu-Cys donors in the first phase of the catalytic cycle is conserved through the formation of a two-site-substituted \(\gamma\)-Glu-Cys acyl-enzyme intermediate whose hydrolysis provides the energy required for the formation of the new peptide bond required for the extension of PC chain length by one \(\gamma\)-Glu-Cys repeat per catalytic cycle.

First discovered in the fission yeast Schizosaccharomyces pombe and termed cadystins (1), phytochelatins (PCs)\(^1\) (poly-(\(\gamma\)-Glu-Cys)\(_n\)-Xaa polymers) have since been found in all plant species investigated, a few fungal species, and some marine diatoms (2), where they act as high affinity metal chelators and facilitate the vacuolar sequestration of heavy metals, most notably \(\text{Cd}^{2+}\) ions. PC-dependent vacuolar \(\text{Cd}^{2+}\) sequestration is perhaps best understood in S. pombe, in which the hmt1 gene product, a PC-selective ATP-binding cassette transporter, pumps cadmium-PC complexes and apo-PCs from the cytosol into the vacuole at the expense of ATP (3, 4).

All known PCs fall into five main classes. These are canonical PCs, homo-PCs (iso-PC(\(\beta\)-Ala)), hydroxymethyl-PCs (iso-PC(Ser)), iso-PCs (iso-PC(Glu)), and des-Gly-PCs, containing \(n\) \(\gamma\)-Glu-Cys repeats capped C-terminally by a Gly, \(\beta\)-Ala, Ser, Glu, or no residue, respectively (5). With the exception of canonical PCs and possibly des-Gly-PCs, the latter of which are found in both plants and fungi, including cadmium-treated grasses (6–8), Rubia tinctorum root cultures (9), Candida glabrata (10), and copper-treated S. pombe (11), the species distributions of these classes differ (12).

PCs are synthesized post-translationally. PC synthases (so-called \(\gamma\)-glutamylcysteine dipeptidyl transpeptidases, EC 2.3.2.15) catalyze the net synthesis of PCs from GSH, from GSH and previously synthesized PCs, or from previously synthesized PCs alone to generate polymers containing 2–11 \(\gamma\)-Glu-Cys repeats. Although it has been almost 15 years since pioneering investigations by Grill et al. (13) yielded partially purified preparations of an enzyme capable of catalyzing these reactions, it is only in the last several years that its molecular identity was determined by the independent cloning and characterization of genes encoding PC synthases (14–16). Originally isolated from Arabidopsis thaliana (AtPCS1), S. pombe (SpPCS), and wheat (Triticum aestivum; TaPCS1), these genes encode 50–55-kDa polypeptides bearing 40–50% sequence similarity to each other (14–16). By all criteria (genetic, molecular, and biochemical), all of these genes encode PC synthases. Arabidopsis cad1 mutants, which are cadmium-hypersensitive and

\(^1\) The abbreviations used are: PCs, phytochelatins; AtPCS1, A. thaliana phytochelin synthase-1; SpPCS, S. pombe phytochelin synthase-1; TaPCS1, T. aestivum phytochelin synthase-1; cadmium-GS\(_2\), bis(glutathionato)cadmium; PC\(_n\), phytochelin containing \(n\ \gamma\)-Glu-Cys repeats; NEM-GS, \(S\)-N-ethylmaleimidylglutathione; RP-HPLC, reverse-phase high pressure liquid chromatography; DTNB, 5,5'-dithio-\(\text{bis}(2\text{-nitrobenzoic acid})\); NEM-PC, \(N\)-ethylmaleimidyldiphophothelatin; NEM, \(N\)-ethylmaleimide; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
PC-deficient (17), are mutated in AtPCS1 (14); SpPCS gene disruptants are hypersensitive to heavy metals and deficient in cellular PCs (14); and heterologous expression of AtPCS1 in Saccharomyces cerevisiae, an organism that lacks PCS homologs and does not otherwise synthesize PCs, confers increased heavy metal tolerance concomitant with Cd\(^{2+}\)-dependent intracellular PC accumulation (16). The capacity of cell-free extracts from AtPCS1- or SpPCS-transformed cells of Escherichia coli (14) and immunopurified epitope-tagged AtPCS1 to mediate the heavy-metal-activated synthesis of both short- and long-chain PCs from GSH in vitro (16) establishes that AtPCS1 and SpPCS (and by implication, TaPCS1) and their equivalents from the same or different sources, e.g. AtPCS2 (18), are not only necessary, but also sufficient for PC biosynthesis. To the inventory of functionally defined PC synthase genes identified can be added ce-pcs1 from an animal source, the model nematode Caenorhabditis elegans (19, 20), whose translation product is likewise not only sufficient for the synthesis of PCs in vitro, but is also necessary for the detoxification of heavy metals in the intact organism (20). This was completely unexpected because never before had the involvement of PCs in heavy metal detoxification in animals been even cursorily speculated.

The facility with which recombinant PC synthases, as exemplified by AtPCS1, can now be purified to apparent homogeneity to yield PC synthase preparations with catalytic activities exceeding those of previous preparations from plant sources (13) by >10\(^{5}\)-fold (16, 22) for detailed mechanistic analyses has exposed some basic deficiencies in our understanding of PC biosynthesis. Notable among these is the realization that heavy metals do not bind directly to the enzyme to activate PC biosynthesis, but instead act as substrate ligands for a bisubstrate-substituted enzyme transpeptidation reaction in which free GSH and its corresponding heavy metal thiolate are cosubstrates (22). Although AtPCS1 is primarily activated post-translationally in the intact plant and the purified enzyme is able to bind heavy metals directly, metal binding per se is not responsible for catalytic activation (22). As illustrated by Cd\(^{2+}\)- and Zn\(^{2+}\)-dependent AtPCS1-mediated catalysis, the kinetics of PC synthesis approximate a substituted enzyme mechanism in which micromolar concentrations of one heavy metal-GS thiolate (e.g. bis(glutathionato)cadmium (cadmium-GSh) or bis(glutathionato)zinc and millimolar concentrations of free GSH act as low and high affinity cosubstrates, respectively. Furthermore, as demonstrated by the capacity of AtPCS1 to mediate the net synthesis of S-alkyl-PCs from S-alkylglutathiones with biphasic kinetics, consistent with the sufficiency of S-alkylglutathiones as both high and low affinity substrates in media devoid of metals, even heavy metal thiolates are dispensable for core catalysis.

On the one hand, these findings reveal that the dependence of AtPCS1 and other PC synthases on the provision of heavy metal ions for activity in media containing GSH and other thiol peptides is a reflection of this enzyme’s requirement for GSH-like peptides containing blocked thiol groups for activity. On the other hand, they highlight two other major shortcomings in our understanding of PC biosynthesis. The first is that it is not known unequivocally if PC synthase is a dipeptidyl- or tripeptidyltransferase. The second, which is related to the first, is that the identity of the substituted intermediate formed by the enzyme during catalysis has not been defined. Whether PC synthases are dipeptidyl- or tripeptidyltransferases is not only of relevance for understanding the catalytic mechanism of this class of transpeptidase, but may also be critical in determining the types of PCs these enzymes are capable of synthesizing.

In most studies of partially purified preparations of the enzyme, PC synthase-catalyzed GSH-dependent PC synthesis has been considered to proceed by the transpeptidation of a γ-Glu-Cys unit from one GSH molecule to another to form PC\(_{2}\) and, after the accumulation of sufficient (substrate) levels of PCs, by the transpeptidation of a γ-Glu-Cys unit from GSH to a PC (PC\(_{n}\)) molecule to generate PC\(_{n+1}\) (13, 23). Appropriately, PC synthase has been defined as a γ-glutamylcysteine dipeptidyl transpeptidase and presumed to catalyze a reaction of the type shown in Equation 1,

\[
(γ-EC)G + (γ-EC)G \rightarrow (γ-EC)(γ-EC)G + G \quad (\text{Eq. 1})
\]

in which chain extension proceeds from the C to N terminus with cleavage of the Cys–Gly peptide bond of the donor, not the acceptor. However, inspection of the literature reveals that the notion that PC synthase is a tripeptidyl transpeptidase is equally tenable. There are no published data to refute a scheme in which PC synthase catalyzes the transfer of γ-Glu-Cys-Gly units in a reaction of the type shown in Equation 2,

\[
(γ-EC)G + (γ-EC)G \rightarrow (γ-EC)(γ-EC)G + G \quad (\text{Eq. 2})
\]

in which case, chain extension would proceed from the N to C terminus, not from the C to N terminus, after cleavage of the Cys–Gly peptide bond of the acceptor, not the donor. An implication of the possibility that PC synthases are tripeptidyl transpeptidases, when combined with their capacity to synthesize PCs from other PCs without the direct participation of GSH is a simple mechanism for the synthesis of des-Gly-PCs (see Fig. 1). If γ-Glu-Cys is construed as the limiting case of a des-Gly-PC (des-Gly-PC\(_{1}\)) and GSH as the limiting case of a PC (PC\(_{1}\)), Equation 1 assumes the general form (see Fig. 1A) shown in Equation 3,

\[
PC\(_{n}\) + PCs \rightarrow PC\(_{n+1}\) + PC\(_{n-1}\) \quad (\text{Eq. 3})
\]

and Equation 2 assumes the general form (see Fig. 1B) shown in Equation 4.

\[
PC\(_{n}\) + PCs \rightarrow PC\(_{n+1}\) + des-Gly-PC\(_{n-1}\) + G \quad (\text{Eq. 4})
\]

It is not known if a by-product model of the type shown in Equation 4 and Fig. 1B could be the sole mechanism for the synthesis of des-Gly-PCs, one that might account for the synthesis of this class of thiol peptides in vitro, but two properties of des-Gly-PCs are at least consistent with such a possibility. The first is the seemingly broad distribution of this PC subclass in that it has been detected in many species of plants and fungi after long-term exposure to heavy metals (see above). This might indicate, as would be predicted by the by-product model, that most organisms able to synthesize PCs also synthesize des-Gly-PCs. The second property is that the levels of des-Gly-PCs, in the few cases in which they have been determined, increase most appreciably after several days of exposure to heavy metals, after the initial burst of PC\(_{2}\) and PC\(_{3}\) accumulation (7). This would be expected if, as implied by the by-product model, des-Gly-PCs arise as a result of the synthesis of longer chain PCs from previously synthesized shorter chain PCs.

The formation of a covalent enzyme intermediate during catalysis is implicit in the finding that the steady-state kinetics of AtPCS1-catalyzed PC synthesis from GSH in media containing heavy metals approximate a scheme in which heavy metal thiolate and GSH interact via a substituted enzyme intermediate, not via a ternary complex, to form PC\(_{2}\) (22). Specifically, given that at least one peptide bond must be cleaved and that at least one new peptide bond forms for each molecule of PC\(_{2}\) synthesized, regardless of whether the PC synthase is a dipeptidyl- or tripeptidyltransferase (Equation 1 or 2; see above), and that the initial attack on the carbonyl carbon of the peptide bond to be cleaved must be by a nucleophile, it is almost
inevitable that the substituted enzyme intermediate is a γ-Glu-Cys acyl intermediate. If correct, two important corollaries follow. The first is that the initial nucleophilic attack on the scissile bond is by an enzyme hydroxyl-derived oxyanion or thiol-derived thiolate anion to generate an enzyme–γ-Glu-Cys oxyester or thioester, respectively. The second, a consideration that has been neglected in the literature despite the status of PC synthase as both a peptidease and a peptide synthase, is that at least some of the energy required for subsequent condensation of the two substrate molecules is derived from acylation of the enzyme coincident with cleavage of the first substrate. A mechanism for PC synthase analogous to those of serine proteinases (24), cysteine proteinases (25), and cystine hydrolases (26–28) is therefore invoked, except that, instead of mediating a dissipative hydrolysis reaction, at least some of the energy required for condensation of the γ-Glu-Cys unit from one substrate with the other substrate during the second PC synthetic phase of the catalytic cycle is derived from an enzyme oxyester of intermediate energy or an enzyme thioester of high energy formed during the first phase of the cycle.

In this study, we have described investigations directed at determining whether AtPCS1 is a dipeptidyl or tripeptidyl transeptidase and elucidation of the identity of the substituted enzyme intermediate formed during catalysis. In so doing, we have established that PC synthase is a dipeptidyl-transferase that undergoes γ-Glu-Cys acylation by GSH at two sites during catalysis concomitant with the release of Gly. Free GSH alone was capable of acylating the first site on the enzyme, but not the second unless Cd$^{2+}$ was added to the reaction medium. Of the two conserved Ser and five conserved Cys residues in the catalytic N-terminal half of AtPCS1, only one (Cys$^{56}$) appears to be essential for catalysis and acylation of the first site by free GSH. We propose that the energy required for each cycle of PC chain extension is derived from the formation of an enzyme cysteinyl thioester at position 56 and presumably a non-seryl, non-cysteinyl oxyester at another position upon the Cys–Gly bond cleavage of at least two incoming GSH molecules.

**MATERIALS AND METHODS**

**Heterologous Expression of AtPCS1-FLAG**—The mutant *S. cerevisiae* ycf1Δ strain DTY167 (MATa ura3-52 leu2-3,112 his3-11,152 trp1-901 lys2-801 sac2-28 cys2-300 his3Δ112 his Δ-2000 tcp1 Δ-901) was transformed with wild-type and in *vitro* mutated AtPCS1-FLAG. In all cases, AtPCS1 was purified by RP-fast protein liquid chromatography on a C18 PepRPC column (Amersham Biosciences). The column was developed with a linear (20 min) gradient of water and 0.05% (v/v) phosphoric acid to acetonitrile and phosphate buffer (pH 7.6) (33). For the quantitation of NEM-PCs, free amino groups were estimated fluorometrically by reacting aliquots of the column fractions with 0.4 mM diethyl maleimide (NEM) to generate NEM-PCs and/or CdCl$_2$ in 100 mM HEPES/BTP buffer (pH 8.0) (22). For RP–HPLC, the reactions were made 5% (w/v) with sulfoalicylic acid and centrifuged before loading aliquots of the supernatants onto a Varian Microbore RP column (250 × 4.6 mm). The column was developed with a linear (20 min) gradient of water and 0.05% (v/v) phosphoric acid to acetonitrile and 0.05% phosphoric acid at a flow rate of 1 ml/min. For the quantitation of PCs, thiols were estimated spectrophotometrically at 412 nm by reacting aliquots (500 μl) of the column fractions with 0.8 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 250 mM phosphate buffer (pH 7.6) (33). For the quantitation of NEM-PCs, free amino groups were estimated fluorometrically by reacting aliquots of the column fractions with 0.4 M sodium borate (pH 9.7) (200 μl) and fluorescamine (20 μl of a 3 mg/ml solution dissolved in acetone) (32). Fluorescence was measured at excitation and emission wavelengths of 390 and 470 nm, respectively, as described before (22). Calibration was confirmed by sequencing the coding sequence encompassing the mutation before yeast transformation.

**Measurement of PC Synthesis Activity and PCs**—PC synthase activity was assayed routinely in reaction medium containing purified wild-type or mutant AtPCS1-FLAG and the indicated concentrations of GSH, N-S-ethylmaleimidyglycylglutathione (NEM-GS), PCs and/or CdCl$_2$ in 100 mM HEPES/BTP buffer (pH 8.0) (22). For RP–HPLC, the reactions were made 5% (w/v) with sulfoalicylic acid and centrifuged before loading aliquots of the supernatants onto a Varian Microbore RP column (250 × 4.6 mm). The column was developed with a linear (20 min) gradient of water and 0.05% (v/v) phosphoric acid to acetonitrile and 0.05% phosphoric acid at a flow rate of 1 ml/min. For the quantitation of PCs, thiols were estimated spectrophotometrically at 412 nm by reacting aliquots (500 μl) of the column fractions with 0.8 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) dissolved in 250 mM phosphate buffer (pH 7.6) (33). For the quantitation of NEM-PCs, free amino groups were estimated fluorometrically by reacting aliquots of the column fractions with 0.4 M sodium borate (pH 9.7) (200 μl) and fluorescamine (20 μl of a 3 mg/ml solution dissolved in acetone) (32). Fluorescence was measured at excitation and emission wavelengths of 390 and 470 nm, respectively, as described before (22). Calibration was confirmed by sequencing the coding sequence encompassing the mutation before yeast transformation.

**Measurement of Purification of PC**—For the studies of AtPCS1-FLAG-catalyzed PC synthesis from PC$_3$ and GSH, PC$_3$ was synthesized by incubating AtPCS1-FLAG (1 μg) in 1 ml of 200 mM HEPES/BTP buffer (pH 8.0) containing 20 mM GSH, 14 mM 2-mercaptoethanol, and 100 μM CdCl$_2$ at 30°C for 1 h. After terminating the reaction with 5% (w/v) sulfoalicylic acid, PC$_3$ was purified semipreparatively by RP-fast protein liquid chromatography of the supernatant on a 100 × 6.4-mm Resource RPC column (Amersham Biosciences). The column was developed with a linear (40 min) gradient of water and 0.05% (v/v) phosphoric acid to 100 mM acetonitrile and 0.05% phosphoric acid at a flow rate of 0.8 ml/min. Thiol detection was described as the routine assays, except that only 50-μl aliquots of the column fractions were reacted with 50 μl of DTNB in 96-well plates. The fractions corresponding to PC$_3$ were pooled, neutralized with 1 M Tris, lyophilized, and stored at −80°C until used.

**Synthesis of [3H]Gly-labeled NEM-GS**—For investigations of PC synthesis and glycine release from S-alkylglutathione derivatives, [3H]Gly-labeled GSH was S-alkylated with N-ethylmaleimide (NEM) to generate [3H]Gly-labeled NEM-GS. After removal of the antioxidant (dithiothreitol) in which [3H]Gly-labeled GSH was shipped by extraction with acidic ethyl acetate immediately before use and its combination with unlabeled GSH to form a 1:100 solution by mixing in 250 mM HEPES/BTP buffer (pH 8.0), [3H]Gly-labeled NEM-GS was synthesized by the addition of an equimolar concentration of NEM to the solution and reaction on ice for 1 h. Upon completion of the reaction, as assessed from the exhaustion of DTNB-reactive free thiols, the reaction mixture was diluted to a final [3H]Gly-labeled NEM-GS concentration of 25 mM and then subjected to RP-fast protein liquid chromatography on a C4 5 μm column (Amersham Biosciences). Elution was at 0.8 ml/min with water and 0.1% (v/v) phosphoric acid (solvent A) to acetonitrile and 0.1% phosphoric acid (solvent B) using a three-phase gradient: 0–20 min, 0–17% solvent B; 20–25 min, 30% solvent B; and 25–30 min, 0% solvent B. The concentration of [3H]Gly-labeled NEM-GS in the column fractions was estimated by liquid scintillation counting. The quantitation of free amino groups was with fluorescamine using GSH as a calibration standard as described (22).

**Measurements of [3H]Gly Release from [3H]Gly-labeled NEM-GS**—For assays of AtPCS1-FLAG-catalyzed Gly...
release and PC synthesis from GSH, purified enzyme (10 μg) was incubated with 3 mM [3H]Gly-labeled GSH (600 μCi/mol) in 100 mM HEPES/TP buffer (pH 8.0) at 30 °C for the times indicated before terminating the reactions. After subjecting the deproteinized supernatants to RP-HPLC, [3H]Gly in the fractions was determined by liquid scintillation counting. For assays of AtPCS1-FLAG-catalyzed [3H]Gly release and NEM-PC synthesis from [3H]Gly-labeled GSH, purified enzyme (10 μg) was incubated with 3 mM [3H]Gly-labeled GSH at 30 °C for the times indicated before terminating the reactions and subjecting the deproteinized samples to RP-HPLC and liquid scintillation counting as described above for [3H]Gly-labeled GSH.

Measurement of Acyl-enzyme Intermediate Formation—Two methods were employed for measuring the acylation of AtPCS1-FLAG. In the first, radioincorporation of the [35S]Cys-labeled GSH or [3H]Gly-labeled GSH, respectively, into AtPCS1-FLAG was monitored by gel-filtration chromatography. AtPCS1-FLAG (0.5–1.0 nmol) was incubated in 200-μl reaction volumes containing either 3 mM [35S]Cys-labeled GSH (2 mM/cmol) or 3 mM [3H]Gly-labeled GSH (2 mM/cmol) with or without 50 μM CdCl2 in 100 mM buffer at 30 °C for 15 min, after which the reaction was terminated, and the acyl intermediate was stabilized by the addition of 1 ml of 100 mM sodium acetate buffer (pH 4.0) containing 6 μg guanidine HCl. To measure the radiolabeled associated with AtPCS1-FLAG, the terminated reactions were applied to a 1 × 25-cm column packed with Sephadex G-50, which was developed with 100 mM sodium acetate buffer (pH 4.0) containing 4 M guanidine HCl and rapid vacuum filtration through Immobilon-P transfer membrane disks monitored by gel-filtration chromatography. At the times indicated, the fractions were collected, and the [35S]Cys-labeled GSH (30–40 Ci/mmol) or [3H]Gly-labeled GSH (20–50 Ci/mmol) and the liquid scintillation mixture used in these experiments (CytosciNet) were purchased from PerkinElmer Life Sciences and ICN, respectively. All of the general reagents were obtained from Fisher, Research Organics, Inc., and Sigma.

RESULTS AND DISCUSSION

AtPCS1 Is a Dipeptidyltransferase

The question of whether AtPCS1-FLAG is a dipeptidyl- or tripeptidyltransferase was addressed in two ways: by determining whether the Gly residue of GSH is or is not retained during the synthesis of PC2 from PC2 and GSH (Method 1) and by determining whether des-Gly-PCs are or are not an immediate by-product of PC synthesis from PCs (Method 2).

Method 1—If AtPCS1-FLAG is a dipeptidyltransferase, the [3H] label on Gly should undergo elimination during the synthesis of PC3 from unlabeled PC2 and [3H]Gly-labeled GSH (Equation 5).

\[(\gamma\text{-EC})_2\text{G} + [\text{3H}]\text{G} \rightarrow (\gamma\text{-EC})_2\text{G} + [\text{3H}]\text{G}\]  \hspace{1cm} (Eq. 5)

By contrast, if AtPCS1-FLAG is a tripeptidyltransferase, the [3H] label of [3H]Gly-labeled GSH should undergo stoichiometric incorporation into the PC3 product (Equation 6).

\[(\gamma\text{-EC})_3\text{G} + [\text{3H}]\text{G} \rightarrow (\gamma\text{-EC})_3\text{G} + [\text{3H}]\text{G} + G\]  \hspace{1cm} (Eq. 6)

To distinguish between these two alternatives, unlabeled PC2 was incubated with [3H]Gly-labeled GSH, and the elimination or incorporation of [3H]Gly into PC2 was determined by RP-HPLC separation and liquid scintillation counting of the reaction products.

It is important to note that, although this is ostensibly the most direct way to determine whether GSH incorporation into the product is conservative or semi-dispersive, there are potential complications. Principal among these would be the rapid incorporation of [3H]Gly-labeled GSH into the PC2 pool during the incubations and the synthesis of radiolabeled PC2 from this and/or net synthesis of PC3 from PC2 without direct participation of GSH. The former complication would result in the net synthesis of [3H]Gly-labeled PC2 regardless of whether AtPCS1-FLAG is a dipeptidyl (Equation 7) or tripeptidyl (Equation 8) transpeptidase.

\[(\gamma\text{-EC})_2\text{G} + [\text{3H}]\text{G} \rightarrow (\gamma\text{-EC})_2\text{G} + [\text{3H}]\text{G} + [\text{EC}]\]  \hspace{1cm} (Eq. 7)

\[(\gamma\text{-EC})_3\text{G} + [\text{3H}]\text{G} \rightarrow (\gamma\text{-EC})_3\text{G} + [\text{3H}]\text{G} + [\text{EC}] + [\text{G}]\]  \hspace{1cm} (Eq. 8)

The second complication would result in the net synthesis of unlabeled PC3 regardless of whether AtPCS1-FLAG is a dipeptidyl (Equation 9) or tripeptidyl (Equation 10) transpeptidase.

\[(\gamma\text{-EC})_2\text{G} + (\gamma\text{-EC})_3\text{G} \rightarrow (\gamma\text{-EC})_2\text{G} + \gamma\text{ECG}\]  \hspace{1cm} (Eq. 9)

\[(\gamma\text{-EC})_3\text{G} + (\gamma\text{-EC})_3\text{G} \rightarrow (\gamma\text{-EC})_3\text{G} + \gamma\text{EC} + G\]  \hspace{1cm} (Eq. 10)

These complications were minimized and enumerated by measuring initial PC2 synthesis (synthesis before the incorporation of [3H]Gly into the PC2 pool from [3H]Gly-labeled GSH, which was also measured) by adding a 10-fold molar excess of [3H]Gly-labeled GSH over PC2 to favor the formation of PC3 from PC2 plus GSH rather than from PC2 alone and by performing parallel experiments using [35S]Cys-labeled GSH instead of [3H]Gly-labeled GSH as a donor to measure the incorporation of γ-Glu-Cys units into the PC2 and PC3 backbones.

The results of these analyses were conclusive (see Fig. 2). First, it was determined that when incubated in medium containing 1 mM GSH, 100 μM PC2, and 100 μM CdCl2, purified AtPCS1-FLAG catalyzed the net synthesis of PC3 with no detectable time lag, implying that the bulk of the synthesis measured was from the PC2 and GSH initially added to the assay system and not indirectly from PC2 derived from the GSH added. Second, it was determined that for incubation times of <6 min, 33% of the thiols in the PC3 product were [35S]Cys-labeled GSH and unlabeled PC2. Third, it was determined that when the precursors were
A. Dipeptidyl transfer

\[ \text{PC}_2 + \text{GSH} \rightarrow \text{PC}_3 \]

B. Tripeptidyl transfer

\[ \text{PC}_2 + \text{GSH} \rightarrow \text{des-Gly-PC}_2 \]

Fig. 1. PC synthase-catalyzed synthesis of PC$_3$ from PC$_2$ by dipeptidyl (A) or tripeptidyl (B) transfer. In dipeptidyl transfer (A), PC chain extension proceeds in the C- to N-terminal direction and is not associated with the production of des-Gly-PCs according to the general equation PC$_n$ + PC$_m$ + PC$_{m-1}$ + PC$_{m-2}$, where PC$_3$ = GSH. In tripeptidyl transfer (B), PC chain extension proceeds in the N- to C-terminal direction and is associated with the production of des-Gly-PCs according to the general equation PC$_n$ + PC$_m$ + PC$_{m-1}$ + des-Gly-PC$_{m-1}$ + G, where des-Gly-PC$_3$ = γ-Glu-Cys.

[3H]Gly-labeled GSH and PC$_2$, the PC$_3$ product was unlabeled over the same time span.

This pattern of synthesis and radioincorporation is uniquely attributable to a dipeptidyl transpeptidation reaction. The dipeptidyl model predicts that the net synthesis of PC$_3$ from PC$_2$ and [3H]Gly-labeled GSH would result in the total elimination of radiolabel concomitant with the liberation of free [3H]Gly, whereas the initial synthesis of PC$_3$ from PC$_2$ and [35S]Cys-labeled GSH would result in a 1:1 molar ratio of [35S]Cys to PC$_3$, indicating that one in three of the thiols in the product are labeled. This is precisely what was found for the shorter incubation times. By contrast, the tripeptidyl transpeptidase model predicts a 1:1 stoichiometric incorporation of [3H]Gly from [3H]Gly-labeled GSH and of [35S]Cys from [35S]Cys-labeled GSH into PC$_3$ concomitant with the liberation of unlabeled Gly from the outset of the reaction.

The incorporation of [3H]Gly into the PC$_3$ pool from the combination of [3H]Gly-labeled GSH with itself was evident from the outset, whereas the incorporation of [3H]Gly into the PC$_3$ pool was a later event (see Fig. 2), implying that the incorporation of [3H]Gly into PC$_3$ is contingent on its synthesis from a [3H]Gly-labeled PC$_2$ backbone. Thereafter, the [3H]Gly/PC$_2$, [3H]Gly/PC$_3$, and [35S]Cys/Cys in PC$_3$ ratios approached limiting values of 1.0 (see Fig. 2).

Method 2—The abstraction of a γ-Glu-Cys unit from any PC$_n$ by dipeptidyl transfer would yield another PC (PC$_{n-1}$) (Equation 3 and Fig. 1A), whereas the abstraction of a GSH unit from any PC$_n$ by tripeptidyl transfer would yield a des-Gly-PC (des-Gly-PC$_{n-1}$) (Equation 4 and Fig. 1B). Despite the potential of tripeptidyl transfer as a simple mechanism for the synthesis of des-Gly-PCs, in no case were these PC derivatives detectable when AtPCS1-FLAG catalyzed the synthesis of PCs from PCs in vitro, whether it was the synthesis of PC$_4$ (and PC$_2$) from PC$_3$ or PC$_5$ (and PC$_3$) from PC$_4$ (data not shown). This observation, in combination with the results of the stoichiometric analyses of [3H]Gly-labeled GSH and [35S]Cys-labeled GSH incorporation into PCs (Fig. 2), substantiates the dipeptidyl-transferase model and refutes the tripeptidyltransferase model.

AtPCS1 Undergoes γ-Glu-Cys Acylation

A logical extension of the demonstration that AtPCS1 is a dipeptidyltransferase and the notion of a substituted enzyme mechanism for the reaction catalyzed is the probable formation of a γ-Glu-Cys acyl-enzyme intermediate. That this is indeed the case is evident from the results shown in Figs. 3 and 4. Incubation of purified AtPCS1-FLAG with [35S]Cys-labeled GSH yielded radiolabeled protein, which, upon acid stabilization with 100 mM sodium acetate buffer (pH 4.0) and denaturation with 6 M guanidine HCl, eluted in the void volume after Sephadex G-50 gel-filtration chromatography (Fig. 3). As determined by SDS-PAGE and Western analysis of the column fractions with anti-FLAG monoclonal antibody M2, the 58-kDa AtPCS1-FLAG protein strictly comigrated with the [35S] label in...
the void volume at an approximate $[35S]$Cys/AtPCS1-FLAG ratio of 0.75 mol of γ-Glu-Cys/mol of AtPCS1-FLAG.

Incorporation of the $[35S]$ label into AtPCS1-FLAG was evident regardless of whether or not Cd$^{2+}$ was included in the reaction medium, but omission of AtPCS1-FLAG or substitution of $[35S]$Cys-labeled GSH with $[3H]$Gly-labeled GSH with or without Cd$^{2+}$ did not yield radiolabeled protein (Fig. 3). The dispensability of Cd$^{2+}$ for labeling with $[35S]$Cys-labeled GSH, despite its capacity to increase labeling to yield a ratio of $\sim 0.96$ mol of γ-Glu-Cys/mol of AtPCS1-FLAG (Fig. 3), implies that the metal ion is not an obligate requirement for primary acylation.

The inability of $[3H]$Gly-labeled GSH to substitute for $[35S]$Cys-labeled GSH implies, as would be predicted for a dipeptidyltransferase reaction, that the intermediate generated is a γ-Glu-Cys acyl-enzyme derivative, not a γ-Glu-Cys-Gly or Cys-Gly derivative.

Application of a methodology with increased time resolution, the membrane filter binding procedure, showed that the concentration of $[35S]$Cys-labeled GSH necessary for half-maximal acylation of AtPCS1-FLAG (2.95 ± 0.35 mM) is commensurate with the $K_{m}$ (GSH) for the low affinity site for steady-state PC$_{2}$ synthesis by the same preparation (13.6 ± 3.3 mM) (22). Under these conditions, when the time between the removal of the enzyme from the reaction medium and the enumeration of bound radiolabel was minimized by rapid filter binding of the samples, the apparent stoichiometry for acylation by free $[35S]$Cys-labeled GSH approached a value of 1 (0.95 ± 0.35 mol of γ-Glu-Cys/mol of AtPCS1-FLAG) (Fig. 4).

Cd$^{2+}$ Increases the Stoichiometry of $\gamma$-Glu-Cys Acylation

Despite their dispensability for the primary acylation of AtPCS1-FLAG by $[35S]$Cys-labeled GSH, heavy metal ions such as Cd$^{2+}$ were nevertheless required for net PC synthesis and maximal acylation of the enzyme. When measured against time, the stoichiometry of labeling of AtPCS1-FLAG with 3 mM free $[35S]$Cys-labeled GSH approached a value of 1 with only very low net PC synthesis (<0.1 nmol/mg), but the addition of 50 μM CdCl$_{2}$ to the same medium increased the stoichiometry of labeling to a value of >1.5 concomitant with the onset of PC synthesis (Fig. 5).

Two alternative schemes for AtPCS1-catalyzed PC synthesis are consistent with these results. In the first of these (Scheme A), acylation of the enzyme with γ-Glu-Cys by the low affinity substrate (free GSH), which is not accompanied by appreciable net PC synthesis, is considered to yield enzyme acylated at a single site that is not competent in net PC synthesis unless a second site undergoes covalent modification (γ-Glu-Cys acylation or otherwise) by the high affinity substrate (cadmium-GS$_{2}$) upon the addition of Cd$^{2+}$ to the reaction medium. In the second scheme (Scheme B), by extension of the analogy with the catalytic mechanism of serine and cysteine proteases, it is envisaged that the provision of cadmium-GS$_{2}$ promotes the net synthesis of PCs by direct condensation of the incoming $[35S]$-labeled GSH molecule with the acyl-enzyme substituent to form a doubly $[35S]$-labeled γ-Glu-Cys γ-Glu-Cys tetrahedral intermediate.

These two schemes are most readily distinguished by deter-
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mining whether the provision of [3H]Gly-labeled GSH instead of [35S]Cys-labeled GSH results in the labeling of enzyme at a 1:1 ratio in the presence (but not absence) of Cd2+. Second site-Glu-Cys acylation (Scheme A) would not result in labeling by [3H]Gly-labeled GSH, but the condensation of [3H]Gly-labeled GSH with Glu-Cys on the first site (Scheme B) would. When this criterion was applied, the outcome predicted from Scheme A was obtained. AtPCS1-FLAG did not undergo radiolabeling by [3H]Gly-labeled GSH whether Cd2+ was or was not present in the medium throughout the incubation (Fig. 2) or was added 10 min after the start of the incubation with [3H]Gly-labeled GSH when Cd2+ was present in the medium throughout the incubation (Fig. 2). AtPCS1-FLAG did not undergo radiolabeling by [3H]Gly-labeled GSH whether Cd2+ was or was not present in the medium throughout the incubation (Fig. 2) or was added 10 min after the start of the incubation with [3H]Gly-labeled GSH when Cd2+ was present in the medium throughout the incubation (Fig. 2).

AtPCS1-catalyzed Substrate Deglycylation

Cd2+-elicited acylation of the enzyme with γ-Glu-Cys and net PC synthesis were accompanied by accelerated deglycylation of the substrate. Provision of 3 mM free [3H]Gly-labeled GSH as the sole substrate resulted in little or no net PC2 synthesis and only relatively low levels of AtPCS1-FLAG-dependent [3H]Gly release (the equivalent of 25.6 nmol of [3H]Gly/nmol of enzyme after 10 min), at which point, further release approached a limit despite retention of 91.6 ± 2.5% of the [3H]Gly-labeled GSH added (Fig. 6C). In marked contrast, provision of the same concentration of [3H]Gly-labeled GSH plus 50 μM Cd2+ resulted in a >100-fold increase in the liberation of [3H]Gly, which approached a limit only after the consumption of >89.0 ± 3.5% GSH when the enzyme had already undergone acylation with γ-Glu-Cys at the first site in medium lacking metal ions (Fig. 5).
Fig. 7. Tentative model for the multisite γ-Glu-Cys acylation of AtPCS1 concomitant with the generation of free Gly, γ-Glu-Cys, and PC₄. The first step is the Cd²⁺-independent formation of enzyme acylated with γ-Glu-Cys at one site coincident with the cleavage of Gly from the first molecule of GSH. The second step is the Cd²⁺-dependent (or blocked thiol-dependent) γ-Glu-Cys acylation of the enzyme at a second site coincident with the cleavage of Gly from a second molecule of GSH (or PC₄). In the third step, which may or may not depend on the provision of Cd²⁺ (or a thiol peptide with a blocked thiol group), one of the γ-Glu-Cys units from the disubstituted enzyme intermediate is transferred to a third molecule of GSH (or PC₄) to generate the product, PC₄ (or PC₄₋₀) containing one additional γ-Glu-Cys repeat. In all three steps, one or more of the γ-Glu-Cys acyl-enzyme intermediates can undergo uncoupled hydrolysis to liberate γ-Glu-Cys and to yield free Gly in excess of the amount specifically required for PC chain extension. In the third step, the simple transfer of one of the two enzyme γ-Glu-Cys acyl groups to water is considered to contribute to the free energy input required for the formation of a new Cys–Glu peptide bond between the other enzyme-bound γ-Glu-Cys unit and GSH (or PC₄) in the PC₄ (or PC₄₋₀) product.

As would be predicted from the substrate requirements for PC synthesis (22), the reaction shown in Fig. 7 is not obligatorily dependent on the provision of heavy metal ions. Other GSH-based peptides, e.g. S-alkylglutathiones such as NEM-GS, will suffice. Not only was [³H]Gly-labeled NEM-GS incorporated into NEM-PC₂ by AtPCS1-FLAG in medium devoid of metal ions, but this reaction was also accompanied by the liberation of [³H]Gly in excess of that expected from the simple combination of two NEM-GS molecules/molecule of NEM-PC₂ generated (Fig. 8). Indeed, as would be expected if NEM-GS goes through the same reaction pathway as GSH and cadmium-GS₂, but with a lower efficiency of transfer of NEM-γ-Glu-Cys to the NEM-GS acceptor versus water, the molar ratio of Gly released to NEM-PC₂ synthesized was consistently >6 (Fig. 8).

Cys⁵⁶ Is Essential for First Site Acylation and Net PC Synthesis

A corollary of the finding that the time and concentration dependence of PC synthesis approximates substituted enzyme kinetics and that AtPCS1 is a dipeptidyl transpeptidase that forms γ-Glu-Cys acyl groups to water, the molar ratio of Gly released to NEM-PC₂ synthesized was consistently >6 (Fig. 8).

Subsumed in this model is the principle that it is the highly
sequence-conserved N-terminal half of AtPCS1 and other PC synthases that catalyzes the core reaction, whereas the more divergent C-terminal half participates in the heavy metal-mediated, but not obligatory augmentation of catalytic activity associated with the direct binding of metals to the enzyme (22). On this basis and the working hypothesis that the mechanism of formation of the acyl intermediate is analogous to those of serine and cysteine proteases, the mutagenesis experiments were directed at determining which Ser or Cys residues (if any) in this segment of the overall sequence might participate in the initial nucleophilic attack on the γ-Glu-Cys donor. Toward this end, each of the five Cys (Cys56, Cys90, Cys91, Cys109, and Cys113) and two Ser (Ser21 and Ser164) residues in the N-terminal half of AtPCS1 that are conserved between this protein and the other PC synthase clones (SpPCS, AtPCS2, TaPCS1, C. elegans PCS1, Brassica juncea PCS1 (GenBank®/EBI accession number CAC37692), Glycine max PCS1 (36), and Athyrium yokoscense PCS1 (accession number BAB64932)) were singly substituted. In all cases, the Cys residues were singly substituted with Ser and the Ser residues with Ala; and whenever an effect was not seen with the Cys-to-Ser substitutions, Cys-to-Ala substitutions were also introduced at the same positions to determine whether, in some instances, a serine hydroxyl group could substitute at least in part for a cysteiny1 sulfhydryl group.

To delineate the necessity of one or more of these residues for catalysis, pYES3-AtPCS1-FLAG constructs containing wild-type or mutant AtPCS1 were transformed into the Cd2+-hypersensitive yeast ycf1Δ strain DTY167 and probed at four levels: (i) by determining whether the pYES3-AtPCS1-FLAG construct in question suppresses the Cd2+-hypersensitive phenotype of yeast strain DTY167, (ii) by determining whether exposure of the yeast transformants to Cd2+ elicits the intracellular accumulation of PCs, (iii) by determining whether, in those cases in which neither Cd2+ tolerance nor Cd2+-dependent PC accumulation is conferred on the transformants, the capacity to synthesize PC in vitro is also abolished, and (iv) by determining whether, in those cases in which all of...
the foregoing are impaired, in vitro enzyme acylation is also affected.

These manipulations demonstrated that substitution of only one of this group of seven conserved residues had an appreciable effect on the PC synthetic activity of AtPCS1-FLAG and that this effect was essentially all or none. None of the Cys-to-Ser (or -Ala) or Ser-to-Ala substitutions, except for the C56S and C56A substitutions, decreased the capacity of heterologously expressed AtPCS1-FLAG to confer Cd\(^{2+}\) tolerance or to mediate PC synthesis (Figs. 9 and 10). C90S, C91S, C106S, and C113S mutant AtPCS1-FLAG conferred similar degrees of Cd\(^{2+}\) tolerance on DTY167 cells as their wild-type equivalent (Fig. 9A), and this was associated with the Cd\(^{2+}\)-dependent accumulation of near wild-type levels of intracellular PCs (Fig. 9B). The same was seen for C90A, C91A, C109A, and C113A mutant AtPCS1-FLAG (data not shown). In strict contrast, C56S AtPCS1-FLAG conferred a degree of Cd\(^{2+}\) tolerance only marginally greater than that of the empty pYES3 vector control (Fig. 9A) and elicited negligible intracellular PC accumulation (Fig. 9B).

The loss of activity associated with the C56S substitution is not attributable to a decrease in the amount, stability, or integrity of the translation product. The DTY167/pYES3-AtPCS1-FLAG transformants containing the C56S mutant contained wild-type steady-state levels of the FLAG-tagged 58-kDa polypeptide as judged by SDS-PAGE and Western analysis of whole cell extracts with anti-FLAG monoclonal antibody M2, and the only mutant to exhibit a decrease in the steady-state levels of this polypeptide (C90S) achieved intracellular PC levels similar to those obtained with the wild-type controls (Fig. 9B). In no case was there any evidence of a change in the electrophoretic mobility of the anti-FLAG antibody-reactive band as a result of mutagenesis (Fig. 9B), excluding these residues from further consideration in this context.

In vitro assays of the capacity of immunoaffinity-purified C56S mutant AtPCS1-FLAG to mediate Cd\(^{2+}\)-dependent PC synthesis from GSH verified that this amino acid substitution abolished catalytic activity (Fig. 11). Whereas wild-type AtPCS1-FLAG synthesized PC\(_2\) and PC\(_3\) at an aggregate rate of 32.1 ± 8.9 µmol/mg/min when assayed in medium containing 3.3 mM GSH and 50 µM CdCl\(_2\), assays of C56S mutant AtPCS1-FLAG under the same conditions yielded rates below the limits of detection (<0.01 µmol/mg/min) (Fig. 11). In combination, these results indicate that Cys\(^{56}\) (but none of the other conserved Cys residues or either of the conserved Ser residues in the N-terminal half of AtPCS1) satisfies the requirements of an active site residue.

A critical property of C56S mutant AtPCS1-FLAG that reinforces the contention that first site -Glu-Cys acylation of the wild-type enzyme is necessary for net PC synthesis is its inability to form this intermediate (Fig. 11). Unlike the wild-type

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**Fig. 10.** Effect of heterologously expressed wild-type and Ser-to-Ala mutant-substituted AtPCS1-FLAG on the Cd\(^{2+}\) hypersensitivity of mutant *S. cerevisiae* ccf1 strain DTY167. Strain DTY167 was transformed with the pYES3-AtPCS1-FLAG vector encoding wild-type AtPCS1 (□) or mutant AtPCS1 in which the Ser residue at position 21 (■) or 164 (□) was singly substituted with Ala or with the empty pYES3 vector (●). Cd\(^{2+}\) hypersensitivity was assessed as described in the legend to Fig. 9.

**Fig. 11.** In vitro PC synthetic activity and susceptibility of wild-type and C56S-substituted AtPCS1-FLAG to -Glu-Cys acylation. PC synthetic activity (□) was assayed by incubating immunoaffinity-purified wild-type AtPCS1-FLAG (WT, 1 µg/ml) or C56S-substituted AtPCS1-FLAG (1 µg/ml) with GSH (3.3 mM) and CdCl\(_2\) (50 µM) at 30°C for 10 min. Acylation of purified wild-type and C56S-substituted AtPCS1-FLAG by GSH in the absence (□) or presence (●) of Cd\(^{2+}\) was assayed by incubation of enzyme (27.3 µg) with [\(^{35}\)S]Cys-labeled GSH (3 mM) in 100 mM HEPES/BTP buffer (pH 8.0) with or without CdCl\(_2\) (50 µM) for 15 min before terminating the reaction and measuring enzyme acylation as described in the legend to Fig. 4.
enzyme, which underwent γ-Glu-Cys acylation by free \[^{35}S\]Cys-labeled GSH at a stoichiometry of 0.75 ± 0.10 mol of γ-Glu-Cys/mol of AtPCS1-FLAG, the C56S-substituted enzyme underwent little or no γ-Glu-Cys acylation (stoichiometry < 0.13 ± 0.02 mol of γ-Glu-Cys/mol of AtPCS1-FLAG) (Fig. 11). Significantly, however, although abolition of this enzyme modification was strictly associated with abolition of PC synthetic activity, it was not accompanied by abolition of acylation at the second site. Incubation of C56S mutant AtPCS1-FLAG in medium containing both GSH and 80 μM Cd\(^{2+}\) resulted in acylation of the enzyme at a stoichiometry of 0.51 ± 0.05 mol of γ-Glu-Cys/mol of AtPCS1-FLAG, a value commensurate with the increment (0.78 mol of γ-Glu-Cys/mol of AtPCS1-FLAG) upon the addition of Cd\(^{2+}\) to enzyme that had already undergone acylation by free \[^{35}S\]Cys-labeled GSH. The implication is that the initial Cys\(^{56}\)-dependent, Cd\(^{2+}\)-independent first site acylation is not a prerequisite for the subsequent Cys\(^{56}\)-independent second site acylation of AtPCS1, but is essential for net PC synthesis, which, in turn, might imply that the condensation of enzyme-bound γ-Glu-Cys with GSH for the synthesis of PC\(_2\) is contingent on acylation of the enzyme at both sites.

**CONCLUSIONS**

Five central conclusions can be derived from these investigations. The first, which is shown directly for the first time rather than by indirect implication, is that PC synthase is a dipeptidyltransf erase (not a tripeptidy ltransf erase) that catalyzes the net transfer of a γ-Glu-Cys unit from one thiol peptide to another or to a previously synthesized PC molecule to mediate chain extension in the N- to C-terminal direction. The second, which is a corollary of the first, is that des-Gly-PCs are not generated as immediate by-products of PC synthase action, or if they are, it is neither an automatic nor necessary consequence of de novo PC synthesis. The third, which is predicted in part from the substituted enzyme kinetics exhibited by AtPCS1 and in part from the necessity for an energy source for the formation of a new peptide bond during each cycle of PC chain extension, is that the enzyme undergoes γ-Glu-Cys acylation at two sites during catalysis. Acylation at both sites is necessary for net PC synthesis, but the requirements for each acylation reaction are distinguishable. The provision of free GSH in media devoid of metal ions is sufficient for acylation of the first site, a reaction that is not accompanied by appreciable net PC synthesis, whereas acylation of the second site necessitates the provision of heavy metal ions, as exemplified by Cd\(^{2+}\), and is accompanied by the net synthesis of PCs. If the contention that AtPCS1 catalyzes a bisubstrate reaction in which free GSH and cadmium-GS\(_2\) act as low and high affinity cosubstrates, respectively (22), is generally applicable, it is likely that acylation of the first site is by GSH and that of the second site is by cadmium-GS\(_2\). The fourth conclusion is that the first site of acylation corresponds to or is at least tightly coupled with Cys\(^{56}\). Of the five conserved Cys residues in the putative catalytic sector of AtPCS1, only one (Cys\(^{56}\)), when substituted with a Ser or Ala residue, abolishes the capacity of heterologously expressed AtPCS1-FLAG to suppress the Cd\(^{2+}\)-hyper-sensitivity of yeast strain DTY167 concomitant with the abolition of both in vivo and in vitro PC synthetic activity and the amenability of the enzyme to direct acylation by free GSH. Cys\(^{56}\) therefore satisfies the requirements of an active site residue responsible for the initial nucleophilic attack on the scissile bond of the GSH (or PC) γ-Glu-Cys donor, in accord with the cysteine protease model, such that AtPCS1-mediated catalysis is initiated by the formation of a high energy thioester intermediate. Acylation at this site, although apparently required for net PC synthesis, does not seem to be a prerequisite for Cd\(^{2+}\)-dependent acylation of the second site in that C56S and C56A mutant AtPCS1-FLAG will incorporate γ-Glu-Cys at this site, albeit at lower efficiency compared with wild-type enzyme. Evidently, since mutation of either of the conserved Ser residues in the N-terminal half of AtPCS1 has little or no effect on the enzyme, conserved residues other than Cys and Ser, e.g. Thr and Tyr residues capable of forming oxyanions, participate in acylation of the second site.

In drawing these conclusions, it is important to take account of an alternative role proposed for Cys residues in the catalytic mechanism of PC synthases. This is the possibility, based on the scheme originally presented by Grill et al. (13) and elaborated by Cobbett (38), that the activation of catalysis depends on the binding of heavy metal ions such as Cd\(^{2+}\) to essential Cys-containing metal-binding sites in the N-terminal catalytic sector of the enzyme. In support of this possibility, Maier et al. (37) provide two classes of finding. The first is that, when synthetic libraries consisting of overlapping 13-residue peptides whose sequences are based on those of SpPCS and TaPCS1 are screened for \(^{109}\)Cd\(^{2+}\) binding, several clusters of contiguous peptides with binding activity are discernible. Of particular relevance to the studies we have described here, most of the sequences that the contiguous overlapping peptides delimit, so-called “core peptides,” encompass the Cys residues that are subject to conservation between different PC synthase sequences. These are the SpPCS and TaPCS1 equivalents of AtPCS1 Cys\(^{56}\), Cys\(^{90}\), Cys\(^{91}\), Cys\(^{109}\), and Cys\(^{113}\). And, in at least a subset of these core peptides, the Cys residues concerned are capable of contributing to binding in so far as Cys-to-Ala substitutions at these positions abolish or diminish \(^{109}\)Cd\(^{2+}\) binding in vitro. The second class of finding is that single substitutions of each of four of five of the conserved Cys residues in the N-terminal sector (Cys\(^{56}\), Cys\(^{90}\), Cys\(^{91}\), and Cys\(^{109}\) in AtPCS1 and their equivalents in SpPCS) with Ala residues yields enzyme that, when expressed in *S. pombe Δpcs* strain Sp27, exerts little or no suppression of the Cd\(^{2+}\)-hypersensitive phenotype whether assayed at the level of both cell growth and intracellular PC accumulation in media containing Cd\(^{2+}\) in the case of SpPCS or at the level of cell growth in media containing Cd\(^{2+}\) alone in the case of AtPCS1. The implication is obvious. These residues behave as if not only essential for metal binding, but also for catalysis, which, in turn, implies that metal binding is an essential prerequisite for catalysis.

Although elegant and in many ways informative, the full significance of the investigations of Maier et al. (37) remains to be determined. At the level of mapping the distribution of metal-binding motifs, it cannot be decided from the results presented whether the Cd\(^{2+}\) binding detected would or could be operative under the conditions that prevail in *in vivo* or even in *vitro* when PC synthetic activity is maximum because the experiments were performed at free Cd\(^{2+}\) concentrations (10 μM) far in excess of those that are achieved cytosolically under physiological conditions or that can be achieved when GSH is present at the millimolar concentrations necessary for net PC synthesis. Given that, in a standard PC synthase reaction medium containing 25 μM CdCl\(_2\) and 3.3 mM GSH, >98% of the total Cd\(^{2+}\) is complexed with GSH as cadmium-GS\(_2\), such that the concentration of free Cd\(^{2+}\) is only of the order of 10\(^{-13}\) M (22), a value some 8 orders of magnitude lower than that used for the peptide binding assays and one that is probably an overestimate of the free concentration in the cytosol, which is replete with soft metal-reactive thiols), it is unlikely that free Cd\(^{2+}\) concentrations in the micromolar range simulate the conditions optimal for PC synthase action. Likewise, although intact AtPCS1-FLAG binds \(^{109}\)Cd\(^{2+}\) at high capacity (\(B_{\text{max}} = 7.09 \pm 0.94\)) (incidentally, at a stoichiometry congruent with the total of seven putative binding sites identified by the pep
tide mapping studies of TaPCS1 under identical buffer conditions (37)), the ligand binding constant (K_L = 0.54 ± 0.21 μM) is 6 orders of magnitude too high to account for activation of the enzyme under standard conditions (22). More probable, therefore, is the possibility that the binding detected in the screens of the PC synthase peptide libraries is instead attributable to the propensity of most free cysteinyl thiolis for rapid reaction with soft ions such as Cd^{2+} to generate metal thiolates. We suspect that the Cd^{2+} binding measured by Maier et al. (37) is more a reflection of the capacity of heavy metals to augment PC synthase activity in the presence of substrate-active S-alkyl derivatives rather than a strict requirement for heavy metals for core catalysis (22). This would reconcile at least in part our findings with those of Maier et al. (37).

Where there seem to be insuperable difficulties, however, is in reconciling the two sets of mutagenesis results. Although Maier et al. (37) found that Cys-to-Ala mutagenesis of each of the residues at positions 56, 90, 109, and 113 yields enzyme that, when expressed heterologously in S. pombe, confers little or no increase in Cd^{2+} tolerance over that conferred by empty expression vector, suggesting that all four residues are required for catalysis, we found that Cys-to-Ala or -Ser mutagenesis of only one of these residues (Cys56) abolished the suppression of Cd^{2+} hypersensitivity and Cd^{2+}-dependent intracellular PC accumulation otherwise conferred by the heterologous expression of wild-type AtPCS1-FLAG in S. cerevisiae. We have repeated our experiments many times, repeatedly double-checked the fidelity of the constructs used by direct sequencing, and consistently obtained the same results. That said, closer inspection of the results published by Maier et al. (37) does, however, reveal that of the four loss-of-function phenotypes noted, that of the C56A mutant is the most striking. Although the C90A, C109A, and C113A mutants confer less tolerance than the wild-type enzyme when expressed in S. pombe, they nevertheless confer some tolerance versus expression vector of the C56A mutant or transformation with empty expression vector.

If substrate peptide bond cleavage is to be the sole source of energy for PC synthetic peptide bond formation, a minimum of two enzyme acylations per catalytic cycle is necessitated. Unlike the two post-translational peptide synthetic reactions immediately upstream, the synthesis of γ-Glu-Cys and GSH catalyzed by γ-glutamylcysteine synthetase and GSH synthetase, respectively, both of which consume 1 mol of ATP/mol of product generated (L-Glu + l-Cys + ATP → γ-Glu-Cys + ADP + P_i, γ-Glu-Cys + L-Gly + ATP → GSH + ADP + P_i), PC synthase-catalyzed PC synthesis is neither sensitive nor subject to direct energization by ATP in vitro (data not shown). It is therefore presumed that, since the energy required for the formation of a peptide bond is ~20 kJ/mol (39) and the thermodynamic efficiency of most biochemical reactions is 30–60%, PC synthase must harness the energy from the deacylation of a minimum of two GSH molecules for each γ-Glu-Cys unit that is added to the N terminus of the growing PC chain. This would explain the necessity for the γ-Glu-Cys acylation of two sites on the enzyme per γ-Glu-Cys unit incorporated per catalytic cycle. To account for GSH deacylation in excess of this amount, it is assumed that, in all steps of the enzyme γ-Glu-Cys acylation cycle, water can serve the role of γ-Glu-Cys acceptor, liberating γ-Glu-Cys and free gly in excess of the amounts required for PC chain extension, a process that is accelerated by the provision of metal-GSH thiolates or S-alkyl-GS derivatives containing blocked thiol groups as exemplified by cadmium-GSs and NEM-GSs, respectively.

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