REVIEW

Phytochelatin synthase: of a protease a peptide polymerase made

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Of the mechanisms known to protect vascular plants and some algae, fungi and invertebrates from the toxic effects of non-essential heavy metals such as As, Cd or Hg, one of the most sophisticated is the enzyme-catalyzed synthesis of phytochelatins (PCs). PCs, \((\gamma\text{-Glu-Cys})_n\text{Gly}\) polymers, which serve as high-affinity, thiol-rich cellular chelators and contribute to the detoxification of heavy metal ions, are derived from glutathione (GSH; \(\gamma\text{-Glu-Cys-Gly}\)) and related thiols in a reaction catalyzed by phytochelatin synthases (PC synthases, EC 2.3.2.15). Using the enzyme from *Arabidopsis thaliana* (AtPCS1) as a model, the reasoning and experiments behind the conclusion that PC synthases are novel papain-like Cys protease superfamily members are presented. The status of \(S\)-substituted GSH derivatives as generic PC synthase substrates and the sufficiency of the N-terminal domain of the enzyme from eukaryotic and its half-size equivalents from prokaryotic sources, for net PC synthesis and deglycylation of GSH and its derivatives, respectively, are emphasized. The question of the common need or needs met by PC synthases and their homologs is discussed. Of the schemes proposed to account for the combined protease and peptide polymerase capabilities of the eukaryotic enzymes vs the limited protease capabilities of the prokaryotic enzymes, two that will be considered are the storage and homeostasis of essential heavy metals in eukaryotes and the metabolism of \(S\)-substituted GSH derivatives in both eukaryotes and prokaryotes.

Introduction

Essential heavy metals, such as Zn and Cu, and non-essential heavy metals, such as As, Cd and Hg, can pose acute problems for organisms. At appropriate levels, essential heavy metals are required as cofactors in ligand interactions and/or redox reactions, as well as for charge stabilization, charge shielding and water ionization in biocatalysis (Voet and Voet 2004). However, supraoptimal levels of essential heavy metals and submicro- to micromolar concentrations of non-essential heavy metals are usually toxic because they displace endogenous metal cofactors, heavy or otherwise, from their cellular binding sites, undergo aberrant capping reactions with the thiol groups of proteins and thiol-containing coenzymes, and promote the formation of active oxygen species (Rea et al. 2004).

Three classes of peptides, glutathione (GSH), metallothioneins and phytochelatins (PCs), have been implicated in heavy metal detoxification and homeostasis in plants (Cobbett and Goldsbrough 2002), but the ones that will be dealt with here are PCs and their immediate

Abbreviations – GSH, glutathione \((\gamma\text{-Glu-Cys-Gly})\); hGSH, homoglutathione \((\gamma\text{-Glu-Cys-}[\beta\text{-Ala}])\); hPC, homo-phytochelatin \([\gamma\text{-Glu-Cys}]_n(\beta\text{-Ala})\); MS, Murashige and Skoog; PC, phytochelatin \([\gamma\text{-Glu-Cys}]_n\text{Gly}\); PCS, gene encoding phytochelatin synthase.
precursor GSH. Derived from GSH and related thiols in a γ-glutamylcysteineyl transpeptidation reaction (Löffler et al. 1989, Vatamaniuk et al. 2004) catalyzed by PC synthases (Grill et al. 1989), PCs have the general structure (γ-Glu-Cys)nXaa, where Xaa is usually Gly. First discovered in the fission yeast Schizosaccharomyces pombe (Kondo et al. 1984) and in cell cultures of Rauvolfia serpentine (Grill et al. 1985), PCs which have since been found in all vascular plants investigated as well as some other fungi, algae, diatoms and invertebrates, are considered to mediate the high-affinity binding and promote the vacuolysosomal sequestration of heavy metals.

It is more than two decades since the partial purification of enzyme preparations from plant sources capable of catalyzing the synthesis of PCs (Grill et al. 1989) and just over a decade since elucidation of the molecular identity of the enzyme through the independent cloning and characterization of genes encoding PC synthases from plant, fungal (Clemens et al. 1999, Ha et al. 1999, Vatamaniuk et al. 1999) and invertebrate sources (Clemens et al. 2001, Vatamaniuk et al. 2001). Originally isolated from Arabidopsis thaliana, S. pombe, wheat (Triticum aestivum) and the nematode worm Caenorhabditis elegans, these genes (designated AtPCS1, SpPCS, TaPCS1 and CePCS1) encode 50–55 kDa polypeptides bearing 40–50% sequence identity to each other. By all criteria these genes encode PC synthases. PC-deficient Arabidopsis cad1 mutants, which are hypersensitive to As, Cd or Hg in the growth medium (Howden et al. 1995), are mutated in AtPCS1 (Ha et al. 1999); heterologous expression of AtPCS1 in the budding yeast Saccharomyces cerevisiae, an organism that lacks endogenous PCS (gene encoding phytochelatin synthase) homologs and does not otherwise synthesize PCs, confers tolerance concomitant with Cd2+-dependent intracellular PC accumulation (Vatamaniuk et al. 1999); SpPCS gene disruptants of S. pombe, which cannot synthesize PCs, are, unlike their relatively metal-tolerant isogenic wild types, acutely sensitive to As, Cd and Hg in the growth medium (Clemens et al. 1999); targeted suppression of expression of the C. elegans ce-pcs-1 gene by RNAi confers a Cdd+-hypersensitive phenotype (Vatamaniuk et al. 2001). The capacity of cell-free extracts from AtPCS1- or SpPCS-transformed cells of Escherichia coli (Ha et al. 1999) and immunopurified epitope-tagged AtPCS1 and CePCS1 for heavy metal-activated synthesis of both short- and long-chain PCs from GSH in vitro (Vatamaniuk et al. 1999, 2001) establishes that AtPCS1 and SpPCSs, and by implication their equivalents from other sources, are not only necessary but also sufficient for PC biosynthesis.

This review is concerned with the fundamentals of PC biosynthesis, what is known of the catalytic mechanism of PC synthases, and of the chemistry underlying and the enzyme residues necessary for PC synthesis and related processes. These considerations represent an unusually fruitful intersection of conventional enzyme kinetic, protein chemical and site-directed mutagenic experiments with computational bioinformatic and X-ray crystallographic approaches to make it plain that what started out as a problem in heavy metal detoxification has its roots in protease biochemistry and the various transactions in which GSH and its derivatives participate. It is against this background that the question of the roles played by PC synthases and their homologs is addressed.

**Blocked substrate thiols and activation by heavy metals**

A biochemically baffling yet physiologically critical property of eukaryotic PC synthases is the susceptibility of the reaction catalyzed to activation by heavy metals. It is through the activation of PC synthase-catalyzed PC synthesis by heavy metals, agents that poison most enzymes, that some eukaryotes can mount a PC-based response to heavy metals. Although, it was originally thought that the activation of eukaryotic PC synthases derives from the direct binding of metal ions to residues, possibly Cys residues, in the sequence-conserved N-terminal domain of the enzyme, this is probably not the case. Unique properties for PC synthase analogous to those of carbonic anhydrase 2 from the marine diatom Thalassiosira weissflogii which deploys Cd2+ instead of Zn2+ as metal cofactor (Lane and Morel 2000), need not be invoked to reconcile the fact that three of the most potent activators of in vitro PC synthesis, Cd2+, Hg2+ and As3+ (Vatamaniuk et al. 2000), also rank among the most noxious thiol-active inhibitors of the majority of enzymes because the dominant effects of the metal ions in question on core catalysis appear to be exerted at the substrate not the enzyme level. Heavy metals ligate substrate, not enzyme, thiols in that AtPCS1-catalyzed PC synthesis approximates bisubstrate kinetics such that micromolar concentrations of heavy metal-GSH thiolate, for instance bis(glutathionato)cadmium (Cd.GS2−), and millimolar concentrations of free GSH, respectively, act as low and high Km cosubstrates even under conditions at which the concentrations of free metal are subpicomolar. As indicated by the facility of AtPCS1 for the net synthesis of S-alkyl-PCs from S-alkylglutathiones at high rates in media devoid of metals, heavy metals are dispensable for catalysis, providing that the thiol groups on at least one of the substrates is(are) blocked (Vatamaniuk et al. 2000). While GSH and its metal...
thiolate are ordinarily required for maximal synthetic activity, other compounds, for instance S-substituted GSH derivatives, can substitute for both in such a way as to overcome the enzyme’s requirement for heavy metals, implying that the decisive factor for core catalysis is the provision of GSH-like substrates containing blocked thiol groups.

A kinetically economical scheme of this type brings with it an implication of considerable potential physiological significance. This is the possibility that the cystolic concentration of free metal ion need not increase even transitorily for net PC synthesis. Given that the complexes formed between heavy metals and thiols are among the most stable known and GSH is an extraordinarily abundant thiol peptide found at steady-state concentrations of between 0.1 and 10 mM (Rabenstein 1989), any soft metal that gains access to the cytosol would be expected to be incorporated into the corresponding thiolate. The GSH thiolates so formed, because of the moderately high and constitutive expression of PCS genes (Cobbett and Goldsbrough 2002), would in turn be incorporated into derivatives, PCs, which bind heavy metals with even higher affinity (Zenk 1996).

On the basis of their investigations of AtPCS1 and its equivalent from soybean (Glycine max), which like many other legumes deploys homoglutathionyl (hGSH; γ-Glu-Cys-β-Ala) instead of GSH for the synthesis of homo-PCs [hPCs: (γ-Glu-Cys)ₙ(β-Ala) polymers], Oven et al. (2002) dispute the notion that blocked thiols, alone, are sufficient for core catalysis. Principal among their concerns are two observations. The first is that, when measured as a function of increasing Cd²⁺ concentration in the range 0.01–0.5 mM at a fixed concentration of GSH, the activity of AtPCS1 is minimal when Cd²⁺ is greatest. While this is the case, it is not because, as Oven et al. (2002) contend, Cd.GS₂ is not a substrate for the enzyme; quite the contrary. It is because, as Vatamaniuk et al. (2000) explain, the enzyme catalyzes a bisubstrate reaction such that when Cd²⁺ is provided in excess, generation of one of the substrates, CdGS₂, at the expense of the other, free GSH, stalls the reaction. The other concern of Oven et al. (2002) is that not only heavy metal-hGSH complexes but also other heavy metal-thiol complexes, for instance, those of Cd²⁺ with 2-mercaptoethanol or Cys, that are not substrates for the enzyme, contribute to the activation of GmhPCS1. While this finding is of interest and might be consistent with the participation of bulk phase thiols in shuttling heavy metals to other, substrate-active thiols or to the auxiliary heavy metal-sensing C-terminal domain of the enzyme discussed below, it does not preclude Cd.GS₂ as a substrate. It is more likely that the activation of GmhPCS1 exerted by thiols other than hGSH under these conditions is attributable to the unusually high (millimolar) concentrations of heavy metal ions that were used, concentrations that would necessitate appreciable chelation by thiols, regardless of whether they could or could not act as substrates in order to alleviate the inhibitory thiol capping to which the enzyme would otherwise be subject.

A γ-Glu-Cys acyl-enzyme intermediate

Steady-state heavy metal-activated PC synthesis from GSH approximates ping-pong kinetics (Vatamaniuk et al. 2000). The reaction catalyzed by PC synthase proceeds through a substituted enzyme intermediate in which the group from the first substrate, the donor (e.g. GSH), is transferred twice, first from the donor to the free enzyme, then from the substituted enzyme to the second substrate, the acceptor (e.g. Cd.GS₂), in a double-displacement reaction, rather than through formation of a ternary, GSH/Cd.GS₂/enzyme, complex and direct transfer of the group in question from the donor to the acceptor (Vatamaniuk et al. 2000). Knowing that at least one peptide bond must be cleaved and one new peptide bond formed for the transfer of a γ-Glu-Cys unit from the donor to the acceptor, it is probable that the substituted enzyme intermediate is a γ-Glu-Cys derivative formed during cleavage of the Cys-Gly peptide bond of the donor. A two-step reaction of the following type might therefore be envisaged for the synthesis of PC₂ from free GSH and Cd.GS₂:

\[
\text{γ-Glu-Cys-Gly} + \text{PCS} \rightarrow \text{γ-Glu-Cys-PCS} + \text{Gly}
\]

\[
\text{γ-Glu-Cys-PCS} + \text{Cd.(γ-Glu-Cys-Gly)} \rightarrow \text{PCS} + \text{Cd.γ-Glu-Cys-γ-Glu-Cys-Gly} + \text{γ-Glu-Cys-Gly}
\]

In agreement with these deductions, PC synthase is susceptible to a γ-Glu-Cys acylation, at two sites (as it turns out) (Vatamaniuk et al. 2004). Whereas provision of GSH in media devoid of metal ions is sufficient for acylation at the first site with kinetics consistent with γ-Glu-Cys transfer consequent on the binding of free GSH to the high Km site inferred from the kinetics of Cd²⁺-activated PC synthesis (Vatamaniuk et al. 2000), γ-Glu-Cys acylation of the second site is seen only after the provision of Cd²⁺.

It should be stressed that although it was initially speculated that Cd²⁺-dependent acylation at the second site is essential for PC synthesis (Vatamaniuk et al. 2004), in that the onset of net synthesis coincides with the provision of Cd²⁺, this interpretation is not correct. The C-terminal domain of PC synthase although
essential for Cd\(^{2+}\)-dependent acylation at the second site is dispensable for core catalysis. Purified truncated AtPCS1 polypeptide, AtPCS1_221tr, containing only the first 221 N-terminal amino acid residues (the sequence-conserved N-terminal domain but not the C-terminal domain; for structure of AtPCS1_221tr refer to Fig. 4) undergoes γ-Glu-Cys acylation at only one site despite its capacity for the net synthesis of PCs from GSH in media containing Cd\(^{2+}\) or the synthesis of S-methyl PCs from S-methylglutathione in media devoid of heavy metals (Romanyuk et al. 2006). The onset of net PC synthesis from GSH after Cd\(^{2+}\)-dependent acylation of the second site is not indicative of a requirement for acylation at this site for catalysis but is instead simply a consequence of provision of the second substrate, Cd.GS\(_2\), for the bisubstrate reaction.

An important corollary follows from the reaction chemistry of AtPCS1, specifically its facility for site-specific peptide bond cleavage and the formation of a γ-Glu-Cys acyl intermediate. Namely, that it is probable initial nucleophilic attack on the scissile bond of the first substrate is by an enzyme hydroxyl-derived oxanion or thiol-derived thiolate anion to yield a γ-Glu-Cys-enzyme oxyester or thioester. In other words, a catalytic mechanism for PC synthase analogous to those of canonical Ser or Cys proteases (Kraut 1977, Kamphius et al. 1985) is invoked.

**A Cys protease-like active site residue**

Site-directed mutagenesis experiments directed at determining which Cys or Ser residues, if any, participate in nucleophilic attack on the γ-Glu-Cys donor by AtPCS1 clearly show the necessity of a Cys residue (Vatamaniuk et al. 2004). Of the five conserved Cys residues in the N-terminal domain of AtPCS1, only one, Cys\(^{56}\), when substituted by a Ser or Ala residue, abolishes the capacity of the heterologously expressed enzyme to suppress Cd\(^{2+}\) hypersensitivity, catalyze PC synthesis and undergo direct Cd\(^{2+}\)-independent acylation by GSH. In contrast, substitution of either of the two conserved Ser residues in this domain of the enzyme has little or no effect on the metal tolerance conferred by or catalytic activity of AtPCS1. It is notable that the abolition of PC synthetic activity and acylation of the first site in C56S and C56A mutants is not accompanied by abolition of acylation at the second site (Vatamaniuk et al. 2004). Evidently, Cys\(^{56}\) satisfies the requirements of a Cys protease-like active site residue, presumably one that undergoes Cd\(^{2+}\)-independent acylation with γ-Glu-Cys to yield an enzyme thioester, but acylation at this site is not a prerequisite for Cd\(^{2+}\)-dependent acylation at the second site.

**PC synthase half-molecules**

PC synthase-like polypeptides are much more widely distributed, albeit sporadically, than was suspected when AtPCS1, TaPCS1, SpPCS and CePCS1 were first cloned. Systematic sequence database searches disclose PCS-like genes in representatives of all of the known eukaryotic kingdoms (Clemens 2006, Clemens and Peršoh 2009). Genes encoding PCS-like polypeptides are to be found in some diatoms, some downy mildews for instance Phytophthora infestans, some ciliates such as Tetrahymena thermophila, as well as representative chordates, echinoderms, annelids and flatworms (Clemens and Peršoh 2009), including those responsible for such prevalent and debilitating human diseases as schistosomiasis (Vatamaniuk et al. 2002). Intriguing as this finding is, there is one other that came from database searches of this type which was to prove to be of considerable strategic significance for understanding the catalytic mechanism of this class of enzymes. This was the realization that PC synthase-like polypeptides are to be found in prokaryotes (Rea et al. 2004). The sequences from prokaryotes, five of cyanobacterial (including two Nostoc species; Rea et al. 2004, Tsuji et al. 2004, Clemens and Peršoh 2009) and five of proteobacterial origin (Rea et al. 2004, Clemens and Peršoh 2009), are approximately one-half the length of their cognates from eukaryotes (220–237 compared to 421–506 amino acid residues) because they lack the more sequence-variable C-terminal domain. And, notwithstanding the relatively low (22–36%) sequence identities between the prokaryotic enzymes and the N-terminal domains of the eukaryotic enzymes, which in of itself does not guarantee a catalytic equivalence, the one prokaryotic homolog whose activity has been examined, the alr0975 protein (‘NsPCS’) from Nostoc sp. PCC 7120, does catalyze a partial reaction catalyzed by canonical PC synthases: the deglycylation of GSH to γ-Glu-Cys (Harada et al. 2004, Tsuji et al. 2004).

**Papain’s distant cousin**

Appreciation of the fundamental similarity of the N-terminal half of eukaryotic PC synthases with their half-molecule cognates from prokaryotes has been instrumental in the assembly of a representative subset of sequences for broader protein sequence and structure database searches. In particular, the FFA03 method for the detection of distant homologies (Rychlewski et al. 2000) when applied to the extended PC synthase family, reveals clear matches with Clan CA Cys proteases as exemplified by the archetype, papain, from Carica
papaya fruit and latex, staphopain A an extracellular enzyme from *Staphylococcus aureus*, and several of the lysosomal cathepsins, for instance, cruzain from animal sources (Fig. 1) (Rea et al. 2004, Romanyuk et al. 2006). While it is important not to overextend the conclusions drawn from alignments of this type, several independent considerations further support the idea that PC synthases and Clan CA Cys proteases are distant cousins. First, the partial reactions catalyzed by PC synthases and Cys proteases bear an uncanny resemblance. Second, both classes contain a Cys residue essential for peptidyl acylation of the enzyme in the first phase of the catalytic cycle. In AtPCS1, this residue appears to correspond to Cys$^{56}$ (Vatamaniuk et al. 2004); in papain, for example, it corresponds to Cys$^{55}$ (Drenth et al. 1976). Third, the enhanced nucleophilicity of the active site Cys residues of Cys proteases arise from their immediate proximity to a His residue in the native protein, and it is the Cys$^{56}$ and His$^{162}$ residues of AtPCS1 that align precisely with the Cys and His residues of the Clan CA Cys proteases, residues 25 and 159, respectively, in papain (Drenth et al. 1976) (Fig. 1). Fourth, the third Cys protease catalytic triad residue, Asn$^{175}$ in papain (Vernet et al. 1995), aligns with a conserved Asp residue in the PC synthase family, residue 180 in AtPCS1 (Fig. 1). This is a permissible substitution from a catalytic standpoint in that in several members of Clan CA the third catalytic triad residue is an Asp instead of an Asn (Barrett and Rawlings 2001). Finally, as would be expected of divergent evolution from a common ancestor rather than convergent evolution from unrelated progenitors, the key residues shared by Clan CA proteases and PC synthases are not only in register but also in the same absolute order in both sets of sequences (Fig. 1).

**AtPCS1 mutants and NsPCS crystals**

The results of directed mutagenesis are in remarkably close agreement with the predictions from FFAS03-based sequence profile matching (Fig. 1). Of a total of 19 residues in the N-terminal domain of AtPCS1 that were substituted, only two in addition to Cys$^{56}$ (above) – His$^{162}$ and Asp$^{180}$ – are essential for catalysis (Vatamaniuk et al. 2004, Romanyuk et al. 2006). Of the three His residues that were substituted with Ala, His$^{162}$ which is fully conserved, and His$^{189}$ and His$^{220}$ which are conserved between AtPCS1 and CePCS1, but not SpPCS, only one His$^{162}$ is essential for Cd$^{2+}$ tolerance and PC synthetic activity. Of the six conserved Asp residues, at positions 71, 84, 89, 174, 180 and 204, that were substituted with Ala only one, the residue at position 180, abolishes Cd$^{2+}$ tolerance and PC synthetic activity (Romanyuk et al. 2006).

It is in this context that crystallographic analyses of AtPCS1’s prokaryotic equivalent, NsPCS, were to prove decisive (Fig. 2) (Vivares et al. 2005). Not only did the results of these seminal investigations yield the first crystal structure for a PC synthase – still the only structure known – but they showed unequivocally that these enzymes belong to the papain superfamily and deploy a Clan CA Cys protease-type catalytic mechanism. The crystal structure of NsPCS is that of a homodimer consisting of two subunits each of which contains a Cys$^{70}$-His$^{183}$-Asp$^{201}$ catalytic triad, the precise equivalent of the putative Cys$^{56}$-His$^{162}$-Asp$^{180}$ triad of AtPCS1 (Fig. 2) (Vivares et al. 2005, Rea 2006). Of the several representatives of the papain superfamily that have been defined structurally, staphopain and bleomycin hydrolase, a Cys hydrolase responsible for deamination of the anticancer agent bleomycin, a glycopeptide (Zheng et al. 1998), bear the closest three-dimensional resemblance to NsPCS. What is more, by elucidating the crystal structures of NsPCS in its native state and after co-crystallization with GS4 it was established that the enzyme’s acyl intermediate is a γ-Glu-Cys thioester of Cys$^{70}$ (Vivares et al. 2005), the strict equivalent of AtPCS1 Cys$^{56}$ (Fig. 2) (Vatamaniuk et al. 2004).
A papain-like reaction scheme

A reaction scheme capable of accounting for the role played by the papain-like Cys-His-Asp catalytic triad (Vatamaniuk et al. 2004, Romanyuk et al. 2006), the requirement that at least one of the thiol groups on one of the two substrates must be blocked for the synthetic reaction to go to completion (Vatamaniuk et al. 2000), the catalytic properties of both full-length and C-terminally truncated eukaryotic PC synthases (Ruotolo et al. 2004, Romanyuk et al. 2006), and the crystallographic data for NsPCS (Vivares et al. 2005, Rea 2006) is depicted in Fig. 3. In this scheme, the first phase of the dipeptidyl transpeptidation reaction, catalyzed by the N-terminal domain of AtPCS1, is one in which the enzyme undergoes acylation by GSH or a PC concomitant with cleavage of the C-terminal Gly residue from the donor. Nucleophilic attack on the carboxyl carbon of the peptide bond that is to be broken in the first substrate is by a thiolate anion derived from Cys\textsuperscript{56} in AtPCS1 to yield an enzyme γ-Glu-Cys thioester. By analogy with papain and other Cys proteases, formation of a thiolate-imidazolium ion pair between Cys\textsuperscript{56} and His\textsuperscript{162}, which are adjacent to each other in the active site, is promoted by the immediately downstream β-carboxylate of Asp\textsuperscript{180} (Fig. 2). Heavy metal substrate thiol complexes are not required in this phase of the reaction in that acylation of both NsPCS and AtPCS1 occurs even when metals are omitted from the reaction medium. In the next phase of the reaction, the second substrate replaces the Gly released from the first substrate. In the simplest case, donor deglycylation – the main reaction catalyzed by NsPCS (Harada et al. 2004, Tsuji et al. 2004, 2005) and a major subsidiary reaction of AtPCS1 (Vatamaniuk et al. 2004) – the second substrate is the solvent, water. The nucleophilicity of the incoming water molecule for attack on the enzyme thioester bond is enhanced by general base catalysis mediated by His\textsuperscript{162} whose electrophilicity is augmented by its immediate proximity to Asp\textsuperscript{180}. When the first substrate is GSH, free γ-Glu-Cys is released from the active site; when PC\textsubscript{n} is the first substrate, des(Gly)-phytochelatin [(γ-Glu-Cys)\textsubscript{n}] is...
Fig. 3. Catalytic mechanism of PC synthase inferred from the results of kinetic analyses, measurements of enzyme γ-Glu-Cys-acylation and site-directed mutagenesis of AtPCS1, and from what is known of the mechanism of distantly homologous Cys proteases of the papain superfamily (Rea et al. 2004). Mechanism corroborated and expanded by crystal structure of native and γ-Glu-Cys-NsPCS (Fig. 2) (Vivares et al. 2005). Refer to text for detailed description.

released. In the case of net PC synthesis or PC chain extension, the second substrate is GSH or another PC molecule and attack on the enzyme thioester is by the N-terminus of the acceptor, GSH or PCn, whose nucleophilicity is enhanced by reconstitution of the thiolate-imidazolium ion pair when the thioester bond is cleaved and a new peptide bond is formed. The catalytic cycle is completed when PC2 or PCn+1 dissociates from the enzyme and is replaced by a new donor molecule. As net PC or S-alkyl-PC synthesis by AtPCS1 has a strict dependence on the provision of Cd.GS2 or S-alkylglutathione it is probably this, the second, phase of the reaction that necessitates the provision of substrate containing a blocked thiol group.

In assigning a papain-like fold to PC synthases, it is also informative to note the identity of potential subsidiary catalytic residues, specifically AtPCS1 Gln50 (Rea et al. 2004, Romanyuk et al. 2006), the equivalent of papain residue Gln19 and NsPCS residue Gln64 (Figs 1 and 2) (Vivares et al. 2005), whose amide side chains in concert with the amide group of the triad Cys likely constitute an oxyanion hole which serves to polarize the carbonyl group of the bond that is to be broken in the first and second phases of the catalytic cycle and stabilize the tetrahedrally distorted transition states of the γ-Glu-Cys donor and enzyme thioester, respectively (Vivares et al. 2005).

### Augmentative metal activation

The properties of C-terminally truncated AtPCS1 reinforce and extend these deductions by suggesting a role for the C-terminal domain in augmentative metal sensing and/or protection of the core catalytic domain from the deleterious effects of free heavy metal ions, for instance when their concentrations are in excess of those required for substrate thiolate formation.

When native AtPCS1 is subjected to limited proteolysis with V8 protease, two N-terminal fragments ending at positions 372 (PCS_Nt1) and 283 (PCS_Nt2) are generated both of which are competent in PC synthesis from GSH at rates only about fivefold lower than those of the full-length polypeptide when Cd2⁺ is the cofactor (Ruotolo et al. 2004). However, both of these truncations are associated with a decrease in the thermal stability of the enzyme and a greater than 100-fold impairment of PC synthesis when Hg2⁺ or Zn2⁺, instead of Cd2⁺ or Cu2⁺, are the metal cofactors (Ruotolo et al. 2004). The properties of the C-terminal domain are those of a structure that contributes to the stability of the full-length polypeptide and plays a role in defining the range of metals to which the enzyme is responsive (Ruotolo et al. 2004).

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direct interaction with the C-terminal domain as ablation of this domain abolishes this effect and renders the enzyme susceptible to the deleterious effects of direct metal binding to the N-terminal domain.

**Concluding remarks**

The most notable difference between the catalytic mechanism of eukaryotic PC synthases and those of their prokaryotic counterparts and papain superfamily members is the identity of the chemical species responsible for resolution of the enzyme-thioester intermediate. During PC synthesis nucleophilic attack on the thioester intermediate is by a second thiol derivative rather than water. Three fundamental questions therefore follow: (1) what needs are met by the eukaryotic enzymes that their prokaryotic homologs do not, or cannot, meet; (2) are there any needs that the enzymes from both sources meet; (3) what structural features do the eukaryotic enzymes have that bias the reaction they catalyze in favor of net synthesis that the prokaryotic enzymes lack?

Another way of asking the first question is to ask why PCS genes should be so widespread and expressed constitutively at such high levels in plants? There is no getting away from the fact that plants synthesize PCs when exposed to non-essential heavy metals and none of us would contest the capacity of PC synthase for contributing to the detoxification of As, Cd and Hg. What is perplexing, however, is why intermittent exposure to these heavy metals whose release is often tightly coupled to the industrial activities of humans, an extremely recent event on the evolutionary landscape, should have driven fixation of PCS genes throughout the plant kingdom and beyond (Rea et al. 2004)? It is as if Cd and As detoxification by PC synthase is an example of a capability – a by-product of the enzyme’s action – that happens to meet a specific unmet need but not a need common to all organisms that have the enzyme. Of the various schemes proposed to account for this phenomenon, one of the most persuasive is the idea that PCs participate in the homeostasis and storage of essential heavy metals.

As an idea this scheme is not new. Steffens et al. (1986), who noted that low levels of PCs are detectable in cell cultures of tomato (Solanum lycopersicum) that had not been exposed to non-essential metals, considered this possibility, as did Grill et al. (1987, 1988) who described PC synthesis elicited by Zn$^{2+}$ and Cu$^{2+}$ salts when cultured cells were transferred into fresh medium. What is new though is recognition of a pitfall associated with certain metal screens; namely, that the usual reagents for plant metal hypersensitivity screens, Murashige and Skoog (MS) medium and standard plant tissue agars, obscure the effects otherwise exerted by the essential heavy metal Zn$^{2+}$ (Tennstedt et al. 2009). When screened on standard, for instance Gelrite® (CP Kelco Inc., GA, USA), agar plates containing one-half-strength MS medium, Arabidopsis AtPCS1 null mutants, as exemplified by cad1-3 and cad1-6 mutants, show little or no hypersensitivity to Zn$^{2+}$; growth is indistinguishable from that of wild-type seedlings (Ha et al. 1999, Cobbett and Goldsbrough 2002, Tennstedt et al. 2009). However, if the same screens are repeated with low ionic strength Hoagland medium without added micronutrients on plates prepared from an agar carrying copper and zinc salts...
Zn$^{2+}$ homeostasis might satisfy the requirements of an unmet need common to all organisms containing the eukaryotic full-length version of the enzyme (Clemens and Peršoh 2009, Tennstedt et al. 2009).

The findings reported by Clemens and colleagues are not only of considerable interest from a basic mechanistic standpoint but also because they raise the possibility that the PC biosynthetic machinery might be suitable as a target for biofortification strategies directed at enhancing the Zn$^{2+}$ content of the edible parts of crop species (Tennstedt et al. 2009). While there may be uncertainties about the exact role played by PC synthases, there can be little doubt of the worldwide prevalence of human dietary Zn$^{2+}$ deficiencies (Welch and Graham 2004).

Subsumed in the second question is the question of what the prokaryotic enzymes do if they do not synthesize PCs and is this a capability that the enzymes from eukaryotes share? In considering the role played by prokaryotic PCS-like polypeptides, it is likely, when account is taken of the overall degree of mechanistic similarity within the PC synthase family, that even if the prokaryotic enzymes are not true synthases their substrates resemble those of their eukaryotic relatives. If there is an activity that all members of their substrates resemble those of their eukaryotic relatives, then there is a distinct possibility that the wide distribution and high levels of expression of PC synthases and existence of PCS-like polypeptides in eukaryotes and prokaryotes, respectively, has as much, perhaps more, to do with the papain-like protease activity of this class of enzymes than the facility of some for PC synthesis.

These are important advances and ones that will almost certainly spawn new approaches to investigations of the exact roles played by members of the PC synthase family. However, when it comes to the third question of what it is that eukaryotic enzymes have that the prokaryotic enzymes do not in order to satisfy the structural requirements for PC synthesis there is still a lot to be learned. All that can be said is that as the N-terminal domain, alone, is sufficient for PC synthesis (Ruotolo et al. 2004, Romanyuk et al. 2006) there must be features, not least a second substrate-binding site, unique to this domain in the eukaryotic enzymes which enhance their capacity to use a second incoming GSH derivative, one with a blocked thiol, over water as an acceptor of the γ-Glu-Cys unit derived from the first substrate. It is as true today as it was 5 years ago (Rea 2006) to state – and this is written as much as a call to action as a statement of fact – that it will be imperative to acquire structural information for eukaryotic PC synthases either as C-terminal truncates or as full-length molecules before and after primary acylation, and, if practicable, before and after interaction with the second substrate to determine what it is about the N-terminal domain that makes these enzymes so much better at PC synthesis than their prokaryotic counterparts.

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