

Chapter 16

Cd(II)-Activated Synthesis of Phytochelatins

Etsuro Yoshimura

16.1 Introduction

Sequestering metal ions is a strategy adopted by organisms to ameliorate metal toxicity. Although their biological function has not been fully elucidated, phytochelatins (PCs), a family of peptides with the general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (PC_n), where $n \geq 2$ (Fig. 16.1), play such a role (Zenk 1996). Thiol groups coordinate metal ions, particularly soft metal ions such as Cd(II) and Cu(I), thereby reducing metal ion activity. The peptides, which are designated as cadystins and have the same structure as PC_2 and PC_3 , were first identified in the fission yeast *Schizosaccharomyces pombe* grown in Cd(II)-containing medium (Kondo et al. 1984). Shortly after the initial identification, the peptides were found to occur in suspension culture cells of the higher plant *Rauvolfia serpentina* exposed to Cd(II) (Grill et al. 1985). Currently, PCs have been identified in higher plants, algae, and some fungi exposed to toxic levels of heavy metal ions (Zenk 1996).

It was suggested early on that the enzyme(s) are involved in PC synthesis, as the peptide possesses γ -glutamyl peptide bonds. PC synthesis in vitro was first demonstrated via reactions catalyzed by the enzyme designated PC synthase (PCS), which was purified from *Silene cucubalus* cell suspension cultures (Grill et al. 1989). The reaction depends on Cd(II) (Loeffler et al. 1989). Thus, adding Cd(II) to a solution containing GSH and PCS initiates PC synthesis, and supplementing the solution with Cd(II) chelators, such as uncomplexed PC_n mixtures or ethylenediaminetetraacetic acid, immediately terminates PC synthesis. These observations led to the PC synthesis mechanism in which the enzyme is activated through binding of Cd(II) to the enzyme, although these observations are insufficient to support this mechanism in the strict sense.

E. Yoshimura (✉)

Department of Applied Biological Chemistry, School of Agricultural and Life Sciences,
The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
e-mail: ayoshim@mail.ecc.u-tokyo.ac.jp

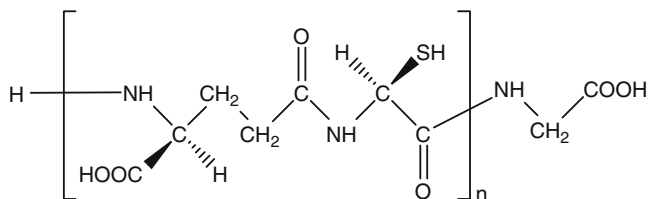


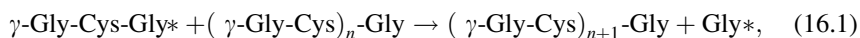
Fig. 16.1 Structure of phytochelatin. A general structure of the peptides is denoted by $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ with n being greater or equal to two, and $\gamma\text{-Glu-Cys}$ indicates a γ -glutamyl linkage, which is a peptide bond formed between the amino group of Cys and the γ -carboxyl group of Glu

It took more than a decade from the first PCS demonstration to identify the genes encoding PCSs, which was performed by three groups independently. Screening Cd (II) tolerant mutant cells from *Saccharomyces cerevisiae* cells carrying wheat (*Triticum aestivum*) or *Arabidopsis thaliana* cDNAs led to the isolation of higher plant PCSs (Clemens et al. 1999; Vatamaniuk et al. 1999). Positional cloning of the *CAD1* gene, which is responsible for PC-dependent Cd(II) tolerance in *A. thaliana*, also resulted in the isolation of a gene encoding a PCS (Ha et al. 1999). Subsequently, PCS sequence data have accumulated, making it feasible to prepare recombinant PCS enzymes in large quantities with a high degree of purity. Due to the use of recombinant enzymes, considerable progress has been made regarding the synthetic mechanisms of PCSs. In this chapter, the enzymatic mechanisms of action for PCS in relation to metal ion activation are reviewed, focusing on the metal activation enzyme reaction.

16.2 Dipeptidyl Transfer of PCS

16.2.1 Direction of PC Synthesis

The time course of in vitro PC synthesis is shown in Fig. 16.2 (Grill et al. 1989). PC_2 was produced immediately after adding Cd(II) to a solution containing glutathione ($\gamma\text{-Glu-Cys-Gly}$; GSH) and PCS purified from *S. cucubalus* cell suspension cultures. After a time lag of 15 min, PC_3 began to be formed, followed by PC_4 . These observations indicate that Cd(II) is required for PC synthesis, and that PC synthesis proceeds through PC_2 production by a transpeptidation of the $\gamma\text{-Glu-Cys}$ group of GSH to another GSH, following a subsequent peptide elongation by stepwise acquisition of a $\gamma\text{-Glu-Cys}$ unit (n) to the peptides. Two mechanisms can be proposed for this peptide elongation, as shown below.



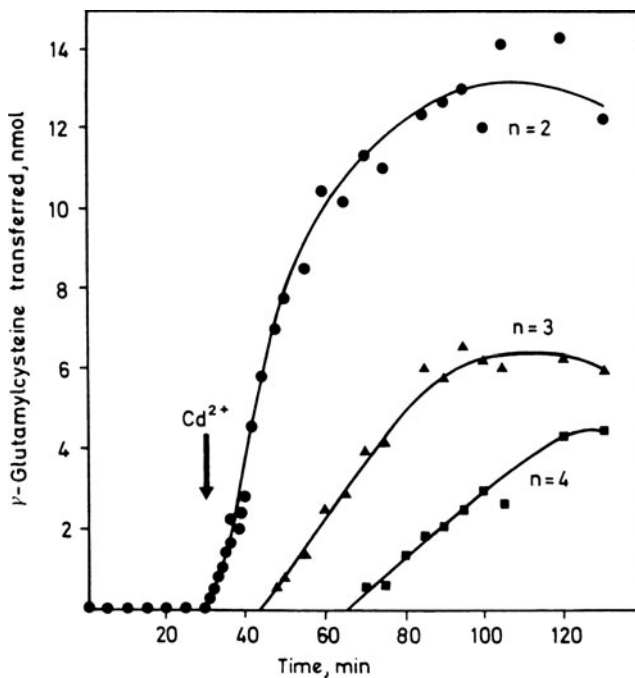
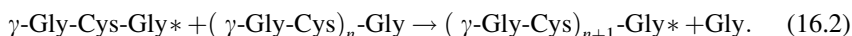


Fig. 16.2 Time course of in vitro phytochelatin synthesis. Adding Cd(II) into a solution containing glutathione (GSH) and phytochelatin synthase (PCS) purified from *Silene cucubalus* cell suspension cultures immediately produced PC₂, followed by PC₃ and PC₄ with lag periods of 15 and 20 min, respectively (taken from Grill et al. 1989, with permission)



Equation (16.1) indicates peptide elongation in a C to N direction, in which the γ -Gly-Cys moiety of GSH is transferred to form PC_n, thereby releasing the Gly attached to the GSH molecule. Conversely, (16.2) demonstrates an N to C elongation in which the Gly attached on PC_n is replaced by GSH with the release of Gly.

The early observation that GSH and PC₃ were formed when PC₂ was used as a sole substrate implies the dipeptide transfer mechanism shown by (16.1) (Grill et al. 1989). In support of this notion, evidence was afforded using PC₂ and GSH with isotopically labeled Gly as substrates in a PC₃ synthetic reaction catalyzed by AtPCS1 C-terminally tagged with FLAG (AtPCS1-FLAG), together with a parallel experiment in which PC₂ and GSH with isotopically labeled Cys were used (Vatamaniuk et al. 2004). An analysis of radioisotope incorporation into the peptides in a Cd(II)-activated reaction substantiates that PCs are synthesized through the dipeptide transfer reaction (16.1).

16.2.2 Acylation of PCS

Acylation of PCS with a γ -Glu-Cys moiety has been demonstrated using radiolabeled GSH in the absence of Cd(II) (Vatamaniuk et al. 2004). Incubating AtPCS1-FLAG and GSH with radiolabeled Cys and subsequent Sephadex G-50 gel-chromatography of the acid-treated mixture showed radioactivity in the void volume, and protein elution was ascertained by a Western blot analysis using anti-FLAG monoclonal antibody, implying that the protein was radiolabeled. By contrast, no radioactivity was recovered in the void volume in a parallel experiment using AtPCS1-FLAG and GSH with radiolabeled Gly. Thus, PCS was acylated by the γ -Glu-Cys moiety of GSH irrespective of the presence of Cd(II).

Accumulating information about the amino acid sequences of PCSs from a range of organisms has uncovered the features of this protein class. A prominent finding is the resemblance of the amino acid sequences of PCSs with those of papain-type proteases, particularly the conservation of a protease catalytic triad in PCSs (Rea et al. 2004). The catalytic triad, which consists of Cys, His, and Asp (or Asn), is essential to the protease, in which an acylated-enzyme intermediate is formed through the thiolate anion of a Cys residue from the proteases, with the aid of a His residue to withdraw H^+ from the Cys thiol group and of the Asp residue to stabilize the resultant positive charge of the His residue, enhancing the nucleophilicity of the thiol. A logical extension of the conservation of the triad in PCSs will be the formation of a similar acylated-enzyme intermediate during PC synthesis.

A point mutation of AtPCS1-FLAG and heterologous expression of the proteins demonstrated the PCS triad to be practically functional (Romanyuk et al. 2006). Among the yeast cells carrying PCS genes with a Cys residue mutated with a Ser, a C56S mutant alone was unable to grow under increasing concentrations of Cd(II). Furthermore, no apparent Cd(II)-dependent PC synthesis activity was found in the cell supernatant of the C56S transformant alone, despite expression of the protein at a level similar to that of other transformants. These results imply the essentiality of the Cys56 residue, a member of the hypothesized catalytic triad. Similarly, the mutation experiments unequivocally demonstrated the association of His162 and Asp189 during PC synthesis. It was therefore demonstrated that the PCS triad plays a catalytic role with the Cys56 residue as a γ -Glu-Cys acylation site, a reasonable deduction from the catalytic reaction of the proteases.

16.3 Kinetic Analysis of Enzyme Reaction

16.3.1 Assignment of the Substrates in a PC Synthetic Reaction

PC synthesis reaction rates have been determined as a function of GSH concentration at a constant total Cd(II) concentration. However, some complexity arises from the nature of the GSH to form complexes with Cd(II). There are two known types of

GSH complexes, namely, 1:1 and 1:2 Cd(II) GSH complexes, which are denoted as Cd(II)–GSH and Cd(II)–GSH₂, respectively. The respective conditional affinity constants are defined by the following equations:

$$K'_1 = \frac{[\text{Cd(II)}-\text{GSH}]}{[\text{Cd(II)}][\text{GSH}]}, \quad (16.3)$$

$$\beta'_2 = \frac{[\text{Cd(II)}-\text{GSH}_2]}{[\text{Cd(II)}][\text{GSH}]^2}, \quad (16.4)$$

where [Cd(II)–GSH] and [Cd(II)–GSH₂] denote the concentrations of Cd(II)–GSH and Cd(II)–GSH₂, respectively, and [Cd(II)] and [GSH] represent the concentrations of free Cd(II) and GSH, respectively. Therefore, it is apparent that while an increase in GSH concentration means an elevated substrate concentration, it results in a decrease in free Cd(II) concentration concomitantly. To avoid this complexity, an enzyme assay system was designed in which the free Cd(II) level was maintained constantly against a change in GSH concentration. For this purpose, the concentration of total Cd(II) was changed with GSH concentration in such a way that the $[\text{Cd(II)}]_t/[\text{GSH}]_t^2$ ratio was kept constant, where [Cd(II)]_t and [GSH]_t denoted the total concentrations of Cd(II) and GSH, respectively. Thus, it was demonstrated from the conditional stability constants that the majority of Cd(II) added to the assay solution is in the form of Cd(II)–GSH₂ complexes (Ogawa and Yoshimura 2010). In addition, as the GSH concentration bound to Cd(II) is negligible with respect to that of total GSH, it follows that [GSH] can be equated to [GSH]_t. This leads to a free Cd(II) level, which is shown by the following equation:

$$[\text{Cd(II)}] = \frac{[\text{Cd(II)}]_t}{\beta'_2[\text{GSH}]_t^2}. \quad (16.5)$$

Figure 16.3a shows PCS activity as a function of total GSH concentration at a ratio of $[\text{Cd(II)}]_t/[\text{GSH}]_t^2$ of 0.05 M⁻¹ (i.e., a free Cd(II) level of 122 pM). The recombinant enzyme that possesses the *A. thaliana* PCS1 amino acid sequence was used (Ogawa et al 2011). The reaction rate does not follow simple Michaelis–Menten-type kinetics, because a Hanes plot of $[\text{GSH}]_t/\text{activity}$ versus [GSH]_t demonstrated nonlinearity under the conditions employed: the plots fell on a linear line at GSH concentrations greater than 25 mM, whereas they turned upward from the line at GSH concentrations less than 25 mM (Fig. 16.3b).

Possible substrates of a PCS-mediated reaction are GSH, Cd(II)–GSH, and Cd(II)–GSH₂. The reaction seems to proceed via a substituted-enzyme mechanism, because the enzyme acylated by a γ -Glu-Cys group has been identified as an intermediate (Vatamaniuk et al. 2004). Using the maximal reaction rate, V_{max} , the enzyme reaction rate, v , for a substituted-enzyme mechanism can be expressed as:

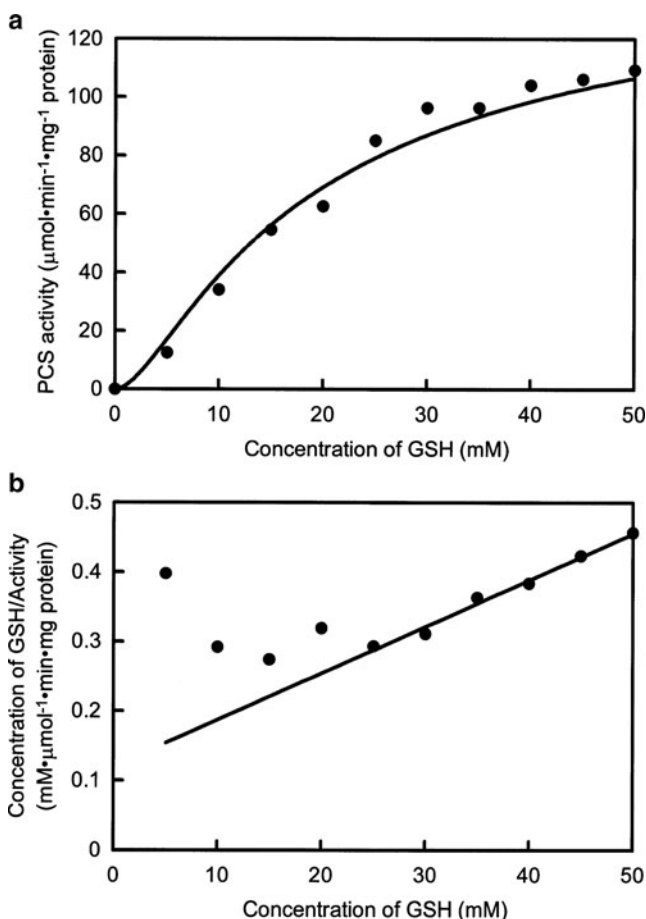


Fig. 16.3 Effect of phytochelatin synthase (PCS) activity on glutathione (GSH) concentration in the presence of a constant free Cd(II) (a), and a Hanes plot of $[\text{GSH}]_0/\text{activity}$ as a function of $[\text{GSH}]_0$ (b). The ratio of $[\text{Cd(II)}]_i/[\text{GSH}]_i^2$ was kept at 0.05 M^{-1} with an increase in $[\text{GSH}]_i$, and a free Cd(II) level of 122 pM was attained. The nonlinearity nature of the Hanes plot, together with the finding that GSH reacts with PCS to form a γ -Glu-Cys acylated intermediate in the absence of Cd(II) demonstrated that the GSH and Cd(II)–GSH₂ complex are the substrates in a PCS-mediated PC synthesis reaction. A nonlinear least-square analysis established that the Michaelis constants for GSH and Cd(II)–GSH₂ were $18.0 \pm 3.2\text{ mM}$ and $5.14 \pm 1.22\text{ }\mu\text{M}$, respectively. The simulated activity (a solid line in a) and the determined activity were in agreement (black circles in a) (Ogawa et al. 2011)

$$v = \frac{V_{\max}[\text{A}][\text{B}]}{K_{\text{mB}}[\text{A}] + K_{\text{mA}}[\text{B}] + [\text{A}][\text{B}]}, \quad (16.6)$$

where A and B denote substrates bound first and second to the enzyme, respectively, and K_{mA} and K_{mB} represent the Michaelis constants for substrates A and B, respectively (Bisswanger 2008). During PCS-mediated PC synthesis, γ -Glu-Cys

acylation of the enzyme occurs in a Cd(II)-independent manner, demonstrating that the substrate first bound to the enzyme should be free GSH. If GSH or Cd(II)-GSH act as a second substrate, then the reaction will follow simple Michaelis-Menten-type kinetics. When GSH is the second substrate, the reaction rate can be reduced to

$$v = \frac{V_{\max}[\text{GSH}]_t}{(K_{\text{mA}} + K_{\text{mB}}) + [\text{GSH}]_t}. \quad (16.7)$$

When Cd(II)-GSH is the second substrate, the concentration of the Cd(II)-GSH complex can be shown as:

$$[\text{Cd-GSH}] = K'_1[\text{Cd(II)}][\text{GSH}]_t. \quad (16.8)$$

Therefore, the rate can be derived by:

$$v = \frac{V_{\max}[\text{GSH}]_t}{\{K_{\text{mA}} + K_{\text{mB}}/(K'_1[\text{Cd(II)}])\} + [\text{GSH}]_t}. \quad (16.9)$$

In contrast, simple Michaelis-Menten-type kinetics likely would not be realized if the Cd(II)-GSH₂ complex were a second substrate. Thus, the concentration of the Cd(II)-GSH₂ complex can be given by the following equation:

$$[\text{Cd-GSH}_2] = \beta'_2[\text{Cd(II)}][\text{GSH}]_t^2. \quad (16.10)$$

Therefore, the PC synthesis rate can be expressed by:

$$v = \frac{V_{\max}'[\text{GSH}]_t^2}{\frac{K_{\text{mB}}}{\beta'_2[\text{Cd(II)}]} + K_{\text{mA}}[\text{GSH}]_t + [\text{GSH}]_t^2}. \quad (16.11)$$

The derivation of this equation leads to the relationship of [GSH]_t/activity vs. [GSH]_t as follows:

$$\frac{[\text{GSH}]_t}{v} = \frac{1}{V_{\max}'} \left\{ [\text{GSH}]_t + K_{\text{mA}} + \frac{K_{\text{mB}}}{\beta'_2[\text{Cd(II)}][\text{GSH}]_t} \right\}. \quad (16.12)$$

This equation is consistent with the Hanes plot of enzyme activity determined at a constant free Cd(II) level (Fig. 16.3b). A nonlinear least-square analysis of the PCS activity data to fit (16.11) yielded $V_{\max}' = 149 \pm 9$, $K_{\text{mA}} = 18.0 \pm 3.2$ mM, and $K_{\text{mB}} = 5.14 \pm 1.22$ μM, which gave a simulated profile significantly consistent with the measured rates. These observations unequivocally demonstrate that PC synthesis proceeds using a GSH molecule and a Cd(II)-GSH₂ complex as substrates that bind first and second to rAtPCS1, as suggested by Vatamaniuk et al. (2000).

16.3.2 Binding of Cd(II) to Activate PCS

Although Cd(II) is essential for PCS-mediated PC synthesis to proceed, the role of Cd(II) in the reaction is equivocal. One of the possible mechanisms is that Cd(II) is solely required to form the substrate Cd(II)–GSH₂ complex in which the enzyme is already in an active form without Cd(II) binding, while another mechanism is that the enzyme needs to bind Cd(II) for activation. The evidence supporting the latter notion was obtained from a PCS assay at a constant total Cd(II) concentration (Fig. 16.4) (Ogawa et al. 2011). When the assay solution contained 10 μM total Cd(II), activity increased and reached a maximum with an increase in GSH concentration up to 15 mM, and then decreased with further increases in GSH concentration (Fig. 16.4, black squares). A similar trend was observed for the enzyme assay system containing 1 or 5 μM Cd(II) (Fig. 16.1, black circles and triangles, respectively). It was also evident that an increase in Cd(II) concentration shifted the maximal activity toward higher GSH concentrations. Thus, activity was maximized at GSH concentrations of approximately 10 and 15 mM in the presence of 5 and

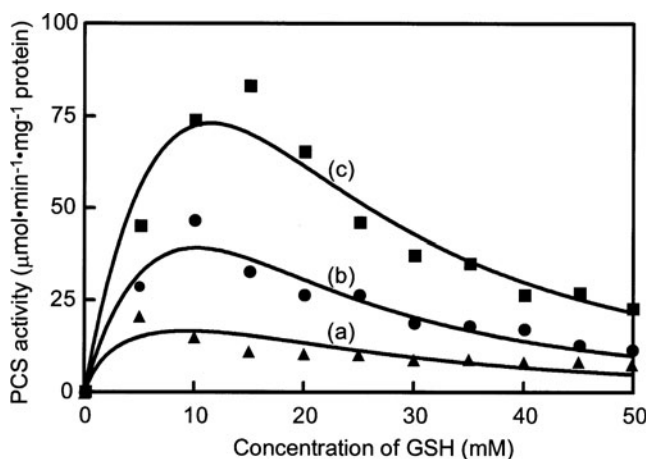


Fig. 16.4 Effect of glutathione (GSH) concentration on phytochelatin synthase (PCS) activity in the presence of total Cd(II) concentrations of 1 μM (black triangles), 5 μM (black circles), and 10 μM (black squares) in the assay solution. Decreased activity at higher GSH concentrations can be attributed to a reduced free Cd(II) level arising from the enhanced formation of Cd(II)–GSH and Cd(II)–GSH₂ complexes, thereby lowering a fraction of active Cd(II)-bound enzyme. A nonlinear least-square analysis using the one-site model (16.15) yielded a V_{max} of $193 \pm 17 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$ and a K_{E1} of $(28.4 \pm 5.1) \times 10^9 \text{ M}^{-1}$ with an R^2 of 0.926 when the activity data at 5 μM Cd(II) were used and a V_{max} of $302 \pm 25 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$ and a K_{E1} of $(15.9 \pm 2.7) \times 10^9 \text{ M}^{-1}$ with an R^2 of 0.938 when the activity data at 10 μM Cd(II) were used. Simulated activity (solid lines b and c) was in good agreement with determined activity. In contrast, a nonlinear least-square analysis of the data determined at 1 μM Cd(II) produced a low correlation coefficient ($R^2 = 0.681$) with a rather inconsistent result between determined activity (black triangles) and simulated activity (solid line a), probably due to the poor activity data set (Ogawa et al. 2011)

10 μM total Cd(II), respectively. At 1 μM total Cd(II), the maximal activity seemed to occur at a concentration less than or equal to 5 mM GSH, although the maximum did not appear unequivocally.

The observation that PCS activity decreased at higher GSH concentrations is consistent with the notion that the enzyme possesses Cd(II)-binding site(s) at which Cd(II) ions bind to activate it. An increase in GSH concentration would decrease free Cd(II) levels by enhancing the formation of Cd(II)–GSH and Cd(II)–GSH₂ complexes, thereby reducing the level of the enzyme bound to Cd(II). Given the binding constant of Cd(II) to rAtPCS1, K_{E1} , the active enzyme concentration can be expressed by the following equation:

$$[\text{Cd(II)-rAtPCS1}] = K_{E1}[\text{Cd(II)}][\text{rAtPCS1}]. \quad (16.13)$$

As the free Cd(II) level in an assay solution is expressed by (16.5), a fraction of PCS bound by Cd(II), f_1 , is shown by:

$$f_1 = \frac{1}{1 + \frac{\beta'_2}{K_{E1}} \cdot \frac{[\text{GSH}]_t^2}{[\text{Cd(II)}]_t}}. \quad (16.14)$$

Assuming that only rAtPCS1 bound to Cd(II) has PCS activity, the reaction rate can be derived as a one-site model, as follows:

$$v = \frac{f_1 V_{\max} [\text{GSH}]_t}{K_{mA} + \left(1 + \frac{K_{mB}}{[\text{Cd(II)}]_t}\right) [\text{GSH}]_t}. \quad (16.15)$$

Using the K_{mA} and K_{mB} values obtained, V_{\max} and K_{E1} were optimized by a nonlinear least-square analysis using the PCS activity data shown in Fig. 16.4. The V_{\max} and K_{E1} values for the activity data sets at total concentrations of 5 and 10 μM were consistent, as revealed by the correlation coefficients, which were close to 0.93. Furthermore, the parameters obtained under both conditions were parallel to each other. In contrast, a low correlation coefficient was obtained for activity at a total Cd(II) concentration of 1 μM ($R^2 = 0.681$), probably due to poor data quality, in which all of the activity plots except that of the origin revealed a decreasing profile. Therefore, omitting the parameters for 1 μM total Cd(II), it can be concluded that a PCS molecule possesses a Cd(II)-binding site with dissociation constants ($1/K_{E1}$) of 35–63 pM to which the ion binds to activate the enzyme.

A possible alternative to this notion may be that the enzyme possesses an inhibitory GSH-binding site(s) at which GSH binds to inhibit PC synthesis. However, this possibility can be ruled out, as PCS activity steadily increased with GSH concentration in the presence of a constant free Cd(II) level (Fig. 16.3). This is in contrast with the reaction rate determined at constant total Cd(II) concentrations in which a significant reduction in rate was found despite the fact that the free GSH

concentrations were substantially equal to those at a constant free Cd(II) concentration.

16.3.3 Inhibitory Second Cd(II) Binding Site of *rAtPCS1*

As shown in Fig. 16.5, PCS activity at a constant total Cd(II) concentration still had a maximum for the assay solution containing higher levels of total Cd(II): the activity maximized at 30 and 60 mM GSH for the assay solution containing 50 μM (black squares) and 500 μM Cd(II) (black circles), respectively. However, a sigmoidal increase in activity was observed at GSH concentrations less than 20 mM. A one-site Cd(II)-binding model appeared to be inappropriate to rationalize the activity at higher Cd(II) concentrations, which can be recognized by the poor correlation coefficient ($R^2 = 0.870$ at 50 μM Cd(II) and $R^2 = 0.771$ at 500 μM Cd(II)). In addition, PCS activity determined as a function of the concentration of Cd(II) revealed a maximum (Fig. 16.6). These findings led to the assumption that *AtPCS1* possesses an inhibitory second Cd(II)-binding site. Binding of Cd(II) to this site

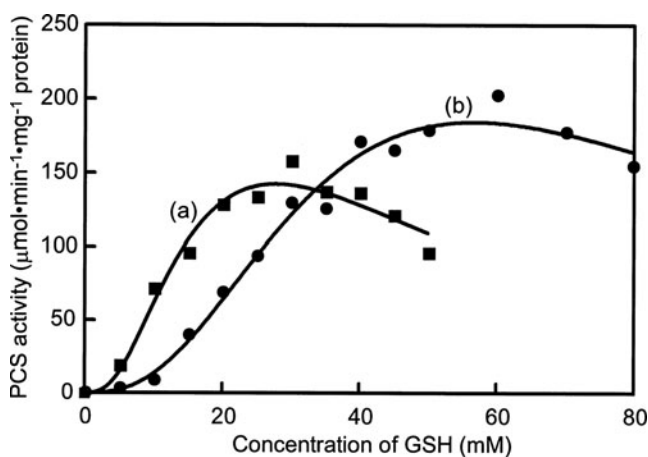


Fig. 16.5 Effect of glutathione (GSH) concentration on phytochelatin synthase (PCS) activity in the presence of total Cd(II) concentrations of 50 μM (black squares) and 500 μM (black circles) in the assay solution. A sigmoidal increase in the activity is apparent at lower GSH concentrations, implying that the enzyme possesses a second Cd(II)-binding site to which Cd(II) binds to inactivate the enzyme. Although the one-site model resulted in a poor simulation, a nonlinear least-square analysis using the two-site model (16.18) yielded consistent results and showed a V_{\max} of $399 \pm 62 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$, a K_{E1} of $(12.4 \pm 4.6) \times 10^9 \text{ M}^{-1}$, and a K_{E2} of $(0.961 \pm 0.442) \times 10^9 \text{ M}^{-1}$ with an R^2 of 0.971 when activity data determined at 50 μM Cd(II) were used and a V_{\max} of $472 \pm 94 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$, a K_{E1} of $(4.55 \pm 1.88) \times 10^9 \text{ M}^{-1}$ and a K_{E2} of $(0.924 \pm 0.333) \times 10^9 \text{ M}^{-1}$ with an R^2 of 0.984 when activity data determined at 500 μM were used. Simulated activity was in fair agreement with determined activity (solid lines in (a) for 50 μM total Cd(II) and (b) for 500 μM total Cd(II)) (Ogawa et al. 2011)

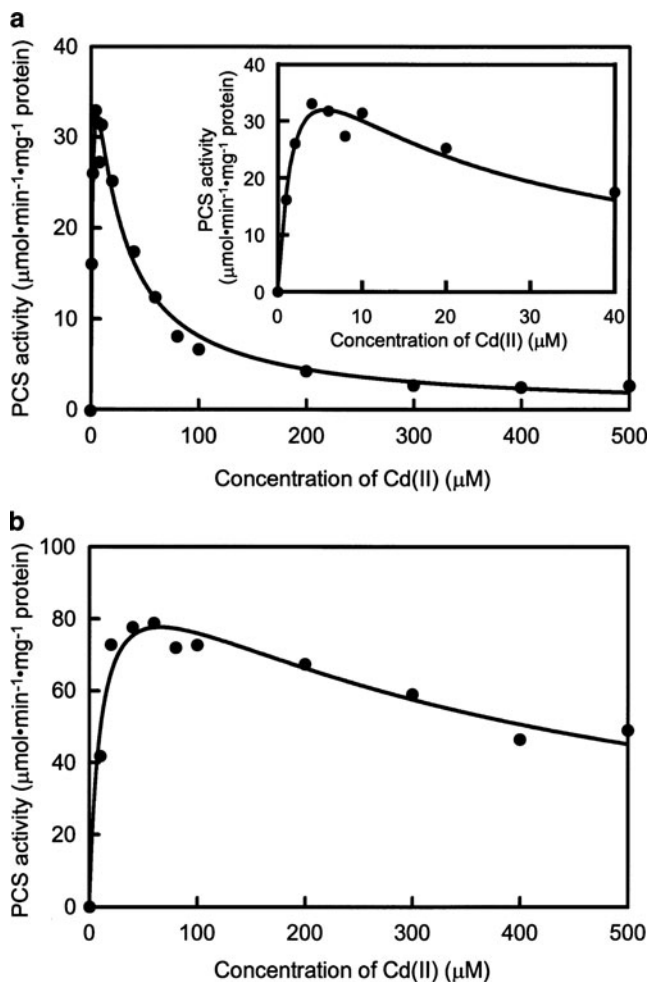


Fig. 16.6 Effects of total Cd(II) concentration on phytochelatin synthase (PCS) activity of AtPCS1 in the presence of 5 mM (a) and 20 mM (b) glutathione (GSH). The insert in (a) is an expansion of the figure at Cd(II) concentrations from 0 to 40 μM. The observation that the activity decreased at higher total GSH concentrations suggested that the enzyme possesses a second Cd(II)-binding site to which Cd(II) binds to inactivate the enzyme. The two-site model (16.18) was applied to a nonlinear least-square analysis, which yielded a V_{\max} of $244 \pm 17 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$, a K_{E1} of $(23.4 \pm 9.8) \times 10^9 \text{ M}^{-1}$, and a K_{E2} of $(0.557 \pm 0.081) \times 10^9 \text{ M}^{-1}$ with an R^2 of 0.986 when activity data determined at 5 mM Cd(II) were used, and a V_{\max} of $194 \pm 14 \mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$, a K_{E1} of $(25.3 \pm 8.6) \times 10^9 \text{ M}^{-1}$, and a K_{E2} of $(0.403 \pm 0.085) \times 10^9 \text{ M}^{-1}$ with an R^2 of 0.968 when activity data determined at 20 mM were used. Simulated activity was in fair agreement with determined activity (a) for 5 mM total GSH and (b) for 20 mM total GSH (Ogawa et al. 2011)

would inhibit PC synthesis. Given the binding constant of Cd(II) to Cd(II)-AtPCS1 at the second site, K_{E2} , the enzyme concentration with two Cd(II) binding sites occupied by Cd(II) (Cd(II)₂-AtPCS1) can be expressed by the following equation:

$$[\text{Cd(II)}_2\text{-rAtPCS1}] = K_{E1}K_{E2}[\text{Cd(II)}]^2[\text{rAtPCS1}]. \quad (16.16)$$

Using (16.13) and (16.14), a fraction of the enzyme with a Cd(II) on the first site alone is shown by:

$$f_2 = \frac{1}{1 + \frac{\beta'_2}{K_{E1}} \cdot \frac{[\text{GSH}]_t^2}{[\text{Cd(II)}]_t} + \frac{K_{E2}}{\beta'_2} \cdot \frac{[\text{Cd(II)}]_t}{[\text{GSH}]_t^2}}. \quad (16.17)$$

Assuming that rAtPCS1 with two Cd(II) binding sites fully occupied by Cd(II) ions completely loses activity, the following equation from a two-site model can be expressed:

$$v = \frac{f_2 V_{\max} [\text{GSH}]_t}{K_{mA} + (1 + \frac{K_{mB}}{[\text{Cd(II)}]_t}) [\text{GSH}]_t}. \quad (16.18)$$

The binding of Cd(II) to the second site would also account for the sigmoidal profile of the activity assayed in the presence of higher concentrations of Cd(II). Free Cd(II) levels may be elevated with GSH concentrations of 0–20 mM at 50 and 500 μM total Cd(II) (Fig. 16.5), in which some of the enzyme is presumed to be inactivated by bringing the Cd(II) ion onto the second site. Equation 16.18 was used to determine the Cd(II) affinity constants for the enzyme, which yielded the dissociation constants for the first site ($1/K_{E1}$) to be 81–220 pM and for the second site ($1/K_{E2}$) to be 1.0–1.1 nM. The parameters optimized using the activity data shown in Fig. 16.5 were consistent with the experimentally determined PCS activity in the presence of 5 and 20 mM GSH.

The binding constants K_{E1} and K_{E2} were also optimized to fit PCS activity determined as a function of total Cd(II) concentration according to (16.7), which gave dissociation constants for the first site ($1/K_{E1}$) of 40–43 pM and for the second site ($1/K_{E2}$) of 1.8–2.5 nM. The PCS activity estimated from the V_{\max} , K_{E1} , and K_{E2} values thus obtained agreed with the experimentally obtained PCS activity at 5 and 20 mM GSH, as shown by the solid lines in Fig. 16.6a, b. Furthermore, the parameters estimated from PCS activity at 5 and 20 mM GSH were consistent with each other. This also supports the suggestion that AtPCS1 possesses two Cd(II) binding sites per molecule.

16.4 Conclusion

Producing PC peptides and complexing metal ions are crucial mechanisms for ameliorating metal toxicity in plant species. It was unequivocally demonstrated that PCS possesses a Cd(II)-binding site with a dissociation constant of a few tens to two hundred pM. Therefore, PCS activity was regulated in such a way that the enzyme was activated by Cd(II) ions by binding to the site and was deactivated by removing the ion from the activated enzyme through complexing the product PCs with Cd(II). This defense mechanism would respond promptly to a challenge of toxic metal ions, as the enzyme is constitutively expressed in cells. Although the physiological relevance of metal binding to PCS has been elucidated, the mode of metal binding to the enzyme must be resolved. A range of metal cations and metalloid oxoanions can function in the reaction initiator: metals and metalloids belonging to groups 11–15 in the fourth, fifth, and sixth periods of the periodic table are assigned to activate PCS-mediated PC synthesis (Oven et al. 2002). It is of interest that these ions with a specific chemical nature are capable of activating PCSs. Further studies will shed light on the detailed mechanisms.

References

- Bisswanger H (2008) Enzyme kinetics, second, revised and updated edition. WILEY-VCH Verlag GmbH and Co., Weinheim, Germany
- Clemens S, Kim EJ, Neumann D, Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J* 18:3325–3333
- Grill E, Löffler S, Winnacker E-L, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ -glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proc Natl Acad Sci USA* 86:6838–6842
- Grill E, Winnacker E-L, Zenk MH (1985) Phytochelatins: the principal heavy-metal complexing peptides of higher plants. *Science* 230:674–676
- Ha S-B, Smith AP, Howden R, Dietrich WM, Bugg S, O'Connell MJ, Goldsbrough PB, Cobbett CS (1999) Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. *Plant Cell* 11:1153–1163
- Kondo N, Imai K, Isobe M, Goto T (1984) Cadystin A and B, major unit peptides comprising cadmium binding peptides induced in a fission yeast – separation, revision of structures and synthesis. *Tetrahedron Lett* 25:3869–3872
- Loeffler S, Hochberger A, Grill E, Winnacker E-L, Zenk MH (1989) Termination of phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. *FEBS Lett* 258:42–46
- Ogawa S, Yoshimura E (2010) Determination of thermodynamic parameters of cadmium(II) association to glutathione using the fluorescent reagent FluoZin-1. *Anal Biochem* 402:200–202
- Ogawa S, Yoshidomi T, Yoshimura E (2011) Cadmium(II)-stimulated activation of *Arabidopsis thaliana* phytochelatin synthase I. *J Inorg Biochem* 105:111–117
- Oven M, Page JF, Zenk MH, Kutchan TM (2002) Molecular characterization of the homo-phytochelatin synthase of soybean *Glycine max*. *J Biol Chem* 277:4747–4754
- Rea PA, Vatamaniuk OK, Rigden DJ (2004) Weeds, worms, and more. Papain's long-lost cousin, phytochelatin synthase. *Plant Physiol* 136:2463–2474

- Romanyuk ND, Rigden DJ, Vatamaniuk OK, Lang A, Cahoon RE, Jez JM, Rea PA (2006) Mutagenic definition of a papain-like catalytic triad, sufficiency of N-terminal domain for single-site core catalytic enzyme acylation, and C-terminal domain for augmentative metal activation of a eukaryotic phytochelatin synthase. *Plant Physiol* 141:858–869
- Vatamaniuk OK, Mari S, Lang A, Chalasani S, Demkiv LO, Rea PA (2004) Phytochelatin synthase, a depeptidyltransferase that undergoes multisite acylation with γ -glutamylcysteine during catalysis. *J Biol Chem* 279:22449–22460
- Vatamaniuk OK, Mari S, Lu Y-P, Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: isolation and *in vitro* reconstitution. *Proc Natl Acad Sci USA* 96:7110–7115
- Vatamaniuk OK, Mari S, Lu Y-P, Rea PA (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase. *J Biol Chem* 275:31451–31459
- Zenk MH (1996) Heavy metal detoxification in higher plants – a review. *Gene* 179:21–30