Molecular basis for cysteine oxidation by Plant Cysteine Oxidases from Arabidopsis thaliana

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Abstract

Plant Cysteine Oxidases (PCOs) play important roles in controlling the stability of Group VII ethylene response factors (ERF-VIIs) via N-Arg/degron pathway through catalyzing the oxidation of their N-Cys for subsequent Arginyl-tRNA--protein transferase 1 (ATE1) mediated arginine installation. Here we presented structures of PCO2, PCO4, and PCO5 from Arabidopsis thaliana (AtPCOs) and examined their in vitro activity by MS. On the basis of Tris-bound AtPCO2, we modelled the Cys-bound AtPCO2 structure and identified key residues involved in N-Cys oxidation. Alanine substitution of potential N-Cys interaction residues impaired the activity of AtPCO5 remarkably. The structural research, complemented by mutagenesis and mass spectrometry experiments, not only uncovers the substrate recognition and catalytic mode by AtPCOs, but also sheds light on the future design of potent inhibitors for plant cysteine oxidases.
Introduction

In Animals and plants, different mechanisms are exploited to sense oxygen concentration and regulate gene expression in response to low oxygen stress (hypoxia)[1]. Animals utilizes hypoxia-inducible transcription factor (HIF) to increase the expression levels of growth factors [2], while Plant Cysteine Oxidases (PCOs) play a critical role in oxygen homeostasis by serving as oxygen-sensing proteins[3, 4]. PCOs were found to control the turnover of Group VII ethylene response factors (ERF-VIIs) starting with Met-Cys- (\(^{1}\)MC\(^{2}\)), via N-degron pathway[4-7]. Once the N-terminal Met (N-Met) of target protein is excised by Met amino peptidase[8], the exposed N-terminal Cys (N-Cys) is subject to oxidization by PCOs, which covert the Cys to Cys-sulfinic acid (CysO\(^{2}\))[7]. After the oxidation of N-Cys, Arginyl-tRNA--protein transferase 1 (ATE1) installs an arginine before N-CysO\(^{2}\) to promote the proteasomal degradation through the Arg/N-degron pathway[4, 7].

The activity of PCOs were greatly compromised in the hypoxic condition, which promotes the expression of hypoxia-response genes by increasing the in vivo stability of the ERF-VIIs it mediated[5, 9]. Five ERF-VII family members were identified in Arabidopsis thaliana, including AtRAP2.2, AtRAP2.3, AtRAP2.12, AtHRE1, and AtHRE2[5, 10]. The turnovers of above response factors mediated by PCOs provide a new layer in understanding how gene expression during stress response is regulated in a oxygen-dependent manner.

PCOs from Arabidopsis thaliana (AtPCOs) has been found to catalyze the N-Cys oxidization of AtHRE1 (\(^{2}\)CGGAVIS\(^{8}\)), AtHRE2 (\(^{2}\)CGGAIIS\(^{8}\)), and AtRAP2.2 (\(^{2}\)CGGAIIS\(^{8}\)), etc [6, 10]. Despite their important biological functions in response to low-oxygen stress, how PCOs recognize the substrate and catalyze the subsequent N-Cys oxidation remain elusive because they display low sequence identity with the dioxygenases that have known structures. Here we solved several crystal structures of PCOs from Arabidopsis thaliana (AtPCOs), including those of AtPCO2, AtPCO4, and
AtPCO5. Although the three PCOs belong to two subfamilies, they display conserved catalytic pocket, suggesting the conserved catalytic mechanism among PCOs. Specifically, the structure of Tris-bound AtPCO2 allows us to model an N-Cys in the catalytic pocket, which reveals the N-Cys specific recognition by PCOs. The PCOs-mediated N-Cys oxidization was complemented by mass spectrometry (MS) experiments. Therefore, our structure research not only provides structural insights into the N-Cys catalytic mechanism by PCOs, but also sheds light on the future design of chemical inhibitors in interfering with the activities of PCOs.

Results

AtPCO2, AtPCO4, and AtPCO5 exhibit activities towards peptides derived from ERF-VII family members

To study the activities of AtPCOs in vitro systematically, we cloned, expressed and purified the full-length or core fragment of several AtPCOs, including AtPCO2-48-276, AtPCO41-241, and AtPCO51-242 (Fig. 1A). Then we synthesized two peptides derived from AtHRE1 and AtRAP2.2, AtHRE12-10 and AtRAP2.22-8, Respectively, and examine the activities of AtPCOs towards the two peptides by MS experiments.

Consistent with previous reports about the control experiments[5, 11], after long incubation without enzyme, the peptides either dimerized via N-Cys (Fig. 2A and 3A) or formed thiazolidine (Fig. 2B and 3B). The dimerized peptide and formation of thiazolidine were confirmed by the peaks correspond to the ~2-fold molecular weight and the +12 Da, respectively. In contrast, both peptides were efficiently catalyzed by AtPCO2-48-276, AtPCO41-241, and AtPCO51-242 in 30 minutes at 37˚C, which generated a peak of +32 Da (Fig. 2C-2E, and Fig. 3C-3E)

Crystal structure of AtPCO51-242, AtPCO41-241, and AtPCO248-276

To understand the substrate binding and catalytic mechanism of AtPCOs, we solved the crystal structure of selenomethionine (SeMet) AtPCO51-242 at a resolution of 2.50 Å (Table 1). Most residues of AtPCO51-242 are visible except the residues at N-, C-
terminus or some loop regions owning to internal flexibility (Fig. 1B). \( \text{ArPCO5} \) adopts a jelly roll-like fold with a chamber constituted by two \( \beta \)-sheets, \( \beta14-\beta1-\beta2-\beta9-\beta4-\beta7 \) and \( \beta6-\beta5-\beta8-\beta3 \), with the former \( \beta \)-sheet packed against \( \alpha1-\alpha3 \) (Fig. 1B, 1C). There is a long bended insertion between \( \beta9 \) and \( \beta14 \) consisting of \( \beta10-\beta13 \), in which \( \beta10 \) and \( \beta11 \) form anti-parallel strands with \( \beta13 \) and \( \beta12 \), respectively. Before crystallization, we added excess ferrous ion in the buffer and the density map of a ferrous ion is found in the catalytic center, which is chelated to His98, His100, His164, and three water molecules. His98 and His100 reside in the loop between \( \beta3 \) and \( \beta4 \), while His164 is localized in \( \beta8 \) (Fig. 1).

Next we solved structures of \( \text{ArPCO4}_{1-241} \) and \( \text{ArPCO2}_{48-276} \), at resolutions of 2.44- and 1.56- Å, respectively (Fig. 4A, 4B and Table 1). Both \( \text{ArPCO4} \) and \( \text{ArPCO2} \) adopt jelly roll-like fold (Fig. 4A, 4B). Superposition of three \( \text{ArPCO} \) structures show that their overall architecture are very similar, as evidenced by the root-mean-square deviations (RMSDs) in a range of 0.57-0.81 Å (Fig. 4C). Especially the three histidines that are chelated to the ferrous ion are conserved in three structures, as an indicative of the conserved ferrous ion chelation mode in \( \text{ArPCO} \)s (Fig. 4A, 4B). On the basis of sequence alignment, it is very likely that above \((\text{His})_3 \) chelation mode also applies for their human homolog, 2-aminoethanethiol dioxygenase (ADO) (Fig. 1A).

Intriguingly, in the structure of \( \text{ArPCO2} \), we found that a Tris molecule, mimic of the N-Cys, occupies the catalytic pocket. In the Tris-bound structure, besides the three histidines, the central ferrous ion is also chelated to the nitrogen and the two carboxyl groups of the Tris molecule to complete the six coordination (Fig. 4D). The nitrogen group of Tris is hydrogen bonded to the hydroxyl group of the \( \text{ArPCO2} \) Tyr215. Tris also makes van del Walls interactions with Phe123, Ile131, Phe199, and Asp209 of \( \text{ArPCO2} \) (Fig. 4D).

**Modeled structure of Cys-bound \( \text{ArPCO2} \)**

Given the similarity between the Tris and the apo cysteine, we modeled the Cys-bound
structure of \( \textit{AtPCO2} \) on the basis of the Tris-bound structure and compared its Cys recognition mode with that of recently solved Cys-bound human CDO (PDB ID: 6N42)[12]. Despite the similar architecture and Ferrous ion chelation mode, the jelly-roll fold of CDO deviates remarkably from that of \( \textit{AtPCO2} \) (Fig. 5A). At the catalytic center, the modelled Cys is chelated to the \textit{Fe}^{2+} via its main chain amino and side chain thiol groups, similar to that observed in the structure of Cys-bound CDO (Fig. 5B, 5C). Despite the similarity, their Cys recognition and catalytic modes are different in several aspects. Firstly, Cys93 and Tyr157 of CDO forms the cysteinyltyrosine bridge to increase the catalytic efficiency (Fig. 5B) [12, 13], whereas the crosslinked moiety is not found in any of \( \textit{AtPCO} \) structures (Fig. 5C). Secondly, the nitrogen group of Cys in the Cys-CDO complex is free, whereas the nitrogen group of Cys is hydrogen bonded to the side chain of Tyr215 in the modeled \( \textit{AtPCO2} \) complex (Fig. 5B, 5C). Thirdly, in both structures, the Cys interaction residues in two structures are distinct (Fig. 5B, 5C). In the CDO structure, the Cys makes van der Waals interactions with the side chains of Leu75, Ser83, Val142, and His155 (Fig. 5B). In the modeled complex structure of \( \textit{AtPCO2} \), the Cys makes van der Waals interactions with the side chains of Phe123, Ile131, and Phe199 of \( \textit{AtPCO2} \) and its side chain makes one hydrogen bond with the side chain carboxyl group of Asp209, positioning the Cys in a favorable conformation for oxidation (Fig. 5C).

The Cys interaction residues of \( \textit{AtPCO2} \) are absolutely conserved in \( \textit{AtPCO4} \) and \( \textit{AtPCO5} \) (Fig. 1A), suggesting a common Cys recognition and catalytic mode shared by \( \textit{AtPCO} \)s. The counterparts of \( \textit{AtPCO2} \) Asp209 and Tyr215 in \( \textit{AtPCO5} \) are Asp176 and Tyr182, respectively (Fig. 1A). We then generated several single mutants for \( \textit{AtPCO5}_{1-242} \), including H164A, D176A, Y182F and C190A, and examined their activities towards the peptide RAP2.2\(^{2-8}\) (CGGAIIS). MS data show that while H164A abolished the activity by disrupting the ferrous chelation, both D176A and Y182F demonstrated compromised activities towards the \( \textit{AtHRE1}_{2-10} \) and \( \textit{AtRAP2.2}_{2-8} \) peptides than wild type \( \textit{AtPCO5} \). In contrast, C190A exhibit comparable activity towards the substrate peptide as the wild type (Supplementary Fig. S1). Collectively,
the mutagenesis experiments and MS data further confirmed the key roles of the Cys binding residues in cysteine oxidation.

**Discussion**

Previous work on human CDO reveal that an cysteinyltyrosine bridge is formed to lower the oxidation potential of tyrosine for efficient catalysis[12, 13]. Whether cysteinyltyrosine bridge is also formed in human ADO and PCOs remains unknown because of the absence of structure evidence. By analyzing the structure of AtPCOs, we proposed that although Cys190 of AtPCO2 is spatially adjacent to Tyr215 and Tyr225 and the three residues are absolutely conserved in the structures of AtPCO4 and AtPCO5 (Fig. 6), it could not form the cysteinyltyrosine bridge with either of them. Firstly, the cysteinyltyrosine bridge of CDO (Cys93-Tyr157) does not have a spatial counterpart in AtPCO2. The Cys190 of AtPCO2 is not the counterpart of CDO Cys92 in CDO (Fig. 5B, 5C). Secondly, the aromatic ring of AtPCO2 Tyr225 is far from the thiol group of Cys223, implied that they are not likely to form crosslinked moiety. (Fig. 6). Thirdly, as for the Tyr215 of AtPCO2, it is not likely to cross link with Cys223, either, because its aromatic ring of Tyr215 is parallel to the side chain plain of Cys223 and it would be energetically unfavorable for the Try215 to rotate its aromatic right at least ~90 degree to form cysteinyltyrosine bridge. Also the main chain carbonyl group of Pro214 and the main chain amino group of Ser216 are hydrogen bonded to the side chain of Arg221 and the main chain carbonyl group of Arg221, respectively (Fig. 6), which does not allow the Tyr215 main chain to endure remarkable change. Above mentioned spatial pattern of the Cys223, Tyr215, and Tyr225 of AtPCO2 are also conserved in AtPCO4 and AtPCO5 (Fig. 6), suggesting that AtPCOs probably exploit a different mechanism rather than cysteinyltyrosine bridge to catalyze cysteine oxidation. We further mutating the Cys190 of AtPCO4, the counterpart of the AtPCO2 Cys223, to an Ala, and found that C190A does not impair the AtPCO4 activity towards RAP2.2-8 (Supplementary Fig. S1).

During the preparation of the manuscript, the structures of AtPCO4 and AtPCO5 from
Arabidopsis thaliana were reported[14]. In that study, the presented structures, as well as the conclusions drawn on the basis of the structures, are similar to ours. In near future, the substrate- or inhibitor- bound structures of AtPCOs would further uncover the catalytic mechanism of N-Cys oxidation.

Conclusion
Plant Cysteine Oxidases from Arabidopsis thaliana have been reported to play an important role in controlling the turnover of response factors via Arg/N-degron pathway. Our presented structures of AtPCO2, AtPCO4, and AtPCO5 unraveled the architecture of PCOs, and the structure of ligand-bound AtPCO2 revealed the potential substrate binding and oxidation residues in the catalytic pocket. Besides, sequence alignment indicates that human ADO might utilize a mechanism to catalyze the oxidation of N-Cys similar to those of AtPCOs. Overall our research not only provides insight into the N-Cys oxidation mechanism by PCOs, but also sheds light on the design of inhibitors for PCOs.

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Declarations of interest: The authors declare that they have no conflict of interest

Author contributions: Q.G. and C.X. designed and conceived the study. Q.G., Z.C., S.L. purified, crystallized the protein, solved the structure, and analyzed the data. Q.G. and G.W. performed MS experiments. C.X. wrote the paper with the help from all authors. J.W., S.L., and C.X. supervised the experiments.
Materials and Methods

Cloning, mutation, protein expression and purification

Genes encoding full-length AtPCO2, AtPCO4, and AtPCO5 were synthesized by Sangon Biotech (Shanghai). AtPCO248-276, AtPCO41-241, and AtPCO51-242 was amplified by polymerase chain reactions (PCR), and cloned into pET28-MHL (Genbank accession number: EF456735). The plasmids were then transformed into E. coli BL21 (DE3) and the recombinant proteins were overexpressed at 16 °C for 20 h in the presence of 0.2 mM isopropyl b-D-1-thiogalactopyranoside (IPTG).

Cells were harvested at 3600 × g, 4 °C for 15 min, and then were resuspended using a buffer containing 20 mM Tris-HCl (pH 7.5), 400 mM NaCl (suspension buffer) and were lysed by sonication. Lysates were centrifuged at 18000 × g, 4 °C for 30 min and supernatants were collected. Recombinant proteins were purified with a fast flow Ni-NTA column (GE Healthcare) and eluted by 20 mM Tris-HCl (pH 7.5), 400 mM NaCl, 500 mM imidazole. Gel filtration and ion-exchange were employed for further purification. Gel filtration experiments were performed on a HiLoad™ 16/600 superdex™ 75 pg column (GE healthcare) with suspension buffer, the fractions containing target recombinant proteins were collected and dialyzed to ion exchange buffer A (20 mM Tris-HCl pH 7.5, 50 mM NaCl). Ion exchange experiments were performed on a Hitrap™ Q HP (1 mL) column (GE healthcare) with ion exchange buffer A and ion exchange buffer B (20 mM Tris-HCl pH 7.5, 1 M NaCl), fraction corresponding to target proteins were collected and concentrated to 20-40 mg/mL and stored at -80°C before further use. Seleno-Methionine (SeMet)-labeled AtPCO51-242 was purified in the same way, except that cells were cultured in M9 medium supplied with 50mg/L Seleno-Methionine. The mutants were constructed by conventional PCR using a MutanBEST kit (TaKaRa) and further verified by DNA sequencing. The mutants were expressed and purified in the same way as the wild type proteins.

Crystallization, data collection and structure determination
Before crystallization, recombinant proteins were pre-incubated with Fe$^{2+}$ at a molar ratio of 1:3 at 4 °C for 30 min. For crystallization of AtPCOs, 1 µl protein was mixed with 1 µl crystallization buffer using the sitting drop vapor diffusion method at 18 °C. SeMet-labeled AtPCO5$_{1-242}$ was crystallized in a buffer containing 0.1 M MES monohydrate, pH 6.5, 12% (w/v) PEG20000. AtPCO4$_{1-241}$ was crystallized in a buffer containing 0.1 M Sodium citrate tribasic dehydrate, pH 5.0, 0.2 M Ammonium acetate, and 20% (w/v) PEG 3350. AtPCO2$_{48-276}$ was crystallized in a buffer containing 0.1 M Tris hydrochloride, pH 8.5, 0.2 M sodium acetate, and 30% w/v polyethylene glycol 4000. Before flash-freezing crystals in liquid nitrogen, all crystals were soaked in a cryo-protectant consisting of 90% reservoir solution plus 10% glycerol.

The diffraction data were collected on beam line BL18U1 at the Shanghai Synchrotron Facility (SSRF). Data sets were collected and processed using the HKL3000 program[15]. The initial model of SeMet AtPCO5$_{1-242}$ was solved by CRANK2[16], built manually by COOT[17] and refined by Phenix[18]. The structures of AtPCO2$_{48-276}$ and AtPCO4$_{1-241}$ were solved by molecular replacement by using the structure of SeMet AtPCO5$_{1-242}$ as the search model. The structures were built manually by Coot[17] and were further refined by Phenix [18].

The atomic coordinates and structure factors of AtPCO2, AtPCO4, and AtPCO5 have been deposited in the Protein Data Bank with PDB ID codes 7CHJ, 7CHI, and 7CXZ.

**Mass spectrometry experiments**

Reversed-phase microcapillary/tandem mass spectrometry (LC/MS/MS) was performed using an Easy-nLC nanoflow HPLC (Proxeon Biosciences) with a self-packed 75 mm 15 cm C18 column connected to a QE-Plus (Thermo Scientific) in data-dependent acquisition and positive ion mode at 300 nL/min. Passing MS/MS spectra were manually inspected to ensure that all b-and y-fragment ions aligned with the assigned sequence and modification sites. A 25 µl reaction mixture contained 1 µM AtPCO2/4/5 (final concentration) and 50 µM peptide (final concentration) in a buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM NaCl, 20 µM FeSO$_4$, 5 mM TCEP, and
1 mM Ascorbic acid. The reaction was incubated at 37°C for 30 min at atmospheric oxygen before being quenched (at 70°C for 10–15 mins). Then, the tubes were centrifuged at 15000 × g for 10 min. Supernatants were analyzed by LC-MS/MS and Proteomics Browser software, with the relative abundances of substrate and product reflecting the cysteine oxidation activities of proteins.

References

oxygen-sensing plant cysteine oxidases 4 and 5 enable targeted manipulation of their activity. Proc Natl Acad Sci U S A


## Table 1 Data collection and refinement statistics

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Values in parentheses are for highest-resolution shell.
Figure legends

Figure 1. Structure of AtPCO5. (A) Sequence alignment of AtPCOs and human ADO (hADO). Secondary structures of AtPCO5 were labelled in blue at the top of aligned sequences. Three Fe^{2+} chelating histidines (His98, His100, and His164), and residues potentially involved in N-Cys recognition are labeled. (B) Overall structure of AtPCO5_{1-242}. AtPCO5 is colored in blue, with three Fe^{2+} chelating histidines (His98, His100, and His164) shown in sticks. Fe^{2+} and three water molecules are shown in orange and red, respectively. (C) Topology diagram of AtPCO5, with secondary structures colored in blue.

Figure 2. Mass spectra data of peptide AtHRE1^{2-10} (CGGAVISDY) catalyzed by AtPCOs. (A)-(B) Spectra of peptide without enzyme as the controls. Spectra the peptide CGGAIIS oxidized by (C) AtPCO5_{1-242}, (D) AtPCO4_{1-241}, and (E) AtPCO2_{48-276}.

Figure 3. Mass spectra data of peptide AtRAP2.2^{2-8} (CGGAIIS) catalyzed by AtPCOs. (A)-(B) Spectra of peptide without enzyme as the controls. Spectra the peptide CGGAIIS oxidized by (C) AtPCO5_{1-242}, (D) AtPCO4_{1-241}, and (E) AtPCO2_{48-276}.

Figure 4. Structures of AtPCO4_{1-241}, and AtPCO2_{48-276}. (A) Overall structure of AtPCO4_{1-241}. The structure of AtPCO4 is shown in red cartoon, with three Fe^{2+} chelating histidines (His98, His100, and His164) shown in sticks. Fe^{2+} and three water molecules are shown in orange and red, respectively. (B) Overall structure of Tris-bound AtPCO2_{48-276}. The structure of AtPCO2 is colored in cyan cartoon, with three Fe^{2+} chelating histidines (His134, His136, and His197) shown in sticks. Fe^{2+} and Tris are shown in orange and yellow, respectively. (C) Superposition of the structures of AtPCO2 (cyan), AtPCO4 (red), and AtPCO5 (blue). The structures are shown in ribbon, with Fe^{2+} chelation residues shown in sticks. (D) Detailed interactions between Tris and AtPCO2. Tris is shown in yellow sticks. Fe^{2+} chelating residues and the Tris-binding residues are shown in cyan sticks. Intermolecular hydrogen bond is indicated by black dashes.
**Figure 5.** Comparison of the catalytic mechanism of ArPCOs with that of human CDO. (A) Superposition of the structure of hCDO (red) with that of ArPCO2 (cyan). The proteins are shown in ribbon, with Fe$^{2+}$ chelating residues shown in sticks. (B) The catalytic center of Cys-bound hCDO, with the Cys and Cys binding residues shown sticks. (C) The catalytic center of Cys-bound ArPCO2, with the Cys and Cys binding residues shown sticks.

**Figure 6.** Spatial pattern of Cys223, Tyr215, and Tyr225 of ArPCO2. Pro214 and Ser216 form main chain hydrogen bonds with Arg221. Fe$^{2+}$ chelating residues, Arg221, Cys223, Pro214-Ser216, Tyr225, as well as their counterparts in ArPCO4 and ArPCO5, are shown in sticks.
Figure 1
Figure 2
Figure 3
Supplementary Fig. S1. Mass spectra data of peptide AtRAP.2.2-8 (CGGAIIS) catalyzed by the variants of AtPCO5. Spectra of peptide catalyzed by (A) AtPCO5 1-242 H164A, (B) AtPCO5 1-242 D176A, (C) AtPCO5 1-242 Y182F, and (D) AtPCO5 1-242 C190A.