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The C-Terminal Extension of Chloroplast 2-Cys Peroxiredoxin Is Critical for Interaction with ATP

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Supporting Information

ABSTRACT: 2-Cys peroxiredoxins (2-Cys Prxs) are ubiquitous enzymes that have been implicated in peroxide-mediated signaling of markedly different processes, such as cancer and photosynthesis. A highly conserved C-terminal extension of eukaryotic homologues modulates both the overoxidation of cysteines and the formation of oligomers. Here, we reveal that the plant counterpart regulates the self-polymerization of 2-Cys Prx triggered by ATP and Mg^{2+}. This feature is of particular importance under oxidative stress because the interaction of ATP with 2-Cys Prx rapidly integrates nonredox chemistry of signaling pathways into a network hub governed by multiple redox transformations at cysteine residues.

Peroxiredoxin (Prx) is a large family of ubiquitous peroxidases that convert H_2O_2, alkyl hydroperoxides, and peroxynitrite to water, alcohol, and nitrite, respectively, using a catalytic cysteine in the N-terminal region, peroxidatic cysteine (Cys_P).[1−3] The known primary structures, available through the PREX database (http://www.csb.wfu.edu/prex/), are grouped into six distinct subfamilies that have been implicated as regulatory hubs in diverse biological processes.[4−9] An additional cysteine in the C-terminal region, resolving cysteine (Cys_R), is the relevant feature of the subfamily of typical 2-Cys peroxiredoxins (2-Cys Prxs) (Figure S-1A of the Supporting Information).[10] Notably, eukaryotic 2-Cys Prxs exhibit a highly conserved C-terminal extension that embraces the prokaryotic homologues in the green lineage of oxygenic photosynthesis (Figure S-1B,C of the Supporting Information).[11,12] Although this YF-containing signature is not part of the redox active site, its function has been linked to H_2O_2-mediated inactivation of the peroxidatic cycle.[12] We recently found that low concentrations of ATP rapidly and reversibly drive decameric 2-Cys Prx (polypeptide, 23.5 kDa) into large soluble assemblies (>2 MDa) that proceed to insoluble aggregates at nucleotide concentrations of >5 mM. Moreover, ATP caused the oligomerization of rapeseed 2-Cys Prx irrespective of the presence or absence of a His_6 tag at the C-terminus (Figure S-2 of the Supporting Information). This dynamic self-polymerization (decamers ⇔ soluble oligomers ⇔ insoluble aggregates) indicates that 2-Cys Prx not only acts as a redox regulatory protein but also effectively responds to nonredox processes that occur in the cellular milieu.[13,14]

Our previous studies revealed that ATP interacts with Cys_R,[15,16] but nucleotide binding motives in 2-Cys Prx were not identified in an exhaustive search of protein databases. Therefore, we used QUANTUM version 3.3 that screens the interactions of proteins with drugs and metabolites to explore docking of ATP to different regions of 2-Cys Prx (Protein Data Bank entry 1qmv). Thus, the binding of ATP to the flexible loop occurring very close to the C-terminal α-helix ($\Delta G_{binding}$ = −32.2 kJ/mol) was stronger than the binding to other parts of the protein ($\Delta G_{binding}$ > −24 kJ/mol). This prediction was consistent with the ATP-mediated quenching of the emission intensity of Trp179, an intrinsic fluorophore located in the C-terminal extension.[15] To unravel the role of the C-terminal extension, we employed a subtractive approach in which mutants of His_6-tagged 2-Cys Prx lacked 9 (Δ192) and 19 (Δ183) residues but retained all the components of the Trx fold (Figure S-1D of the Supporting Information).[17] The analysis of truncated mutants by dynamic light scattering (Figure 1) revealed that 3 mM ATP/Mg (i) drove the monodisperse decameric form of wild-type and Δ192 2-Cys Prx (hydrodynamic diameter of 13 nm) to the formation of large polydisperse oligomers (hydrodynamic diameter of >200 nm) but (ii) did not alter the size of the Δ183 counterpart (hydrodynamic diameter of 13 nm).

We further tested whether soluble species coalesce into insoluble aggregates at high concentrations of ATP/Mg by following the aggregation of His_6-tagged 2-Cys Prx through (i) the turbidity of the suspension (light scattering) and (ii) the semiquantification of proteins in soluble and insoluble fractions [sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE)].

As illustrated in Figure 2, 5 mM ATP caused the immediate formation of insoluble forms of wild-type and Δ192 2-Cys Prx while the solubility of the Δ183 counterpart was not perturbed. These findings complemented a recent study in which Arg129, an amino acid residue close to the peroxidatic site, is necessary for the assembly of large protein complexes (Figure S-3 of the Supporting Information).[16] The investigation of the intrinsic fluorescence of His_6-tagged 2-Cys Prx further supported the idea that the C-terminal extension plays a causative role in the binding of ATP/Mg to 2-Cys Prx. If ATP/Mg quenches the emission of Trp179 but not that of Trp88,[15] the nucleotide should cause global changes in the intensities of Δ192 2-Cys

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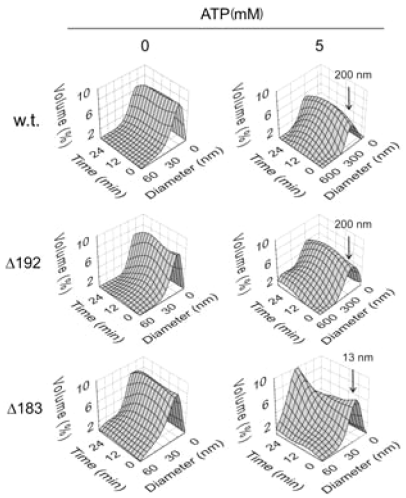

Figure 1. Effect of ATP/Mg on the hydrodynamic diameter of truncated His_6-tagged 2-Cys Prx. As indicated, ATP (3 mM) was added to His_6-tagged 2-Cys Prx (10 μM) in 20 mM Tris-HCl buffer (pH 8) containing 3 mM $MgCl_2$. Dynamic light scattering analyses were conducted at 25 °C, as described in the Experimental Procedures (Supporting Information).

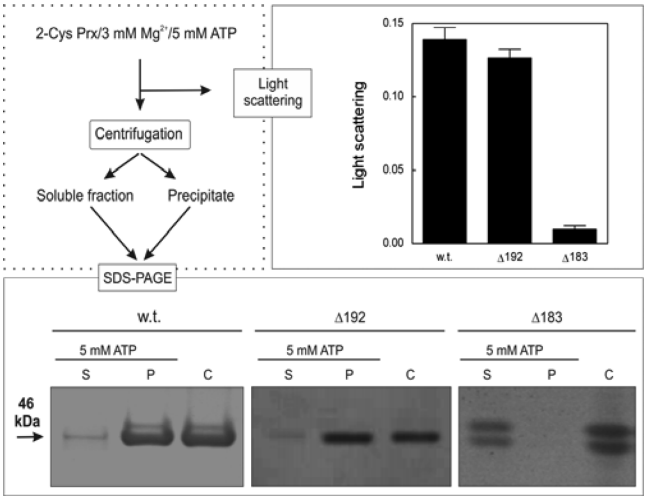

Figure 2. ATP/Mg-mediated aggregation of truncated His_6-tagged 2-Cys Prx. The truncated variants of His_6-tagged 2-Cys Prx (10 μM) in 30 mM Tris-HCl buffer (pH 8) and 3 mM $MgCl_2$ were incubated for 5 min at 25 °C in the presence and absence of 5 mM ATP. For light scattering, the turbidity of samples was quantified at 600 nm in a spectrophotometer. Data represent mean values ± the standard deviation (n = 4). For centrifugation, after centrifugation of protein samples (C), the supernatant fraction (S) and the precipitate (P) were analyzed by nonreducing SDS−PAGE.

Prx and should be ineffective on Δ183 2-Cys Prx. As predicted (Figure 3), the emission intensities of wild-type and Δ192 2-Cys Prx declined markedly in response to ATP/Mg while those of Δ183 2-Cys Prx were totally insensitive.

Recently,[16] circular dichroism (CD) spectroscopy revealed that changes in the secondary structure of 2-Cys Prx accompany the self-polymerization mediated by ATP/Mg. Again, if the C-terminal region is critical for the binding of ATP to His_6-tagged 2-Cys Prx, the length of this extension should condition the response of the secondary structure to the binding of the nucleotide. To overcome the high extinction coefficient of ATP that precludes the direct use of CD

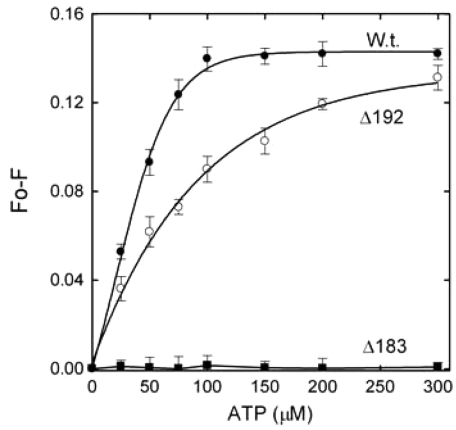

Figure 3. Effect of ATP/Mg on the intrinsic fluorescence of truncated His_6-tagged 2-Cys Prx. Tryptophan emission spectra were scanned by increasing the concentration of ATP while keeping constant the concentrations of His_6-tagged 2-Cys Prx variants (2 μM) and Mg^{2+} (2 mM) (see the Experimental Procedures in the Supporting Information). After correction for the inner filter effect, the differences in the emission intensities (at 340 nm) between His_6-tagged 2-Cys Prx (F_o) and [His_6-tagged 2-Cys Prx]·[ATP-Mg^{2+}] complexes (F) were fit to a saturation curve by nonlinear least-squares regression analyses.

spectroscopy in the far-UV region, ATP was photochemically cross-linked to all variants of His_6-tagged 2-Cys Prx, allowing the close contact of the adenine ring with the protein (Figure S-4A of the Supporting Information). CD spectra of untreated variants used in our study had negative and large positive Cotton effects near 220 nm and below 200 nm, respectively (Figure S-4B of the Supporting Information), suggesting the predominance of α-helical structures. However, the content of α-helices fell from 33.2 to 10.0%, while the content of β-strands increased from 17.6 to 38.8% when wild-type and Δ192 2-Cys Prx were illuminated in the presence of ATP/Mg (Figure S-4B,C of the Supporting Information). Notably, these secondary structures remained unchanged in the Δ183 counterpart. Although this trend is not shown, deconvolution of CD spectra revealed that ATP/Mg did not alter the proportion of unordered and β-turn structures in all variants. On the other hand, the hydrophobic probe 8-anilinonaphthalene-1-sulfonic acid exhibited a marked increase in fluorescence emission upon binding of ATP/Mg to both wild-type and Δ192 2-Cys Prx, but the change was much less pronounced in the Δ183 counterpart (Figure S-5 of the Supporting Information). Taken together, these observations suggest that the interaction of ATP with the C-terminal extension promotes the intrinsic conformational flexibility of His_6-tagged 2-Cys Prx with respect to reversible oligomerization and aggregation.

Despite missing 9 and 19 amino acid residues in their C-terminal regions, rapeseed Δ192 and Δ183 2-Cys Prxs, respectively, had all the elements of a competent peroxidase, i.e., the catalytic Cys53 (Cys_P) and Cys175 (Cys_R) that act concertedly with Thr50 and Arg129 in the reduction of hydroperoxides.[18−21] Therefore, the ability of truncated mutants to reduce H_2O_2 was similar to that of the wild-type counterpart (Figure S-6A of the Supporting Information). However, 3 mM ATP/Mg lowered by 90, 75, and 50% the catalytic capacity of wild-type, Δ192, and Δ183 2-Cys Prx, respectively (Figure S-6B of the Supporting Information). Thus, although the present variants of His_6-tagged 2-Cys Prx are fully competent peroxidases, truncated mutants are less

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sensitive than the wild-type counterpart to inactivation by ATP/Mg.[15] These peroxidase activities were particularly striking and consistent with the interaction between the C-terminal extension of His_6-tagged 2-Cys Prx and ATP.

The C-terminal extension of His_6-tagged 2-Cys Prx had been hitherto linked to regulation of the formation of the Cys_R–Cys_P transient disulfide during the peroxidatic cycle.[10,13] This mechanism of action suggests that the peculiar sequence is a critical determinant of Cys_P overoxidation under oxidative stress. The studies detailed herein revealed that the binding of endogenous metabolites to the C-terminal extension regulates the fast and reversible modification of the quaternary structure. In this context, the novel function has emerged as a very effective mechanism that puts in concert rapidly nonredox chemistries of the cell, via binding of nucleotides, with transformations in the oxidation states of cysteines.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, C-terminal extension, the effect of the His_6 tag on 2-Cys Prx, site-directed mutagenesis, CD spectroscopy, ANS binding, and peroxidase activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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