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Regulation of plant alternative oxidase activity: A tale of two cysteines

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Abstract

Two Cys residues, Cys_I and Cys_{II}, are present in most plant alternative oxidases (AOXs). Cys_I inactivates AOX by forming a disulfide bond with the corresponding Cys_I residue on the adjacent subunit of the AOX homodimer. When reduced, Cys_I associates with α-keto acids, such as pyruvate, to activate AOX, an effect mimicked by charged amino acid substitutions at the Cys_I site. Cys_{II} may also be a site of AOX activity regulation, through interaction with the small α-keto acid, glyoxylate. Comparison of *Arabidopsis* AOX1a (AtAOX1a) mutants with single or double substitutions at Cys_I and Cys_{II} confirmed that glyoxylate interacted with either Cys, while the effect of pyruvate (or succinate for AtAOX1a substituted with Ala at Cys_I) was limited to Cys_I. A variety of Cys_{II} substitutions constitutively activated AtAOX1a, indicating that neither the catalytic site nor, unlike at Cys_I, charge repulsion is involved. Independent effects at each Cys were suggested by lack of Cys_{II} substitution interference with pyruvate stimulation at Cys_I, and close to additive activation at the two sites. However, results obtained using diamide treatment to covalently link the AtAOX1a subunits by the disulfide bond indicated that Cys_I must be in the reduced state for activation at Cys_{II} to occur.

Keywords: Plant alternative oxidase; Plant mitochondria; Disulfide redox regulation; Enzyme activation

1. Introduction

The alternative oxidase (AOX) of plant mitochondria is a homodimeric, diiron-carboxylate protein [1] that accepts electrons directly from the ubiquinone pool and reduces oxygen to water. Unlike the cytochrome pathway, with which it competes for electrons, the alternative pathway translocates no protons across the inner mitochondrial membrane and therefore conserves no energy. While the particulars of AOX interaction with plant metabolism are not clear, a variety of evidence suggests that, rather than being a purely wasteful enzyme, AOX can act to decrease formation of harmful reactive oxygen species from an over-reduced ubiquinone pool, help to balance the redox state of the cell especially with respect to reductant produced by photosynthesis, and allow the TCA cycle to proceed under conditions of cytochrome pathway impairment or when levels of intracellular ATP are high [2,3].

The AOX monomer can be divided approximately into an N-terminal third, and the more C-terminal two thirds that constitute a four-helical diiron binding structure (Fig. 1A). Most plant AOXs have two highly conserved cysteine residues, termed Cys_I and Cys_{II} (nomenclature of Berthold et al. [4]). Cys_I is located in the structurally undefined N-terminus, whereas Cys_{II} is located at the N-terminal end of the hydrophilic portion of the first diiron-binding helix (Fig. 1A). Biochemical regulation is known to occur at Cys_I. When the Cys_I residues of the AOX dimer interact with α-keto acids, perhaps forming a thiohemiacetal, the enzyme becomes activated [5,6]. This activation evidently arises not from a direct effect on the active site, but through a charge-induced conformational change, because substitution of Cys_I with either a positively or a negatively charged amino acid results in a constitutively active enzyme [7]. When this conformational change is prevented, either by oxidation of Cys_I residues in the native homodimer to form an intermolecular disulfide bond [8] or by substitution of Cys_I with a hydrophobic amino acid residue ([7]; unpublished results in [1,4]), an inactive enzyme results. These regulatory features allow the plant AOX's activity to be influenced by intermediates of carbohydrate metabolism and cellular redox state, consistent with its hypothesized functions listed above.

Although the large majority of plant AOX protein sequences conserve Cys_I, some do not (Table 1). Two of these, in which a

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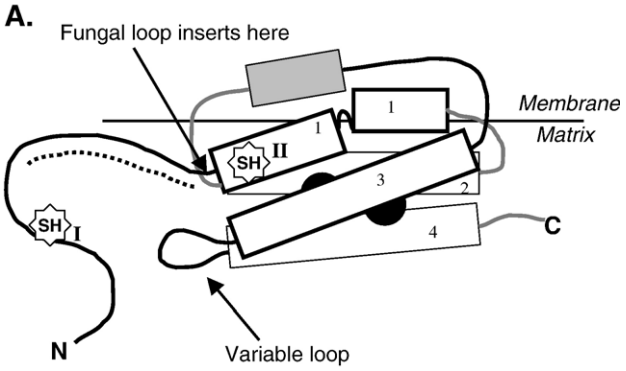

B.

Most Cys_I/Cys_{II} AOXs	RRYGCRAMMLET
Ser_{II} AOXs	RR**HASHA**LL**L**ET
Lycopersicon AOX1b	RR**Hm**C**H**AM**L**LET
Sauromatum AOX	RRY**A**CRAMMLET
Glycine max AOX3	**e**RYGC**H**AMMLET

Fig. 1. Structural and sequence characteristics of the plant AOX protein. (A) Diagrammatic representation of the structure of the plant AOX monomer. The four diiron-binding helices of the active site are shown as numbered rectangles. The structure of the N-terminus is unknown. The conserved Cys residues (designated "I" and "II") are shown as sulfhydryl groups. The dotted line segment in the N-terminal region shows the location of a possible quinone-binding motif [34], and sites of a fungal sequence insertion and a variable loop region are also indicated (see text). Drawn after [23,32,38]. (B) Alignment of residues in the region surrounding Cys_{II} (marked with a star). Top line shows residues common to AOXs having both Cys_I and Cys_{II}. Second line is from sequences of Ser_{II} AOXs (all the Ser_{II} "Plant AOX" sequences of Table 1). The last three sequences illustrate the variability in this region, one noted by Crichton et al. [32] for *Sauromatum*, in AOXs with Cys_{II}. Note that *Lycopersicon* AOX1b has a Ser at the Cys_I position. Accession numbers for *Sauromatum* and *Glycine max* AOX3, respectively: P22185, O03376. Residues in upper case bold are conserved relative to the Ser_{II} sequence. Residues in lower case bold are unique substitutions.

Ser residue (Ser_I) occupies the Cys_I position, have been studied, one from tomato [9] and one from maize [10]. For these AOX proteins, inactivation through formation of the intersubunit disulfide bond is not possible [9,10]. Further, the native tomato Ser_I isoform is stimulated, not by α-keto acids, but by succinate [9]. Similarly, for soybean and *Arabidopsis* Cys_I-type AOXs, substitution of Ser (soybean; [11]) or Ala (*Arabidopsis*; [7]; unpublished results in [11]) for Cys_I also confers succinate activation. While the basis for activation by succinate may also involve a conformational change, the nature of succinate interaction with the AOX protein most likely differs from that between Cys_I and α-keto acids [9,12].

The second highly conserved plant AOX Cys residue, Cys_{II}, may also be involved in modulating AOX activity. Two observations suggest this. Substitution of Ala at Cys_{II} has the effect of increasing basal activity of *Arabidopsis thaliana* AOX1a (AtAOX1a) [5]. In addition, 5 mM glyoxylate further stimulates AtAOX1a previously activated either with pyruvate or by substitution of Cys_I with a charged amino acid ([7]; unpublished results in [1]). This stimulation was traced to Cys_{II} because a substitution of Ala for Cys_{II} removed the additional glyoxylate stimulation [7]. However, because the sulfhydryl reagent iodoacetate was used to block glyoxylate effects at Cys_I in this mutant AtAOX1a [7], the possibility of an iodoacetate effect elsewhere in the protein could not be discounted. To explore the apparent activation of AOX by glyoxylate at Cys_{II} further, we have used site-directed mutagenesis of both Cys_I and Cys_{II} of AtAOX1a, combined with its heterologous expression in *E. coli*. By this approach, we have confirmed Cys_{II} as an activating site for AtAOX1a, and have explored the nature of that activation and the relationship between activation at Cys_I versus Cys_{II}.

2. Materials and methods

2.1. Materials

Bacterial growth media were prepared using Difco components (Becton Dickinson, Sparks, MD, USA). Restriction endonucleases and DNA ligase were from Promega (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). Chemicals were from Sigma (St. Louis, MO, USA).

Table 1
Examples of variant residues found at the Cys_I and Cys_{II} locations in AOXs and related proteins

Accession number[a]	Organism/source	Cys_I residue	Cys_{II} residue
Bacterial AOX			
YP_203961	*Vibrio fischeri*	n.a.[b]	Lys
ZP_00334281	*Thiobacillus denitrificans*	n.a.	His
ZP_00303905	*Novosphingobium aromaticivorans*	n.a.	His
	Sargasso Sea dataset[c]	n.a.	His
PTOX			
CAA06190	*Arabidopsis thaliana* (At4g22260)	?[b]	Ala
AAG18450	*Lycopersicon esculentum*	?	Ala
AAG02288	*Capsicum annuum*	?	Ala
AAC35554	*Oryza sativa*	?	Ala
AAG00450	*Triticum aestivum*	?	Ala
Plant AOX			
AAK58483	*Lycopersicon esculentum*, AOX1b[d].	Ser	Cys
AAL27797	*Zea mays*, AOX3[e].	Ser	Ser
XP_473758	*Oryza sativa*, AOX1b[f].	Ser	Ser
AAU11468	*Saccharum officinarum,* AOX1b	Cys	Ser
AAU11470	*Saccharum officinarum*, AOX1d	Cys	Ser
TC140366[g]	*Hordeum vulgare*	Cys	Ser
TC140365[g]	*Hordeum vulgare*	Cys	Ser
TC267599[g]	*Triticum aestivum*	Cys	Ser
TC267600[g]	*Triticum aestivum*	Ser	Ser

[a] Numbers are NCBI (National Center for Biotechnology Information) accession numbers unless otherwise noted.
[b] n.a. = the N-terminal region containing Cys_I or its analog is absent; ? = sequences differ from plant AOX N-terminus such that assigning a residue corresponding to Cys_I is difficult.
[c] Taken from the analysis of McDonald et al. [37]; there are nine accessions in this bacterial AOX type group: EAI62226,EAK49986, EAI66229, EAI79090, EAH004433, EAJ022071, EAI41828, EAK46738, EAH88150 [37].
[d] [9].
[e] [10].
[f] [31].
[g] TIGR (The Institute for Genomic Research) database designations.