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

Ann L. Umbach *, Vicki S. Ng [1], James N. Siedow

DCMB Group/Biology Department, Box 91000, Duke University, Durham, NC 27708-1000, USA

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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

* Corresponding author. Tel.: +1 919 613 8181; fax: +1 919 613 8177.
E-mail address: umbacha@duke.edu (A.L. Umbach).
[1] Current address: P.O. Box 922450, La Jolla, CA 92092-2450, USA.

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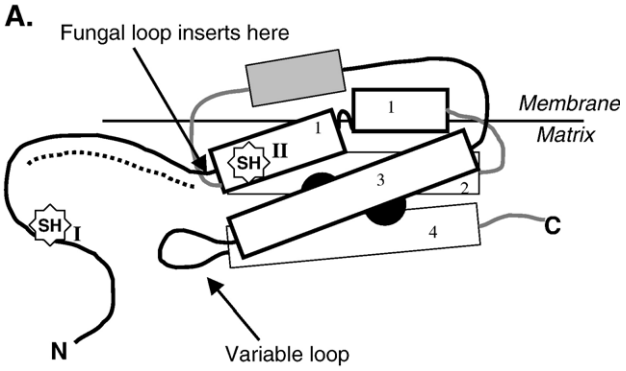

B.

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.

2.2. Site-directed mutagenesis and bacterial transformation

Substitution of amino acid residues at the Cys_I and Cys_{II} residue positions was accomplished as described [7] with the *Arabidopsis thaliana AOX1a* (At3g22370) clone contained in pAOX [13] as the template for single mutations at Cys_{II}. This clone corresponds essentially to the mature AtAOX1a protein sequence with Cys_I at position 78 (position 127 in the full-length sequence) and Cys_{II} at position 128 (position 177 in the full-length sequence). Two previously described *AtAOX1a* clones mutated at Cys_I (substitutions of Glu and Ala, [5]), also derived from pAOX, were used as templates to make double mutants at Cys_{II}. A total of seven mutants were generated. Mutants are designated showing: residue at site I/residue at site II, e.g. the wild-type AtAOX1a is AOX-Cys/Cys. The seven mutant AtAOX1as made were: AOX-Glu/Glu, -Glu/Ala, -Ala/Ala, -Ala/Glu, -Cys/Glu, -Cys/Lys, and -Cys/Trp. Mutagenesis was performed using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA, USA) according to the manufacturer's instructions. Primers used for the different substitutions at Cys_{II} were as follows, with the mutant codon shown in bold italics. For Cys_{II} changed to Glu: Forward 5′CGATTTGTTC-TTCCAGAGGAGATATGGA***GAA***CGAGCTATGATGC3′, Reverse 5′GCAT-CATAGCTCG***TTC***TCCATATCTCCTCTGGAAGAACAAATCG3′; For Cys_{II} changed to Ala: Forward 5′CTTCCAGAGGAGATATGGA***GCT***CGAGCTAT-***AGC***TCCATATCTCCTCTGGAAG3′; For Cys_{II} changed to Lys: Forward 5′ CCGATTTGTTCTTCCAGAGGAGATATGGA***AAG***CGAGCTATGATG3′, Reverse 5′GCATCATAGCTCG***CTT***TCCATATCTCCTCTGGAAGAACAAA-TC3′. For changing Cys_{II} to Trp, the same primers as for Lys were used except that the mutant codons were ***TGG*** in the forward primer and ***CCA*** for the reverse.

The mutated clones were used to transform XL-1 Blue *E. coli* from which the plasmids were re-isolated to confirm, by sequencing, the presence of the mutations and the lack of any nonspecific changes elsewhere. The sequences were analyzed by the Duke University DNA Core Facility, using the PRISM™ ABI system.

2.3. Alternative oxidase protein expression in SASX41B cells and activity assays

The plasmids containing wild-type or mutated *AtAOX1a* were introduced into SASX41B *E. coli*, a heme-deficient, and therefore cytochrome *c* oxidase deficient, strain [14] that can be induced to functionally express AOX protein under the proper growth conditions [13,15].

SASX41B cells were grown to induce AtAOX1a expression as described by Berthold [15]. Membranes were isolated in the presence of reductant, diothiothreitol (DTT), using a French pressure cell (ThermoSpectronic, Rochester, NY, USA) according to [5]. Membrane protein was determined by the method of Lowry et al. [16].

Activity of wild-type and mutated AtAOX1a protein in the isolated membranes was assayed as previously described [5,7] using 1 mM NADH as the substrate and a Clark-type oxygen electrode to monitor oxygen consumption. Nearly complete inhibition of the oxygen consumption rate by 2 mM SHAM confirmed that activity was due to AOX. Any small residual rate occurring in the presence of SHAM was subtracted from the uninhibited rate to provide the corrected rates reported in the Results. Before SHAM addition, pyruvate, glyoxylate, or succinate were added individually or sequentially to the assays, usually at 5 mM final concentration, from 1 M or 0.5 M (glyoxylate) stock solutions, kept as frozen aliquots. This assay concentration was chosen because of previous work indicating it to be either saturating for activation or sufficient to observe a strong activation [7,11,17].

2.4. Immunoblotting and densitometry

Samples of *E. coli* membranes were analyzed by SDS-PAGE followed by immunoblotting with the "AOA" antibody [18] as described [7] to determine the level of expression of AtAOX1a. Gels consisted of 10–17% gradients, and 100 mM DTT was included in the sample buffer, except for samples treated with the oxidant azodicarboxylic acid bis(dimethylamide) (diamide; see below).

Because AtAOX1a protein expression varied with the type of mutant protein and between different induction experiments (for example, see [7]), measured AOX activities had to be adjusted based on the relative amount of AOX protein present in the membranes. Films from immunoblots for AtAOX1a obtained by a chemiluminescent method and derived from gels run with equal amounts of total membrane protein per sample were analyzed by densitometry [7,8]. Membrane samples from any single induction experiment were always analyzed on the same gel. An average density value was obtained for AtAOX1a protein level from the cell lines of a single experiment. A fractional value was then calculated from the AtAOX1a protein density of each cell line relative to the mean. This value was used to adjust the rate of oxygen consumption. The final specific activity units were nmol O_2/ min/ mg membrane protein, adjusted for within-experiment AtAOX1a expression level.

2.5. DTT and diamide treatments

Cells were grown to induce AtAOX1a production by the method described above. Cell batches were divided in half. Membranes were isolated from one half by the usual method, i.e., with 5 mM DTT present in isolation buffers [5], and from the other half in the same way except that DTT was omitted. Membranes isolated without DTT were further treated with 3 mM diamide, added from a fresh 300 mM stock in DMSO, in a 200-μl volume on ice for 30 min. Subsequently, the membranes were diluted with 4.0 ml resuspension buffer before centrifugation at 100,000×*g* for 1 h. The resulting pellets were resuspended in 200 μl buffer and used in activity assays in the usual manner.

3. Results

3.1. Activation by substitution at Cys_{II}

Amino acid substitutions were made at the Cys_{II} residue of AtAOX1a and the activities of the mutated proteins were measured, after preparation under reducing conditions, in the presence or absence of the α-keto acid pyruvate. Glu, Lys and Trp introduce, respectively, a negatively charged, a positively charged, and a bulky hydrophobic side-chain into the Cys_{II} site. The previously-described Ala substitution [5] introduces a small non-polar side chain. Of the four substitutions, all but Trp caused a marked increase in basal, i.e., without pyruvate being present, AOX activity (Fig. 2; "no addition"). In comparison, at the Cys_I site, basal activation by charged substitutions, but not by Ala, occurred ([7]; this study). All of the AtAOX1a mutants were activated by pyruvate (Fig. 2) although for AOX-Cys/Lys, the amount of activation was less.

3.2. Pyruvate acts only at Cys_I; glyoxylate acts at both Cys_I and Cys_{II}

Substitutions of charged (Glu) or uncharged (Ala) amino acids were made at Cys_I and Cys_{II} to determine the site(s) of glyoxylate and pyruvate activation of AtAOX1a. Similar to the results of Fig. 2, substitution of Cys_{II} with Ala resulted in increased basal AOX activity, even when Ala was present at the Cys_I site (Table 2, AOX-Cys/Ala and AOX-Ala/Ala).

Regardless of the substitution at the Cys_{II} site, if Cys_I was present, AtAOX1a was stimulated by pyruvate (Table 2). Conversely, when Cys_I was mutated and Cys_{II} was present, essentially no pyruvate stimulation occurred (Table 2). Glyoxylate stimulated any AtAOX1a in which either Cys was present (Table 2) indicating that glyoxylate can act at both Cys sites. The double mutants AOX-Ala/Ala and AOX-Glu/Glu were negligibly stimulated by either glyoxylate or pyruvate (Table 2) supporting Cys_I and Cys_{II} as the exclusive

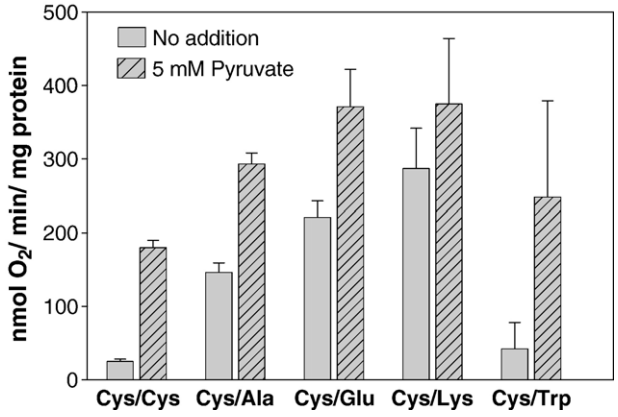

Fig. 2. Activity of AtAOX1a proteins substituted at Cys_{II} compared to wild-type AtAOX1a with and without the α-keto acid activator, pyruvate. Wild-type and mutated AtAOX1a proteins, functionally expressed in heme-deficient *E. coli*, were assayed for oxygen consumption in bacterial membranes isolated in the presence of 5 mM DTT. The substrate was 1 mM NADH, with 5 mM pyruvate added after a steady rate with NADH was established. Residual rates present after addition of 2 mM SHAM were subtracted to obtain adjusted rates which were then normalized on the basis of total membrane protein and level of alternative oxidase protein expression. Grey bars are the rates in the presence of NADH alone; hatched bars are rates after the addition of pyruvate. Graphed values are averages from independent protein induction experiments: 4 for AOX-Cys/Cys (wild-type), 3 for AOX-Cys/Glu, and 2 for the other mutated proteins. The error bars equal half the range, for $n=2$, or the standard error.

sites in the AtAOX1a protein for activation by these two α-keto acids.

Using these same AtAOX1a mutants, the two Cys sites were further distinguished by the magnitude of the pyruvate or glyoxylate concentration needed for activation. In keeping with previous observations ([7,15,17]; unpublished results in [1]), those proteins substituted at Cys_{II}, with Cys_I present, were half-maximally activated by pyruvate or glyoxylate at μM concentrations. For those substituted at Cys_I, but with Cys_{II} present, glyoxylate had no effect on activity at 1 mM or less, but close to maximal activation was achieved at 5 mM (Table 3).

3.3. Independent activation at Cys_I and Cys_{II}

The specificity of pyruvate for Cys_I and glyoxylate at both Cys_I and Cys_{II} was further demonstrated by activity assays in which the two α-keto acids were added sequentially. Glyoxylate added after pyruvate stimulated AOX activity further only when Cys_{II} was present (Fig. 3A, AOX-Cys/Cys,-Glu/Cys,-Ala/Cys), indicating that with pyruvate or a substitution blocking the Cys_I site, glyoxylate interacted with Cys_{II}. The effect was weakest for the wild-type enzyme (Fig. 3A). Addition of glyoxylate first to activity assays for any of the proteins prevented further stimulation of activity by pyruvate (Fig. 3B).

Together with the results in Fig. 2 and Table 2, these sequential addition experiments suggested that activation occurs relatively independently at the two Cys sites. To examine this possibility another way, we exploited the ability of succinate to stimulate AtAOX1a substituted at Cys_I with Ala

Table 2
Comparison of activity of wild-type and mutated *Arabidopsis* AOX1a without or with the α-keto acids pyruvate or glyoxylate

AOX Type[a]		Pyruvate[b]	% Increase	Glyoxylate[b]	% Increase
Cys/Cys	−	25 (3)[c,d]		16 (3)[d]	
	+	180 (10)	620	230 (25)	1338
Glu/Cys	−	220 (61)[e]		174 (40)[e]	
	+	237 (71)	8	342 (103)	97
Cys/Glu	−	221 (22)[e]		212 (28)[e]	
	+	371 (51)	68	312 (26)	47
Glu/Glu	−	267 (34)[e]		266 (36)[e]	
	+	274 (27)	3	289 (37)	9
Ala/Cys	−	35 (3)[e]		34 (8)[e]	
	+	42 (9)	20	83 (8)	144
Cys/Ala	−	146 (13)[f]		130 (32)[f]	
	+	293 (15)	101	277 (31)	114
Ala/Ala	−	154 (5)[e]		119 (11)[e]	
	+	174 (11)	13	136 (17)	14

[a] Wild-type (Cys/Cys) or mutated AtAOX1a; Amino acids given are the residues at the Cys_I/Cys_{II} sites.

[b] AOX activity in *E. coli* membranes isolated in the presence of 5 mM DTT was sequentially measured without (−) and with (+) the designated α-keto acid present at 5 mM. The respiratory substrate was 1 mM NADH. Residual rates in the presence of SHAM were subtracted from the raw rates and the rates were adjusted for the amount of expressed AtAOX1a protein. Rates are nmol O_2/ min/ mg membrane protein and are averages of rates from 2 to 4 separate AtAOX1a protein induction experiments.

[c] Values in parentheses are either standard errors, for $n=3$ or 4, or half the range for $n=2$, where n is the number of separate induction experiments.

[d] $n=4$.

[e] $n=3$.

[f] $n=2$.

([7]; unpublished results in [11]) and performed sequential addition assays with succinate and glyoxylate. AOX-Ala/Cys was stimulated incrementally by succinate and glyoxylate, showing similar increases in activity with the addition of each acid in either order, and approached fully-activated wild-type rates when both acids were present (Fig. 4A and B). For AOX-Ala/Ala and AOX-Ala/Glu, which were not glyoxylate-stimulated but had elevated basal activities, addition of succinate resulted in increased rates that were comparable to

Table 3
Activator concentrations needed for half-maximum AtAOX1a activity[a]

AOX Type[b]	Pyruvate[c]	Glyoxylate[c]
Cys/Cys	50–100 μM	<50 μM[d]
Cys/Glu	<50 μM	<50 μM
Cys/Ala	<50 μM	<50 μM
Glu/Cys	n.s	<5 mM[e]
Ala/Cys	n.s.	<5 mM[e]

[a] AOX activity in *E. coli* membranes was measured essentially as described for Table 2, with NADH as the respiratory substrate. Pyruvate or glyoxylate was added to the assays from a stock solution. When a constant rate of oxygen uptake had been achieved, another stock addition was made to reach the next desired activator concentration. After all concentration levels had been tested, SHAM was added to stop respiration.

[b] Wild-type (Cys/Cys) or mutated AtAOX1a; Amino acids given are the residues at the Cys_I/Cys_{II} positions.

[c] Concentrations tested: 0, 0.05, 0.10, 0.50, 1.00, 5.00, 10.00 mM.

[d] An additional stimulation (18–20%) was observed at 5 mM glyoxylate.

[e] Note that at 1 mM, glyoxylate did not stimulate, whereas at 5 mM, stimulation was close to the apparent maximum reached at 10 mM.

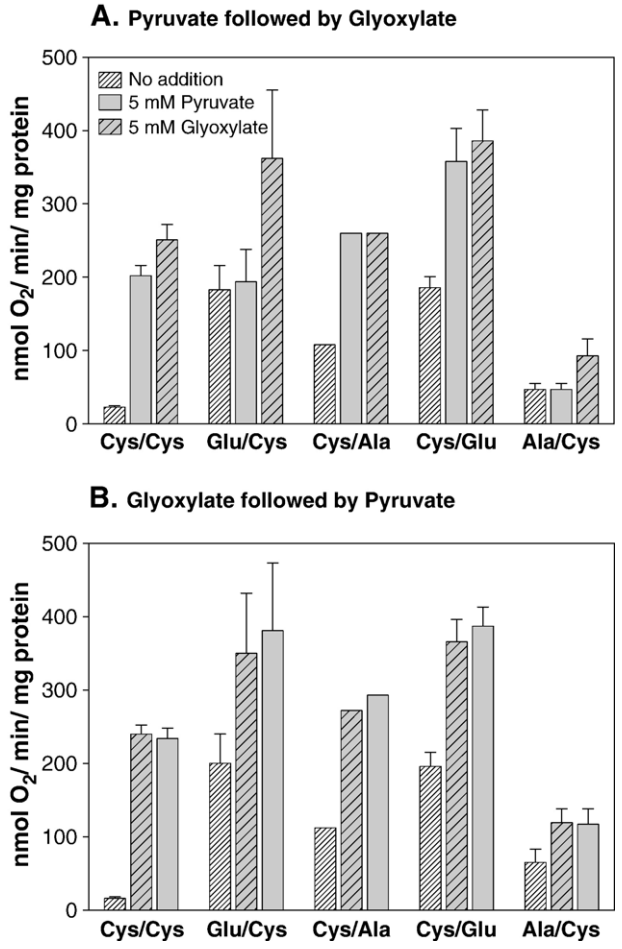

Fig. 3. Activity response of AtAOX1a proteins, mutated at Cys_I and/or Cys_{II}, to sequential additions of the α-keto acids pyruvate and glyoxylate. Assays were performed as described for Fig. 2. Glyoxylate was used at 5 mM final concentration. In A, after NADH addition, pyruvate was added to the assay mixture. When a new constant rate had been established, glyoxylate was added. In B, glyoxylate addition was followed by pyruvate addition. Bars correspond to the assay additions as shown; the white cross-hatched bars are the rates in the presence of NADH alone. Graphed values are averages of separate induction experiments: 4 for AOX-Cys/Cys; 3 for AOX-Glu/Cys and AOX-Cys/Glu; 2 for AOX-Ala/Cys; 1 for AOX-Cys/Ala. Error bars where shown are the standard error or, for $n=2$, half the range.

those of the fully activated wild-type enzyme (Fig. 4A and B). Addition of succinate or glyoxylate in either order did not markedly affect activity of the double mutant AOX-Glu/Ala (data not shown). Therefore, substitution of Ala at Cys_{II} does not appear to have the same effect as Ala at Cys_I with respect to conferring succinate activation, and succinate can be considered to activate only when Ala is located in the Cys_I position in these particular assays.

3.4. The Cys_I disulfide bond blocks the Cys_{II} glyoxylate effect

Activation at the two Cys sites appeared to occur largely independently. However, for the preceding experiments, the Cys_I site was always in a free state due to amino acid substitutions and because isolation of the membranes in the presence of DTT prevented formation of the intersubunit disulfide bond (see Materials and methods; [5]). To determine what effect the disulfide bond might have on activation at Cys_{II}, activities of AtAOX1a wild-type and mutants in membranes isolated without DTT and then treated with diamide to oxidize the AOX disulfide bond were measured. Diamide treatment prevented pyruvate activation of the wild-type enzyme, AOX-Cys/Cys (Fig. 5), consistent with over 90% of the protein dimer being in the covalently associated, i.e., oxidized, form and Cys_I being inaccessible. Diamide treatment also prevented glyoxylate stimulation of the wild-type enzyme (Fig. 5). In contrast, the basal activity of AOX-Glu/Cys, which cannot form a covalent dimer through the Cys_I disulfide bond, was not affected by diamide treatment (Fig. 5, Glu/Cys, "no addition", compare to Fig. 3A) and this mutant was stimulated by glyoxylate following pyruvate addition (Fig. 5). Therefore, by analogy, it seems unlikely that diamide might have interfered directly with Cys_{II} in the wild-type enzyme. The Cys_{II}-substituted AtAOX1a, AOX-Cys/Ala, was also diamide-treated. In the absence of diamide, this mutant had high basal activity and was stimulated by pyruvate (Figs. 2 and 3). This pyruvate stimulation was blocked by diamide treatment (Fig. 5). However, AOX-Cys/Ala retained substantial basal activity (Fig. 5, AOX-Cys/Ala, "no addition"), 47% and 71% of the standard-isolation AOX rate for two induction experiments, even though,

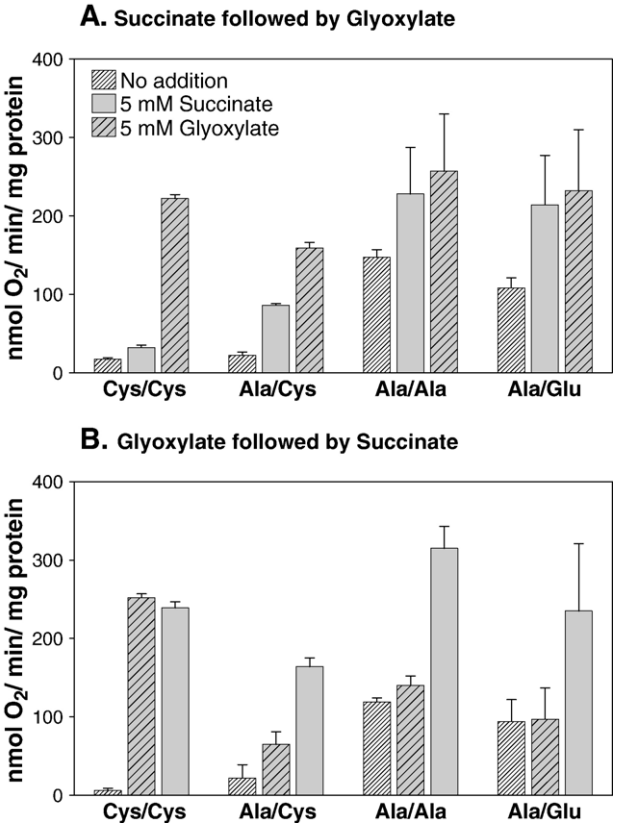

Fig. 4. Activity responses of AtAOX1a proteins mutated at Cys_I and/or Cys_{II} to sequential additions of succinate and glyoxylate. Assays were performed as described for Figs. 2 and 3 except that 5 mM succinate was used instead of pyruvate. Bars are as described for Fig. 3 and as illustrated in the figure. Graphed values are averages of two separate induction experiments. Error bars show half the range.

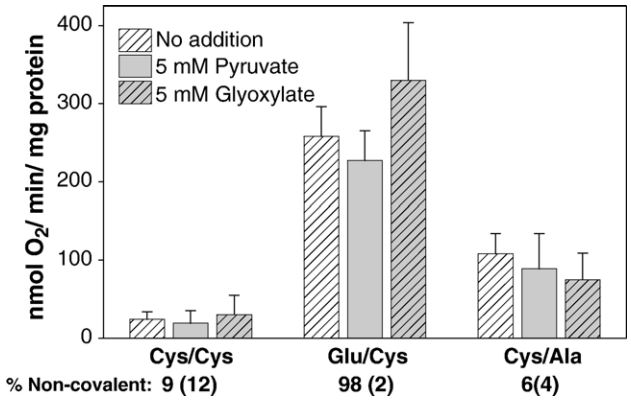

Fig. 5. Effect of oxidation by diamide on the activities of wild-type and mutated AtAOX1a. Diamide treatment drives intersubunit disulfide bond formation. Activities are shown for the wild-type enzyme (Cys/Cys), and AtAOX1a substituted with Glu at Cys_I (Glu/Cys) or with Ala at Cys_{II} (Cys/Ala). Membranes were isolated without DTT and were further treated with 3 mM diamide before activity was assayed. Sequential additions of pyruvate and glyoxylate were made to the activity assays as described for Fig. 3. White cross-hatched bars are activities in the presence of NADH alone. Averages from three (Cys/Cys) or two (Glu/Cys, Cys/Ala) separate inductions for each AtAOX1a, and the standard deviation (Cys/Cys) or half the range, are shown. The average percentages of the AOX protein in the non-covalently associated state (migrating as the monomer) after diamide treatment, as determined by immunoblot densitometry from SDS-PAGE gels run under non-reducing conditions, are shown with the standard deviation (Cys/Cys) or half the range in parentheses.

at most only 10% of the protein was in the non-covalent, reduced state with Cys_I free (Fig. 5).

4. Discussion

Most known plant AOX protein sequences contain two conserved Cys residues, Cys_I and Cys_{II}. Previous work has established Cys_I, located in the N-terminal third of the protein, as a site of AOX activity regulation through its interaction with α-keto acids and as the component of an inactivating intersubunit disulfide bond in the homodimeric enzyme [5,6,8,19]. Here, we extend previous observations ([7]; unpublished results in [1]) that Cys_{II} can also be a site for regulation of AOX activity.

By making amino acid substitutions at Cys_{II} of AtAOX1a, either independently or together with substitutions at Cys_I, we have been able to separate the sites of action of two α-keto acids, glyoxylate and pyruvate. The smaller glyoxylate activates through interaction with AtAOX1a at both Cys_I and Cys_{II}, while pyruvate acts only at Cys_I (Table 2). By analogy with Cys_I, the glyoxylate association with Cys_{II} may be through thiohemiacetal formation [12]. The concentration dependence for activation at the two Cys residues differs, which may indicate different modes of interaction, but could also reflect differing accessibilities of the two cysteines to the respective α-keto acids. Activities of the double-mutant proteins AOX-Glu/Glu and AOX-Ala/Ala were not increased by glyoxylate or pyruvate (Table 2, Fig. 4), consistent with the Cys_I and Cys_{II} sites being the only regions of the protein where α-keto acid activation takes place.

The near-additive activation at the two Cys sites, arising from either residue substitution or addition of activators (Figs. 3, 4), indicates that changes induced in the AOX protein at Cys_I and Cys_{II} occur independently. The mutant AOX-Ala/Cys, which responded incrementally to sequential additions of succinate and glyoxylate in either order, particularly demonstrated this (Fig. 4). However, the wild-type enzyme is something of an exception to additive activation, as the additional stimulation at Cys_{II} was relatively small (Fig. 3, Table 3). One possibility is that if a bulky thiohemiacetal moiety formed at Cys_I, it could effectively block reactivity of glyoxylate with Cys_{II} due to steric hindrance. Less bulky residues at the Cys_I site might only reduce the accessibility of Cys_{II}, requiring a high concentration of glyoxylate to activate there, but not preventing the activation as completely. Whatever its basis, the weak stimulation of wild-type AtAOX1a by glyoxylate at Cys_{II} and the high concentration needed calls the physiological significance of this latter activation into question. The concentration of glyoxylate might increase during photorespiration, even in mitochondria, leading to some additional stimulation of AOX under a condition where its activity would be needed to regenerate NAD^+ [20,21]. However, Cys_{II} may have another role in the AOX protein, as suggested by results discussed below.

An intriguing feature of the Cys_{II}-substitution AtAOX1a mutants was that most were constitutively active (Ala, Gly, Lys; Ser, unpublished results in [1]) suggesting activation at Cys_{II} does not involve the AOX catalytic site directly, given the wide range of different chemical entities that allow retention of activity when placed at that site. This result is consistent with the location of Cys_{II} on the side of helix I nearly opposite from its diiron coordinating Glu (Fig. 1A). Unlike at Cys_I, where charge repulsion appears to be the driving force for activation [7], at Cys_{II} uncharged as well as charged substitutions resulted in a high level of basal activity. This variety of activating residues mirrors the range of residues found naturally at the Cys_{II} site among AOXs from different taxa, and related proteins. Among all these proteins, in contrast to its conservation as a Cys among most plant AOXs, the residue at the Cys_{II} position is strikingly unconserved in a region of otherwise high homology, illustrated by a conserved-domain alignment for AOXs and relatives (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=cd01053; [22]). Other residues include Ala, found in the chloroplast plastid terminal oxidase sequences (PTOX or IMMUTANS; Table 1), and Lys or His found in the recently-identified AOX relatives of bacteria [23–26] (Table 1). Fungal AOXs have a variety of residues at the Cys_{II} site, none of which are Cys (see [27] for some examples) and there is a growing list of plant AOX sequences (Table 1) in which Cys_{II} is substituted with Ser.

Given the range of substitutions at the Cys_{II} site that constitutively activate AtAOX1a, the relatively small stimulation of the wild-type enzyme by glyoxylate at Cys_{II}, and the general lack of conservation of Cys_{II} overall in AOXs and related proteins, perhaps the predominance of Cys_{II} in plant AOX proteins is due to its non-activating nature. This feature may be of critical importance. Results obtained using wild-type

AtAOX1a treated with diamide to covalently link the AOX dimer subunits by the disulfide bond indicate that Cys_I must be in the reduced state for complete activation at Cys_{II} to occur (Fig. 5). Formation of the disulfide bond either blocks access of glyoxylate to $Cys_{II,}$ or allows glyoxylate binding but prevents subsequent activation. Consequently, when Cys_{II} is present, AtAOX1a activity is primarily under control of the redox state of Cys_I, regardless of glyoxylate concentration. However, if an activating residue is already present at the Cys_{II} site, as was the case when Cys_{II} was substituted with Ala (Fig. 5), the inhibitory effect of the disulfide bond is incomplete and considerable AOX activity is possible, short-circuiting the regulatory bond mechanism. The strong control of activity by the disulfide/sulfhydryl redox system for perhaps all Cys_I/Cys_{II} AOXs fits well with the likelihood that modulation of the AOX bond redox state is by an isocitrate dehydrogenase-driven system [28,29] possibly operating via thioredoxin [30] in vivo.

Studies with plant AOX isoforms that are naturally substituted at the Cys_I and Cys_{II} sites (Table 1) will be essential for elucidating the role of these regions in AOX regulation. Unlike the substitution mutants of AtAOX1a used here, these enzymes have native sequence and structural contexts. By analogy with the AtAOX1a mutants, Ser_I/Ser_{II} AOXs identified in maize (AOX3; [10]) and rice (AOX1b; [31]) would be expected to have high basal activity, even without succinate, due to the presence of Ser at the Cys_{II} site. Similarly, Cys_I/Ser_{II} AOXs (Table 1), should be partly active, even when the Cys_I residues are oxidized. However, extrapolating results of activating substitutions or glyoxylate effects in AtAOX1a to other enzymes is risky. AOX1b of tomato, a Ser_I/Cys_{II} AOX (Table 1) and the only native substituted AOX to be characterized with respect to acid activators [9], has no basal activity and, although it is activated by succinate at Ser_I, it is not activated by glyoxylate alone even though Cys_{II} is present [9].

For plant AOXs with Cys_I and Cys_{II}, the residues surrounding Cys_{II} are highly conserved (Fig. 1B). In contrast, all the native Ser_{II} enzymes listed in Table 1 have a different amino acid motif surrounding Ser_{II}, regardless of the residue at the Cys_I position (Fig. 1B). Significantly, tomato AOX1b has three of six residues in common with the Ser_{II}-type motif around its Cys_{II} (Fig. 1B), perhaps accounting for the inability of glyoxylate to stimulate its activity. As Crichton et al. [32] and Holtzapffel et al. [9] point out for the Cys_I site, other residues, perhaps contained in these specific motifs, are likely involved in the events occurring around the Cys_{II}/Ser_{II} site. Therefore it cannot be concluded that the native Ser_{II} AOXs would be constitutively active. A more important feature for the native substituted AOXs is probably the lack of redox control when Ser_I is present, suggestive of an altered physiological role [9].

Much of the structural variability in eukaryotic AOX and related prokaryotic sequences occurs around one end of the diiron-binding four-helical bundle: the presence or absence of a long N-terminal segment [23], the insertion common to PTOX and to the bacterial sequences in the interhelical loop between helices three and four [23,24], and an extra segment, or loop, present in AOXs of fungi [27] and *Chlamydomonas* [33] that inserts between helix one and a putative quinone-binding site [34] (Fig. 1A). The plant AOX regulatory Cys_I is also potentially associated with this side of the protein (Fig. 1A). The substitution mutants studied here have revealed another region in this general vicinity, represented by Cys_{II}, that can affect activity. Perhaps not coincidentally, three of the four sequence regions identified by Crichton et al. [32] as potentially involved in the constitutive activity of the *Sauromatum* Cys_I/Cys_{II}-type AOX are located at this same end of the diiron bundle, two occurring around Cys_I and Cys_{II}, respectively, and one in the loop between helices 3 and 4. A possible function of activated Cys_I and Cys_{II}, as well as these other variable features, could be to modulate ubiquinone binding [7,35], either by enhancing binding of the reduced substrate or by reversing binding of an inhibitory oxidized product [36]. Comparing effects of amino acid substitutions within a native Cys_{II}-type AOX context versus within a Ser_{II}-type AOX context will help to develop a picture of the processes occurring in this region of the protein that influence AOX catalysis.

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