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

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Abstract

Two Cys residues, CysI and CysII, are present in most plant alternative oxidases (AOXs). CysI inactivates AOX by forming a disulfide bond with the corresponding CysI residue on the adjacent subunit of the AOX homodimer. When reduced, CysI associates with α-keto acids, such as pyruvate, to activate AOX, an effect mimicked by charged amino acid substitutions at the CysI site. CysII 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 CysI and CysII confirmed that glyoxylate interacted with either Cys, while the effect of pyruvate (or succinate for AtAOX1a substituted with Ala at CysI) was limited to CysI. A variety of CysII substitutions constitutively activated AtAOX1a, indicating that neither the catalytic site nor, unlike at CysI, charge repulsion is involved. Independent effects at each Cys were suggested by lack of CysII substitution interference with pyruvate stimulation at CysI, 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 CysI must be in the reduced state for activation at CysII to occur.

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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 CysI and CysII (nomenclature of Berthold et al. [4]). CysI is located in the structurally undefined N-terminus, whereas CysII 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 CysI. When the CysI 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 CysI 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 CysI residues in the native homodimer to form an intermolecular disulfide bond [8] or by substitution of CysI 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 CysI, some do not (Table 1). Two of these, in which a
Ser residue (SerI) occupies the CysI 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 SerI isoform is stimulated, not by \( \alpha \)-ketoads, but by succinate [9]. Similarly, for soybean and Arabidopsis CysI-type AOXs, substitution of Ser (soybean; [11]) or Ala (Arabidopsis; [7]; unpublished results in [11]) for CysI 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 CysI and \( \alpha \)-ketoads [9,12].

The second highly conserved plant AOX Cys residue, CysII, may also be involved in modulating AOX activity. Two observations suggest this. Substitution of Ala at CysII 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 CysI with a charged amino acid ([7]; unpublished results in [1]). This stimulation was traced to CysII because a substitution of Ala for CysII removed the additional glyoxylate stimulation [7]. However, because the sulphydryl reagent iodoacetate was used to block glyoxylate effects at CysI 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 CysII further, we have used site-directed mutagenesis of both CysI and CysII of AtAOX1a, combined with its heterologous expression in \( E.\ coli \). By this approach, we have confirmed CysII as an activating site for AtAOX1a, and have explored the nature of that activation and the relationship between activation at CysI versus CysII.

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

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Organism/source</th>
<th>CysI residue</th>
<th>CysII residue</th>
</tr>
</thead>
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<tr>
<td><strong>Bacterial AOX</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YP_203961</td>
<td>Vibrio fischeri</td>
<td>n.a.</td>
<td>Lys</td>
</tr>
<tr>
<td>ZP_00334281</td>
<td>Thiolobacillus denitrificans</td>
<td>n.a.</td>
<td>His</td>
</tr>
<tr>
<td>ZP_00303905</td>
<td>Novosphingobium</td>
<td>n.a.</td>
<td>His</td>
</tr>
<tr>
<td></td>
<td>aromaticivorans</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sargasso Sea dataset</td>
<td>n.a.</td>
<td>His</td>
</tr>
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<td><strong>PTOX</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CAA06190</td>
<td>Arabidopsis thaliana</td>
<td>?</td>
<td>Ala</td>
</tr>
<tr>
<td></td>
<td>(At4g22260)</td>
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<td></td>
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<td>Lycopersicon esculentum</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>AAG02288</td>
<td>Capsicum annumum</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>AAC35554</td>
<td>Oryza sativa</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>AAG00450</td>
<td>Triticum aestivum</td>
<td>?</td>
<td>?</td>
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<td><strong>Plant AOX</strong></td>
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<td>Cys</td>
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<td>Ser</td>
</tr>
<tr>
<td>TC140366 ^6</td>
<td>Hordeum vulgare</td>
<td>Cys</td>
<td>Ser</td>
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<td>TC267600 ^6</td>
<td>Triticum aestivum</td>
<td>Ser</td>
<td>Ser</td>
</tr>
</tbody>
</table>

\(^a\) Numbers are NCBI (National Center for Biotechnology Information) accession numbers unless otherwise noted.

\(^b\) n.a. = the N-terminal region containing CysI or its analog is absent; ? = sequences differ from plant AOX N-terminus such that assigning a residue corresponding to CysI is difficult.

\(^c\) Taken from the analysis of McDonald et al. [37]; there are nine accessions in this bacterial AOX type group: EA162226, EAK49986, EA166229, EAI79090, EAH04433, EAJO22071, EAJ41828, 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 CysI and CysII 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 CysII. This clone corresponds essentially to the mature AToAOX1a protein sequence with CysII at position 78 (position 127 in the full-length sequence) and CysI at position 128 (position 177 in the full-length sequence). Two previously described AToAOX1a clones mutated at CysII (substitutions of Glu and Ala, [5]), also derived from pAOX, were used as templates to create double mutants at CysII. A total of seven mutants were generated. Mutants are designated showing: residue at site I/residue at site II, e.g. the wild-type AToAOX1a is AOX-Cys/Cys. The seven mutant AToAOX1as 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 CysII were as follows, with the mutant codon shown in bold italics. For CysII changed to Glu: Forward 5′ CGAATGGTC- TTCCAGAGGAGATATGGAGAAGACGGCTATGATGCG, Reverse 5′ GCATCATAAAGCCTTCTCCATATCTCCTCCTGAGAAGAACAATGCG; For CysII changed to Ala: Forward 5′ TTCCAGAGGAGATATGGAGAAGACGGCTATGATGCG, Reverse 5′ GCATCATAAAGCCTTCTCCATATCTCCTCCTGAGAAGAACAATGCG. For changing CysII to Trp, the same primers as for Lys were used except that the mutant codons were TGG in the forward primer and CCG 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 AToAOX1a 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 AToAOX1a expression as described by Berthold [15]. Membranes were isolated in the presence of reductant, diithiothreitol (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 AToAOX1a 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 the method of Lowry et al. [16].

Activity of AToAOX1a 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 O2/min/mg membrane protein, adjusted for within-experiment AtAOX1a expression level.

2.6. DTT and diamide treatments

Cells were grown to induce AToAOX1a 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 CysII

Amino acid substitutions were made at the CysII 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 CysII 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 CysII site, basal activation by charged substitutions, but not by Ala, occurred (Table 2; 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 CysI; glyoxylate acts at both CysI and CysII

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

Regardless of the substitution at the CysII site, if CysI was present, AtAOX1a was stimulated by pyruvate (Table 2). Conversely, when CysI was mutated and CysII was present, essentially no pyruvate stimulation occurred (Table 2). Glyoxylate stimulated any AtAOX1a in which either CysI 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 CysI and CysII as the exclusive
sites in the AtAOX1α protein for activation by these two \( \alpha \)-keto acids.

Using these same AtAOX1α 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 CysII, with CysI present, were half-maximally activated by pyruvate or glyoxylate at \( \mu \)M concentrations. For those substituted at CysI, but with CysII 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 CysI and CysII

The specificity of pyruvate for CysI and glyoxylate at both CysI and CysII was further demonstrated by activity assays in which the two \( \alpha \)-keto acids were added sequentially. Glyoxylate added after pyruvate stimulated AOX activity further only when CysII was present (Fig. 3A, AOX-Cys/Cys-Glu/Cys-ALA/Cys), indicating that with pyruvate or a substitution blocking the CysI site, glyoxylate interacted with CysII. 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 AtAOX1α substituted at CysI with Ala (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 those observed with the wild-type enzyme.

### Table 3

Comparison of activity of wild-type and mutated *Arabidopsis* AOX1α without or with the \( \alpha \)-keto acids pyruvate or glyoxylate

<table>
<thead>
<tr>
<th>AOX Type</th>
<th>Pyruvate</th>
<th>Glyoxylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys/Cys</td>
<td>50–100 ( \mu )M</td>
<td>&lt;50 ( \mu )M</td>
</tr>
<tr>
<td>Cys/Glu</td>
<td>&lt;50 ( \mu )M</td>
<td>&lt;50 ( \mu )M</td>
</tr>
<tr>
<td>Cys/Ala</td>
<td>&lt;50 ( \mu )M</td>
<td>&lt;50 ( \mu )M</td>
</tr>
<tr>
<td>Glu/Cys</td>
<td>n.s.</td>
<td>&lt;5 ( \mu )M</td>
</tr>
<tr>
<td>Ala/Cys</td>
<td>n.s.</td>
<td>&lt;5 ( \mu )M</td>
</tr>
</tbody>
</table>

* Wild-type (Cys/Cys) or mutated AtAOX1α; Amino acids given are the residues at the CysI/CysII sites.

* AOX activity in *E. coli* membranes isolated in the presence of 5 mM DTT was sequentially measured without (−) and with (+) the designated \( \alpha \)-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 AtAOX1α protein. Rates are nmol O₂/ min/mg membrane protein and are averages of rates from 2 to 4 separate AtAOX1α protein induction experiments.

* 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.

* \( n = 4 \).

* \( n = 3 \).

* \( n = 2 \).
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 CysII does not appear to have the same effect as Ala at CysI with respect to conferring succinate activation, and succinate can be considered to activate only when Ala is located in the CysI position in these particular assays.

3.4. The CysI disulfide bond blocks the CysII glyoxylate effect

Activation at the two Cys sites appeared to occur largely independently. However, for the preceding experiments, the CysI 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 CysII, 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 CysI 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 CysII 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 CysII in the wild-type enzyme. The CysII-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, 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 CysII does not appear to have the same effect as Ala at CysI with respect to conferring succinate activation, and succinate can be considered to activate only when Ala is located in the CysI position in these particular assays.

Fig. 3. Activity response of AtAOX1a proteins, mutated at CysI and/or CysII, 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.

Fig. 4. Activity responses of AtAOX1a proteins mutated at CysI and/or CysII 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.
at most only 10% of the protein was in the non-covalent, reduced state with CysI free (Fig. 5).

4. Discussion

Most known plant AOX protein sequences contain two conserved Cys residues, CysI and CysII. Previous work has established CysI, located in the N-terminal third of the protein, as a site of AOX activity regulation through its interaction with \( \alpha \)-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 CysII can also be a site for regulation of AOX activity.

By making amino acid substitutions at CysI of AtAOX1a, either independently or together with substitutions at CysI, we have been able to separate the sites of action of two \( \alpha \)-keto acids, glyoxylate and pyruvate. The smaller glyoxylate activates through interaction with AtAOX1a at both CysI and CysII, while pyruvate acts only at CysI (Table 2). By analogy with CysI, the glyoxylate association with CysII may be through thiohemicatalytic 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 \( \alpha \)-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 CysI and CysII sites being the only regions of the protein where \( \alpha \)-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 CysI and CysII occur independently. The mutant AOX-Ala/CysI, 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 CysII was relatively small (Fig. 3, Table 3). One possibility is that if a bulky thiohemicatalytic moiety formed at CysI, it could effectively block reactivity of glyoxylate with CysII due to steric hindrance. Less bulky residues at the CysI site might only reduce the accessibility of CysII, 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 CysII 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, CysII may have another role in the AOX protein, as suggested by results discussed below.

An intriguing feature of the CysII-substitution AtAOX1a mutants was that most were constitutively active (Ala, Gly, Lys; Ser, unpublished results in [1]) suggesting activation at CysII 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 CysII on the side of helix I nearly opposite from its diiron coordinating Glu (Fig. 1A). Unlike at CysI, where charge repulsion appears to be the driving force for activation [7], at CysII 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 CysII 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 CysII 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=ed01053; [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 CysII 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 CysII is substituted with Ser.

Given the range of substitutions at the CysII site that constitutively activate AtAOX1a, the relatively small stimulation of the wild-type enzyme by glyoxylate at CysII, and the general lack of conservation of CysII overall in AOXs and related proteins, perhaps the predominance of CysII 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 CysI must be in the reduced state for complete activation at CysII to occur (Fig. 5). Formation of the disulfide bond either blocks access of glyoxylate to CysII or allows glyoxylate binding but prevents subsequent activation. Consequently, when CysII is present, AtAOX1a activity is primarily under control of the redox state of CysII, regardless of glyoxylate concentration. However, if an activating residue is already present at the CysII site, as was the case when CysII 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/sulphydryl redox system for perhaps all CysII/CysII 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 CysII and CysII 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, SerI/SerII 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 CysII site. Similarly, CysI/SerII AOXs (Table 1) should be partly active, even when the CysII residues are oxidized. However, extrapolating results of activating substitutions or glyoxylate effects in AtAOX1a to other enzymes is risky. AOX1b of tomato, a SerI/CysII 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 SerI, it is not activated by glyoxylate alone even though CysII is present [9].

For plant AOXs with CysI and CysII, the residues surrounding CysII are highly conserved (Fig. 1B). In contrast, all the native SerII enzymes listed in Table 1 have a different amino acid motif surrounding SerII, regardless of the residue at the CysII position (Fig. 1B). Significantly, tomato AOX1b has three of six residues in common with the SerII motif around its CysII (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 CysII site, other residues, perhaps contained in these specific motifs, are likely involved in the events occurring around the CysII/SerII site. Therefore it cannot be concluded that the native SerII AOXs would be constitutively active. A more important feature for the native substituted AOXs is probably the lack of redox control when SerI 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 CysII 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 CysIII, 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 CysII/CysII-type AOX are located at this same end of the diiron bundle, two occurring around CysII and CysIII, respectively, and one in the loop between helices 3 and 4. A possible function of activated CysII and CysIII, 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 CysII-type AOX context versus within a SerII-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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References


