Regulation of the Cyanide-resistant Alternative Oxidase of Plant Mitochondria

IDENTIFICATION OF THE CYSTEINE RESIDUE INVOLVED IN α-KETO ACID STIMULATION AND INTERSUBUNIT DISULFIDE BOND FORMATION

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The cyanide-resistant alternative oxidase of plant mitochondria is a homodimeric protein whose activity can be regulated by a redox-sensitive intersubunit sulfhydryl/disulfide system and by α-keto acids. After determining that the Arabidopsis alternative oxidase possesses the redox-sensitive sulfhydryl/disulfide system, site-directed mutagenesis of an Arabidopsis cDNA clone was used to individually change the two conserved Cys residues, Cys-128 and Cys-78, to Ala. Using diamide oxidation and chemical cross-linking of the protein expressed in Escherichia coli, Cys-78 was shown to be: 1) the Cys residue involved in the sulfhydryl/disulfide system; and 2) not required for subunit dimerization. The C128A mutant was stimulated by pyruvate, while the C78A mutant protein had little activity and displayed no stimulation by pyruvate. Mutating Cys-78 to Glu produced an active enzyme which was insensitive to pyruvate, consistent with α-keto acid activation occurring through a thiohemiacetal. These results indicate that Cys-78 serves as both the regulatory sulfhydryl/disulfide and the site of activation by α-keto acids. In light of these results, the previously observed effects of sulfhydryl reagents on the alternative oxidase of isolated soybean mitochondria were re-examined and were found to be in agreement with a single sulfhydryl residue being the site both of α-keto acid activation and of the regulatory sulfhydryl/disulfide system.

In all plants and many fungi, a cyanide-resistant, alternative respiratory pathway branches from the cytochrome pathway at the ubiquinone pool in mitochondria (1). Electron flow through this alternative pathway is not coupled to ATP synthesis at two of the three proton translocation sites. In specific thermogenic floral tissues of some plants, a high rate of electron flow through the alternative pathway generates heat and volatilizes compounds that attract insect pollinators (2). In non-thermogenic plant tissues, the alternative pathway may allow continued turnover of carbon skeletons through glycolysis and the tricarboxylic acid cycle when the ATP concentration is high and inhibits ATP synthase activity (3) so that the pathway can function as an energy overflow mechanism (4). However, because the alternative pathway can be active even when the cytochrome pathway is not saturated (5–7), it is likely that the two pathways are regulated in concert to balance ubiquinone pool oxidation/reduction and carbon skeleton turnover in response to cytosolic ATP levels (8, 9).

In recent years, a great deal of progress has been made in understanding the post-translational regulation of the alternative oxidase, the terminal oxidase that comprises the alternative respiratory pathway. The alternative oxidase is a homodimeric protein in the inner mitochondrial membrane and the oxidation/reduction state of an intersubunit disulfide bond has been demonstrated to regulate its activity (10, 11). A second mechanism of activation of the alternative oxidase involves α-keto acids, notably pyruvate (12), which significantly activate the enzyme when the regulatory sulfhydryl/disulfide is in the reduced state, but have less effect on the oxidized form of the enzyme (11, 13, 14). Recently, use of sulfhydryl reagents showed that the site of α-keto acid action also involves a sulfhydryl residue, probably through the formation of a thiohemiacetal by reaction between the cysteine sulfhydryl and the α-keto acid (15). The same study indicated that this sulfhydryl is different from the one involved in disulfide bond formation.

Based on homology alignment of amino acid sequences deduced from cDNA sequences, the alternative oxidases of higher plants contain only two conserved Cys residues (9). Both occur in the NH₂-terminal domain of the protein, which is believed to be located within the mitochondrial matrix before the first putative membrane-spanning helix. These conserved Cys residues are the likely candidates for formation of the intersubunit disulfide bond and the site of α-keto acid activation, and correspond to Cys-78 and Cys-128 in the deduced sequence of the Arabidopsis thaliana alternative oxidase used in this study. Based on assumptions about the enzyme’s structure, it was predicted that the more NH₂-terminal Cys-78, which may be exposed to the mitochondrial matrix, was involved in the sulfhydryl/disulfide system, and Cys-128, which may lie closer to the postulated catalytic site near the membrane, was the site of α-keto acid action (15). In the present study, we have used site-directed mutagenesis, heterologous expression in Escherichia coli cells, and subsequent cross-linking and activity assays to determine that the cysteine residues involved in regulatory disulfide bond formation and α-keto acid stimulation are identical.
EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and DNA ligase were from Promega (Madison, WI) or New England Biolabs (Beverly, MA), and all other chemicals were from Sigma unless otherwise noted.

Site-directed Mutagenesis and DNA Sequence Analysis—An Arabidopsis alternative oxidase cDNA clone in the vector pcdNAl (Invitrogen, San Diego, CA), designated pAOX (16), was used to make changes in specific codons of the alternative oxidase gene. The deduced amino acid sequence of this clone corresponds to that of AOX1a, a member of the alternative oxidase gene family described by Saisho et al. (17). The Sculptor Mutagenesis Kit (Amersham Corp., Arlington Heights, IL) was used to create two mutations. Sequences of the primers used to change the codons for Cys-78 or Cys-128 to codons for Ala were 5'-CCATGGCCCTAAAAGCGTTCCACTTCCATTCAGAACC-3' and 5'-CCACGGATCCTGAGCTCATATCATCCTCTCCG-3', respectively. The plasmids containing these changes were designated pAO-T78A and pAO-A128A, and the proteins expressed from these plasmids were designated AO-C78A and AO-C128A, respectively. DNA sequence analysis was performed using fluorescent dye-labeled nucleotides (PRISM™ Ready Reaction Dye Deoxy™ Terminator Cycle Sequencing Kit, Perkin-Elmer, Foster City, CA) and an automated sequencer (ABI Model 373A DNA Sequencer; ABI, Columbia, MD) as described by the manufacturer.

An additional mutation was created in the Arabidopsis cDNA clone to change the codon for Cys-78 to the codon for Glu using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA) according to the manufacturer's specifications. The following primers were used: 5'-GGTCCTGATGGAGATGGGACGAAATCAGGCGCTATGGG-3' and 5'-CCATGGCGCTAAATCGTCTGCTAAGAC. The mutated plasmid was designated pAO-T78E, and the expressed protein, AO-C78E. DNA sequencing to confirm the presence of the mutation and absence of nonspecific mutations was done by the Duke University DNA Analysis Facility using the PRISM™ ABI system.

Expression of Arabidopsis Alternative oxidase in E. coli Cells, Spheroplast Preparation, and Cell Membrane Isolation—Wild-type and mutant forms of the alternative oxidase were expressed from the pcdNAII constructs described above in either DH5α E. coli cells for cross-linking experiments, or in SAXS41B cells for alternative oxidase activity measurements. Protein expression by DH5α cells was induced following a procedure based on that of Kumar and Soll (16). A colony from a freshly streaked plate was used to inoculate 3 ml of M9 medium (18) containing 100 μg/ml ampicillin and the culture was grown overnight in a 37 °C shaker. The overnight culture was used to inoculate 50 ml of M9 medium containing 100 μg/ml ampicillin and this was also grown overnight in a 37 °C shaker. The cells were then pelleted and resuspended in ice-cold M9/phosphate buffer. This suspension was used to inoculate 100 ml of LB medium in a 250-ml flask containing 100 μg/ml ampicillin and 0.2 μM cyanide. Alternatively, 2 mM azide, instead of cyanide, was added to the cultures, including the empty vector controls. These cultures were grown for 16 h in a 37 °C shaker. Spheroplasts were prepared as described previously (19).

Induction of protein expression by SAXS41B E. coli, autotrophic for δ-aminolevulinic acid and therefore heme deficient (20), was described by Berthold (21). Alternative oxidase protein production was induced by growth of cells without the heme precursor and with vigorous aeration (21).

Cell membranes were prepared for alternative oxidase activity assays using a French Press. Cells were pelleted from the medium in which they had grown and were resuspended and washed in PBS1 (137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4). The cells were washed a second time in PBS to which 5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride had been freshly added. DTT was included to keep the alternative oxidase protein in the reduced state throughout membrane isolation. The pellets were resuspended in 5 ml of PBS/DTT/phenylmethylsulfonyl fluoride/w/v bovine serum albumin. This suspension was passed once through a French pressure cell at 20,000 psi. The lysed cell mixture was spun at 10,000 × g for 10 min to pellet intact cells. Cell membranes were isolated from the supernatant by spinning at 100,000 × g for 1.5 h. The final pellet was resuspended in PBS with 0.1% (w/v) bovine serum albumin. All operations took place at 4 °C. Protein concentrations were determined as described in Larson et al. (22).

Isolation of Mitochondria—Mitochondria were isolated and purified using a Percoll gradient according to Umbach and Siedow (10). For Arabidopsis (A. italiana (L.) Heyn, ecotype Columbia) mitochondria, rosette leaves were harvested from 4-week-old plants grown at 22 °C with 16 h light. Soybean (Glycine max (L.) Merr. cv FFR 583) mitochondria were isolated from 11- to 12-day-old expanding cotyledons of seedlings grown in a greenhouse.

Alternative Oxidase Activity Assays—Oxygen consumption by isolated E. coli membranes was measured using a Clark-type oxygen electrode in 2 ml of a buffer consisting of 150 mM NaCl, 50 mM KF, 10 mM Tris, 5 mM MgCl2, and 5 mM HEPES, adjusted to pH 7.0. Respiratory chain activity was initiated by the addition of 1 mM NADH. For α-keto acid stimulation of the expressed plant alternative oxidase, 5 mM pyruvate was used. The enzyme was inhibited with 1.5 mM SHAM added from a fresh 1.0 M stock in dimethyl sulfoxide. No KCN-sensitive bacterial oxidase activity was detectable in the SAXS41B cell membranes.

Alternative oxidase activity of isolated soybean mitochondria was measured using a Clark-type oxygen electrode in 2 ml of a buffer consisting of 10 mM KP, pH 7.0, 10 mM KCl, 5 mM MgCl2, 0.3 mM mannitol, 1 mM EDTA, and 0.1% (w/v) bovine serum albumin. Additions made during the assays were (final concentration): 0.5 mM durenoquinol, 6 μM myxothiazol, 5 mM pyruvate, iodoacetate, or NEM, and 2 mM SHAM. Sulphydryl reagents were added from concentrated stocks prepared fresh on the day of the experiment.

Oxidation with Diamide and Cross-linking with EGS—Disulfide bond formation was induced by adding diamide at a final concentration of 5 mM to 0.5 ml of sonicated DH5α E. coli spheroplasts at a protein concentration of 1.0 mg/ml in PBS, then mixing at room temperature for 30 min on a nutator mixer (VWR Scientific, West Chester, PA). The reaction was quenched by the addition of excess cysteine. Cross-linking with EGS (Pierce, Rockford, IL) was the same except that the final EGS concentration was 1 mM and quenching was with excess glycine.

Immunoblot Analysis—For cross-linked E. coli samples, spheroplasts at 1.0 mg of protein/ml were solubilized by adding an equal volume of SDS-PAGE sample buffer (23) without (diamide-treated samples only) or with 5% 2-mercaptoethanol, boiled for 5 min, and separated by SDS-PAGE. For Arabidopsis and soybean mitochondrial samples, 10–17% gradient gels were used and samples were prepared with or without 100 mM DTT as reductant in the sample buffer. Immunoblotting was performed using a monoclonal antibody, AOA, raised against the alternative oxidase of Saurornatum guttatum Schott (24). The secondary antibody was an anti-mouse horseradish peroxidase conjugate (Pierce) and alternative oxidase protein bands were visualized using a chemiluminescence system (NEN Life Science Products, Boston, MA).

Wild-type and mutant alternative oxidase protein expression by the SAXS41B cells varied both within and between experiments. To normalize enzyme activity data to account for this variability, densitometry (10) was performed on immunoblots from gels run using cell membrane samples of equal total protein amounts in sample buffer containing DTT. The presence of reductant in the sample buffer promoted uniform reactivity of the antibody with the protein (see below). The highest density sample was given the arbitrary density unit of 1.0 alternative oxidase density unit/mg of membrane protein, with other densities assigned fractional units. These density units, averaging 0.64/mg of protein, were used to adjust the original data (nanomole of O2/min/mg of protein) to nanomole of O2/min/AO density unit.

RESULTS

The Alternative Oxidase of Arabidopsis Displays Typical Oxidized and Reduced Forms—The alternative oxidase of Arabidopsis was present in isolated leaf mitochondria predominantly in the oxidized state (i.e. as disulfide-linked dimers; Fig. 1, lane 1) with a significant increase in disulfide-linked dimers seen after treatment of isolated mitochondria with the oxidant, diamide (Fig. 1, lane 2). Treatment of isolated mitochondria with the reductant DTT resulted in a nearly complete loss of disulfide-linked dimer, concomitant with an increase in the amount of protein in the reduced state (i.e. seen as monomers on SDS-PAGE gels; Fig. 1, lane 3). (Note that the reduced form is less visible in gel samples lacking sample buffer reductant, but is readily visible when sample buffer reductant is present.) The resulting reduced alternative oxidase protein could be reoxidized

1 The abbreviations used are: PBS, phosphate-buffered saline; DTT, dithiothreitol; SHAM, salicylhydroxamic acid; NEM, N-ethylmaleimide; diafine, azidocarboxylic acid bis(dimethylamide); EGS, ethylene glycol bis(succinimidylsuccinate); PAGE, polyacrylamide gel electrophoresis.
Plant species (10, 13, 25, 26), making the reversible intersubunit disulfide bond observed for isolated by Kumar and Soll, is abundant in leaf tissue, whereas cDNA clone of Kumar and Soll (16) used in these mutagenesis studies (10, 13, 25, 26), making the Arabidopsis gene a good candidate for site-directed mutagenesis to determine the cysteine residue involved.

Although there are at least four copies of the alternative oxidase gene in Arabidopsis (17), the protein isozyme produced in leaf mitochondria probably corresponds to the product of the cDNA clone of Kumar and Soll (16) used in these mutagenesis studies. The mRNA of AOX1a, identified by Saisho et al. (17) through sequence comparison as corresponding to the clone isolated by Kumar and Soll, is abundant in leaf tissue, whereas mRNAs of the other alternative oxidase genes are present only at very low levels in leaf tissue (17).

The yield of mitochondria from the leaves was insufficient for convincing activity assays, so α-keto acid stimulation of the Arabidopsis alternative oxidase activity in organello could not be unambiguously confirmed. However, α-keto acid stimulation of the bacterial expressed enzyme was observed, as reported below.

Cys-78 is the Cysteine Residue of the Arabidopsis Alternative Oxidase Involved in Disulfide Bond Formation—Growth of DH5a E. coli cells containing the wild-type alternative oxidase in the presence of KCN or azide resulted in the expression of functional alternative oxidase protein, as shown previously by Kumar and Soll (16). We used this heterologous system to study mutated forms of the alternative oxidase expressed from the Arabidopsis cDNA clone. Site-directed mutagenesis was employed to individually change the codons for Cys-78 and Cys-128 to Ala codons. Sequencing verified that the desired mutations were the only mutations in the Arabidopsis alternative oxidase cDNA clones (data not shown). When membranes from E. coli cells expressing the wild-type alternative oxidase, the alternative oxidase with Cys-78 changed to Ala (AO-C78A), or the alternative oxidase with Cys-128 changed to Ala (AO-C128A) were treated with diamide, both the wild-type and AO-C128A proteins formed disulfide-linked dimers (Fig. 2, lanes 1 and 2), which could be reduced if the samples were prepared in gel sample buffer containing reductant (Fig. 2, lanes 7 and 8). However, no intersubunit disulfide bond was formed by AO-C78A (Fig. 2, lane 3), suggesting Cys-78 is the cysteine involved in forming the disulfide bond.

Cys-78 is a regulatory cysteine of the cyanide-resistant oxidase. Alternative oxidase expression was induced in DH5a E. coli cells by growth in the presence of 0.2 mM KCN. The membrane fractions were untreated (lanes 7–9), incubated in 3 mM diamide (lanes 1–3), or treated with 1 mM EGS (lanes 4–6). Expressed alternative oxidases were from wild-type (AO), lanes 1, 4, and 6; AO-C128A, lanes 2, 5, and 8; and AO-C78A, lanes 3, 6, and 9. Proteins were analyzed by immunoblotting. Samples in lanes 4–9 were prepared with reductant in the gel sample buffer. Twenty micrograms of total membrane protein were loaded onto each lane. The alternative oxidase proteins were detected with the alternative oxidase monoclonal antibody, AOA. Ox., the oxidized, disulfide linked form of the alternative oxidase; Red., the reduced form of the alternative oxidase. Molecular weight standards are shown to the right.

Cross-linking with the Lys-Lys-specific cross-linker EGS showed that AO-C78A formed dimers (Fig. 2, lane 6), as did AO-C128A (Fig. 2, lane 5) and the wild-type protein (Fig. 2, lane 4). These results indicate that dimerization of the alternative oxidase is not dependent on the presence of the disulfide bond-forming Cys-78.

Mutations at Cys-78, Not Cys-128, Affect α-Keto Acid Stimulation of the Arabidopsis Alternative Oxidase—Alternative oxidase activity assays of SASS41B E. coli membranes were used to assess the effect of the Cys mutations on the ability of the expressed alternative oxidase to be stimulated by α-keto acids. SASS41B cells were used for these experiments because alternative oxidase expression was found to occur at reliably higher levels in these cells under inductive conditions than in the DH5a cells. Alternative oxidase protein expression levels were quantified from immunoblots by densitometry (10) and were used to normalize the measured activities to the amount of immunoreactive protein present as described under “Experimental Procedures.”

The expressed wild-type alternative oxidase from Arabidopsis displayed typical α-keto acid stimulation with pyruvate such that activity was very low unless pyruvate was present (Fig. 3). This observation has also been made recently by Berthold (21) using the SASS41B expression system. Mutation of Cys-128, predicted to be the site of α-keto acid action (15), to Ala had no effect on the ability of the enzyme to be stimulated by pyruvate (Fig. 3). This mutation, however, caused a pronounced overall increase in activity, relative to wild-type, whether or not pyruvate was present (Fig. 3). Conversely, the alternative oxidase in which Cys-78 was mutated to Ala was only minimally active and showed no response to added pyruvate (Fig. 3), suggesting that the site of action of α-keto acids is likely at this Cys. Similar results have recently been obtained below.
with tobacco alternative oxidase (27).

The possibility existed that the lack of pyruvate stimulation in the mutant AO-C78A was symptomatic of a general decrease in enzyme activity, rather than loss of the α-keto acid-binding site. To test this, an additional mutant was constructed in which Cys-78 was changed to a Glu residue, positioning a Glu carboxylate within one methylene of the carboxylate moiety formed by the proposed thiohemiacetal (15), as diagrammed below (R = CH$_3$ for pyruvate):

\[
\begin{align*}
R & \\
\text{Thiohemiacetal: } & H-C-CH$_2$-S-C-CO$_2$ & \text{(Eq. 1)} \\
& | & \text{OH} \\
\text{Glutamate: } & H-C-CH$_2$-CH$_2$-CO$_2$ & 
\end{align*}
\]

If the carboxyl group of the thiohemiacetal is the activating moiety, the Glu mutation might display significant activity. The resulting mutant protein, AO-C78E, had substantial activity, relative to AO-C78A, equaling 58% of wild-type activity in the presence of pyruvate. Furthermore, AO-C78E displayed no pyruvate sensitivity (Fig. 3), suggesting that the Glu side chain was able to substitute for the thiohemiacetal. As with AO-C78A, the AO-C78E dimer was cross-linked by EGS, but was not oxidized by diamide (data not shown). Because the modifications to Cys-78 affected both disulfide bond formation and the activation by an α-keto acid, the most parsimonious interpretation is that both regulatory features are located at this Cys residue.

**Re-examination of Sulfhydryl Reagent Effects Supports Cys-78 as the Site of Pyruvate Action**—The results of experiments using isolated soybean mitochondria and iodoacetate, a sulfhydryl reagent that mimics the action of pyruvate, originally indicated that the site of α-keto acid action was a sulfhydryl group distinct from the residue involved in intersubunit disulfide bond formation (15). This conclusion was reached largely on the apparent ability of iodoacetate to bind to the alternative oxidase site of α-keto acid activation even when the enzyme was in the oxidized state and the constituent bond sulfhydryls were not available to react with iodoacetate. Clearly, the results of the site-directed mutagenesis studies reported above are at odds with these experimental results. To reconcile these data, additional experiments with soybean mitochondria were done.

A major assumption in the earlier experiments (15) was that iodoacetate would be washed from the mitochondria between experimental treatments, and that any residual iodoacetate remaining would be inactivated by the subsequent addition of DTT. To test this assumption, soybean mitochondria were first treated with 3 mM diamide to oxidize the alternative oxidase disulfide bond and then treated with either 5 mM pyruvate or iodoacetate under assay conditions, replicating the previous study (15). Subsequently, the mitochondria were washed twice, then treated on ice with 20 mM DTT for 1 h to reduce the alternative oxidase disulfide bond, and washed again. Finally, the mitochondria were re-treated with 3 mM diamide for 30 min. Samples for SDS-PAGE were taken at each of these steps (Fig. 4A). The alternative oxidase was nearly completely oxidized at the beginning of the experiments (Fig. 4B, lanes 1 and 4), making Cys-78 inaccessible to iodoacetate. After pyruvate or iodoacetate addition, DTT treatment reduced the alternative oxidase disulfide bond (Fig. 4B, lanes 2 and 5, respectively). Subsequent addition of diamide caused a reoxidation of the alternative oxidase that had been exposed to pyruvate (Fig. 4B, lane 3). This reoxidation was possible both because pyruvate had probably been washed from the mitochondria and because, if pyruvate were associated with Cys-78, the thiohemiacetal is readily reversed by reactions such as diamide-driven oxidation. However, reoxidation did not occur with the alternative oxidase that had been initially exposed to iodoacetate (Fig. 4B, lane 6). Therefore, iodoacetate did not wash out of the mitochondria before DTT treatment. Instead, it was present and apparently able to react with the disulfide bond sulfurs (Cys-78) as they were reduced to their component sulfhydryls by DTT treatment.

As an alternative method of testing the involvement of Cys-78 in α-keto acid stimulation, the ability of diamide treatment to protect the alternative oxidase from NEM inactivation was determined. NEM, another sulfhydryl reagent, inhibits the alternative oxidase and its site of action was shown to be the same as that of pyruvate and iodoacetate (15). DTT- or diamide-treated mitochondria were exposed to 5 mM NEM under assay conditions. NEM had an inhibitory effect on alternative oxidase activity (Fig. 5), as shown in Fig. 5A, which demonstrates that NEM had an inhibitory effect on alternative oxidase activity (Fig. 5). The mitochondria were removed from the assay cuvette, spun down, and washed. Then, both types of mitochondria were re-assayed for alternative oxidase activity. As Fig. 5B shows, 5 mM DTT addition to the assay allowed recovery of activity and pyruvate stimulation by the diamide-treated mitochondria. However, DTT addition had no such effect on activity of the DTT-treated mitochondria (Fig. 5A). Thus, NEM must have bound to Cys-78 of the DTT-treated alternative oxidase and caused inactivation of the enzyme in the initial step of the experimental series. Conversely, the oxidized Cys-78 of the diamide-treated mitochondria was protected and the enzyme activity was regained upon reduction by DTT of the disulfide bond to the constituent sulfhydryls.
As shown above, Cys-128 is not involved in disulfide bond formation and, therefore, in either DTT- or diamide-treated mitochondria would be free to bind NEM. If this did happen, it had little effect on final enzyme activity (Fig. 5, B, after washing, +Pyr). Thus, the sites of NEM inhibition and α-keto acid stimulation both appear to be associated with the same sulfhydryl, Cys-78.

DISCUSSION

The activity of all plant alternative oxidases investigated so far can be regulated by a redox-sensitive intersubunit sulfhydryl/disulfide system (10, 11, 13, 14). Here we show the alternative oxidase of *A. thaliana* leaf mitochondria also forms homodimers that can be linked by a disulfide bond, making the *Arabidopsis* alternative oxidase gene a reasonable candidate for site-directed mutagenesis to determine which residues are involved in formation of the disulfide bond.

Using the heterologous bacterial expression system first demonstrated by Kumar and Still (16), we determined the effects of changing the two conserved Cys residues of the plant alternative oxidase on the ability of the mutated proteins to form the intersubunit disulfide bond. The disulfide linkage between alternative oxidase monomers was abolished in the mutant AO-C78A but not in the mutant AO-C128A (Fig. 2).

![Diagram](image-url)
indicating the more NH₂-terminal of the two conserved cysteine residues, Cys-78 (Fig. 6), is involved in the sulfhydryl/disulfide redox regulatory system of the alternative oxidase. This cysteine was originally predicted to be the disulfide bond site based on its potential exposure to the mitochondrial matrix where it could interact with the naturally occurring sulfhydryl reductant, thioredoxin (15). Similarly, for the tobacco alternative oxidase, the more NH₂-terminal homologous Cys residue has recently been shown to be involved in disulfide bond formation (27). One exception to the conservation of Cys-78 is now known. In one of a pair of tandem-arranged alternative oxidase genes of rice, the residue corresponding to Cys-78 is a Ser (28). It is not yet known if this is a pseudogene, or if a functional protein is produced which is free of redox control (28).

Although mutation of Cys-78 abolished disulfide bond formation, it did not disrupt the homodimeric association of the protein (Fig. 2), indicating that other features of the protein are involved in the protein-protein interaction. It may be that while Cys-78 itself is not necessary for dimerization, the protein domain surrounding it is, but this requires further investigation.

The bacterial expression system allowed us to demonstrate α-keto acid stimulation of the wild-type Arabidopsis alternative oxidase, a result technically difficult to obtain with Arabidopsis mitochondria, and to examine the effects of Cys mutations on this stimulation. Our previous work showed that α-keto acids act at a sulfhydryl group, most likely through formation of a thiohemiacetal (15). However, contrary to our earlier conclusion, Cys-78, not Cys-128, is the site of α-keto acid action (Fig. 3). When Cys-78 was replaced by the carboxylate-bearing Glu, enzyme activity approached that of wild-type in the presence of pyruvate, and pyruvate stimulation was abolished (Fig. 3). The change of Cys-78 to a Glu residue was designed to approximate the adduct generated following thiohemiacetal formation between the cysteine sulfhydryl and an α-keto acid. However, the Glu side chain does not exactly match the structural properties of a thiohemiacetal. Glu is one methylene shorter than the thiohemiacetal formed by reaction between the cysteine residue and an α-keto acid. In contrast, the adduct formed by the reaction of iodoacetate with a sulfhydryl is equal in length to the thiohemiacetal. Iodoacetate treatment stimulates alternative oxidase activity to levels close to those achieved with pyruvate (15). Thus, the shorter side chain of Glu might account for the lower level of activity, relative to wild-type plus pyruvate, exhibited by the C78E mutant. Another possibility exists, where Cys-78 is not the exact site of α-keto acid interaction with the protein, but has spatial proximity to it. If this were the case, then the C78E mutation would not be expected to recover enzyme activity to wild-type plus pyruvate levels. However, the simplest interpretation of the mutant protein results is that Cys-78 and the site of α-keto acid activation are one and the same. Consequently, the C78E mutation created an active alternative oxidase which is no longer subject to control by redox regulation or α-keto acid levels.

The elevated activity of AO-C128A was unexpected. The predicted position of Cys-128 is at the base of the first membrane spanning helix of the alternative oxidase. This helix may contribute residues to the enzyme’s postulated quinone-binding site (29). If so, a mutation at Cys-128 could conceivably affect quinone binding and hence activity, but whether or not this is the basis of the greater activity of AO-C128A remains to be shown.

The earlier conclusion that the Cys involved in α-keto acid activation was different from the Cys involved in the sulfhydryl/disulfide system was based on experiments using soybean mitochondria and iodoacetate, a sulfhydryl reagent which can act as an α-keto acid mimic (15). In these previous experiments, even when the alternative oxidase protein was fully oxidized, iodoacetate appeared able to bind to the α-keto acid site, so that when the protein was subsequently reduced, it was fully activated without the addition of pyruvate. To the contrary, the results presented here demonstrate that iodoacetate remained present throughout the experimental protocol, not having been completely washed out of the mitochondria, and was able to react during DTT treatment to activate the alternative oxidase (Fig. 4B).

Also reported in this article is the observation that diamide treatment (oxidation) protects the activity of the alternative oxidase of soybean mitochondria from inhibition by NEM, whereas the activity of the reduced form of alternative oxidase is highly susceptible to NEM (Fig. 5). Thus, NEM inhibition apparently derives from its binding to the redox-regulatory Cys, Cys-78. NEM could bind at a different site that is sterically inaccessible in the oxidized form of the enzyme, but this more complex scenario is unnecessary to explain the other results of this study. Use of the sulfhydryl reagents with the mutant forms of alternative oxidase would supply a more complete picture of these interactions. For instance, NEM should not be able to inhibit AO-C78E activity. However, that reagent strongly inhibits NADH oxidation by the E. coli membranes, precluding these experiments.

Part of the rationale for the earlier proposal that Cys-128 is the site of pyruvate action was the supposed proximity of this residue to the postulated active site of the enzyme (Fig. 6). As the structure of the alternative oxidase is currently concep-
alized, Cys-78, now shown to be the site of \( \alpha \)-keto acid activation, appears to be farther from the postulated active site, compared with Cys-128 (Fig. 6). In this context, it is interesting to note that a mutation influencing alternative oxidase sensitivity to its inhibitor SHAM, and therefore presumably the enzyme's quinol-binding site, has been recently identified in the extreme COOH terminus of the enzyme (21). A question still to be answered is how thiohemiacetal formation exerts such a profound effect on alternative oxidase activity, the answer to which should lead to further insights regarding the structure of this enzyme.

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