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Activation of the plant mitochondrial alternative oxidase: insights from site-directed mutagenesis

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Abstract

The homodimeric cyanide-resistant alternative oxidase of plant mitochondria reduces oxygen to water without forming ATP. *Arabidopsis thaliana* alternative oxidase AOX1a is stimulated by pyruvate or other α-keto acids associating with a regulatory cysteine at position 78, by succinate in a serine-78 mutant, and by site-directed mutation of position 78 to glutamate. The mechanism of activation was explored with additional amino acid substitutions made at Cys-78 in AOX1a, which was functionally expressed in *Escherichia coli*. Oxidases with positively charged substitutions (Lys and Arg) were insensitive to pyruvate or succinate but were more active than the wild type without pyruvate. Uncharged substitutions (Gln, Leu) produced an inactive enzyme. These results indicate that activation may be due to conformational changes caused by charge repulsion between the dimer subunits and not through a direct role of α-keto acids in catalysis. Oxygen isotope fractionation experiments suggest that the charge of the amino acid at position 78 also affects the entry of oxygen into the active site. Therefore, the N-terminal portion of the protein containing residue 78 can indirectly affect both catalysis at the diiron active site and the path of oxygen to that site. In addition, both positively and negatively substituted alternative oxidases were stimulated by glyoxylate, suggesting the presence of a second activation site, possibly Cys-128.

Keywords: Alternative oxidase; Regulatory cysteine; α-Keto acid; Activity regulation; Plant mitochondria; Cyanide-resistant respiration

1. Introduction

A cyanide-resistant respiratory pathway, while apparently lacking in animals, is present in the mitochondria of many organisms, including all examined higher plants, many fungi, and some protists [1,2]. The alternative oxidase protein, which constitutes the pathway, uses electrons from the ubiquinone pool of the inner mitochondrial membrane to reduce oxygen to water. In contrast with the ubiquitous Complex III to cytochrome *c* oxidase pathway, no energy for ATP synthesis is conserved by this reaction [1,2]. The alternative pathway's consequent freedom from control by the mitochondrial proton motive force should allow the alternative oxidase to dissipate excess reducing power generated in the cell under a variety of conditions (e.g., Refs. [3–6]).

In isolated plant mitochondria, two interrelated biochemical regulators of alternative oxidase activity have been identified. One of these is the presence or absence of a disulfide bond that can form between the two subunits of the homodimeric oxidase. When the subunits are linked by the disulfide bond, the enzyme is inactive [7]. When the bond is reduced such that the monomers are non-covalently associated, the enzyme can be activated by the second biochemical regulator, α-keto acids, which must be present for full activity [8–11].

The amino acid residue at which these regulatory features operate has been established by site-directed mutagenesis of the *Arabidopsis* [12] and *Nicotiana* [13] alternative oxidase proteins. The residue responsible is the more N-terminal of two conserved cysteines (the Cys_I of Berthold et al. [14], corresponding to Cys-78 in the truncated *Arabidopsis* AOX1a clone used in this study and Cys-127 in the full-length *Arabidopsis* AOX1a protein). Additional site-direc-

Abbreviations: Diamide, azodicarboxylic acid bis(dimethylamide); DTT, dithiothreitol; DU, densitometry unit; EGS, ethyleneglycol bis(succinamidylsuccinate); IOA, iodoacetate; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHAM, salicylhydroxamic acid

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ted mutagenesis studies in combination with studies of the response of alternative oxidase activity to sulfhydryl reagents showed that α-keto acids interact with the alternative oxidase protein at this same residue [12,15]. This interaction likely takes the form of a thiohemiacetal in which the carbonyl carbon of the α-keto acid bonds to the sulfur of the cysteine sulfhydryl. Such an association results in the introduction of a negatively charged carboxylate at that location in the protein [15]. Evidence supporting this structure was derived from a site-directed mutant in which the critical Cys was replaced with a Glu, which introduced a permanent negative charge into the protein at approximately the same site. This Glu-substituted form of the alternative oxidase neither required the presence of an α-keto acid to achieve high levels of activity nor responded to pyruvate [12].

The importance of these regulatory features for the functioning of the alternative oxidase *in vivo* is still unclear, but there is no doubt that they can profoundly affect alternative oxidase activity in isolated systems [10,11]. With respect to the α-keto acid effect, when mitochondria have been depleted of pyruvate by treatment with lactate dehydrogenase, alternative oxidase activity is no longer detectable [9,16]. Similarly, alternative oxidase protein expressed in the membranes of *Escherichia coli* cells, even with the regulatory Cys reduced, is only weakly active until pyruvate is added to the preparation [12]. Because activity without an α-keto acid is so low, it has been suggested that the α-keto acid takes part directly in the catalytic mechanism of the alternative oxidase [16], perhaps by effecting deprotonation of the substrate ubiquinol [15].

Despite the occurrence of the regulatory Cys in the vast majority of alternative oxidase genes sequenced to date, exceptions exist. Two sequences, one from tomato [17] and one from rice [18], show a substitution of Ser for Cys at the regulatory site. While these native proteins have not been examined, *Arabidopsi*s or soybean alternative oxidases in which a Ser was introduced by site-directed mutagenesis at this site are not activated by α-keto acids, but are activated by the dicarboxylic acid succinate [17]. No thiohemiacetal could form with a Ser residue, although an interaction between succinate and this Ser via hydrogen-bonding could result in the introduction of a negative charge near the same site on the protein as thiohemiacetal formation or the Glu substitution.

To gain further insight into the mechanism by which plant alternative oxidase activators, particularly α-keto acids, have their effect, we continued with site-directed mutagenesis of *Arabidopsis* AOX1a at the site of the regulatory Cys. Two substitutions were made in the context of previous work: an Asp substitution to compare with the previously characterized Glu substitution [12] and a Ser substitution as a basis for comparison with the work of Djajanegara et al. [17]. In addition, two new classes of substitutions were made, one consisting of amino acids with positively charged side chains (Lys, Arg) and the other consisting of uncharged side chains (Gln, Leu). The mutated forms of the protein were functionally expressed in *E. coli* and the effects of the substitutions on activity, response to pyruvate, succinate and other organic acids, and ^{18}O fractionation were assessed.

2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA ligase were obtained from either New England Biolabs (Beverly, MA, USA) or Promega (Madison, WI, USA). Lactate dehydrogenase (LDH) and other chemicals used in buffers and assays were from Sigma (St. Louis, MO, USA). Additional components for bacterial growth media were from Difco (Becton Dickinson, Sparks, MD, USA).

2.2. Site-directed mutagenesis and DNA sequence analysis

The cDNA clone that was used to generate alternative oxidases mutated at the site of the regulatory Cys was contained in pAOX, described by Kumar and Söll [19], consisting of an *Arabidopsis* alternative oxidase sequence (accession number M96417) in the vector pcDNAII (Invitrogen, San Diego, CA). The alternative oxidase deduced amino acid sequence corresponds to that of AOX1a (Ref. [20], accession number D89875) except that it is truncated at the N-terminal end, about where the signal sequence is likely to be cleaved. Consequently, the regulatory Cys in pAOX is at position 78, corresponding to position 127 in the full-length AOX1a sequence. A second highly conserved Cys closer to the C-terminus, the Cys_{II} of Berthold et al. [14], is at position 128 in pAOX (position 177 in the full-length sequence). The method for introduction of the changes of Cys-78 to Ala and Glu and Cys-128 to Ala was as described previously [12]. Additional mutations at the Cys-78 codon (for Asp, Ser, Arg, Lys, Gln, and Leu) were introduced using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA) according to the manufacturer's instructions. The primers used for the change to Asp were: 5′-GGTTCTGAATGGAAGTGGAAC-***GAT***TTCAGGCCATGGG-3′ and 5′-CCCATGGCCT-GAA***ATC***GTTCCACTTCCATTCAGAACC-3′, forward and reverse, respectively. The altered codons are shown in bold italics. The same primers were used to make the other substitutions, except that the altered codons, for forward and reverse, respectively, became: Ser-TCT, AGA; Arg-CGT, ACG; Lys-AAG, CTT; Gln-CAG, CTG; Leu-TTG, CAA. The mutated proteins so generated are referred to throughout the paper as, for example, AOX1a-C78D for the Asp substitution. DNA sequencing to confirm the presence of the mutation and the absence of nonspecific mutations was done by the Duke University DNA Analysis Facility using the PRISM™/ABI system.

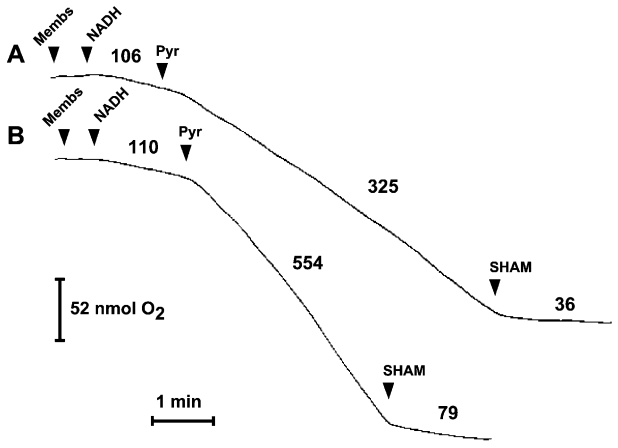

Fig. 1. Representative traces of O_2 consumption by *E. coli* membranes isolated from cells expressing the wild-type *Arabidopsis* alternative oxidase, AOX1a. Trace A is with membranes isolated without DTT in the isolation buffers. Trace B is with membranes isolated in the presence of 5 mM DTT, the standard experimental condition. One batch of cells was divided for the two types of isolations, therefore, the level of alternative oxidase protein expression is identical between the two. Resuspended membranes ("membs"; 0.107 and 0.119 mg membrane protein for traces A and B, respectively) were added to the oxygen electrode cuvette at 100% oxygen saturation and respiration was initiated by addition of 1.0 mM NADH, followed by 5 mM pyruvate ("pyr"). SHAM (2 mM) was added to inhibit alternative oxidase activity. The residual rate was not KCN-sensitive. Values shown are nmol O_2/min/mg protein.

2.3. Alternative oxidase protein expression in E. coli, and membrane preparation

The constructs containing mutated or wild-type alternative oxidases in the pcDNAII vector described above were used to transform an *E. coli* strain, SASX41B, that is auxotrophic for δ-aminolevulinic acid, and therefore heme-deficient unless δ-aminolevulinic acid is supplied [21]. Alternative oxidase protein expression by these cells was induced as described by Berthold [22], under conditions of growth without δ-aminolevulinic acid and with vigorous aeration. SASX41B cells grown in this manner have no main pathway activity; instead, they require the plant alternative oxidase to grow aerobically [19,22].

Membranes were isolated from the *E. coli* cells using a French pressure cell (ThermoSpectronic, Rochester, NY, USA) as described [12]. Membrane protein was measured according to Lowry et al. [23].

2.4. Alternative oxidase activity assays

Alternative oxidase activity of the isolated *E. coli* membranes was assessed as previously described [12] using 1.0 mM NADH as the respiratory substrate and monitoring oxygen consumption. Inhibition by salicylhydroxamic acid (SHAM), at a final concentration of 2 mM added from freshly prepared 1 M stock in DMSO, was used to confirm alternative oxidase activity. In general, only SHAM-sensitive oxygen uptake due to the alternative oxidase was present in the membrane preparations, with no KCN-sensitive activity detectable. All reported rates were adjusted for the residual rate in the presence of SHAM (Fig. 1). Most of the organic acids and aldehydes added to activity assays were prepared as either 0.5 or 1 M stock solutions in assay buffer adjusted to pH 7.0, and stored as frozen aliquots. Glutaric acid was dissolved at 1 M in ethanol. For some assays, duroquinol, prepared according to Rich [24] from duroquinone, was used as the respiratory substrate at 0.5 mM.

To determine whether residual pyruvate was affecting the activity of any of the alternative oxidases, LDH (5 units per ml) was included in the assay buffer in the oxygen electrode cuvette to scavenge residual pyruvate. The effect on activity was observed over a 10-min period.

2.5. Oxidation and cross-linking

Disulfide bond formation, induced with 3 mM azodicarboxylic acid bis(dimethylamide) ("diamide"), and cross-linking, with 0.5 mM ethyleneglycol bis(succinamidylsuccinate) (EGS; Pierce, Rockford, IL), was carried out as described [12].

2.6. Immunoblotting

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after the method of Laemmli [25], sample buffer with 100 mM dithiothreitol (DTT) was added to the membrane samples, except for those treated with

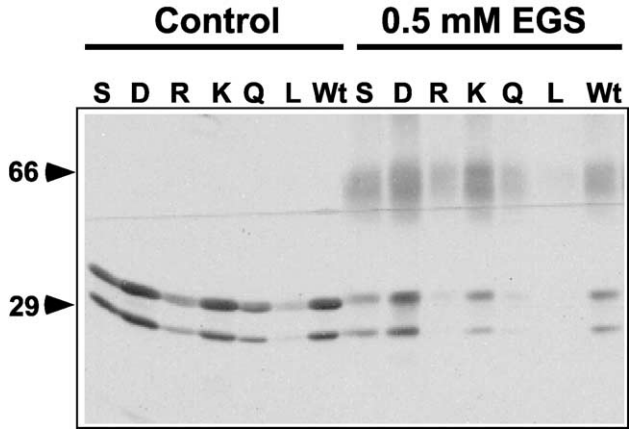

Fig. 2. Immunoblot showing cross-linking of wild-type and mutant alternative oxidases in *E. coli* membranes. Membranes were either untreated ("Control" lanes) or treated for 30 min with 0.5 mM EGS. The reaction was stopped by the addition of excess glycine, and SDS-PAGE sample buffer containing reductant was added. Following electrophoresis and transfer, the blots were probed with the monoclonal "AOA" antibody [26]. Twenty micrograms total membrane protein was loaded in each lane, except for AOX1a-C78L for which 40 μg was loaded. The letters above the lanes signify the substitutions made at Cys-78; "Wt" = wild-type protein. The positions of molecular mass markers are shown at the left.

diamide where DTT was omitted. The proteins were separated on 10–17% gradient gels. Subsequent immunoblotting was as described [12] using the "AOA" antibody [26].

Expression levels of the alternative oxidase protein by *E. coli* varied both among the various alternative oxidase protein types and among different protein induction experiments for any one alternative oxidase type. Thus, measured alternative oxidase rates needed adjustment for this variability in expression. To do this, densitometry was performed [7] on immunoblots detecting the alternative oxidase from SDS-PAGE gels loaded with 15 μg cell membrane protein per sample. Membrane samples from any one induction experiment were always together on the same immunoblot to minimize variability arising from the densitometry procedure. The specific activities of oxygen consumption by the membranes nmol O_2/min/mg protein were divided by the densitometry units (DUs) obtained, so that the final activity units were: nmol O_2/min/DU. Before adjustment with the densitometry values, specific activity of the wild-type oxidase (in the presence of pyruvate) ranged between

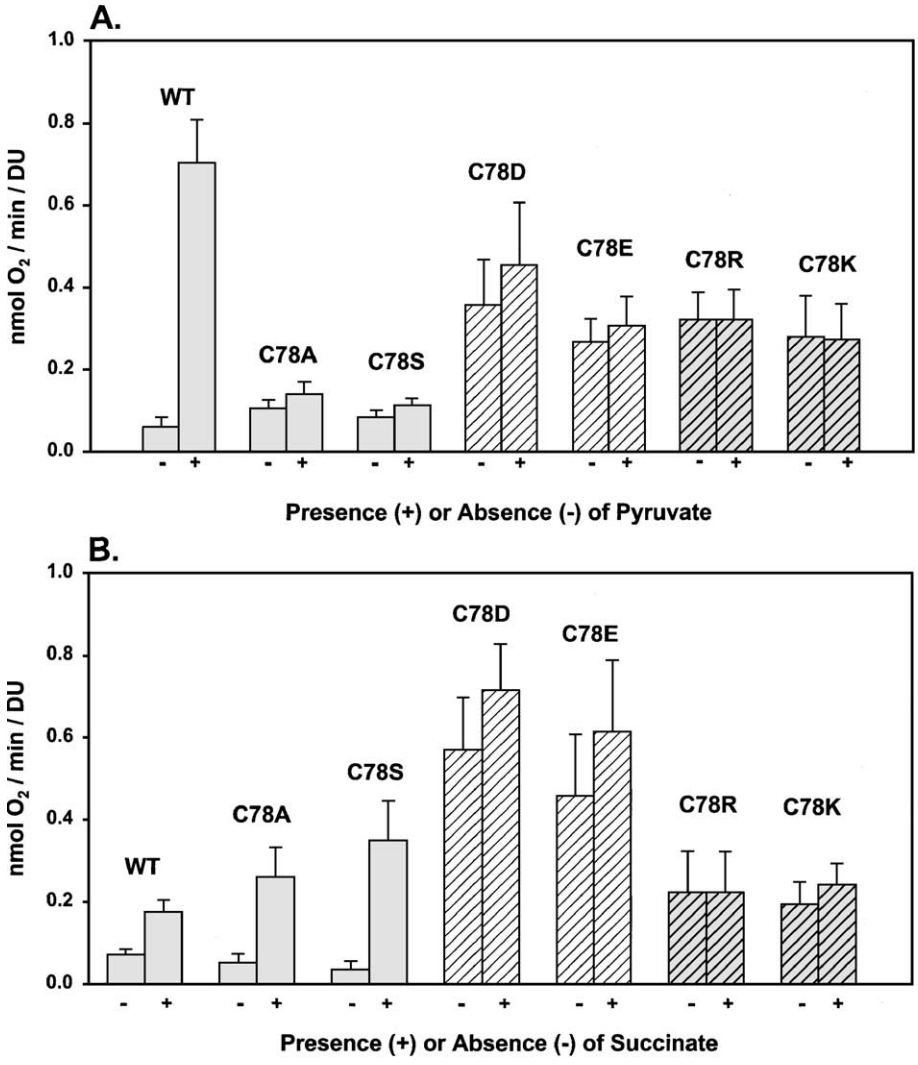

Fig. 3. Activities of wild-type and mutant alternative oxidases in the absence and presence of pyruvate (A) or succinate (B). Isolated *E. coli* membranes were assayed for expressed *Arabidopsis* alternative oxidase activity by measuring oxygen consumption with NADH as substrate. The rates shown are following addition of 1.0 mM NADH, minus and plus 5 mM pyruvate (A) or 5 mM succinate (B). SHAM (2 mM) was added at the end of each assay and the residual rate in its presence was subtracted to yield the presented SHAM-sensitive rate. Specific activity, equal to nmol O_2/min/mg membrane protein was adjusted for different levels of alternative oxidase protein expressed by the bacteria by taking densitometry readings from immunoblots of the alternative oxidase in the corresponding membranes. The graphs show the arithmetic means and their standard errors (see Materials and methods). The standard errors are not appropriate for comparing differences between activator levels within mutants. The white cross-hatched bars indicate the negative-charge substituted mutants, the grey cross-hatched bars indicate the positive-charge substituted mutants. Labels over the pairs of bars indicate wild-type alternative oxidase (WT) or the various mutated alternative oxidase forms. For A, the number of independent protein inductions from which means were derived for each alternative oxidase form were: wild-type, 17; AOX1a-C78A, 5; AOX1a-C78S, 7; AOX1a-C78D, 6; AOX1a-C78E, 9; AOX1a-C78R, 6; AOX1a-C78K, 7. For B, the number of independent protein inductions from which means were derived for each alternative oxidase form were: wild-type, 9; AOX1a-C78A, 5; AOX1a-C78S, 6; AOX1a-C78D, 4; AOX1a-C78E, 3; AOX1a-C78R, 2; AOX1a-C78K, 4.

200 and 500 nmol O_2/min/mg membrane protein (e.g., Fig. 1).

In all membrane preparations, a 29-kDa band that was reactive with the AOA antibody was present on immunoblots (see Fig. 2 for example). This band was not due to a non-specific antibody reaction (data not shown). Possibly a product of proteolysis, although protease inhibitors did not prevent its formation, it could also have been a partial translation product. For purposes of adjusting the activity data for the amount of alternative oxidase protein as described above, only densitometry results from the full-sized (32 kDa) alternative oxidase form were used, since it was not possible to determine if the 29-kDa form was active. However, using the summed densitometry values for the 32- and 29-kDa forms to adjust the data did not affect the relative outcomes of the experiments.

2.7. Statistical analysis of activity data

A total of 25 protein induction experiments was carried out involving the three activators pyruvate, succinate, and glyoxylate, and the wild-type and six mutant alternative oxidases. Because of the logistics of growing the bacteria, for any single induction experiment, a subset of mutants (two to five) and a subset of activators (usually two) were examined. To simplify the statistical analysis, data from each activator were analyzed separately; but all seven alternative oxidase types were included in each analysis. In each induction experiment, activity measurements taken in the presence and absence of the activator for a given mutant were done sequentially on the same aliquot of bacterial membranes (c.f., Fig. 1) while measurements on the different mutants were, naturally, done on separate aliquots. Consequently, differences in activity between presence or absence of activator within a mutant are assessed with more precision than differences in activity between two different mutants (with the activator present or absent). To account for these two levels of precision in our analysis, we used mixed model analysis of variance techniques appropriate for split-plot design [27,28]. We used SAS Statistical Software (SAS Institute, Cary, NC) for the calculations.

In Figs. 3 and 4, the arithmetic means of the activity data and their standard errors, calculated without recourse to the analysis of variance results, are plotted. Those standard errors, which include variation between induction experiments, are not appropriate for assessing significance between activator levels for any given mutant because, as mentioned above, the latter are known with greater precision. The statistical analysis allows us to correctly test for differences in activity between activator levels for a given mutant and to compare such differences between mutants. We report test results as "significant" when $P \leq 0.05$. When estimating fold changes in activity attributable to the presence of activator for any mutant or for comparing such fold changes between mutants, we used estimates derived from the statistical analysis rather than simply from the arithmetic means. These estimates can differ slightly from those based on the arithmetic means because the analysis adjusts for the fact that not every mutant was included in every induction experiment.

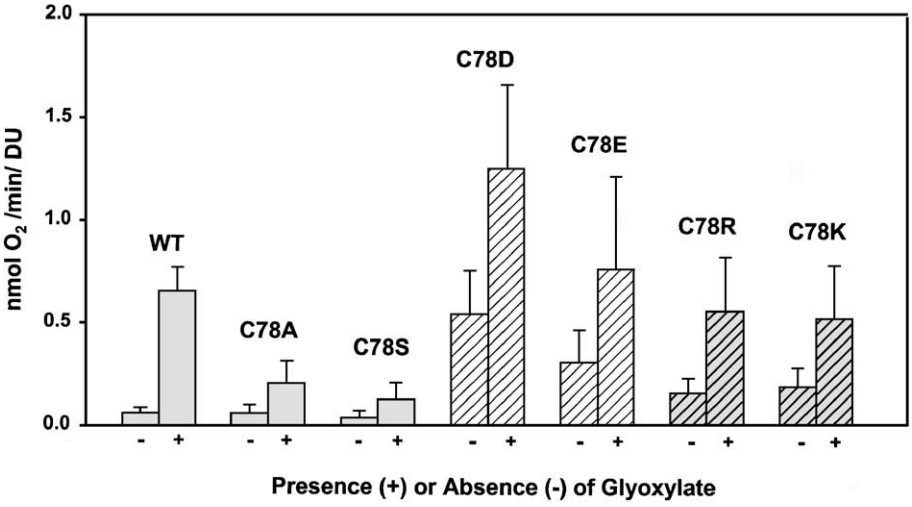

Fig. 4. Activities of wild-type and mutant alternative oxidases in the absence and presence of glyoxylate. Isolated *E. coli* membranes were assayed for expressed *Arabidopsis* alternative oxidase activity by measuring oxygen consumption with NADH as substrate. The rates shown are after addition of 1 mM NADH, minus and plus 5 mM glyoxylate. Rates were adjusted for the residual rate and for different levels of expressed alternative oxidase protein as described for Fig. 3. The graphs show the arithmetic means and their standard errors (see Materials and methods). The standard errors are not appropriate for comparing differences between activator levels within mutants. The white cross-hatched bars indicate the negative-charge substituted mutants; the grey cross-hatched bars indicate the positive-charge substituted mutants. Labels over the pairs of bars indicate wild-type alternative oxidase (WT) or the various mutated alternative oxidase forms. The number of independent protein inductions from which means were derived for each alternative oxidase form were: wild-type, 10; AOX1a-C78A, 2; AOX1a-C78S, 2; AOX1a-C78D, 2; AOX1a-C78E, 4; AOX1a-C78R, 3; AOX1a-C78K, 3.

2.8. Measurement of oxygen isotope fractionation

Oxygen isotope fractionation by wild-type and mutant alternative oxidases in isolated *E. coli* membranes was measured with the aqueous phase oxygen isotope analysis system developed for use with isolated mitochondria [29,30]. The amount of membranes needed ranged from 0.27 mg membrane protein for the most active oxidase forms to 2.25 mg for the least active in 25 ml reaction medium. NADH and SHAM were used at the same concentrations as for the activity assays. Rates in the presence of SHAM were negligible. All regression lines used in calculations had $r^2 > 0.995$.

3. Results

All membrane isolations were performed in the presence of 5 mM DTT to prevent inactivation of the wild-type enzyme through disulfide bond formation. Pyruvate, which can maintain the oxidase in the activated state [11,22], could not be used because this study was concerned with the effect of added pyruvate, among other compounds, on oxidase activity. For the wild-type alternative oxidase, pyruvate had to be added before any appreciable rate was achieved with membranes isolated either with or without DTT (Fig. 1). Activity of the oxidase in the presence of pyruvate for membranes isolated without DTT ranged from 60% (Fig. 1) to as little as 15% of the activity seen in membranes isolated with DTT. For uniformity, DTT was also included in isolations of membranes harboring mutant oxidases. However, omission of DTT during isolation did not result in lower enzyme activity for any of the mutant oxidases (data not shown).

3.1. The mutated alternative oxidases maintain a dimeric structure

As reported in our earlier work [12], substitutions of Glu or Ala at residue 78 had no effect on the dimeric nature of the alternative oxidase. The same held true for the substituted alternative oxidases AOX1a-C78R, -C78K, -C78S, -C78D, -C78Q, and -C78L. When *E. coli* membranes containing these mutated alternative oxidases were incubated with EGS, a lysine-specific, 16.1-Å chemical cross-linker, all oxidases exhibited cross-linked dimeric-weight bands on immunoblots (Fig. 2). As expected, none of the substituted oxidases could be cross-linked by diamide, a mild oxidant that promotes formation of the intersubunit disulfide bond in the wild-type oxidase (data not shown).

3.2. Presence of a positive charge at residue 78 confers activity

Activity of the expressed wild-type and mutant proteins was measured in membranes isolated from the heme$^-$ *E. coli* cells with NADH as respiratory substrate and assessed for stimulation by pyruvate or succinate. Of all the alternative oxidase types, only the wild-type (Cys-78) oxidase was statistically significantly stimulated by 5 mM pyruvate (Fig. 3A). AOX1a-C78S was significantly stimulated by 5 mM succinate (Fig. 3B) in contrast to its minimal response to pyruvate (Fig. 3A), confirming the results of Djajanegara et al. [17]. The level of activity reached by AOX1a-C78S with succinate was about half of the wild-type plus pyruvate rate. Djajanegara et al. [17] indicated 5 mM was the concentration needed for half-maximal stimulation; presumably, higher rates would be achieved with higher succinate concentrations. The degree of stimulation of AOX1a-C78S by succinate seen here, about six-fold based on estimates derived from the analysis of variance, is similar to the five-fold stimulation seen by Djajanegara et al. [17], based on one set of rates reported therein. AOX1a-C78A also showed statistically significant stimulation by succinate (Fig. 3B) and little response to pyruvate (Fig. 3A), consistent with unpublished results mentioned in Ref. [17]. Although wild-type, AOX1a-C78D, and -C78E were statistically significantly stimulated by succinate, the magnitude of these stimulations was smaller (122%, 28%, and 37%, respectively, based on the estimated means) relative to the stimulation of AOX1a-C78A and -C78S (418% and 489%, respectively, based on the estimated means). Similar to the Glu substitution [12], the negative-charged mutant AOX1a-C78D was statistically significantly more active relative to wild type, without an activator being present (Fig. 3A and B). As mentioned, addition of pyruvate had no significant effect on AOX1a-C78D activity (Fig. 3A) and the effect of succinate, although significant, was small in magnitude relative to its effect on AOX1a-C78A and AOX1a-C78S (Fig. 3B).

A new category of substitution mutants, those having positively charged side chains, was found to have activity profiles similar to those of the negative-charge substitutions (Fig. 3A). That is, both AOX1a-C78R and -C78K had activity above that of wild type and the Ser- and Ala-substituted alternative oxidases without pyruvate present. The activities of AOX1a-C78K and AOX1a-C78R were not significantly stimulated by either pyruvate or succinate (Fig. 3A and B).

The activity observed with these positively charged mutants could have been the result of carbamylation of the Lys and Arg side chains by CO_2. Because carbamylation requires a deprotonated amino group [31], its likelihood is greater for Lys than Arg due to the large difference in the pK_as of their amino and guanidinium groups, respectively [32]. Carbamylation would replace the side-chain positive charge with a negative charge, and thus the activation seen could again be the result of a negative charge, as for wild-type oxidase with pyruvate or the C78E and C78D mutants. To address this possibility, the effect of adding bicarbonate or depleting CO_2 from the assay buffer on wild-type and Lys- and Arg-substituted alternative oxidase activities was

assessed. Neither of these experimental manipulations caused an assay buffer pH change. The concentration of added bicarbonate, 5 mM, increased the dissolved CO_2 concentration about four-fold above ambient at pH 7.0. Bicarbonate added to assays run under conditions of ambient CO_2 had no effect on AOX1a-C78R and -C78K activity, while the wild-type AOX1a activity, in the presence of pyruvate, was slightly stimulated (14%). When the assay buffer, the membrane suspension, NADH stock solution, and, for wild-type oxidase assays, pyruvate, had been equilibrated with CO_2-free air by extensive bubbling, AOX1a-C78R activity was still unaffected by addition of 0.5 mM bicarbonate. AOX1a-C78K activity was stimulated minimally (6%), while the wild-type activity in the presence of pyruvate was stimulated 33%. As another test, membranes containing AOX1a-C78K equilibrated with ambient air were added to CO_2-free assay buffer and CO_2-free NADH was added. Rather than activity decreasing due to reversal of carbamylation (which happens rapidly [32]), the activity instead increased by nearly 20% within 5 min. Taken together, these results do not support carbamylation as the basis for the activity seen with the positive-charge substituted alternative oxidases.

The observed activity of any of the charge-substituted mutants, positive or negative, was not the result of residual pyruvate associated with the isolated membranes. Addition of LDH to the assays to metabolize pyruvate caused no change in the rates of any of the alternative oxidases (data not shown). Succinate stimulation was not a result of succinate dehydrogenase (SDH) activity because SDH activity was not detectable in the heme $^-$ *E. coli* membranes nor did inclusion of 5 mM malonate have any effect on activity (data not shown).

3.3. An uncharged substitution creates an apparently inactive enzyme

AOX1a-C78L was the one mutant alternative oxidase unable to support growth of the heme $^-$ *E. coli*. Cells transformed with the AOX1a-C78L construct grew very little and produced little or no mutated alternative oxidase protein as assessed by immunoblotting (Fig. 2). No alternative oxidase activity was detectable when assays were attempted. The presence of the AOX1a-C78L plasmid construct was verified in the cell stocks used for the expression studies (data not shown). The codon used for the Leu substitution was not a low-frequency codon for *E. coli*, occurring at 13.1–13.7 codons per 1000 [33]. All the codons used to make the mutations had at least twice the use frequency of the Cys codon in the wild-type enzyme, which was well expressed by the cells.

The substitution of Gln, which introduced a bulky, uncharged, but hydrophilic, residue at the regulatory Cys position resulted in some occasional cell growth but the AOX1a-C78Q protein was poorly expressed. Only one time, among seven attempted inductions, were cell membranes obtained from this mutant that had an unambiguous, but low, SHAM-sensitive rate, unaffected by pyruvate (nmol O_2/min/DU = 0.17). Membranes containing either AOX1a-C78Q or AOX1a-C78L sometimes had low levels of KCN-sensitive respiratory activity that may have been primarily responsible for cell survival under the induction conditions.

3.4. Effects of other organic acids and aldehydes on alternative oxidase activity

To obtain more information about the factors involved in the stimulation of alternative oxidase activity, acting at or near residue 78, a variety of compounds were tested for their effects on the activities of the wild-type and mutated alternative oxidases. These included mono- and dicarboxylic acids and aldehydes of different carbon chain lengths. All were used at 5 mM final concentration. A number of the compounds tested had minimal or no effect on the activity of the wild-type or mutant forms of the oxidase. These were: formate (1C); glycolate, oxalate, acetate (2C); malonate (3C); fumarate (4C); and glutarate (5C).

Although maleic acid (4C, dicarboxylate in *cis*) did not appreciably stimulate wild type, relative to its activity with pyruvate, or the charge-substituted oxidases, it did stimulate the activity of AOX1a-C78S and AOX1a-C78A to 58% and 66%, respectively (averages of two separate inductions), of the activity measured in the presence of succinate. Citrate and *cis*- and *trans*-aconitate, having various arrangements of two carboxylate groups spaced four carbons apart were also tested for their effects on AOX1a-C78S and AOX1a-C78A. Citrate was without effect. However, both *cis*- and *trans*-aconitate stimulated AOX1a-C78S 40% and 64%, respectively (averages of two separate inductions), and AOX1a-C78A 100% and 70% respectively (results from one induction), relative to their activities in the presence of succinate.

Glyoxylate was unique among the tested organic acids in that, at 5 mM, it had a stimulatory effect on all of the alternative oxidases (Fig. 4). At 1 mM or lower, except for stimulating wild-type enzyme activity, glyoxylate had little effect. While 5 mM glyoxylate stimulated wild-type activity to levels much like those seen in the presence of pyruvate (compare Fig. 3A, wild-type + pyruvate with Fig. 4, wild-type + glyoxylate; note the difference in the *Y*-axis scale between the two figures) which was expected, it also stimulated those oxidases with charged substitutions at Cys-78, increasing their rates over two-fold (Fig. 4). The stimulation was significant for AOX1a-C78D and -C78E and nearly so for AOX1a-C78R ($P = 0.08$). Because, in addition to wild type, the substitution mutants were affected, glyoxylate cannot be forming a thiohemiacetal at the Cys-78 site, suggesting there might be a second site of action for this α-keto acid. Other 2C carboxylic acids were ineffective at stimulating the oxidases (see above), suggesting the importance of the α-keto group for the effect and raising the possibility that another thiohemiacetal could be involved. The second site could be the other highly con-

served Cys in the alternative oxidase sequence, Cys_{II} of Berthold et al. [14] and Cys-128 in the *Arabidopsis* sequence used here.

To examine this possibility, an alternative oxidase mutant previously generated by site-directed mutagenesis, AOX1a-C128A [12], was used. This alternative oxidase has a high level of activity without activators being present, relative to the wild-type oxidase, but is stimulated, as expected, by pyruvate [12]. The Ala substitution at position 128 should abolish the glyoxylate effect, if residue 128 were involved. Membranes bearing wild-type (AOX1a), AOX1a-C128A, or AOX1a-C78E proteins were isolated in the presence of 5 mM iodoacetate (IOA), or of 5 mM DTT, and their responses to glyoxylate observed. IOA binds to Cys-78, introducing a permanent negative charge, resulting in alternative oxidase activation [12]. Thus, AOX1a-C128A treated with IOA should be equivalent to AOX1a-C78E (i.e., negative charge at residue 78) except for the lack of Cys-128. As shown in Table 1, glyoxylate stimulated the activity of wild-type and AOX1a-C128A by large percentages, relative to their activities without glyoxylate. AOX1a-C78E and both wild-type and AOX1a-C78E isolated with IOA, although not stimulated by pyruvate (data not shown), were all stimulated 42–47% by glyoxylate (Table 1). However, isolation of AOX1a-C128A in the presence of IOA dramatically decreased the glyoxylate effect on this oxidase form to a 14% stimulation. Note that the rates achieved by AOX1a + IOA and AOX1a-C128A + IOA in the presence of glyoxylate were lower than their non-IOA-treated counterparts (Table 1). This is because the carboxymethylation of Cys-78 by IOA results in a lower rate stimulation than that occurring when Cys-78 associates with an α-keto acid [12,15].

To exclude the possibility of an upstream site of glyoxylate action, NADH dehydrogenase in particular, duroquinol was used as a respiratory substrate. Although the rates attained were low, glyoxylate stimulation was still observed for the charge-substituted oxidases (data not shown), indicating that the glyoxylate effect is directly through the alternative oxidase. Further, NADH-supported cytochrome *c* oxidase pathway activity of cells grown in the presence of δ-aminolevulinic acid, to allow heme formation, was unaffected by glyoxylate (data not shown).

Table 1
Glyoxylate stimulation of alternative oxidase activity from membranes isolated with and without iodoacetate (IOA)

Alternative oxidase	Activity (nmol O_2/min/AOX unit)		Percentage stimulation by glyoxylate
	+ NADH	+ Glyoxylate	
AOX1a	26 ± 10	317 ± 28	1119
AOX1a + IOA	87 ± 21	128 ± 6	47
AOX1a-C78E	114 ± 10	170 ± 0	49
AOX1a-C78E + IOA	170 ± 25	241 ± 34	42
AOX1a-C128A	149 ± 16	443 ± 65	197
AOX1a-C128A + IOA	256 ± 8	292 ± 11	14

E. coli cell membranes containing wild-type or mutant alternative oxidases were isolated in the presence of either 5 mM DTT or 5 mM IOA. Activity was assayed with 1 mM NADH and following subsequent 5 mM glyoxylate addition. The percentage stimulations shown are from the ratio of the change in activity upon glyoxylate addition to the activity before glyoxylate addition. Each activity value is the average of two measurements (± half of the range between the individual measurements), from a representative experiment. For this table, specific activity values (nmol O_2/min/mg protein) were adjusted by the proportion of the immunoblot densitometry value for each oxidase, relative to that of the wild-type (AOX unit).

Table 2
Oxygen-isotope fractionation by wild-type and mutated alternative oxidases in *E. coli* membranes

Alternative oxidase	Δ (‰)
Wild-type + pyruvate	29.96 (± 0.24)
AOX1a-C78E	29.79 (± 0.29)
AOX1a-C78S + succinate	28.99 (± 0.05)
AOX1a-C78S	25.31 (± 0.46)
AOX1a-C78K	24.53 (± 0.33)

^{18}O fractionation was measured with 1.0 mM NADH as the respiratory substrate. Where indicated, pyruvate or succinate was present in the assay. Fractionation values are expressed as Δ (see Ref. [29]) and are the averages (± half of the range) of determinations made using membranes from two separate alternative oxidase protein induction experiments. At each time, from two to four measurements were taken for each membrane sample.

3.5. Oxygen isotope fractionation by wild-type and mutant alternative oxidases

The ^{18}O fractionation by several of the mutant alternative oxidases was compared with the fractionation by the wild-type oxidase in the presence of pyruvate. AOX1a-C78E and AOX1a-C78S plus succinate had fractionation values similar to that of the wild-type alternative oxidase plus pyruvate (Table 2) and similar to values measured for the alternative oxidase in chlorophyll-containing plant tissues or mitochondria isolated from them [30,34]. These are three alternative oxidase forms where a negative charge should be present in the protein in the vicinity of residue 78. On the other hand, AOX1a-C78S without succinate and AOX1a-C78K had fractionation values similar to each other, and distinctly different from those of the others (Table 2), but similar to fractionation values seen in non-green plant tissues and mitochondria isolated from them [30,34] and a yeast [35]. The activity of the wild-type alternative oxidase without pyruvate was too low to permit an accurate determination of fractionation.

4. Discussion

A variety of amino acid substitutions were made at the regulatory Cys residue of the *Arabidopsis* AOX1a protein, including positively charged and uncharged amino acids. While none of these substitutions affected the dimeric structure of the alternative oxidase, they did have pronounced effects on alternative oxidase activity and its response to activators. Some of these effects have been

observed previously. The Glu-substituted oxidase had been shown to be active without a pyruvate requirement [12]. The second negatively charged substituted enzyme reported here, AOX1a-C78D, has the same properties (Fig. 3A) indicating that the effect of the Glu mutation is not unique. The low basal activities, lack of response to pyruvate [12,13], and stimulation by succinate [17] of AOX1a-C78S and -C78A have been previously reported. These results all underscore the importance of residue 78, or its surroundings, for the activators' effects on the alternative oxidase.

The principal goal of this study was to find clues concerning the mechanism of activation originating from this interaction of an activator, whether α-keto acid or succinate, with residue 78. One approach used was to make positively charged amino acid substitutions at residue 78. These alternative oxidases, AOX1a-C78K and -C78R, have activities well above those of AOX1a-C78A, AOX1a-C78S, and wild-type without activators present (Fig. 3A and B). The fact that alternative oxidases with positively charged substitutions at residue 78 display activity strongly suggests that the activation mechanism of α-keto acids, and likely succinate as well, is not through a direct involvement in catalysis. That a positive charge could substitute in a catalytic mechanism requiring a fixed negative charge, for example, for ubiquinol deprotonation [15], is very unlikely. Instead, the outcome of the positive substitution (an active oxidase) is consistent with activation occurring through charge repulsion between the two adjacent subunits, which in turn suggests that a conformational change may result, enhancing activity. Such charge repulsion could take place if either positive- or negative-charged amino acid side chains are present, or when negatively charged activators are in the vicinity of position 78. Recently, some weakly conserved elements of a proposed quinone binding motif have been identified in the alternative oxidase N-terminal region just to the C-terminal side of Cys-78 [36]. Perhaps a charge-driven conformational change in the N-terminal region could position these residues in the correct orientation to interact with ubiquinone, but this is highly speculative.

The Gln substitution, lacking a charge, resulted in a weakly active enzyme barely able to support heme$^-$ *E. coli* growth while the Leu substitution produced an apparently non-functional enzyme incapable of supporting growth. These results are also consistent with charge repulsion being a part of alternative oxidase activation. Just as the charged amino acids and activators may promote some movement in the protein, the presence of the hydrophobic side chains (Leu) on adjoining surfaces of the two dimer subunits may result in a hydrophobic association between the pair (e.g., analogous to that seen with hemoglobin β-chains containing the sickle cell mutation [37]) that prevents the conformation necessary to activate the enzyme. This situation would also be analogous to the wild-type deactivation of the oxidase through formation of the intersubunit disulfide bond.

In another approach to understanding the mechanism of activation by α-keto acids and succinate, an array of additional compounds was tested for effects on the wild-type and mutant oxidases. These were used at 5 mM, the concentration reported for half-maximum stimulation by succinate of the Ser-substituted alternative oxidase in *E. coli* membrane vesicles [17]. Some of these compounds have been tested before with the wild-type enzyme in isolated mitochondria [38]. With the exception of glyoxylate (see below), none had any particular effect on wild-type or the charge-substituted alternative oxidases. Maleic acid, however, did stimulate AOX1a-C78S and -C78A, the succinate-sensitive oxidase forms. This maleic acid effect is in contrast to the lack of effect by fumarate observed here and by Djajanegara et al. [17]. Fumarate and maleic acid each contains a double bond positioning the two carboxyls *trans* and *cis*, respectively, so their carboxylate groups have distinctly different orientations. Thus, the ability to attain a specific orientation of the two negative charges seems to be important for activation of the C78A and C78S mutants.

In this context, the results with the tricarboxylic acids *cis*- and *trans*-aconitate, both of which were able to activate AOX1a-C78S and -C78A, might initially seem difficult to interpret. But, an examination of their structures indicates that each possesses succinate-like features on different parts of the molecule, possibly accounting for their stimulatory effects. Citrate, which failed to stimulate either AOX1a-C78S or -C78A, also has several dicarboxylates separated by two carbons. Perhaps, because it lacks the double bond of the aconitates, the proper orientation of the two carboxyls is never achieved.

The 2C α-keto acid, glyoxylate, at 5 mM, displayed the unexpected ability to stimulate the oxidases with charged substitutions at the site of the regulatory Cys. This effect is therefore apparently distinct from the α-keto acid/succinate effect at Cys-78. A free Cys at position 128 appears important for the glyoxylate effect because the effect was markedly diminished in a mutant oxidase in which Cys-128 was replaced with Ala and its Cys-78 had been carbamylated by IOA (Table 1). For both the wild-type oxidase and AOX1a-C78E, IOA treatment did not block the glyoxylate effect, perhaps because IOA does not react readily with Cys-128. While glyoxylate is an intermediate in the plant photorespiratory cycle, the physiological relevance of the glyoxylate effect is obscure since millimolar concentrations are required. In contrast, pyruvate has a very low effective concentration for stimulation, less than 5 μM for half-maximal stimulation [38]. However, the possibility that the glyoxylate effect may come about through the formation of a second thiohemiacetal at the other well-conserved Cys of the alternative oxidase protein sequence points toward another region of the protein through which its activity can be modulated *in vitro*. Additional site-directed mutagenesis can be used to explore this phenomenon.

Returning to the nature of the activation mechanism at Cys-78, a further indication of the similarity of activation by

pyruvate of the wild-type alternative oxidase, succinate of AOX1a-C78S, and the negative-charge substitution of AOX1a-C78E, is the near-identity of their ^{18}O fractionation values (Table 2). This similarity suggests that, with fractionation as well as activities, the Glu substitution represents a good analog of the thiohemiacetal formed between Cys-78 and pyruvate. The fractionation of AOX1a-C78K is more like the least-active alternative oxidase form measured, AOX1a-C78S without succinate (Table 2). Thus, while the presence of the fixed positive charge can support oxidase activity, its effect on the pathway of molecular oxygen to the active site is apparently different from the effects produced by the fixed negative charges. Finnegan et al. [39] established that soybean AOX2 and AOX3 are the abundant alternative oxidase forms in light-grown cotyledons and roots, respectively, providing the apparent basis for the differences in alternative oxidase oxygen fractionation observed for these tissues [39]. That AOX2 and AOX3 can have different fractionation values is perhaps not surprising. Our data indicate that a single amino acid change can have a pronounced effect on fractionation.

One interpretation of the larger fractionation values (greater discrimination against ^{18}O) for the negative-charge forms of the oxidase is that the proposed conformational change in these oxidases opens the oxygen pathway. A relatively constricted oxygen pathway could lead to less diffusional exchange of heavy and light oxygen isotopes, requiring the enzyme to use a larger proportion of the heavy isotope (smaller fractionation value). Upon opening of the pathway, diffusional exchange would occur more readily, allowing reaction with the preferred isotope, ^{16}O ($^{32}O_2$).

Both old and new models proposing a diiron center as the active site of the alternative oxidase [14,40,41] place the N-terminal third of the protein, which contains Cys-78, outside of the diiron-binding domain. The results from the substituted alternative oxidases of this study suggest that a conformational change may originate in this domain, due to the presence of a positive or negative charge, and have an indirect effect on activity at the diiron site and on the path of oxygen to that site. In addition, the second highly conserved Cys, Cys-128, which is located near the junction of the N-terminal third of the protein with the diiron-binding domain [14,41], may also be capable of modulating alternative oxidase activity. While the disposition of the N-terminal portion of the alternative oxidase relative to the active site remains unknown, evidence is increasing that this portion of the protein can interact in important ways with the active site.

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