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Molecular Localization of a Redox-Modulated Process Regulating Plant Mitochondrial Electron Transport

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Using in organellar assays, we found that significant tobacco alternative oxidase (AOX) activity is dependent on both reduction of a putative regulatory disulfide bond and the presence of pyruvate, which may interact with a Cys sulfhydryl. This redox modulation and pyruvate activation thus may be important in determining the partitioning of electrons to AOX in vivo. To investigate these regulatory mechanisms, we generated tobacco plants expressing mutated AOX proteins. Mutation of the most N-terminal Cys residue (Cys-126) to an Ala residue produced an AOX that could not be converted to the disulfide-linked form, thus identifying this Cys residue as being responsible for redox modulation. Although this mutation might be expected to produce an AOX with constitutive high activity in the presence of pyruvate, we found it to have minimal in organellar activity in the presence of pyruvate. Nonetheless, the Cys-126 mutation did not appear to have compromised the catalytic function of AOX, given that cells expressing the protein displayed high rates of cyanide-resistant respiration in vivo. The striking difference between in vivo and in organellar results suggests that an additional mechanism(s), as yet unidentified by in organellar assays, may promote activity in vivo. Mutation of the Cys residue nearest the presumptive active site (Cys-176) to an Ala residue did not prevent disulfide bond formation or affect the ability of AOX to be stimulated by pyruvate, indicating that this Cys residue is involved in neither redox modulation nor pyruvate activation.

INTRODUCTION

In aerobic photosynthetic organisms, it is well established that a reversible oxidation and reduction of enzyme Cys sulfhydryls is a significant direct mode of regulation of a metabolic process in vivo. The activities of several chloroplast enzymes involved in photosynthetic carbon metabolism are modulated by reduction of regulatory disulfides in response to light and their subsequent oxidation in the dark. The process is mediated by photosynthetic electron transport and the ferredoxin–thioredoxin system (Scheibe, 1991; Buchanan, 1992). There are increasing indications that other metabolic enzymes in photosynthetic organisms are also subject to such redox modulation, either to coordinate other metabolic processes with photosynthesis (Huppe et al., 1994; Sasaki et al., 1997) or to regulate processes that are largely independent of photosynthesis (Kiss et al., 1991; Umbach and Siedow, 1993; Anderson et al., 1995; Besse et al., 1996).

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In plant mitochondria, there are two paths of respiratory electron transport from ubiquinone to O_2 (Day et al., 1980; Vanlerberghe and McIntosh, 1997). Electron transfer through the cytochrome pathway is coupled (through the generation of a proton motive force) to ATP synthesis, and the terminal oxidase (cytochrome oxidase) is inhibited by cyanide. Alternatively, electron flow from ubiquinone to alternative oxidase (AOX) is not coupled to ATP production. The AOX is cyanide resistant but sensitive to substituted hydroxamic acids, such as salicylhydroxamic acid (SHAM) and *n*-propyl gallate. The partitioning of electrons between these two pathways may be dependent on a redox modulation of AOX activity because the protein exists in the inner mitochondrial membrane as either a noncovalently linked or covalently linked dimer (Umbach and Siedow, 1993). The dimer, when covalently linked by a putative intermolecular Cys disulfide bond(s) between the two subunits, is a less active form of AOX (as determined by in organellar assays), whereas reduction of the disulfide bond(s) produces a more active form (Umbach and Siedow, 1993). These forms can be interconverted by treatment with the reductant DTT and the oxidant diamide and visualized by nonreducing SDS-PAGE and

protein gel blot analysis, in which the oxidized form has an "apparent" molecular mass twice that of the reduced form (Umbach and Siedow, 1993).

We have shown that reduction of tobacco AOX to its active form is mediated in isolated mitochondria by the oxidation of specific tricarboxylic acid (TCA) cycle substrates, notably isocitrate and malate (Vanlerberghe et al., 1995). A likely explanation is that intramitochondrial reducing power generated by the oxidation of these organic acids supports the reduction of AOX. Based on the organic acid specificity of this effect, we speculated that NADPH may be specifically required. Reduction may then be mediated by a thioredoxin system requiring NADPH. Such a system has been identified in plant mitochondria but has not yet been ascribed any specific function (Moller and Rasmusson, 1998).

A redox-modulated regulation of AOX would couple AOX activity to TCA cycle metabolism and the redox state of the mitochondrial matrix. It is often postulated that AOX functions to modulate respiratory carbon flow (Lambers, 1985), and recent evidence suggests that the pathway may function to alleviate the generation of harmful reactive oxygen species in the mitochondrion, presumably by preventing overreduction of particular electron transport chain components (Wagner, 1995; Vanlerberghe and McIntosh, 1996; Popov et al., 1997; Purvis, 1997). Hence, the proposed redox modulation of AOX activity is consistent with proposed functions of the pathway.

AOX activity is strongly stimulated by pyruvate (Millar et al., 1993; Day et al., 1994), and significant AOX activity in isolated tobacco mitochondria is dependent on both reduction of the putative regulatory disulfide bond(s) and the presence of pyruvate (Vanlerberghe et al., 1995). Studies with soybean AOX suggest that pyruvate action is due to its interaction with a Cys sulfhydryl to form a thiohemiacetal because the activation is mimicked by iodoacetate (Umbach and Siedow, 1996).

In a diverse range of species and tissues, the highest rates of in organellar AOX activity are measured when the enzyme is in its reduced form and pyruvate is present (Vanlerberghe et al., 1995; Umbach and Siedow, 1996; Hoefnagel et al., 1997; Millar et al., 1997). With these more optimal in organellar assay conditions, soybean AOX can compete with the cytochrome pathway for electrons (Hoefnagel et al., 1995; Ribas-Carbo et al., 1997), indicating that electron flow through this nonphosphorylating pathway may be significantly greater than previously thought. To investigate these novel mechanisms of regulation of electron transport, we have employed site-directed mutagenesis of the cloned tobacco gene encoding AOX and have generated transgenic plants expressing mutated AOX proteins. Using this approach, we identify Cys-126 as being responsible for the putative redox modulation of plant AOX, and we show that a second Cys residue (Cys-176) is not involved in pyruvate activation of tobacco AOX. Furthermore, our results suggest that AOX activity in vivo may be promoted by a factor(s) not yet identified by in organellar assays.

RESULTS

Based on the *Aox1* cDNA sequence (Vanlerberghe and McIntosh, 1994), tobacco AOX has two cysteines in its N-terminal hydrophilic domain (Cys-126 and Cys-176). These are candidates to be involved in the putative redox modulation and/or pyruvate activation of AOX because they are both predicted to reside in the mitochondrial matrix and are the only two cysteines completely conserved among the known plant sequences (Vanlerberghe and McIntosh, 1997). Using site-directed mutagenesis, we have generated classes of transgenic plants overexpressing different mutated AOX proteins in which one or both of these Cys residues have been changed to an Ala (see Methods for an explanation of the naming of these plants).

Each of the Mutated Tobacco AOX Proteins Could Be Overexpressed in Tobacco Mitochondria

Analysis of multiple independent transformants of each class of transgenic plants showed that each of the different AOX proteins could be overexpressed in tobacco. Figure 1A indicates the large increase in the level of immunoreactive AOX protein present within purified leaf mitochondria. As expected, the level of overexpression of any one AOX protein could vary dramatically between independent transformants (Figure 1A). The analysis also suggested that replacement of Cys-176 decreased the amount of overexpression typically seen. This can be observed by comparing the range of overexpression in class B and C plants (which have Cys-176) with class D and E plants (which lack Cys-176; Figure 1A). We have not conducted investigations to attempt to explain these differences in the level of overexpression typically achieved. Nonetheless, each of the classes of plants containing an AOX construct had dramatically higher levels of AOX protein than did wild-type plants (in which levels under these growth conditions were near the limit of detection) or plants transformed with only the kanamycin resistance gene (class A plants). It should be kept in mind, however, that all of the transgenic plants contain a low level of native AOX protein and activity as a result of transforming wild-type tobacco.

Cys-126 Is Responsible for the Covalent Redox Modulation of Tobacco AOX

When mitochondria overexpressing the native AOX protein (class B) or AOX protein in which Cys-176 alone had been mutated (class D) were isolated and analyzed by nonreducing SDS-PAGE and protein gel blot analysis, the majority of the AOX protein was in the oxidized (less active) form (Figure 1B). The result with class B mitochondria confirms our previous observations that native tobacco AOX is largely oxidized after mitochondrial isolation (Vanlerberghe et al., 1995). The

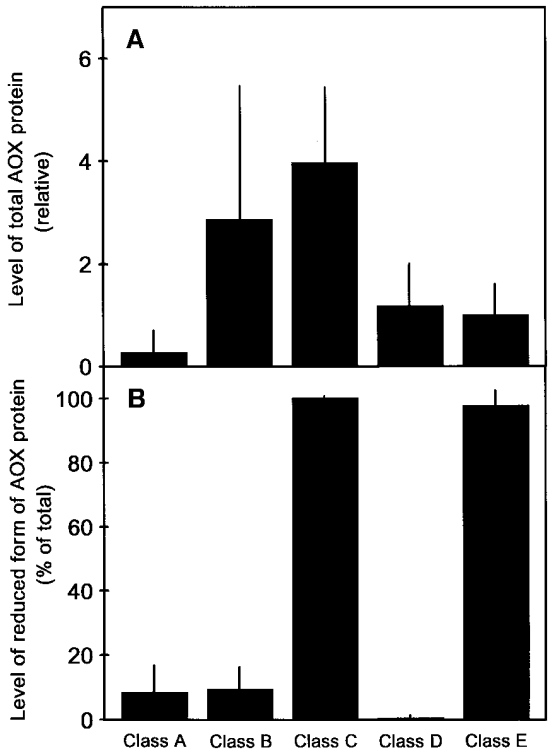

Figure 1. Analysis of Transgenic Tobacco Overexpressing Different AOX Proteins.

Leaf mitochondria were isolated, and mitochondrial protein (50 μg) was separated by SDS-PAGE, transferred to nitrocellulose, and probed with a monoclonal antibody raised to AOX. The AOX protein was then quantified by densitometry. See Methods for an explanation of the naming of transformants.

(A) Reducing SDS-PAGE was used to compare total AOX protein in the different classes of plants. Data are the average ±SD from seven (class A), four (class B), 14 (class C), 13 (class D), and 11 (class E) independent transformants.

(B) Nonreducing SDS-PAGE was used to compare the level of the reduced (more active) and oxidized (less active) forms of AOX in the different classes of plants. Data are the average ±SD from two (class A), three (class B), nine (class C), nine (class D), and nine (class E) independent transformants.

result with class D mitochondria indicates that the oxidized form of AOX can exist in the absence of Cys-176. However, little or no oxidized AOX was detected in mitochondria after mutation of Cys-126 (C and E classes), indicating that Cys-126 is responsible for the intermolecular disulfide bond formation (Figure 1B). Table 1 reinforces this conclusion, showing that even after treatment with the oxidant diamide, all of the AOX protein in C and E mitochondria remains in the reduced (more active) form. Conversely, the AOX in B or D mitochondria was effectively oxidized by diamide, after reduction by DTT (Table 1).

Mutation of Cys-126 Results in a Significant Loss of Tobacco AOX Activity in Isolated Mitochondria

Figure 2 provides a comparison of AOX protein and activity in typical C and D class mitochondria. The C class mitochondria contain a high level of AOX protein, and because it lacks Cys-126, all of this protein is present in the reduced (more active) form after mitochondrial isolation. We expected, therefore, to see large amounts of AOX activity in the presence of pyruvate, but this was not the case; the subsequent addition of DTT (which should not be necessary to increase activity) had little effect. Conversely, in the D class (in which AOX is mostly in the oxidized, less active form after mitochondrial isolation), high activity was seen in the presence of pyruvate once AOX was converted to its reduced (more active) form by DTT (Figure 2).

Figure 3 provides a detailed comparison of AOX protein levels and activities in isolated mitochondria from different transgenic plants. Although the level of AOX protein in the C transformants analyzed was very high (the highest of all transformants analyzed, in fact; Figure 3A), the level of AOX activity after pyruvate addition was very low (Figure 3B). Subsequent addition of DTT had a little stimulatory effect on this activity (Figure 3C), but this may be due to the low level of native AOX protein expected in all lines and expected to become active after DTT addition. This background activity would be expected to be of similar magnitude to that seen in wild-type plants (Figures 3B and 3C). Both B and D transformants, on the other hand, had high levels of AOX activity in the presence of pyruvate and DTT, as expected given their high AOX protein levels. Other α-keto acids besides pyruvate (such as glyoxylate) have also been shown to activate

Table 1. Effect of DTT and Diamide on the Level of the Reduced (More Active) Form of AOX in Native and Mutated Proteins

	Reduced Form of AOX Protein (% of Total Protein)			
Treatment[a]	B9	C3	D3	E6
None	2	100	0	100
+DTT	100	100	100	100
+Diamide	0	100	0	100
+DTT, +diamide	33	100	0	100
+Diamide, +DTT	95	100	91	100

[a] Leaf mitochondria were isolated from transgenic plants B9, C3, D3, and E6, and treated with DTT (10 mM), diamide (3 mM), DTT followed by diamide, or diamide followed by DTT, using a protocol similar to that described by Umbach and Siedow (1993). Treated mitochondrial proteins (50 μg) were then separated by nonreducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody raised to AOX. The level of the AOX protein was quantified by densitometry. Representative results are shown. See Methods for an explanation of the naming of transformants.

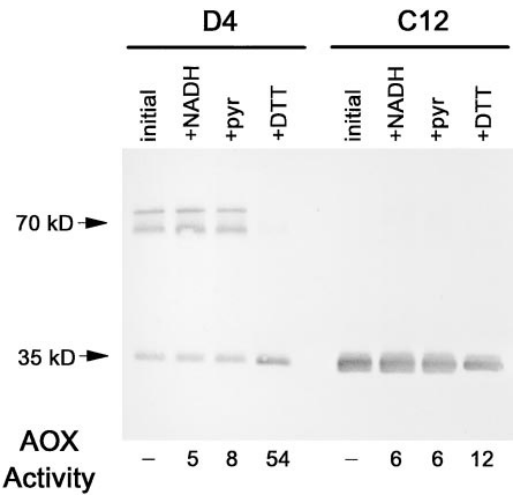

Figure 2. AOX Protein and Activity in Leaf Mitochondria Isolated from Transgenic Tobacco Overexpressing a Mutated AOX Protein Lacking Cys-176 (D4) or Cys-126 (C12).

The O_2 electrode analysis of AOX activity and the nonreducing SDS-PAGE and gel blot analysis of the AOX protein were performed at the same time. The initial treatment included freshly isolated mitochondria in a reaction buffer without any substrate. The +NADH treatment was performed after the subsequent addition of NADH (2 mM), followed by ADP (1 mM) and then myxothiazol (16 μM). The +pyr treatment was performed after the subsequent addition of 2 mM pyruvate, and the +DTT treatment was performed after subsequent addition of 10 mM DTT. AOX activity is expressed as nanomoles of O_2 per milligram of protein per minute. Numbers at left indicate apparent molecular mass. See Methods for an explanation of the naming of transformants.

soybean AOX, albeit at higher concentrations (Millar et al., 1996). We found that both B and D transformants had high levels of AOX activity in the presence of 10 mM glyoxylate and DTT, but activity in the C transformants was again very low (data not shown).

Table 2 shows that in both the B and D mitochondria, high rates of AOX activity also could be achieved if citrate or malate was added to bring about reduction of the AOX protein (rather than DTT). The result with B mitochondria is consistent with our previous observations that native tobacco AOX is reduced in response to the metabolism of these substrates (Vanlerberghe et al., 1995). However, under these conditions, the AOX activity of C and E mitochondria again remained much lower than expected (Table 2).

Results of in vivo experiments (see below) suggested that the Cys-126–mutated AOX may be susceptible to loss of activity during mitochondrial isolation. We attempted to protect against this loss of activity by including pyruvate or iodoacetate in the mitochondrial isolation buffers. However, we found that these additions to several mitochondrial isolations had no impact on the level of activity seen in C12 or C3 transformants (data not shown).

Cys-176 Is Not Necessary for Pyruvate or Iodoacetate Activation of Tobacco AOX

The reduced (more active) form of native tobacco AOX shows little activity in the absence of pyruvate (Vanlerberghe et al., 1995). Figure 4 shows that activation of reduced AOX by pyruvate occurred rapidly in both B and D mitochondria. Also, this pyruvate activation could be mimicked in both types of mitochondria by iodoacetate, although the level of AOX activity that could be achieved with iodoacetate was always less than the level that could be achieved by pyruvate (Figure 4). This indicates that Cys-176 (absent in D mitochondria) is not necessary for the pyruvate or iodoacetate activation of native tobacco AOX.

The Cys-126–Mutated AOX Is Active in Vivo

Although mutation of Cys-126 in C plants results in a significant loss of AOX activity in isolated mitochondria (see above), we found that this mutated AOX protein does have considerable activity in vivo. Table 3 shows the respiratory characteristics of tobacco suspension cells derived from either wild-type leaves or leaves of primary transformants. Control respiration rates were similar in all three cell types. In wild-type cells, the level of cyanide-resistant, SHAM-sensitive respiration (the AOX capacity) was ~17% of the control respiration rate. This value is similar to that previously reported for AOX capacity in wild-type tobacco cells (Vanlerberghe et al., 1994). As expected, the AOX capacity in B9 cells (overexpressing native tobacco AOX) was much higher (92% of the control respiration rate). Similarly, the AOX capacity in C12 cells was also much higher (91% of the control respiration rate), indicating that the Cys-126–mutated AOX protein does have considerable activity in vivo.

Figure 5 is a nonreducing SDS-PAGE and gel blot analysis of AOX protein from mitochondria of the different suspension cells. As expected, the native AOX protein (from wild-type cells and from B9 cells) is largely in the oxidized form after mitochondrial isolation, and the level of AOX protein is much higher in B9 than in the wild type. In C12, the level of AOX protein is also very high but, as expected, is present entirely in the reduced form after mitochondrial isolation. This analysis also shows that the high AOX capacity seen in C12 cells (Table 3) is not due to an unpredictably high level of background native AOX protein in these cells because such native AOX protein would be seen largely in the oxidized form after mitochondrial isolation. However, as expected, we see little oxidized AOX protein in the C12 mitochondria (Figure 5).

The studies described above were done in suspension cells to overcome inherent problems with the use of inhibitors

(cyanide and SHAM) and the measurement of AOX capacity in intact tobacco leaves. We have previously shown that these inhibitors can be successfully used to estimate AOX capacity both in wild-type tobacco suspension cells and in transgenic cells with either increased or decreased levels of the native AOX protein due to the introduction of sense or antisense *Aox1* constructs (Vanlerberghe et al., 1994).

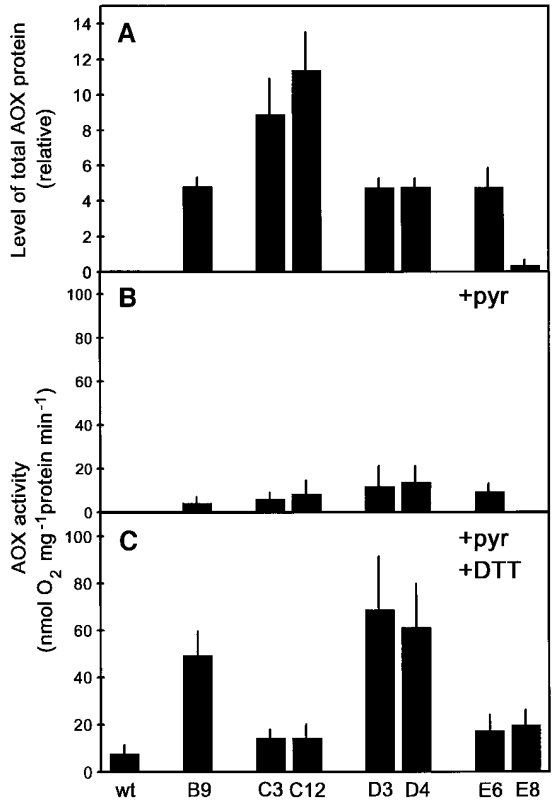

Figure 3. AOX Protein and Activity in Leaf Mitochondria Isolated from Wild-Type Tobacco or from Transgenic Tobacco Overexpressing Different AOX Proteins.

Data are the average ±SD of four (wild-type [wt]), four (B9), four (C3), four (C12), three (D3), four (D4), four (E6), and three (E8) separate mitochondrial preparations. See Methods for an explanation of the naming of transformants.

(A) Mitochondrial protein (75 μg) was separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody raised to AOX. The AOX protein was then quantified by densitometry.

(B) AOX activity (+pyr) was measured in the O_2 electrode after sequential additions of 2 mM NADH, 1 mM ADP, 16 μM myxothiazol, and 2 mM pyruvate. AOX activity is the rate of O_2 uptake after the addition of pyruvate and minus any O_2 uptake after the addition of 100 μM *n*-propyl gallate. In most cases, the latter was zero.

(C) AOX activity (+pyr and +DTT) was measured after sequential additions of 2 mM NADH, 1 mM ADP, 16 μM myxothiazol, 2 mM pyruvate, and 10 mM DTT. AOX activity is the rate of O_2 uptake after the addition of DTT and minus any O_2 uptake after the addition of 100 μM *n*-propyl gallate. In most cases, the latter was zero.

Table 2. AOX Activity and Protein Levels in Leaf Mitochondria Isolated from Wild-Type Tobacco and from Transgenic Tobacco Overexpressing Different AOX Proteins

	AOX Activity[b] (nmol O_2 mg^{-1} protein min^{-1})			
Class[a]	Control	+Citrate	+Malate	Protein Level[c] (Relative)
Wt	0.0	3 ± 2	3 ± 2	—
B	5 ± 2	51 ± 13	64 ± 21	4.8 ± 0.8
C	8 ± 5	20 ± 9	22 ± 12	10.7 ± 2.3
D	14 ± 8	54 ± 18	96 ± 37	4.8 ± 0.4
E	5 ± 6	17 ± 11	19 ± 16	3.1 ± 2.7

[a] The data are the average ±SD of at least four separate mitochondrial isolations. The plants used were wild type (Wt), B9, C3, C12, D3, D4, E6, and E8. See Methods for an explanation of the naming of transformants.

[b] The control AOX activity was measured after sequential additions of 2 mM NADH, 1 mM ADP, 16 μM myxothiazol, and 2 mM pyruvate. The +citrate and the +malate activity were measured in the same way, except that either 10 mM citrate or 10 mM malate (and 10 mM glutamate) were added along with the NADH. In all cases, activity is the rate of O_2 uptake after the addition of pyruvate and minus any O_2 uptake after the addition of 100 μM *n*-propyl gallate. In most cases, the latter was zero.

[c] Mitochondrial protein (75 μg) was separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody raised to AOX. The level of the AOX protein was then quantified by densitometry. The level of the AOX protein in the wild type was near the limit of detection of the densitometer.

DISCUSSION

We have identified Cys-126 as the redox-active regulatory Cys responsible for the interconversion of the noncovalently linked more active form and covalently linked less active form of tobacco AOX (Figure 1B and Table 1). Figure 6 outlines our working model, showing that this interconversion is dependent on the redox state of the mitochondrial pyridine nucleotide pool, thereby providing a mechanism to couple TCA cycle metabolism with AOX activity. Mutation of Cys-126 produces an AOX that no longer can be converted to the disulfide-linked less active form. Although this mutation might be expected to produce an enzyme with constitutive high activity in the presence of pyruvate in isolated mitochondria, we found that this is not the case. Rather, we found the Cys-126–mutated protein has minimal in organellar activity in the presence of pyruvate (Figures 2 and 3B and Table 2). One possibility is that mutation of Cys-126 by

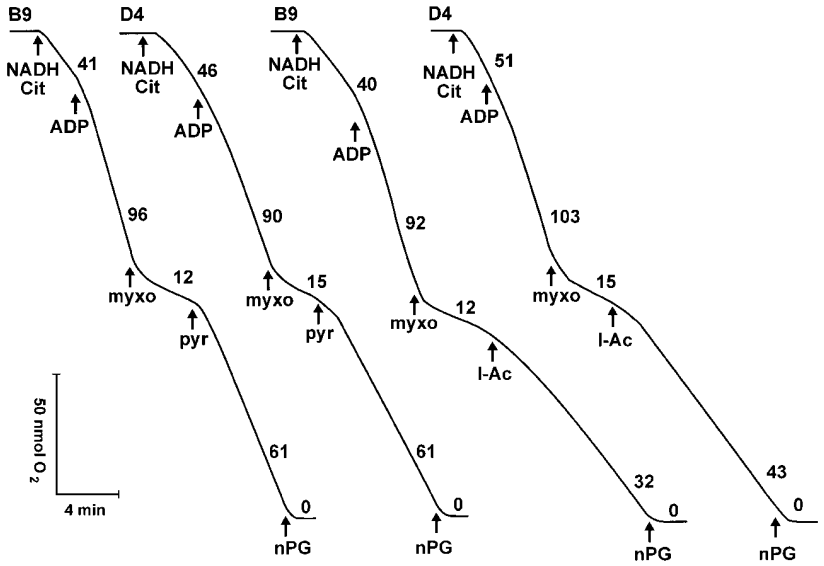

Figure 4. O_2 Electrode Traces Showing the Effect of Pyruvate and Iodoacetate on Native AOX Activity (B9) and on the Activity of a Mutated AOX Lacking Cys-176 (D4).

Additions were made at the following final concentrations: 2 mM NADH, 10 mM citrate (Cit), 1 mM ADP, 16 μM myxothiazol (myxo), 2 mM pyruvate (pyr), 5 mM iodoacetate (I-Ac), and 100 μM *n*-propyl gallate (nPG). Higher concentrations of pyruvate or iodoacetate were shown to have no additional stimulatory effect on AOX activity (data not shown). Numbers on traces refer to rates of O_2 uptake (nanomoles of O_2 per milligram of protein per minute). Representative results are shown. See Methods for an explanation of the naming of transformants.

an Ala residue had compromised the enzyme's catalytic function. However, this does not appear to be the case, because tobacco suspension cells overexpressing this mutated AOX have high rates of cyanide-resistant, SHAM-sensitive respiration, which can only be attributed to the Cys-126–mutated AOX (Table 3 and Figure 5).

One possible explanation for the difference seen between in vivo and in organellar activity is that the Cys-126 mutation makes AOX more susceptible to a loss of activity during the mitochondrial isolation. Because the presence of pyruvate has been shown to protect both soybean and *Arum* AOX against a dramatic loss of activity during solubilization and partial purification (Zhang et al., 1996; Hoefnagel et al., 1997), we tried isolating mitochondria in the presence of pyruvate or iodoacetate. However, this approach had no apparent effect on the in organellar activity we could measure in the Cys-126–mutated AOX (data not shown).

Studies using sulfhydryl reagents indicated that pyruvate stimulation of soybean AOX was dependent on its interaction with a Cys residue (Umbach and Siedow, 1996). The ability of the sulfhydryl reagent iodoacetate to stimulate AOX activity in a manner similar to that of pyruvate suggested that pyruvate stimulation involved the formation of a thiohemiacetal (Umbach and Siedow, 1996). The Cys residue involved was predicted to be that nearest the presumptive active site (Umbach and Siedow, 1996), a binuclear iron center (Siedow et al., 1995). The predicted Cys corresponds to Cys-176 in tobacco. However, we have shown here that mutation of Cys-176 to an Ala residue does not affect the ability of tobacco AOX to be stimulated by either pyruvate or iodoacetate (Figure 4). Given this result, pyruvate stimulation must either involve interaction with a different Cys or not be dependent on interaction with a Cys residue.

Other potential Cys residues for interaction with pyruvate would be Cys-126 and Cys-198. Cys-198 is thought to reside in the intermembrane space (Vanlerberghe and McIntosh, 1997). Involvement of this Cys residue in pyruvate activation could make the activity of AOX more responsive to the level of cytosolic pyruvate. However, this Cys residue is not present in several plant species (Vanlerberghe and McIntosh, 1997), including soybean AOX3 and potato AOX. Both of these proteins have been reported to be stimulated by pyruvate (Day et al., 1994; Hiser et al., 1996). Evidence also suggests that the pyruvate stimulation takes place from within the mitochondrial matrix (Day et al., 1994; Millar et al., 1996). These factors make it unlikely that Cys-198 is involved in pyruvate activation.

We have shown that Cys-126 is the redox-active regulatory Cys residue, but presumably it could also be the Cys residue necessary for pyruvate stimulation. If pyruvate inter-

action with Cys-126 were dependent on the presence of a Cys-126 sulfhydryl (such as for the formation of a thiohemiacetal), then it would be expected, as has been observed, that the oxidized form of native AOX is not responsive to pyruvate but that the reduced form (with its component sulfhydryls) is pyruvate stimulated (Vanlerberghe et al., 1995). Also, if Cys-126 is the residue that interacts with pyruvate, it would explain the substantial loss of AOX activity in isolated mitochondria when this Cys is mutated (C and E mitochondria), because in the absence of pyruvate stimulation, the reduced native tobacco AOX shows little in organellar activity (Vanlerberghe et al., 1995). However, if a pyruvate–Cys-126 interaction is responsible for the pyruvate activation in isolated mitochondria, then some other mechanism must be capable of substituting for this interaction in vivo, because the Cys-126–mutated AOX shows substantial in vivo activity (Table 3). The discovery of the biochemical control of AOX by redox modulation and pyruvate activation has allowed much higher rates of AOX activity to be measured in isolated mitochondria than had been seen previously. Continued efforts toward activation of the Cys-126–mutated AOX may lead to the discovery of yet additional mechanisms to optimize in organellar AOX activity.

In conclusion, we have shown that Cys-126 is the redox-active Cys responsible for the interconversion of the covalently and noncovalently linked forms of tobacco AOX and that Cys-176 does not play any apparent role in either redox modulation or pyruvate activation. In transgenic plants and suspension cells overexpressing the Cys-126–mutated AOX, the AOX protein is always present in the reduced (more active) form (Figure 5). These transgenic plants will provide a powerful approach to assess the importance of the redox modulation of AOX to respiratory metabolism in vivo. For example, the oxygen isotope fractionation technique developed by Guy et al. (1989) could be used to determine whether the presence of AOX protein that cannot be converted to its disulfide-linked less active form alters the partitioning of electrons between the cytochrome pathway and AOX in vivo.

Table 3. Respiratory Characteristics of Wild-Type and Transgenic (B9 and C12) Tobacco Suspension Cells

Cells[a]	Respiration[b] (nmol O_2 mg^{-1} dry weight hr^{-1})	AOX Capacity[c]
Wild type	533 ± 48	93 ± 25
B9	615 ± 64	569 ± 40
C12	723 ± 142	657 ± 122

[a] Data are the average ±SD from four separate experiments using cells from different subcultures and at 3 days after subculture. See Methods for an explanation of the naming of transformants.
[b] Respiration is O_2 uptake in the absence of any inhibitors. The rate of residual O_2 uptake (in the presence of KCN and SHAM) has not been subtracted from this rate. This residual rate was wild type, 32 ± 5; B9, 24 ± 2; C12, 45 ± 11 nmol O_2 mg^{-1} dry weight hr^{-1}.
[c] AOX capacity is defined as that O_2 uptake resistant to 1 mM KCN and sensitive to 2 mM SHAM.

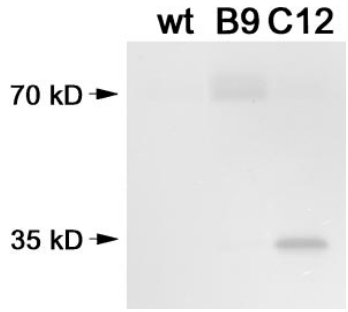

Figure 5. AOX Protein in Wild-Type (wt) Tobacco Suspension Cells and Transgenic Cells Overexpressing the Native AOX Protein (B9) or a Mutated AOX Protein Lacking Cys-126 (C12).

Mitochondrial protein (75 μg) was separated by nonreducing SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody raised to AOX. Numbers at left indicate apparent molecular mass. See Methods for an explanation of the naming of transformants.

METHODS

Site-Directed Mutagenesis

Standard recombinant DNA techniques were performed according to Sambrook et al. (1989). The cDNA clone pAONT1 (EcoRI fragment) contains the complete coding region of tobacco *Aox1* (1059 bp) along with an additional 64 nucleotides at the 5′ end and 273 nucleotides at the 3′ end (Vanlerberghe and McIntosh, 1994). This cDNA was subcloned into the M13 vector pUC119 (in *Escherichia coli* XL-1 Blue) and used to isolate single-stranded DNA template (Vieira and Messing, 1987) for mutagenesis.

Oligonucleotide-directed in vitro mutagenesis was performed according to the kit manufacturer's instructions (Amersham, Oakville, Canada). The oligonucleotide 5′-CATGGCCTAAAGGCATTCCATTTCC-3′ was used to generate mutations to change Cys-126 to Ala and to introduce the unique restriction site BsmI. The oligonucleotide 5′-AACATCATAGCTCTAGCACCATAATC-3′ was used to generate mutations to change Cys-176 to Ala and remove a BsrDI restriction site (silent mutation). Putative mutant clones generated separately by these oligonucleotides were subjected to restriction analysis (by using either BsmI or BsrDI), and clones verified to contain the correct restriction site mutations were then subjected to DNA sequence analysis (Vanlerberghe and McIntosh, 1994) to confirm the Cys mutations. Sequence analysis of the full length of the clones confirmed that all of the correct mutations were introduced and that no other unexpected mutations were present. These clones were named pAO126 (with the Cys-126 mutation) and pAO176 (with the Cys-176

mutation). Plasmid DNA from pAO126 and pAO176 was then cut with the restriction enzymes NsiI and BglII. The small NsiI-BglII fragment from pAO126 was then purified and ligated with the purified large NsiI-BglII fragment from pAO176. This clone (named pAO126176) was then confirmed by DNA sequence analysis to contain both of the Cys mutations (and the silent mutation) but no other changes.

Plant Transformation

Native or mutated *Aox1* cDNA constructs were ligated in the EcoRI site of the binary expression vector pGA748 (An et al., 1988) in *E. coli* MC1000. Restriction analysis (with BglII) was used to select clones with *Aox1* in sense orientation to the 35S promoter of cauliflower mosaic virus. These clones were used to transform *Agrobacterium tumefaciens* LBA4404 (carrying Ti plasmid pAL4404) by a direct DNA uptake method (An et al., 1988). Tobacco (*Nicotiana tabacum* cv Petit Havana SR1) was then transformed by the leaf disc method, and kanamycin-resistant plants were regenerated as described previously (Horsch et al., 1988). Primary transformants were propagated in GA-7 vessels (Magenta Corp., Chicago, IL) on a modified Murashige and Skoog medium (Horsch et al., 1988) supplemented with 100 μg mL^{-1} kanamycin and under 24 hr of fluorescent light.

Several classes of transformed plants were generated and named as follows: class A, transformed with the pGA748 vector containing no AOX insert; class B, overexpresses the native AOX protein; class C, overexpresses a mutated AOX in which Cys-126 is changed to an Ala; class D, overexpresses a mutated AOX in which Cys-176 is changed to an Ala; and class E, overexpresses a mutated AOX in which Cys-126 and Cys-176 are changed to Ala. Within each class, plants are also given a number to distinquish independent transformants (e.g., B1, B2, or B3).

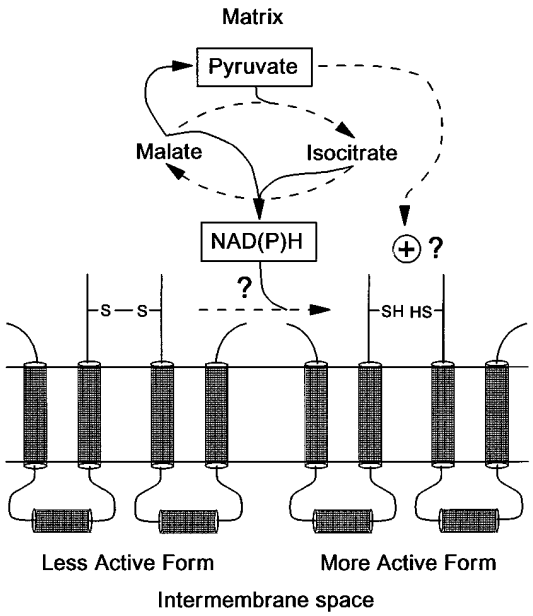

Figure 6. A Working Model of AOX Regulation in Tobacco Mitochondria.

The AOX dimer, when covalently linked by an intermolecular disulfide bond at Cys-126 (-S-S-), is a less active form of AOX; reduction of this regulatory disulfide to its component sulfhydryls (-SH HS-) produces the more active form. The active form is much more responsive to stimulation by pyruvate (+). Reduction of the Cys-126 regulatory disulfide occurs in response to the oxidation of isocitrate or malate by the TCA cycle and may be mediated by a thioredoxin system requiring NADPH. Pyruvate stimulation may involve the formation of a thiohemiacetal, but Cys-176 is not involved in this activation. The question marks indicate that the mechanism of stimulation of AOX by pyruvate and the mechanism of reduction of the AOX regulatory disulfide bond are not completely understood. The AOX protein features are not drawn to scale.

Isolation and Assay of Leaf Mitochondria

All analyses were done with primary transformants. For the analyses presented in Figure 1 only, Percoll gradient–purified mitochondria were isolated from 1 g fresh weight of leaf tissue by using a miniprep procedure (Boutry et al., 1984; Vanlerberghe et al., 1994). For all other analyses, mitochondria were isolated by adapting the method of Day et al. (1985). Leaves (20 to 40 g fresh weight) were thinly sliced, ground thoroughly with a mortar and pestle (in homogenization medium containing 0.3 M sucrose, 25 mM Tes, 2 mM EDTA, 10 mM KH_2PO_4, 1% [w/v] PVP-40, 1% [w/v] BSA, 20 mM ascorbic acid, and 4 mM Cys, pH 7.5), and filtered through four layers of Miracloth (Calbiochem, La Jolla, CA). Washed mitochondria were obtained from the homogenate, as described by Day et al. (1985), except that the wash medium consisted of 0.3 M sucrose, 10 mM Tes, and 0.1% (w/v) BSA, pH 7.2. Washed mitochondria were then purified on a PVP–Percoll gradient, as described by Day et al. (1985). In some cases, mitochondria were isolated in the presence of pyruvate or iodoacetate. In this case, fresh compounds were added to the homogenization, wash, and PVP–Percoll gradient media just before use. Mitochondrial protein was quantified by a modified Lowry assay (Larson et al., 1986).

Oxygen uptake by mitochondria (~0.2 to 0.25 mg of protein in a final volume of 1 mL) was measured in a Clark-type oxygen electrode cuvette (Hansatech Ltd., Norfolk, UK) at 25°C in a reaction medium containing 10 mM Tes, pH 7.2, 0.25 M sucrose, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0.1% (w/v) BSA, and 0.1 mM each of NAD, NADP, ATP, and thiamine pyrophosphate. Other additions were as described in the legends of Figures 2 to 4 and Table 2. Pyruvate, iodoacetate, and DTT were made fresh on the day of use. The O_2 concentration in air-saturated H_2O at 25°C was assumed to be 240 μM.

Protein Analysis of Leaf Mitochondria

For reducing SDS-PAGE, we added mitochondria (50 to 75 μg of protein) to sample buffer (2% [w/v] SDS, 2% [v/v] 2-mercaptoethanol, 10% [v/v] glycerol, and 42 mM Tris, pH 6.8) and boiled for 2 min before adding 0.08% (w/v) bromophenol blue tracking dye. Nonreducing SDS-PAGE analysis was performed by omitting the 2-mercaptoethanol from the sample buffer. SDS-PAGE analysis was performed with an SE 600 electrophoresis unit (Hoefer Pharmacia Biotech, San Francisco, CA) and the buffer system of Laemmli (1970). A 5% (w/v) polyacrylamide stacking gel and a 10 to 17.5% polyacrylamide gradient resolving gel were used. The resolved pro-

teins were then transferred to a nitrocellulose membrane (Schleicher and Schuell) by using a TE 50X electrotransfer unit (Hoefer Pharmacia Biotech) and a buffer consisting of 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. After transfer, the blot was washed twice for 15 min in PBS–Tween (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2, and 0.3% Tween 20) and allowed to dry.

For immunoblot analysis, the blot was incubated 1 hr in PBS–Tween containing a 1:200 dilution of a monoclonal antibody (AOA) raised against the *Sauromatum guttatum* AOX (Elthon et al., 1989). Then, after two washes for 5 min with PBS–Tween, the blot was incubated 1 hr in PBS–Tween containing a 1:2000 dilution of phosphatase-labeled affinity-purified antibody raised to mouse IgG (H+L; Kirkegaard and Perry Laboratories, Gaithersburg, MD). After washing twice for 5 min with PBS–Tween followed by two washes for 5 min with color buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, and 0.3% Tween 20), the blot was developed by reaction with color buffer supplemented with 0.4 mM *p*-nitro blue tetrazolium chloride and 0.4 mM 5-bromo-4-chloro-indolyl phosphate. The color development was stopped by two 5-min washes in PBS–Tween. Relative amounts of AOX protein on protein gel blots were quantified using a GS-700 scanning densitometer with Molecular Analyst software (Bio-Rad).

Transgenic Suspension Cells

Fresh leaf tissue from wild-type (nontransformed) and some primary transformants was placed on a modified Murashige and Skoog medium (Horsch et al., 1988) supplemented with 0.2 $\mu g/mL^{-1}$ naphthaleneacetic acid and, in the case of transgenic lines, 100 $\mu g/mL^{-1}$ kanamycin. The tissue was incubated in the dark (28°C) to generate callus tissue, which was then transferred to a previously described liquid medium (Linsmaier and Skoog, 1965) containing 3% (w/v) sucrose as a carbon source. After several months of growth in liquid medium, a fine suspension of heterotrophic cells was obtained. Cultures (200 mL) were then routinely grown in the dark on a rotary shaker (140 rpm at 28°C) and were subcultured every 7 days by 14-fold dilution of the cells in fresh medium. Transgenic lines were grown in medium supplemented with 75 $\mu g/mL^{-1}$ kanamycin. All experimental analyses of the cells occurred once they had been propagated in liquid culture for 9 months. Respiratory characteristics of suspension cells and isolation of washed mitochondria from suspension cells were performed as previously described (Vanlerberghe et al., 1995).

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