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Covalent and Noncovalent Dimers of the Cyanide-Resistant Alternative Oxidase Protein in Higher Plant Mitochondria and Their Relationship to Enzyme Activity[1]

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Evidence for a mixed population of covalently and noncovalently associated dimers of the cyanide-resistant alternative oxidase protein in plant mitochondria is presented. High molecular mass (oxidized) species of the alternative oxidase protein, having masses predicted for homodimers, appeared on immunoblots when the sulfhydryl reductant, dithiothreitol (DTT), was omitted from sodium dodecyl sulfate-polyacrylamide gel sample buffer. These oxidized species were observed in mitochondria from soybean (*Glycine max* [L.] Merr. cv Ransom), *Sauromatum guttatum* Schott, and mung bean (*Vigna radiata* [L.] R. Wilcz). Reduced species of the alternative oxidase were also present in the same mitochondrial samples. The reduced and oxidized species in isolated soybean cotyledon mitochondria could be interconverted by incubation with the sulfhydryl reagents DTT and azodicarboxylic acid bis(dimethylamide) (diamide). Treatment with chemical cross-linkers resulted in cross-linking of the reduced species, indicating a noncovalent dimeric association among the reduced alternative oxidase molecules. Alternative pathway activity of soybean mitochondria increased following reduction of the alternative oxidase protein with DTT and decreased following oxidation with diamide, indicating that electron flow through the alternative pathway is sensitive to the sulfhydryl/disulfide redox poise. In mitochondria from *S. guttatum* floral appendix tissue, the proportion of the reduced species increased as development progressed through thermogenesis.

A cyanide-resistant respiratory pathway is found in the inner membrane of mitochondria from many organisms, including a number of fungi, certain protozoa, and all higher plants thus far examined (Moore and Siedow, 1991). When this pathway is engaged, electrons diverge from the main, cyanide-sensitive electron transport pathway at the UQ pool and, instead of continuing to the terminal Cyt *c* oxidase, flow through an "alternative oxidase" that catalyzes the reduction of molecular O_2 to water (Moore and Siedow, 1991). Energy conservation (i.e. proton translocation) sites 2 and 3 are thus bypassed by the alternative oxidase (Moore and Siedow, 1991). The engagement of the alternative pathway is governed largely by the redox poise of the UQ pool, such that electron flow through the pathway only occurs when the UQ pool reduction state exceeds a certain threshold (Siedow and Moore, 1993), beyond which engagement of the pathway increases as a nonlinear function of the UQ pool reduction state (Siedow and Moore, 1993). The alternative respiratory pathway can be distinguished from the main pathway and other mitochondrial O_2-consuming processes by its sensitivity to specific inhibitors, such as salicylhydroxamic acid (Moore and Siedow, 1991).

A nuclear gene coding for an alternative oxidase protein has been identified (Rhoads and McIntosh, 1991), and expression of an alternative oxidase gene is sufficient to confer alternative pathway activity in *Escherichia coli* (Kumar and Söll, 1992). To date, alternative oxidase cDNAs from the aroid *Sauromatum guttatum* Schott (Rhoads and McIntosh, 1991), a yeast, *Hansenula anomala* (Sakajo et al., 1991), *Arabidopsis thaliana* (L.) Heyn. (Kumar and Söll, 1992), and *Glycine max* (L.) Merr. (Whelan et al., 1993) have been isolated and sequenced. Antibodies developed against the plant alternative oxidase protein (Elthon and McIntosh, 1987; Elthon et al., 1989) reveal that the protein can be present as more than one molecular mass species on SDS-PAGE gels. In soybean cotyledon mitochondria, two species (33–39 kD; Obenland et al., 1990; Kearns et al., 1992) are present. Several molecular mass species occur in *S. guttatum* floral appendix mitochondria as well. During the onset of appendix thermogenesis, a 37-kD species, the only species present early in appendix development, is joined by 35- and 36-kD species (Elthon and McIntosh, 1987; Rhoads and McIntosh, 1992).

Despite knowledge of the relationship of the alternative oxidase to the main mitochondrial respiratory pathway and the identification of both a gene and a protein associated with the alternative oxidase, the role of alternative respiration in plant metabolism and physiology is unclear. The oxidase has been postulated to provide an energy overflow pathway, allowing the tricarboxylic acid cycle to operate independently of ATP synthesis (Lambers, 1982). However, more conclusive evidence is needed to substantiate this theory. At the biochemical level, little is known about the structure of the alternative oxidase protein. The enzyme's reaction with molecular O_2 to produce water indicates that a metal cofactor must be present, but it remains unidentified (Moore and Siedow, 1991). Whether the alternative oxidase associates with other proteins in a multisubunit complex, as do other

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Abbreviations: diamide, azodicarboxylic acid bis(dimethylamide); EGS, ethylene glycolbis-(succinimidylsuccinate); UQ, ubiquinone; UQ_o, oxidized ubiquinone; UQ_r, reduced ubiquinone; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

mitochondrial electron transport proteins (Hatefi, 1985) is not known, and the modification(s) that give rise to the multiple molecular mass species seen on SDS-PAGE gels (Elthon and McIntosh, 1987; Obenland et al., 1990; Kearns et al., 1992) are uncharacterized. In addition to these protein structural features, any factors within the mitochondrion that might regulate interaction of the alternative oxidase with the main electron transport chain are unknown. Understanding the nature of both structure and regulation of the alternative oxidase will be necessary to clarify its function.

In this paper we present results demonstrating that the alternative oxidase protein in mitochondria is present in a dimeric state in the membrane and that a portion of the dimeric population is covalently linked through a disulfide bond. Furthermore, at a given UQ pool reduction state, the oxidized, covalently associated dimeric form of the alternative oxidase exhibits markedly lower activity relative to activity of the noncovalently associated form. Thus, the structure of the alternative oxidase, through the reversible nature of the intermolecular disulfide linkage, provides a means of regulating enzyme activity.

MATERIALS AND METHODS

Plant Material

Soybean seedlings (*Glycine max* [L.] Merr. cv Ransom) were grown in flats either on a soil mixture in a greenhouse with a natural photoperiod or, for root mitochondria, on sand: Perlite (1:1, v/v) in a growth chamber with a 14-h, 27°C light (700 μmol of photons m^{-2} s^{-1}) period and a 10-h, 23°C dark period. Soybean seeds were soaked for 10 min in a 10% Clorox solution, rinsed extensively, immersed in water, and bubbled with air for 2 h before planting. The day of imbibition and planting is designated day 0.

Etiolated mung bean (*Vigna radiata* [L.] R. Wilcz) hypocotyls were obtained from seeds treated similarly to soybean seeds and grown in the dark on vermiculite for 4 d at 20°C. *Sauromatum guttatum* Schott plants were maintained in a greenhouse with a natural photoperiod. Appendix tissue was harvested at different floral stages.

Isolation of Mitochondria

Isolation of soybean cotyledon or root mitochondria was adapted from the procedure of Day et al. (1985). Except for harvesting, all steps were at 4°C. Cotyledons or roots were harvested, weighed, rinsed with distilled water, and then cut into small pieces with scissors. Ice-cold grinding buffer (0.3 M Suc [0.4 M for roots], 25 mM tetrasodium PPi, 2 mM EDTA, 10 mM KH_2PO_4, 1% PVP-40 [w/v], 1% BSA [w/v], and 20 mM ascorbic acid [pH 7.5]) was added at a 2:1 ratio with respect to tissue weight. Tissue was ground for 20 s with a hand-held Braun homogenizer and filtered through four layers of cheesecloth. The filtrate was centrifuged for 5 min at 1000*g*, and the resulting supernatant was spun at 14,500*g* for 8 min. Pellets were resuspended to half the grinding buffer volume with wash buffer (0.3 M Suc, 10 mM Tes, and 0.1% [w/v] BSA [pH 7.5]) and centrifuged at 1000*g* for 5 min. The supernatant was spun at 17,000*g* for 20 min. Each pellet was thoroughly homogenized, resuspended in 5 mL of wash buffer, and layered onto a 35-mL solution consisting of 28% (v/v) Percoll, with a linear gradient of 0 to 4.4% PVP-25 (w/v) (Aldrich, Milwaukee, WI) in wash buffer. One gradient was needed for each 100 g of cotyledon tissue or 200 g of root tissue. The gradients were spun at 45,000*g* for 40 min. Subsequently, the mitochondrial layer near the bottom of the gradient was removed and diluted with a 10-fold volume of wash buffer. The mitochondria were spun at 27,000*g* for 15 min. This wash step was repeated two to three times until a compact mitochondrial pellet was obtained. Final resuspension was in 1 to 2 mL of wash buffer. Mung bean hypocotyl mitochondria were isolated and gradient was purified by the same method.

Washed mitochondria from *S. guttatum* floral appendix tissue were isolated according to the method of Berthold et al. (1988) except that the Suc density gradient step was omitted. Total mitochondrial protein was determined by the method of Lowry et al. (1951) with BSA as a standard.

SDS-PAGE and Immunoblotting

Standard and gradient SDS-PAGE gels were modified from the system of Laemmli (1970). The procedure described by Berthold and Siedow (1993) was followed, either with or without the inclusion of urea, except that 100 mM DTT was used as the sample buffer reductant. Urea gel samples were denatured without reductant for 30 min at room temperature, and DTT was added just before loading. For non-urea gels, samples were denatured in sample buffer with or without DTT by heating for 5 min at 90°C. High molecular mass standards from Sigma were used as molecular mass markers. Protein transfer from the gels to nitrocellulose and subsequent incubation with antibodies were as described by Harlow and Lane (1988). The monoclonal antibody AOA, developed against the alternative oxidase protein of *S. guttatum* (Elthon et al., 1989), was used at dilutions of either 1:800 (for *S. guttatum* blots) or 1:500 (for soybean and mung bean blots). Visualization was with the ECL chemiluminescent reagent system (Amersham, Arlington Heights, IL). For densitometry, films were scanned using the Stratagene Eagle Eye still video system. Subsequent analysis was with National Institutes of Health Image 1.45 software using 20-frame summing to reduce background.

Treatment of Mitochondria with DTT and Diamide

DTT, in mitochondrial wash buffer, was added to an aliquot of gradient-purified mitochondria to give a final concentration of 20 mM. An equivalent volume of wash buffer (without DTT) was added to a second and third aliquot. After the three samples were incubated for 30 min on ice, 0.4 M diamide, in DMSO, was added to the third aliquot to a final concentration of 3 mM. DMSO was added to the first (DTT treated) and second (DMSO control) aliquots to give the final DMSO concentration in the diamide-treated aliquot. Incubation on ice continued for 30 min more. The mitochondria were then spun down and washed twice in wash buffer at 2-fold the original aliquot volume. Final resuspension was in wash buffer. For incubations in which diamide treatment followed DTT treatment, DTT-treated mitochondria were

washed and then treated with diamide as described before. For DTT treatment following diamide treatment, 20 mM (final) DTT was added directly to diamide-treated mitochondria without washing. Incubation continued for 1 h, after which the mitochondria were washed and resuspended. Fresh DTT and diamide stocks were made on the day of each experiment.

Cross-Linking with SMCC and EGS

The cross-linking procedure was based on the method developed by Korth et al. (1991) for EGS. Freshly isolated or previously frozen mitochondria were treated with 20 mM DTT or kept as controls, as described before, except that no DMSO was added during the 1-h incubation. At the end of the treatment, the mitochondria were washed twice in 2-fold the initial volume with 0.3 M Suc, 0.1% (w/v) BSA, and 10 mM K phosphate (pH 7.5). Resuspension was in the same buffer to 1 mg mL^{-1} mitochondrial protein. For cross-linking, SMCC or EGS (Pierce, Rockford, IL) was added from a concentrated stock in DMSO to the washed, resuspended mitochondria to produce final concentrations of 0.5 and 0.25 mM (SMCC) or 1.0 mM (EGS). After the suspension was mixed at room temperature for 15 min, either 100 mM Gly (with EGS) or 100 mM Cys plus 100 mM Gly (with SMCC) was added at a final concentration of 15 mM each to stop the cross-linking reaction. Mixing continued for an additional 15 min. As controls, the stop solutions were added before EGS or SMCC, which completely prevented cross-linking.

Determination of UQ Pool Reduction State and Alternative Oxidase Activity

The reduction state of the UQ pool and the rate of O_2 consumption of isolated soybean cotyledon mitochondria were measured simultaneously using a combined O_2 and voltametric ("Q electrode") apparatus and method similar to that described by Dry et al. (1989). Control (DMSO treated), DTT-, or diamide-treated mitochondria (0.3–0.4 mg mL^{-1}) were incubated at 25°C in 2.2 mL of reaction medium (Siedow and Girvin, 1980), 0.15 mM ATP to activate succinate dehydrogenase, and 2 μM UQ-1 as the exogenous indicator to measure the mitochondrial UQ pool redox poise. Respiration was initiated by addition of 5 mM succinate. Two subsequent additions of 50 μM ADP resulted in two state 3/state 4 transitions. Myxothiazol (6 μM) was then added to inhibit the main respiratory pathway. After a steady rate of O_2 uptake by the alternative pathway was established, 1.0 mM NADH was added, and the new O_2 uptake rate was measured. For each mitochondrial treatment, the residual respiration rate was determined after NADH addition, with the electrode depolarized, in the presence of 2.0 mM salicylhydroxamic acid.

For calculations of UQ_r, the maximum mitochondrial UQ_o level was taken as the stable position of the electrode trace after mitochondria were added to the reaction mixture, but before substrate addition. The UQ_r for succinate-supported alternative oxidase activity was the trace deflection from UQ_o observed after myxothiazol addition, and UQ_r for NADH oxidation was the trace deflection from UQ_o at the time at which O_2 uptake was measured. Maximum mitochondrial levels of UQ_r, attained under anaerobic conditions, were found to be essentially the same among the treatments for a given mitochondrial isolation. Drift in the electrode signal during the course of the day was corrected for by monitoring changes with time in the trace deflection of a reduced UQ-1 standard. NADH alone caused some deflection of the electrode trace (Day et al., 1991), and this deflection was subtracted as background from UQ_r measurements. Residual O_2 uptake rates were subtracted from rates in the presence of myxothiazol to give net alternative pathway activity.

For measurement of alternative oxidase activity in *S. guttatum* mitochondria using duroquinol, the procedure described by Berthold and Siedow (1993) for *Symplocarpus foetidus* L. was followed.

Chemicals

Urea, bis-acrylamide, and ammonium persulfate were from Bio-Rad (Melville, NY). Duroquinol was from ICN Biomedicals (Irvine, CA). Except where noted, all other chemicals were from Sigma.

RESULTS

Presence of Disulfide-Linked Alternative Oxidase Species in Isolated Mitochondria

When isolated plant mitochondria were disrupted in SDS-PAGE sample buffer, from which reductant (100 mM DTT) was omitted, and subsequently immunoblotted using the AOA monoclonal antibody against the alternative oxidase protein (Elthon et al., 1989), bands were detected in the 60- to 75-kD range, in addition to the molecular mass species of 30 to 39 kD described previously (Fig. 1). The higher molecular mass species were detected in mitochondria from several sources, including *S. guttatum* floral appendices, soybean cotyledons and roots, and mung bean hypocotyls. Quantification by densitometry of the high and low molecular mass species showed an increase in the amount of the lower molecular mass species approximately equivalent to the decrease in the amount of higher molecular mass species upon addition of DTT (data not shown). Because their appearance was caused by lack of reductant in the gel sample buffer, the linkage creating these high molecular mass species is most probably a disulfide bond. For clarity, the disulfide-linked higher molecular mass species will be designated the "oxidized" species, and the lower molecular mass species will be designated the "reduced" species.

BSA, the 66-kD molecular mass marker, migrated above the oxidized alternative oxidase species when DTT was present in the sample buffer and below the oxidized species when DTT was omitted (Fig. 1). This is probably because of the presence of 17 internal disulfide bonds in the BSA molecule (Koch and Raleigh, 1991). However, migration of the other mass markers shown in Figure 1 did not differ with or without DTT, and alternative oxidase protein masses were calculated based on a regression using these markers.

The oxidized species are probably dimers of the reduced species, as shown by molecular mass calculations. For example, the mass of the single reduced species in mung bean

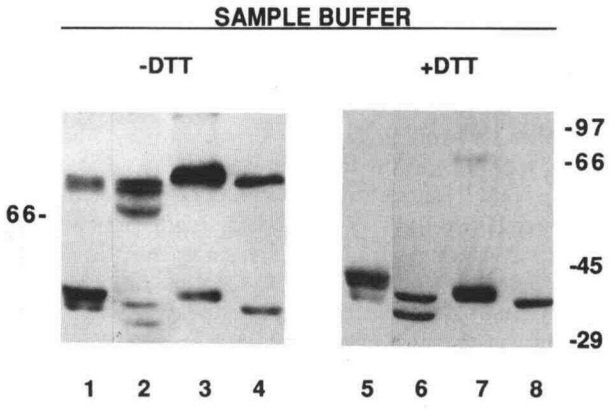

Figure 1. Presence of high molecular mass species of the alternative oxidase protein in isolated plant mitochondria. An immunoblot from a 10% SDS-PAGE gel probed for the alternative oxidase protein using the AOA monoclonal antibody (Elthon et al., 1989) is shown. Gel samples for lanes 1 through 4 were prepared without (−) DTT in the sample buffer; samples for lanes 5 through 8 were prepared in the presence (+) of 100 mM DTT. Protein was loaded: lanes 1 and 5, 9 μg; lanes 2, 3, 6, and 7, 25 μg; lanes 4 and 8, 50 μg. Mitochondria were isolated from a thermogenic *S. guttatum* floral appendix (lanes 1 and 5), 9-d-old soybean (*G. max*) cotyledons (lanes 2 and 6), 11-d-old soybean roots (lanes 3 and 7), and mung bean (*V. radiata*) hypocotyls (lanes 4 and 8). Molecular masses (kD) of markers prepared in the presence of DTT in the sample buffer are shown on the right, and the position of BSA prepared without DTT is shown on the left.

hypocotyls was 33 kD, and the oxidized species mass was 69 kD (Fig. 1, lanes 4 and 8). In soybean cotyledon mitochondria, two reduced species with masses of 30 and 34 kD were observed, along with three oxidized species of masses 60, 67, and 71 kD (Fig. 1, lanes 2 and 6). Recently, immunoblots of soybean cotyledon mitochondria have shown a third reduced species, intermediate in molecular mass between the 30- and 34-kD bands, along with the three oxidized species (data not shown). This species was only observed when DTT was omitted from the SDS-PAGE gel sample buffer. It has not been possible to determine whether this additional band's presence (or absence) results from a procedural artifact or is a band that was previously poorly resolved. Thus, the presence of the three high molecular mass oxidized species in soybean cotyledon mitochondria (Fig. 1, lane 2) can be explained by either the random association of two reduced species into the three possible oxidized dimeric species (30/30, 30/34, and 34/34 kD) or the self-association of each of the three reduced species.

Kumar and Davidson (1992) have recently shown that disulfide-linked dimers, particularly of metal-containing proteins, can arise as artifacts of sample preparation for SDS-PAGE in the absence of reducing agent when heat is used to denature the protein. Two criteria can help to establish whether or not the observed dimers are such artifacts. A true dimer will also be present in an unheated sample, and its presence will be independent of protein concentration (Kumar and Davidson, 1992). When soybean cotyledon mitochondria samples were prepared for a 10% SDS-PAGE gel with and without heating, the oxidized (dimeric) species of the alternative oxidase protein were present in comparable amounts in both unheated and heated samples (Fig. 2, lanes 2 and 3), indicating that the dimers were not artifacts due to heating. In addition, in samples in which total protein concentration ranged from 0.9 to 0.1 mg mL^{-1}, the ratio of the oxidized to reduced species, as determined by densitometry, either remained constant (root mitochondria, data not shown) or increased slightly (cotyledon mitochondria; Fig. 2, lanes 4–7). For a purified protein over a similar protein concentration range, artifactual disulfide cross-linking decreases markedly with decreasing sample protein concentration (Kumar and Davidson, 1992), with only monomers being present at low concentrations and dimers appearing only at high concentrations. Thus, the alternative oxidase oxidized species met the criteria for true dimers because they were not created by heating or high protein concentration.

Effect of DTT and Diamide on Intact Mitochondria

Given the effect of reductant on the alternative oxidase protein under the denaturing conditions of SDS-PAGE sample preparation, it was of interest to know whether the alternative oxidase protein disulfide bond could be reduced or formed in intact mitochondria. For these experiments, DTT as reductant, and diamide, a reagent that oxidizes thiols to disulfides (Zanotti et al., 1992), were used. When freshly isolated soybean cotyledon mitochondria were incubated

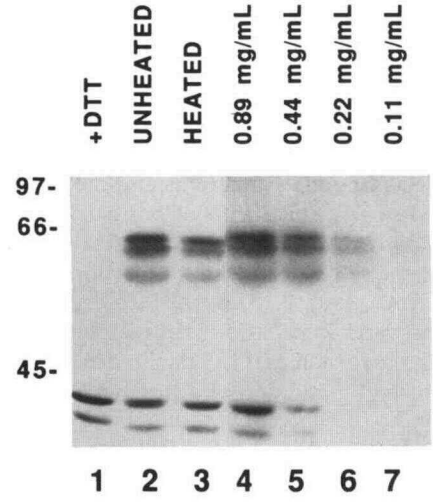

Figure 2. Effects of heating and protein concentration on the oxidized alternative oxidase protein species of 8-d-old soybean cotyledon mitochondria. An immunoblot from a 10% SDS-PAGE gel probed with the AOA monoclonal antibody is shown. All samples were prepared without DTT in the sample buffer except for the lane 1 sample and the molecular mass markers, which were prepared with 100 mM DTT. All samples were heated for 5 min at 90°C except for lane 2, which was held at room temperature. The amount of protein loaded for lanes 1 through 3 was 26 μg. For lanes 4 through 7, the final protein concentrations in the sample buffer were 0.89, 0.44, 0.22, and 0.11 mg mL^{-1}, respectively, and 40 μL was loaded onto each lane. Molecular masses (in kD) are indicated on the left.

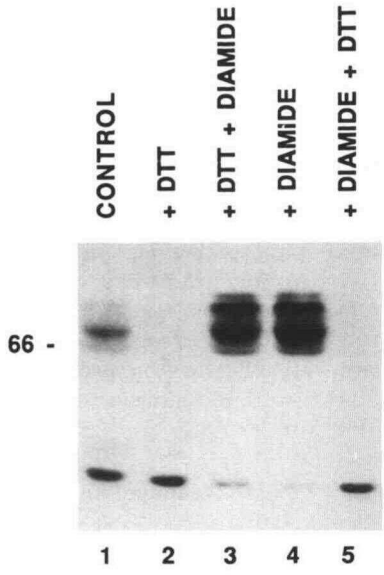

Figure 3. Interconversion of reduced and oxidized alternative oxidase protein species by DTT and diamide. Freshly isolated mitochondria from 8-d-old soybean cotyledons were treated on ice with 20 mM DTT, 3 mM diamide, or both reagents sequentially. An immunoblot from a 10% SDS-PAGE gel probed with the AOA monoclonal antibody is shown. All samples, including the molecular mass marker shown at left, were prepared without DTT in the sample buffer. Lanes 1 to 4 were loaded with 50 μg of protein, and lane 5 was loaded with 35 μg of protein. Mitochondrial incubations were with: lane 1, DMSO (control); lane 2, 20 mM DTT; lane 3, 20 mM DTT followed by 3 mM diamide; lane 4, 3 mM diamide; lane 5, 3 mM diamide followed by 20 mM DTT.

with 20 mM DTT on ice for 1 h, the oxidized species were almost completely converted to the reduced species (Fig. 3, lanes 1 and 2). A time course indicated that reduction of the oxidized species was essentially complete after a 30-min incubation with DTT (data not shown). Conversely, treatment of mitochondria with 3 mM diamide for 30 min on ice resulted in oxidation of almost all of the reduced species (Fig. 3, lanes 1 and 4). The effect of either DTT or diamide treatment could be reversed by subsequent incubation of the mitochondria with the other reagent (Fig. 3, lanes 3 and 5).

Chemical Cross-Linking of the Alternative Oxidase Protein

The relatively high reagent concentration used for oxidation of the alternative oxidase by diamide made it unclear whether the reduced species associated simply by random collisions of the monomers in the membrane or were actually stable associations of noncovalently linked dimers. To distinguish between these possibilities, the chemical cross-linker SMCC was used.

SMCC is a hydrophobic, heterobifunctional reagent with two reactive groups. One group reacts with Lys, and the other reacts with Cys. Thus, SMCC cross-links these two amino acids when they are in proximity (Yoshitake et al., 1979). Isolated soybean cotyledon mitochondria exhibiting a mixture of reduced and oxidized species were treated without (control) or with (DTT treated) 20 mM DTT to reduce the alternative oxidase protein and then were incubated with submillimolar concentrations of SMCC. For both control and DTT-treated mitochondria, cross-linking of the reduced species by SMCC occurred, with most of them being converted to covalently linked dimers by either 0.5 or 0.25 mM SMCC (Fig. 4A). Some molecular mass species heavier than dimers appeared, but only in limited amounts, following SMCC cross-linking (Fig. 4A). Experiments in which soybean root mitochondria were used also resulted in SMCC cross-linking of the reduced protein species (data not shown).

The cross-link formed by SMCC, unlike a disulfide bond, is not susceptible to reduction. Therefore, SMCC-linked dimers should be stable in the presence of gel sample buffer containing DTT. This was true for the DTT-treated mitochondria; only low amounts of the reduced species were present after incubation with SMCC when samples were prepared

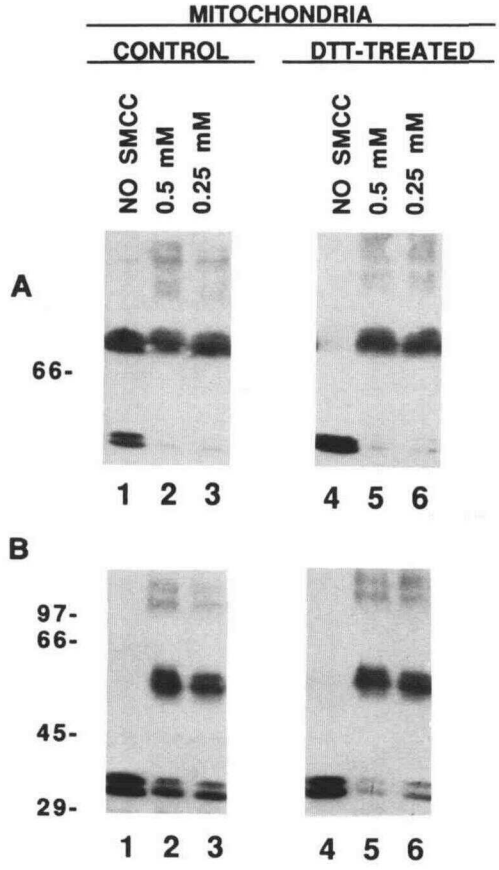

Figure 4. Chemical cross-linking of alternative oxidase protein reduced and oxidized species with SMCC. Mitochondria freshly isolated from 9-d-old soybean cotyledons, after treatment without (control) or with (DTT treated) 20 mM DTT for 1 h on ice, were cross-linked at the indicated SMCC concentrations. Immunoblots from 10% SDS-PAGE urea gels probed for the alternative oxidase protein using the AOA monoclonal antibody are shown. A, No DTT in the gel sample buffer; B, 100 mM DTT in the gel sample buffer. For A and B, Lanes 1 to 3, control mitochondria; lanes 4 to 6, 20 mM DTT-treated mitochondria; lanes 1 and 4, mitochondria incubated without SMCC; lanes 2, 3, 5, and 6, mitochondria incubated at the indicated SMCC concentrations. About 45 μg of protein were loaded in each lane. Molecular masses (in kD) appear on the left.

with 100 mM DTT in the gel sample buffer (Fig. 4B, lanes 5 and 6). However, for control mitochondria, treatment of gel samples with 100 mM DTT revealed a substantial proportion of reduced species on the immunoblot, uncross-linked by SMCC (compare lanes 2 or 3 of Fig. 4, A and B). These results are consistent with SMCC reacting with one of the sulfhydryl groups that participates in the native disulfide cross-linking. Thus, for control mitochondria, SMCC apparently only cross-links the reduced species and not the oxidized, disulfide-linked species, creating a mixed population of SMCC and disulfide cross-linked dimers. The latter are then reduced to monomers in the presence of DTT in the gel sample buffer.

With DTT-treated mitochondria, virtually all of the protein is present as the reduced, low molecular mass species and, hence, is cross-linked by SMCC and stable in the presence of DTT in the sample buffer. When the hydrophobic, homobifunctional, Lys-specific, cross-linker EGS (Abdella et al., 1979) was used, cross-linking of the reduced species of both control and DTT-treated mitochondria occurred as with SMCC (Fig. 5). However, in contrast to the results with SMCC, all EGS cross-linked dimers in both control and DTT-treated mitochondria were stable in the presence of DTT in gel sample buffer (Fig. 5). Unlike SMCC, EGS reacts only with Lys moieties, which are not involved in disulfide bond formation and, therefore, EGS is able to covalently cross-link the oxidized species.

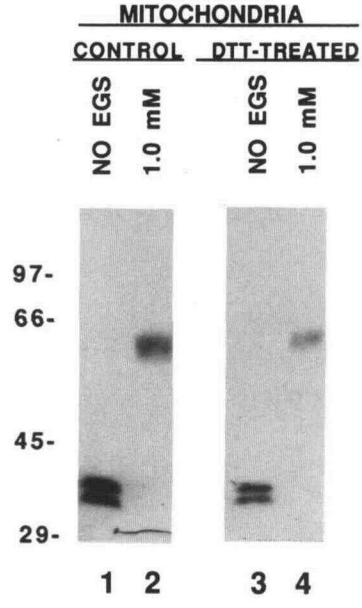

Figure 5. Chemical cross-linking of alternative oxidase protein species with EGS. Mitochondria isolated from 8-d-old soybean cotyledons, after treatment without (control) or with (DTT treated) 20 mM DTT for 1 h on ice, were cross-linked with 1.0 mM EGS. An immunoblot from a 10% SDS-PAGE urea gel probed for the alternative oxidase using the AOA monoclonal antibody is shown. For all samples, 100 mM DTT was present in the gel sample buffer. Lanes 1 and 2, Control mitochondria; lanes 3 and 4, 20 mM DTT-treated mitochondria; lanes 1 and 3, mitochondria incubated without EGS; lanes 2 and 4, mitochondria incubated with 1.0 mM EGS. About 40 μg of protein were loaded per lane. Molecular masses (in kD) appear on the left.

The presence of only two dimeric molecular mass species in SMCC cross-linked samples when DTT was included in the sample buffer was an unexpected result (Fig. 4B). Three dimer species were consistently observed in soybean cotyledon mitochondria when DTT was omitted from the sample buffer in untreated mitochondria (e.g. Fig. 1, lane 2) and in SMCC cross-linked mitochondria (Fig. 4A, lanes 2, 3, 5, and 6). Apparently, some combined effect of SMCC and DTT is responsible for modifying dimer migration or modifying the epitope involved in antibody recognition, resulting in the two-band pattern seen in Figure 4B. The dimeric mass bands appearing with EGS were too indistinct to determine their exact number (Fig. 5).

Effect of DTT and Diamide Treatment on Mitochondrial Alternative Pathway Activity

The presence of both covalently and noncovalently associated dimers of the alternative oxidase protein in native mitochondria suggested a possible role for the disulfide linkage in regulation of alternative oxidase activity (Ziegler, 1985; Buchanan, 1991). Initially, this possibility was examined by measuring O_2 uptake due to alternative oxidase activity in mitochondria treated with 20 mM DTT or 3 mM diamide. Although DTT treatment stimulated and diamide treatment inhibited alternative oxidase activity, these reagents also affected other components of the respiratory pathway. In particular, succinate dehydrogenase and NADH dehydrogenase activities were variably stimulated by DTT and inhibited by diamide (data not shown). Thus, it could not be determined whether DTT or diamide had a direct effect on the alternative oxidase or acted indirectly by regulating flow of reductant to the oxidase.

Use of the voltametric measurement of the UQ redox state in conjunction with measuring O_2 uptake permitted the level of the immediate electron donor to the alternative oxidase, UQ_r, to be determined and the enzyme activity at that UQ_r level to be measured. Variation in the activities of the complexes that donate electrons to UQ will not confound measurements of alternative oxidase activity when UQ_r is measured directly because of the dependence of alternative oxidase activity upon the reduction state of the UQ pool (Siedow and Moore, 1993). Succinate-supported alternative oxidase activity of control and DTT- and diamide-treated soybean cotyledon mitochondria was measured after two state 3/state 4 transitions and subsequent inhibition of the main Cyt oxidase pathway. This was followed by the addition of NADH. Figure 6 shows that levels of UQ pool reduction by each substrate were similar for the different mitochondrial treatments. Nevertheless, marked differences (statistically significant at $\alpha = 0.05$) occurred in alternative oxidase activity among the treatments. Diamide treatment depressed the alternative oxidase activity, whereas DTT treatment increased activity with respect to control mitochondria (Fig. 6). When mitochondria treated with diamide were subsequently treated with DTT, the alternative oxidase activity was restored and exceeded control rates similar to the effect of DTT alone (Fig. 6). A statistical analysis limited to data from the two isolations

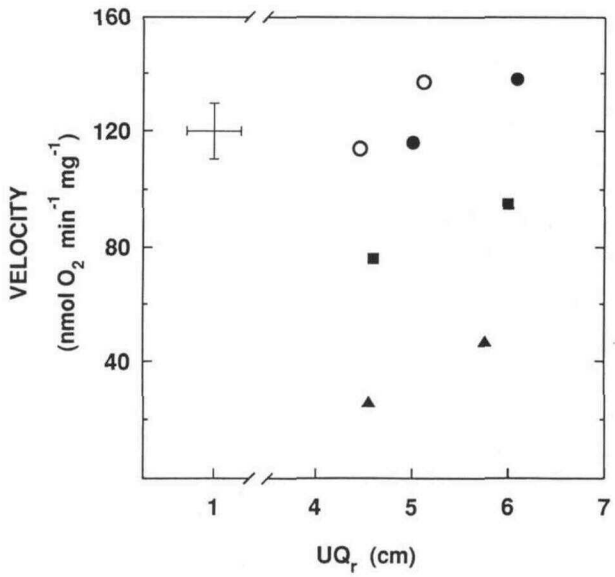

Figure 6. Effect of DTT and diamide treatment on the relationship of alternative pathway activity to the level of UQ pool reduction. Freshly isolated 7- or 8-d-old soybean cotyledon mitochondria were treated with DTT, diamide, or DMSO (control). Alternative pathway activity was measured as O_2 uptake in the presence of 6 μM myxothiazol while the reduction state of the UQ pool was monitored. For each replicate, two levels of UQ pool reduction were achieved by the sequential addition of succinate and NADH. UQ_r is represented by the deflection, in cm, of the electrode trace following each substrate addition. Solid symbols are averages of three separate mitochondrial isolations, one with two replicates per sulfhydryl reagent treatment and the other two with three replicates per treatment. The open symbols are averages from the latter two isolations, with two and three replicates, respectively. Bars at upper left show the minimum separation between solid symbols of different sulfhydryl reagent treatments required for statistical significance at $\alpha = 0.05$. Minimum separation was calculated from a two-tailed *t* test using a pooled estimate of variance (with 23 degrees of freedom) based on an analysis of variance using all data and accounting for the split-unit statistical design (Snedecor and Cochran, 1989). ■, DMSO control; ●, 20 mM DTT; ▲, 3 mM diamide; ○, 3 mM diamide followed by 20 mM DTT.

in which this treatment was performed showed the activities to be significantly different from the control but not significantly different from the DTT treatment.

Immunoblots of control and treated mitochondria confirmed the expected changes in the alternative oxidase protein species. Control mitochondria contained the typical mixture of reduced and oxidized species. Mitochondria treated with DTT alone or treated with diamide followed by DTT contained primarily reduced alternative oxidase species, whereas in diamide-treated mitochondria the alternative oxidase was largely in the oxidized state (e.g. Fig. 3 and data not shown).

Variation in Proportion of Oxidized and Reduced Species with *S. guttatum* Appendix Development

The alternative oxidase protein associated with floral appendix mitochondria of *S. guttatum* undergoes changes in both its amount and the number of low molecular mass species present as the appendix proceeds through the developmental stages up to and including thermogenesis (Elthon and McIntosh, 1987; Rhoads and McIntosh, 1992). As illustrated in Figure 7, the *S. guttatum* appendix alternative oxidase also shows a shift during development in the relative proportions of the oxidized and reduced species present, with the oxidized species being relatively more abundant before thermogenesis and the reduced species being predominant during and following thermogenesis. Alternative oxidase activity showed some correspondence with this change in proportion of oxidized and reduced species (Fig. 7 and data not shown). However, activity levels also corresponded with the total amount of alternative oxidase protein present and appearance of the two additional lower molecular mass forms (which do not resolve well in *S. guttatum* when DTT is omitted from the gel sample buffer).

DISCUSSION

The results described in this paper are the first to indicate that higher plant mitochondria contain disulfide-linked (oxidized) dimers of the alternative oxidase protein. Some dimeric molecular mass species had been observed on immunoblots in an *S. foetidus* enzyme preparation (Berthold and Siedow, 1993), but these were thought to be nonspecific aggregates caused by the hydrophobicity of this integral membrane protein. An alternative explanation for the higher molecular mass species, the linkage of a distinct, nonantigenic protein

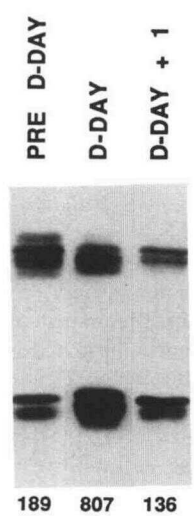

Figure 7. Variation in the proportion of reduced and oxidized species of the alternative oxidase protein during different developmental stages in *S. guttatum* appendix mitochondria. Samples were prepared without DTT in the sample buffer, run on an SDS-PAGE 10 to 17% gradient gel, and immunoblotted using the AOA monoclonal antibody. Mitochondria were isolated from appendices harvested before the day of floral opening and thermogenesis (pre D-day), during thermogenesis (D-day), and the day after thermogenesis (D-day + 1). Representative samples are shown. Each lane contains 10 μg of mitochondrial protein from a different appendix. The numbers below each lane are the corresponding alternative oxidase activities (nmol O_2 min^{-1} mg^{-1} protein) measured with 0.5 mM duroquinol as substrate.

to the individual reduced species, cannot be ruled out, but it seems unlikely. The nonantigenic protein would need to have the same molecular mass as the reduced alternative oxidase protein species in mitochondria having single reduced and oxidized species (e.g. mung bean hypocotyl, soybean roots) to create a "dimer" molecular mass. In mitochondria with multiple reduced and oxidized forms (e.g. soybean cotyledons, *S. guttatum* appendix), it would be necessary not only for the nonantigenic protein to have a mass similar to the reduced species but for its mass to vary to the same extent as those of the reduced species to create oxidized species of the observed molecular masses. Thus, dimerization of the reduced alternative oxidase species is the most parsimonious explanation for the observed oxidized forms.

Reduced and oxidized alternative oxidase species exist simultaneously in mitochondria from *S. guttatum* appendices, soybean cotyledons and roots, and mung bean hypocotyls (Fig. 1). In isolated soybean mitochondria, these reduced and oxidized species can be interconverted by diamide and DTT, demonstrating the characteristic reversibility of disulfide bonds (Fig. 3). In the presence of the chemical cross-linkers EGS and SMCC, the reduced species become covalently coupled dimers (Figs. 4 and 5). These data indicate that most, if not all, of the alternative oxidase protein molecules are present in the mitochondrial inner membrane as dimers, some disulfide cross-linked and the rest noncovalently associated. The noncovalent association is a stable one that persists through freezing and thawing of the mitochondria (data not shown).

Although molecular mass species larger than dimers were evident following addition of the chemical cross-linkers (Fig. 4), their limited amounts and indistinct band patterns suggest that these were nonspecific associations and that the alternative oxidase dimer is not closely associated with other proteins in the mitochondrial membrane. This is consistent with the recent study by Kumar and Söll (1992), in which expression of the *Arabidopsis thaliana* alternative oxidase gene alone in *Escherichia coli* was sufficient to support bacterial growth in the presence of cyanide.

The amino acid sequences deduced from the alternative oxidase cDNA clones for the mature protein (i.e. without the transit sequence) of *S. guttatum* (Rhoads and McIntosh, 1991), *A. thaliana* (Kumar and Söll, 1992), and *Glycine max* (Whelan et al., 1993) contain only three Cys residues each, providing a limited number of candidates for the residue(s) involved in the disulfide linkage(s). Among the three plant mature sequences, only two Cys residues are conserved, corresponding to residues 122 and 172 in the *S. guttatum* sequence, 78 and 128 in *A. thaliana*, and 94 and 144 in *G. max*. A third Cys is found at positions 150 in *A. thaliana* and 166 in *G. max*, respectively, but not in *S. guttatum*. The derived amino acid sequence for the yeast (*Hansenula anomala*) alternative oxidase cDNA (Sakajo et al., 1991) has four Cys residues, none of which are conserved precisely relative to the plant amino acid sequence. However, one Cys appears at position 138, placing it just two residues away from the conserved *S. guttatum* Cys-172 when the sequences are aligned by homology. Just downstream of this location, the yeast sequence becomes increasingly homologous with the sequence of the higher plants (Sakajo et al., 1993). Hydropathy plots of the alternative oxidase protein sequences indicate the start of the first of two putative membrane-spanning helical regions just after the conserved Cys-172 position (Moore and Siedow, 1991; Rhoads and McIntosh, 1991; Sakajo et al., 1991). If Cys-172 were involved in the disulfide linkage, it might help to stabilize a helix-helix interaction between adjacent monomers.

Experimental manipulation of the redox state of the alternative oxidase disulfide bond had a marked effect on soybean alternative oxidase activity. With the bond fully reduced, enzyme activity at a given level of UQ_r was greater than that of control mitochondria that contained a mixture of oxidized and reduced alternative oxidase species. Conversely, with the bond fully oxidized, enzyme activity was considerably below control levels (Fig. 6). This effect of the redox state of the disulfide bond on alternative oxidase activity could only be reliably observed at the level of the UQ pool because DTT and diamide also variably affected succinate dehydrogenase and NADH dehydrogenase activities. Duroquinol, as a respiratory substrate, would have bypassed these complexes, but it is a poor electron donor for soybean mitochondria (Day et al., 1991) and was not used for this reason.

An example in which alternative oxidase activity increases at a given UQ_r level is found in the maturation of the *Arum maculatum* spadix. Mature spadix mitochondria exhibit relatively higher alternative oxidase activity at a given level of UQ_r than do immature spadix mitochondria (Siedow and Moore, 1993). *S. guttatum* is an aroid relative of *A. maculatum*. During maturation of its spadix, the alternative oxidase undergoes a change from having predominantly oxidized to predominantly reduced species (Fig. 7). Preliminary data suggest that diamide treatment decreases alternative oxidase activity in thermogenic *S. guttatum* mitochondria, whereas DTT has little effect on activity, as expected because the alternative oxidase protein is in largely reduced form. Thus, the activity of the *S. guttatum* enzyme, and perhaps of the *A. maculatum* enzyme as well, appears to be sensitive to the redox state of the disulfide bond.

When considered together, all of these data, including the changes in soybean alternative oxidase activity with disulfide bond oxidation and reduction, the developmental change in the oxidized and reduced species seen in *S. guttatum*, and the developmental change in activity observed for *A. maculatum*, suggest that the conversion of oxidized to reduced enzyme species may help to regulate the activity of the alternative oxidase in situ.

No mechanism is currently known that could drive the oxidation and reduction of the alternative oxidase disulfide bond in plant mitochondria. However, the presence of thioredoxin in both plant and animal mitochondria has recently been established (Bodenstein-Lang et al., 1989; Marcus et al., 1991). This small protein catalyzes the reduction of protein disulfide bonds, which frequently results in enzyme activation (Holmgren, 1985; Buchanan, 1991). Although no thioredoxin-activated enzyme has been identified in plant mitochondria, the F_0F_1 ATPase of mammalian mitochondria contains sulfhydryl residues whose reduction state is critical to the activity of the enzyme (Yagi and Hatefi, 1984; Zanotti et al., 1992). The alternative oxidase could be among the enzymes susceptible to regulation by plant mitochondrial

thioredoxin. The relative behavior of the alternative oxidase under conditions of disulfide bond oxidation or reduction is consistent with activation by a thioredoxin-like system and with the proposed role of the alternative oxidase as an energy overflow pathway. Under conditions in which "overflow" might be desirable, limiting levels of ADP would lead to a buildup of reductant within the mitochondrial matrix, and the thioredoxin system could be activated to reduce the disulfides, thereby promoting the activity of the alternative oxidase and permitting the overflow pathway to operate to a greater extent. Under conditions in which ADP is not limiting, oxidation would be favored and intrinsic activity of the alternative oxidase would decrease.

How the disulfide bond affects enzyme activity can only be surmised at this point. Radiation inactivation analysis of the alternative oxidase in *S. guttatum* and *S. foetidus* mitochondria indicates that the functional unit of the enzyme has a molecular mass equivalent to the monomer (Berthold et al., 1988). Thus, the disulfide bond could decrease enzyme activity through simple stearic hindrance of active sites on the monomer subunits or possibly by affecting cofactor binding to the monomers.

In addition to previously reported changes in the amount of alternative oxidase protein (Elthon and McIntosh, 1987; Obenland et al., 1990; Stewart et al., 1990; Vanlerberghe and McIntosh, 1992) and its molecular mass forms (Elthon and McIntosh, 1987; Obenland et al., 1990; Rhoads and McIntosh, 1992), the dimeric protein structure with its reversible disulfide bonds provides another feature of the enzyme that must be reckoned with when investigating mechanisms for regulation of the alternative pathway during respiration.

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