

# Plant Mitochondrial Electron Transfer and Molecular Biology

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## INTRODUCTION

Aerobic respiration, a process performed by all plants, involves the controlled oxidation of metabolites containing reduced carbon to produce carbon dioxide and water as the final products (Taiz and Zeiger, 1991). Several types of reduced carbon compounds, including fatty acids, organic acids, and amino acids, can serve as the primary reducing substrates for plant respiration. However, the most common substrate used by plant tissues for respiration is carbohydrate (CH<sub>2</sub>O). The complete oxidation of a carbohydrate releases a large amount of free energy, much of which is coupled to the conversion of ADP and Pi to ATP. When sucrose (C<sub>12</sub>H<sub>22</sub>O<sub>11</sub>) is the substrate, aerobic respiration can be divided into three distinct phases: glycolysis, the tricarboxylic acid (TCA) cycle, and the coupled processes of mitochondrial electron transfer and ATP synthesis. Except for glycolysis, all of the metabolic steps of aerobic respiration take place in the mitochondrion. Like mitochondria found in other eukaryotes, plant mitochondria are roughly spherical subcellular organelles that usually range from 1 to 2 μm in diameter. They are delimited by a mitochondrial envelope that consists of two phospholipid bilayers, known as the outer and inner mitochondrial membranes (Douce, 1985). The outer membrane is permeable to molecules of 3 kD or smaller (Mannella, 1985), whereas the inner membrane represents the primary physical barrier to the uptake of small molecules and ions by the organelle (Douce, 1985). The presence of two membranes separates the mitochondria into four metabolic compartments: the outer membrane, the volume between the two membranes (intermembrane space), the inner membrane, and the volume within the inner membrane, known as the mitochondrial matrix.

Glycolysis involves a series of soluble enzymes located in the cytosol that bring about the conversion of sucrose to four molecules of pyruvate and the net production of four molecules each of ATP and NADH. The pyruvate generated during glycolysis is transported from the cytosol into the mitochondria, where the enzymes of the TCA cycle oxidize it to three molecules of CO<sub>2</sub>. For each molecule of sucrose oxidized by glycolysis and the TCA cycle, 12 molecules of CO<sub>2</sub>, 20 molecules of NADH (16 in the mitochondria and four in the cytosol), and four molecules of FADH<sub>2</sub> are produced. To allow continued operation of the two respiratory pathways, these two reduced coenzymes need to be reoxidized to NAD<sup>+</sup> and FAD, respectively. This is accomplished by the mitochondrial

electron transfer chain (Figure 1), which oxidizes the reduced coenzymes and transfers the resulting electrons to oxygen, producing water as the final product.

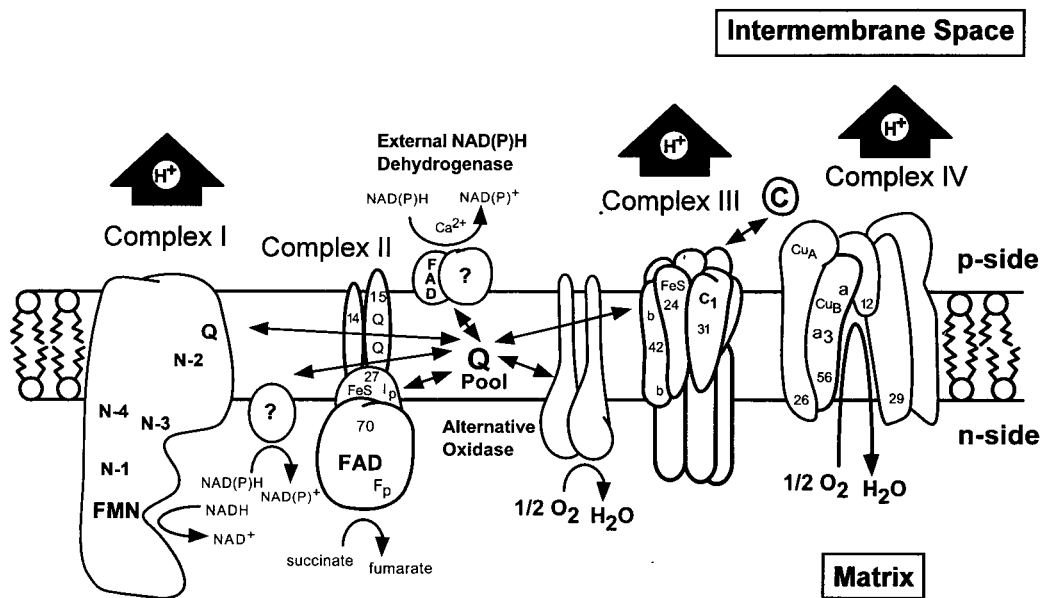
For each molecule of oxygen reduced to water by the oxidation of two molecules of NADH, 435 kJ/mol is released. Roughly half of this free energy is lost as heat, but the remainder is coupled to the translocation of protons across the inner mitochondrial membrane to give rise to an electrochemical proton gradient, commonly referred to as a proton motive force (Nicholls and Ferguson, 1992; Whitehouse and Moore, 1995). This translocation moves protons from the mitochondrial matrix into the intermembrane space, leaving the side of the inner membrane facing the intermembrane space positively charged (p-side) relative to the side facing the mitochondrial matrix (n-side). This nomenclature conforms to that applied to all membranes that participate in bioenergetic processes (Cramer and Knaff, 1990) and should replace the terms c-side and m-side when referring to the two surfaces of the inner mitochondrial membrane. The free energy stored in the proton motive force is subsequently used to drive the synthesis of ATP from ADP and Pi. This reaction is catalyzed by the proton-translocating F<sub>0</sub>F<sub>1</sub>-ATP synthase (Nicholls and Ferguson, 1992; Whitehouse and Moore, 1995). The combined oxidation of the reduced coenzymes by the electron transfer chain and the synthesis of ATP is referred to as oxidative phosphorylation (Nicholls and Ferguson, 1992).

Aerobic respiration serves as the primary source of the ATP used in energy-requiring reactions that take place during the growth and development of plants. In addition, intermediate compounds produced during the operation of glycolysis and the TCA cycle are the primary precursors for a variety of biosynthetic pathways in plants, including those that lead to fats, terpenoids, porphyrins, nucleotides, and amino acids (Taiz and Zeiger, 1991). Although much progress has been made in recent years in understanding the details of many aspects of respiration in plants, in this review we focus on advances directly associated with the plant mitochondrial electron transfer chain, particularly the cyanide-resistant oxidase, and on characterization of the molecular genetics of plant mitochondria.

## PLANT MITOCHONDRIAL ELECTRON TRANSFER CHAIN

The primary features of the electron transfer chain found in plant mitochondria are similar to those of the electron transfer

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**Figure 1.** Organization of the Plant Mitochondrial Electron Transfer Chain in the Inner Mitochondrial Membrane.

Electron transfer complexes I through IV, the two additional NAD(P)H dehydrogenases, and the alternative oxidase are shown in diagrammatic fashion, incorporating what is currently known about their topological orientation in the membrane, some indication of the primary cofactors known to be present (for details, see Nicholls and Ferguson, 1992, pp. 107–135), and the molecular masses of several of the major polypeptides. The Q Pool refers to the large pool of ubiquinone that freely diffuses within the inner membrane and serves as the electron carrier that transfers electrons derived from the four dehydrogenases to either complex III or the alternative oxidase. The bold upward-facing arrows designate the three complexes at which proton translocation takes place, leading to generation of the proton motive force that drives ATP synthesis. See the text for additional details.

chain in mitochondria isolated from other eukaryotes, consisting of four integral multiprotein complexes (complexes I through IV; Figure 1) (Nicholls and Ferguson, 1992; Trumpower and Gennis, 1994). Complex I (NADH:ubiquinone oxidoreductase) is an NADH dehydrogenase that oxidizes the NADH generated in the mitochondrial matrix by the TCA cycle and transfers the resulting electrons to ubiquinone. Complex II (succinate:ubiquinone oxidoreductase) contains the only enzyme of the TCA cycle (succinate dehydrogenase) that is not freely soluble in the matrix; this complex catalyzes the oxidation of succinate to fumarate during the operation of the TCA cycle, transferring the resulting electrons to ubiquinone. Ubiquinone, which is a fully substituted *p*-benzoquinone species having a prenyl side chain consisting of 45 (UQ-9) or 50 (UQ-10) carbon atoms, exists primarily as a free pool within the hydrophobic phase of the inner mitochondrial membrane (Cramer and Knaff, 1990; Siedow, 1995). Complex III (ubiquinone:cytochrome *c* oxidoreductase) oxidizes the ubiquinone reduced by complexes I and II and transfers the resulting electrons to cytochrome *c*. Cytochrome *c* is a small (12.5 kD) peripheral membrane protein located on the p-side of the inner membrane and is the only protein of the electron transfer chain that is not part of an integral membrane protein complex. Reduced cytochrome *c* is oxidized by complex IV (cytochrome *c* oxidase), the terminal electron transfer complex in the series.

During electron transfer, there are three sites of energy conservation, at complexes I, III, and IV, at which protons are translocated across the inner membrane to generate the proton motive force that drives ATP synthesis. The F<sub>0</sub>F<sub>1</sub>-ATP synthase is not part of the electron transfer chain per se, but its association with the inner membrane and its primary role in oxidative phosphorylation have led to its being referred to as complex V (Hatefi, 1985). The F<sub>0</sub> component of the ATP synthase is an integral multiprotein complex located in the inner membrane that provides a hydrophilic channel for the movement of protons back across the inner membrane. The F<sub>1</sub> component is a peripheral multiprotein complex that is bound directly to the F<sub>0</sub> complex and is located on the n-side of the inner membrane. The F<sub>1</sub> complex contains the catalytic site for the conversion of ADP and Pi to ATP, which therefore takes place in the mitochondrial matrix.

One aspect of the standard electron transfer chain in which plant mitochondria appear to differ from those of other eukaryotes involves complex III. In addition to the standard catalytic subunits (cyt *b*, 42 kD; Rieske Fe/S protein, 24 kD; cyt *c*<sub>1</sub>, 31 kD; Figure 1), purified complex III from potato has three proteins of 55, 53, and 51 kD, which make up the high-molecular-weight "core proteins" of complex III. This differs from the two core proteins (50 and 45 kD) seen in complex III in nonplant sources (Braun and Schmitz, 1992; Braun et al., 1993). Furthermore,

the protease activity that cleaves the transit peptides of imported mitochondrial proteins cofractionates with plant complex III as a membrane-bound activity; in contrast, the processing proteases of animal and fungal mitochondria are soluble in the matrix (Braun et al., 1992; Eriksson and Glaser, 1992). The amino acid sequences derived from cDNAs for the core proteins of potato complex III indicate that subunits I and II (55 and 53 kD) are 40 to 50% similar to the  $\beta$ -mitochondrial processing protease ( $\beta$ -MPP) component from *Neurospora* (Emmermann et al., 1993), whereas subunit III shows considerable similarity with the  $\alpha$ -MPP component from yeast (Braun et al., 1992). The observed cross-reactivity of antibodies against the two fungal matrix processing proteases with all three plant mitochondrial core proteins further supports these sequence comparisons (Braun et al., 1993). These results indicate a unique role for the plant mitochondrial complex III core proteins that appears to differ from the as-yet-undefined role played by the complex III core proteins in mitochondria from other organisms.

Plant mitochondria are also unique in having several electron transfer components in the inner membrane that are not found in mitochondria from most other organisms (Figure 1; Douce and Neuburger, 1989; Siedow, 1995). In addition to complex I, most plant mitochondria contain two other NAD(P)H dehydrogenases: one facing the n-side of the inner mitochondrial membrane, and the other facing the p-side of the membrane (Møller and Lin, 1986; Douce and Neuburger, 1989). The additional NAD(P)H dehydrogenase facing the matrix can, in theory, compete with complex I for oxidizing the NADH generated during the operation of the TCA cycle. However, recent evidence suggests that this enzyme more probably acts as an NADPH dehydrogenase in situ (Rasmussen and Møller, 1991; Møller et al., 1993). Although pathways for the formation and utilization of NADPH in plant mitochondria are known (Rasmussen and Møller, 1990), their exact role in metabolism is not well understood at present. The NAD(P)H dehydrogenase facing the p-side of the inner membrane is known as the external NAD(P)H dehydrogenase (Møller and Lin, 1986). It can oxidize both NADH and NADPH generated in the cytosol, given the permeability of the outer mitochondrial membrane to molecules up to 3 kD in size (Douce, 1985; Mannella, 1985).

Both of these dehydrogenases donate electrons directly to the ubiquinone pool. However, neither catalyzes the translocation of protons across the inner mitochondrial membrane, so the free energy released during electron transfer between NAD(P)H and ubiquinone (73 kJ/2e<sup>-</sup>) through the activity of either dehydrogenase is lost as heat and not used for ATP synthesis. The biochemical characterization of these plant-specific dehydrogenase activities is now in progress (Siedow, 1995); considerable insight into the role of these two activities in plant mitochondrial bioenergetics would ultimately accrue from the isolation of a cDNA associated with one or the other activity. One of the more intriguing and unexplained aspects of these plant-specific NAD(P)H dehydrogenases is what functional advantage plants derive from having pathways that appear to

waste the free energy released during aerobic respiration rather than conserving it for the synthesis of ATP.

## THE CYANIDE-RESISTANT RESPIRATORY PATHWAY

As well as having additional pathways for the oxidation of NAD(P)H, plant mitochondria also contain a pathway for the transfer of electrons from reduced ubiquinone to oxygen that bypasses cytochrome *c* oxidase. This "alternative" pathway is associated with the presence of an oxidase found in the inner membrane of mitochondria from all higher plants, many algae and fungi, and certain protozoa (Henry and Nyns, 1975; Siedow, 1982; Lance et al., 1985; Siedow and Berthold, 1986; Moore and Siedow, 1991). The alternative oxidase is distinguished from the normal cytochrome *c* oxidase by its insensitivity to cyanide, azide, and carbon monoxide, all of which inhibit the latter oxidase. However, electron flow through the alternative pathway can be specifically inhibited by several compounds, including salicylhydroxamic acid (SHAM) and *n*-propyl gallate (Siedow, 1982). Most tissues of all higher plants examined to date contain some level of the cyanide-resistant, SHAM-sensitive oxygen uptake that is diagnostic of alternative pathway activity.

With the alternative pathway, electrons are shunted off of the standard electron transfer pathway at the level of the ubiquinone pool (Figure 1; Lance et al., 1985; Moore and Siedow, 1991). The sole enzyme activity of the alternative pathway, ubiquinol oxidase, transfers electrons from reduced ubiquinone (ubiquinol) to molecular oxygen, producing water as the reduced product (Moore and Siedow, 1991; Berthold and Siedow, 1993). No proton motive force is generated during electron flow between ubiquinol and oxygen catalyzed by the alternative pathway (Moore et al., 1978). Therefore, all of the free energy released during electron flow from ubiquinol through the alternative pathway (290 kJ/4 e<sup>-</sup>) is lost as heat and is unavailable for use in ATP synthesis. Thus, during the oxidation of NADH formed by the TCA cycle, two of the three sites of energy conservation on the main electron transfer chain are bypassed when electrons flow through the alternative pathway, leading to a reduction in the resulting proton motive force and in the amount of ATP synthesis that takes place (Moore et al., 1978; Whitehouse and Moore, 1995).

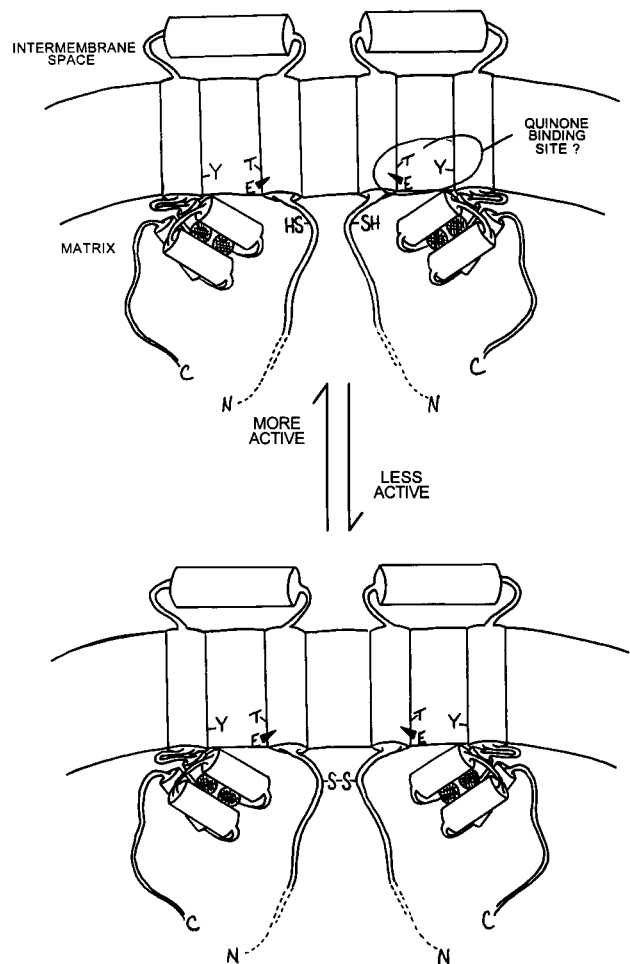
No single area of research relating to plant mitochondria has seen as much progress in recent years as the study of the alternative pathway. The primary features of the alternative pathway previously described have been recognized for many years (Siedow, 1982). However, attempts to characterize the unusual oxidase associated with the pathway met with no real success until Elthon and McIntosh made antibodies against the alternative oxidase of *Sauromatum guttatum* (Elthon and McIntosh, 1987; Elthon et al., 1989a). AOA, the monoclonal antibody that recognizes the *S. guttatum* protein (Elthon et al., 1989a), has proved to be broadly cross-reactive with the

alternative oxidase from all plants tested to date, as well as with that found in several fungi (Lambowitz et al., 1989; Sakajo et al., 1993). On immunoblots of proteins isolated from plant mitochondria, the AOA antibody binds to from one to three immunoreactive protein bands in the range of 35 to 37 kD, depending on the plant and the tissue from which the mitochondria have been isolated (Elthon et al., 1989a; Obenland et al., 1990; Kearns et al., 1992). The pattern of protein bands observed with any given tissue is reproducible, but at present neither the structural nature nor the functional significance of these multiple bands is understood.

The availability of this antibody against the alternative oxidase facilitated the initial isolation from *S. guttatum* of a cDNA clone encoding this enzyme (Rhoads and McIntosh, 1991); this isolation has been followed by isolation of alternative oxidase cDNAs from Arabidopsis (Kumar and Söll, 1992), soybean (Whelan et al., 1993), tobacco (Vanlerberghe and McIntosh, 1994), and an ascomycetous yeast, *Hansenula anomala* (Sakajo et al., 1991). A genomic clone of the *S. guttatum* oxidase has also been isolated and characterized (Rhoads and McIntosh, 1992). Derived amino acid sequence similarities among the different plant oxidases range from ~80 to 87% (Vanlerberghe and McIntosh, 1994). All of these proteins are nuclear encoded, and the presence of up to three alternative oxidase genes in soybean has recently been reported (J. Whelan and D.A. Day, personal communication). The alternative oxidase cDNA from Arabidopsis was isolated based on its ability to rescue a mutant strain of *Escherichia coli* that lacks the terminal cytochrome-containing oxidase and thus cannot grow under aerobic conditions (Kumar and Söll, 1992). This cDNA's ability to complement the *E. coli* mutant confirmed that it encodes the alternative oxidase and further indicated that the appearance of an active alternative pathway requires only the expression of this single protein species.

The amino acid sequences derived from the cDNA sequences have provided the first insights into the structure of the alternative oxidase. The derived amino acid sequences include putative mitochondrial transit peptides at the N terminus, whose lengths vary among different plants (McIntosh, 1994; Vanlerberghe and McIntosh, 1994). The mature alternative oxidase protein (that is, lacking the 6.5-kD transit peptide) from *S. guttatum* contains 283 amino acids and has a calculated molecular weight of ~32,000. The mature alternative oxidases from other plants appear to be this same size. Hydrophathy analysis of the deduced amino acid sequences of all known alternative oxidases indicates that two regions of the protein are hydrophobic, strongly  $\alpha$ -helical, and of sufficient length to span a membrane (Moore and Siedow, 1991; Rhoads and McIntosh, 1991). These two hydrophobic regions are located in roughly the center of the protein and are separated by ~40 amino acids. The remainder of the protein, ~100 amino acids on the N-terminal side of the first membrane-spanning region and ~100 amino acids on the C-terminal side of the second membrane-spanning region, is quite hydrophilic, with charged residues distributed throughout most of its length (Moore and Siedow, 1991). This has led to a model for the

structure of the alternative oxidase in which the protein is anchored to the inner mitochondrial membrane by two membrane-spanning  $\alpha$ -helices, with the hydrophilic domains flanking each membrane-anchoring helix and extending into the matrix (Figure 2; Moore and Siedow, 1991; McIntosh, 1994).



**Figure 2.** Structural Organization of the Alternative Oxidase Dimer in the Inner Mitochondrial Membrane.

Conversion of the dimer between the more active noncovalently associated reduced state ( $-SH HS-$ ) and the less active disulfide-bridged oxidized state ( $-S-S-$ ) is illustrated. The four-helical bundle that forms the postulated binuclear iron center in the C-terminal hydrophilic domain of the protein (Siedow et al., 1995) is shown on the matrix side of the inner membrane. The redox-active cysteine residue that regulates alternative oxidase activity may be the conserved cysteine located near the N terminus of the first membrane-spanning helix, positioning it near the postulated binuclear iron center found at the active site of the enzyme. The indicated T, Y, and E residues are conserved among all known alternative oxidase sequences and may serve as potential ubiquinone binding ligands as a result of their ability to bind to the headgroup of ubiquinone via hydrogen bonds. Except for the N-terminal hydrophilic domain, which has been shortened, protein features are approximately to scale.

Protease studies using right-side-out and inside-out inner membrane vesicles have demonstrated that the alternative oxidase protein is much more susceptible to proteolysis when the matrix face of the membrane is exposed, suggesting that the two hydrophilic domains are located on the n-side of the inner membrane (Rasmusson et al., 1990; Siedow et al., 1992).

The alternative oxidases reported to date show a high degree of amino acid sequence conservation in a region that begins at the first of the two membrane-spanning helices and continues through much of the C-terminal hydrophilic domain. The N-terminal hydrophilic domain shows the least sequence similarity among different plant species and is particularly divergent in the yeast *Hansenula*. No large-scale sequence similarities between the alternative oxidase and other known proteins have been found in computer searches of sequence data bases (Moore and Siedow, 1991; Rhoads and McIntosh, 1991; McIntosh, 1994).

The ability of the alternative oxidase to reduce oxygen to water has led to the prediction that the active site should contain a transition metal, possibly two coupled metals (Siedow, 1982; Moore and Siedow, 1991). In the presence of the  $\text{Fe}^{2+}$  chelator *o*-phenanthroline, induction of the alternative oxidase in *H. anomala* by addition of inhibitors of mitochondrial protein synthesis led to the appearance of the 36-kD alternative oxidase protein but no alternative pathway activity (Minagawa et al., 1990). However, upon addition of  $\text{Fe}^{2+}$  to the medium, alternative pathway activity appeared rapidly. These results make iron the leading candidate for the metal associated with the alternative oxidase active site.

Additional evidence that the alternative oxidase active site contains iron comes from the observation of Siedow et al. (1995) that the alternative oxidase has several unusual physical properties in common with the binuclear iron protein methane monooxygenase. Amino acid sequence comparisons showed that all of the plant alternative oxidases contain two copies of the conserved iron binding motif (E-X-X-H) found in the large family of coupled binuclear iron proteins to which methane monooxygenase belongs (Nordlund et al., 1992; Wilkins, 1992). These motifs appear in the C-terminal hydrophilic domain of the alternative oxidase and begin just after the second proposed membrane-spanning helical region. Based on the presence of these motifs, an iron-binding four-helical bundle, analogous to those found in other binuclear iron proteins, can be constructed for the alternative oxidase (Figure 2; Siedow et al., 1995). Additional characterization of the alternative oxidase active site is needed; the hypothesis that the catalytic site contains a coupled binuclear iron center suggests that specific electron paramagnetic resonance spectroscopic signals should appear when the oxidase is poised at different reduction states (Wilkins, 1992).

Another structural feature of the alternative oxidase was revealed recently when Umbach and Siedow (1993) used chemical cross-linkers to demonstrate that the oxidase exists as a dimer in the inner mitochondrial membrane. In addition, two distinct states of the dimeric alternative oxidase were identified: an oxidized state in which the dimer is covalently

cross-linked by an intermolecular disulfide bridge, and a reduced state in which the disulfide bond is reduced to its component sulfhydryls and the dimeric structure is maintained through noncovalent interactions (Umbach and Siedow, 1993). The activity of the alternative oxidase is dependent on its oxidation state, with the reduced form being four- to fivefold more active than the oxidized form (Umbach and Siedow, 1993; Umbach et al., 1994). The regulatory significance of this redox system *in vivo* has yet to be demonstrated. However, plant mitochondria contain thioredoxin (Bodenstein-Lang et al., 1989; Marcus et al., 1991), which plays a role in maintaining the reduction state of regulatory sulfhydryl/disulfide bonds in other enzymes (Holmgren, 1985), and it could act in plant mitochondria by reducing the oxidized alternative oxidase to the more active reduced state in response to changes in the redox state of the mitochondrial matrix. For example, the matrix becomes more reduced when electron flow through the cytochrome pathway becomes limiting; under such circumstances, the alternative pathway could provide an energy overflow pathway to maintain a threshold level of respiration (Lambers, 1980; Moore and Siedow, 1991).

A second potential regulatory feature of the alternative oxidase involves the observed stimulation of its activity by added pyruvate (Millar et al., 1993). Because the stimulation of alternative oxidase activity is not due to oxidation of added pyruvate, Millar et al. (1993) postulated that pyruvate acts as an allosteric effector of the alternative oxidase. This interpretation is supported by the results of Umbach et al. (1994), who showed that a primary effect of the added pyruvate is to lower the effective  $K_m$  for the reaction of the alternative oxidase with its reducing substrate, ubiquinol. In addition, the response is not specific to pyruvate but is more generally associated with any  $\alpha$ -keto acid (Day et al., 1994; Umbach et al., 1994). Stimulation of alternative pathway activity by organic acids had been reported previously (Wagner et al., 1989; Lidén and Åkerlund, 1993).

The finding that  $\alpha$ -keto acids lower the apparent affinity of the alternative oxidase for reduced ubiquinone is particularly important in light of previous results indicating a relatively high  $K_m$  for the reaction between the alternative oxidase and ubiquinol; significant alternative oxidase activity occurred only when >40% of the ubiquinone pool was reduced (Dry et al., 1989; Siedow and Moore, 1993). The high apparent  $K_m$  is consistent with the notion that the alternative pathway serves an energy overflow function in plant mitochondria (Bahr and Bonner, 1973; Lambers, 1980) and that electrons divert to the alternative pathway only when electron transfer through the main pathway is at, or approaching, saturation. A practical consequence of the energy overflow paradigm is that the level of engagement of the alternative pathway (that is, the percentage of the total alternative pathway activity functioning under any given set of conditions) could be ascertained by the extent to which alternative pathway inhibitors, such as SHAM, inhibit respiration (Møller et al., 1988). That is, because no electron redistribution onto the already saturated main pathway would be possible following inhibition of the alternative pathway, any decrease in the respiration rate would reflect how

much the alternative pathway was engaged in the absence of the inhibitor.

However, an important consequence of the finding that pyruvate shifts the  $K_m$  of the alternative oxidase for ubiquinol closer to that associated with the main pathway (Umbach et al., 1994) is the possibility that, in the presence of pyruvate, the alternative pathway can compete effectively with the main pathway for electrons from the ubiquinone pool. Using indirect assays of electron distribution between the main and alternative pathways in isolated mitochondria following the addition of electron transfer inhibitors, Hoefnagel et al. (1995) recently demonstrated that, in the presence of pyruvate, the two pathways compete for electrons; moreover, engagement of the alternative pathway does not first require saturation of electron flow through the main pathway. Similar results have been obtained from direct measurements of the differential fractionation against the oxygen isotope  $^{18}\text{O}$  by the main and alternative oxidases (Guy et al., 1989) during electron transfer in isolated plant mitochondria in the absence of inhibitors (Ribas-Carbo et al., 1995).

Collectively, these observations challenge the prevailing paradigm that the alternative pathway operates passively in an energy overflow capacity and only when electron flow through the main pathway is saturated. The allosteric effect of  $\alpha$ -keto acids makes it clear that SHAM inhibition of respiration (Møller et al., 1988) is not a useful measure of engagement of the alternative pathway in intact tissues because, if the endogenous level of mitochondrial pyruvate (or other suitable  $\alpha$ -keto acid) is sufficiently high, both pathways can be engaged simultaneously without either being saturated. Inhibition of the alternative pathway by SHAM would simply lead to the redistribution of electron flow onto the main pathway, with little or even no inhibition of respiration (Ribas-Carbo et al., 1995). Under the energy overflow paradigm, this observation would lead to the erroneous conclusion that the alternative pathway had not been engaged. Clearly, many earlier studies using intact plant tissues need to be reevaluated in light of these findings. Recent advances in application of the oxygen isotope discrimination technique to study the partitioning of electron flow between the main and alternative pathways in intact tissues (Robinson et al., 1992) and in isolated mitochondria (Ribas-Carbo et al., 1995) should help define the conditions under which engagement of the alternative pathway does and does not compete directly with electron flow through the main pathway.

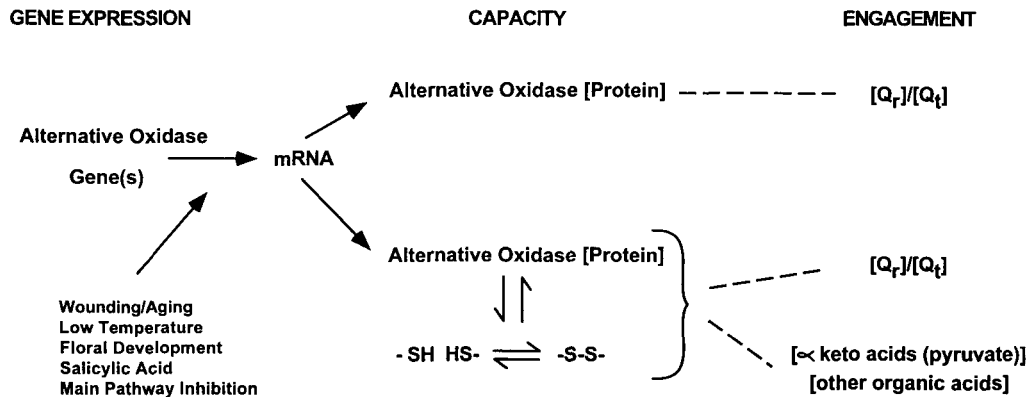
Regulation of the alternative pathway is not limited to post-translational events such as disulfide bond reduction and allosteric regulation but also takes place at the level of gene expression (for review, see McIntosh, 1994). Increases in the steady state level of alternative oxidase protein have been documented as a component of wound-induced aging in potato tubers (Hiser and McIntosh, 1990), in thermogenic spadices during floral development (Elthon et al., 1989b; Rhoads and McIntosh, 1992), and as a response to low, nonfreezing temperatures (Stewart et al., 1990; Vanlerberghe and McIntosh, 1992a). Exogenous salicylic acid has been shown to promote

an increase in alternative oxidase mRNA in thermogenic spadix tissue from *S. guttatum* (Rhoads and McIntosh, 1992) and increased levels of the alternative oxidase protein in tobacco cell suspension cultures (Rhoads and McIntosh, 1993).

Interestingly, inhibition of electron flow through the main pathway with antimycin, which blocks electron transfer at the level of complex III (Nicholls and Ferguson, 1992), leads to enhanced alternative pathway activity in tobacco (Vanlerberghe and McIntosh, 1992b, 1994) and petunia (Wagner et al., 1992) tissue culture cells. In the case of tobacco cells, the increased activity is correlated with an increase in the amount of alternative oxidase protein (Vanlerberghe and McIntosh, 1992b) and mRNA (Vanlerberghe and McIntosh, 1994). A similar induction of the alternative oxidase protein in response to inhibition of the cytochrome pathway has been observed in fungi (Lambowitz et al., 1989; Sakajo et al., 1991). These results suggest the existence of a mechanism whereby signals indicating that electron flow through the main pathway has become restricted are transmitted from the mitochondria to the nucleus, leading to increased expression of the alternative oxidase. It remains for future studies to ascertain the mechanism that lies behind this important aspect of the regulation of expression of a nuclear-encoded mitochondrial gene.

In summary, the regulation of electron flow through the alternative pathway in plant mitochondria is more complex than was envisioned only a few years ago. At that time, the maximum level of alternative pathway activity attainable (that is, the capacity) was viewed as solely a function of the amount of alternative oxidase protein present in the inner mitochondrial membrane, and the actual level of alternative pathway activity seen under any given conditions (that is, the engagement) was viewed as simply a function of the reduction state of the ubiquinone pool (Figure 3, upper pathway). It still appears that metabolic regulation of alternative oxidase activity occurs at two levels, but now it is clear that the *capacity* of the alternative pathway is dependent on both the amount of alternative oxidase protein present and the redox status of the regulatory sulfhydryl/disulfide bond, and the level of *engagement* of the pathway is dependent on the amount of reduced ubiquinone, in conjunction with the protein's allosteric status as determined by the mitochondrial concentration of  $\alpha$ -keto acids, particularly pyruvate (Figure 3, lower pathway). The potential complexity of this regulatory system underscores the need to understand better how regulation by both the sulfhydryl/disulfide redox status of the alternative oxidase and mitochondrial pyruvate levels modulates alternative pathway activity *in vivo*.

These new insights into regulation of the alternative pathway suggest that it may play a more significant role in plant respiration than had been assumed previously, given that the alternative pathway can compete directly with the main pathway for electrons. Under some conditions, operation of this apparently energetically wasteful pathway may be necessary to maintain rates of mitochondrial electron flow. For example, either under conditions in which the absolute flux of reductant



**Figure 3.** Factors That Regulate the Total (Capacity) and Actual (Engagement) Amount of Alternative Oxidase Activity.

As the upper pathway indicates, the amount of alternative oxidase protein (determined by the extent of alternative oxidase gene expression) and the amount of reduced ubiquinone ( $[Q_r]$ ) present were considered until recently to be the only factors affecting capacity and engagement, respectively. As the bottom pathway shows, recent findings indicate that capacity is also dependent on the redox state of the alternative oxidase intermolecular sulfhydryl/disulfide system and that the level of engagement is also modulated by the concentration of  $\alpha$ -keto acids (pyruvate) in the mitochondria as well as by the amount of reduced ubiquinone present.

is high during the operation of the photorespiratory cycle in photosynthesizing tissues or under conditions of cold stress, when electron flow through the main pathway would be restricted, electron flow through the alternative pathway could compete with flow through the main pathway.

## PLANT MITOCHONDRIAL MOLECULAR GENETICS

The endosymbiotic origin of mitochondria has resulted in the existence of a genetic system within the organelle separate from that associated with the nucleus. The plant mitochondrial genome, like that of other eukaryotes, encodes only a small percentage of the proteins located within the organelle, indicating that over the course of evolution, most of the genes that resided in the original endosymbiont have migrated to the nuclear genome (Gray, 1993). Several reviews relating to various aspects of the plant mitochondrial genome have appeared recently (Bonnard et al., 1992; Wissinger et al., 1992; Mackenzie et al., 1994; Schuster and Brennicke, 1994). This section briefly reviews some of the plant-specific features of the mitochondrial genome.

At many hundreds of kilobases in size, the plant mitochondrial genome not only is considerably larger than the 16-kb circular DNA found in most animal mitochondria but also shows a large variability in size (up to 15-fold) among different plants (Hanson and Folkerts, 1992). A major advance in the study of plant mitochondrial genetics came with publication of the complete nucleotide sequence of the mitochondrial genome from the liverwort, *Marchantia polymorpha* (Oda et al., 1992). This sequence confirmed the long-held view that much of the extra

DNA in the plant mitochondrial genome consists of noncoding sequences, including introns and nonfunctional sequences often duplicated throughout the genome. In contrast, the DNA from animal mitochondria contains very few noncoding regions. The introns that exist are found only among a few lower invertebrates, which primarily accounts for why animal mitochondrial DNA (mtDNA) is so much smaller than that found in plant mitochondria. Interestingly, plant mtDNA strictly uses the universal genetic code (Oda et al., 1992; Schuster and Brennicke, 1994), which also distinguishes it from the mtDNA of animals, fungi, and protozoa, all of which show deviations from the universal code in one or more codons (Attardi and Schatz, 1988).

Another feature that sets plant (and fungal) mtDNA apart from animal mtDNA is the extent to which the specific genes encoded by plant mtDNA can vary from one plant to the next. The mitochondrial genome of all plants encodes three rRNAs and 16 or more tRNAs. The exact number of tRNAs varies from species to species, but in no plant does the mtDNA encode a complete complement of tRNAs. Therefore, some of the mitochondrial tRNAs must be encoded in the nucleus and imported from the cytoplasm. Studies of protein synthesis in isolated plant mitochondria indicate that 20 to 30 proteins are encoded by plant mtDNA (Schuster and Brennicke, 1994), which is more than the 13 proteins encoded by animal mtDNA but considerably fewer than expected from the 94 open reading frames (ORFs) present in the liverwort mtDNA sequence (Oda et al., 1992). Although 16 genes that encode potential ribosomal proteins are found in the liverwort mtDNA (Oda et al., 1992), only 10 ribosomal protein genes (seven small subunit genes and three large subunit ones) have been identified to date among higher plant mtDNAs, and all 10 genes have yet to be identified within a single species (Schuster and Brennicke,

1994). It remains for future studies to ascertain whether some of the 16 ribosomal genes in the liverwort mtDNA have been transferred to the nucleus in higher plants (see the following) or simply have not yet been found on the mitochondrial genome.

Among the 13 proteins associated with complexes I through IV and the ATP synthase that are encoded by animal mtDNA, all have been found to be encoded by plant mtDNA in one or more species (Schuster and Brennicke, 1994). These include seven subunits of complex I (*nad1-6* and *nad4L*), cytochrome *b* (*cob*) of complex III, the three largest subunits of cytochrome *c* oxidase (*coxI-III*), and two subunits of the  $F_0$  component of the ATP synthase (*atp6* and *atp9*). In addition, plant mtDNA commonly encodes the  $\alpha$ -subunit of the  $F_1$  component of the ATP synthase and at least two additional subunits of complex I (*nad7* and *nad9*). In the liverwort mtDNA, four ORFs having sequence similarity with prokaryotic genes involved in heme *c* biosynthesis have been identified (Oda et al., 1992). Another 10 ORFs, whose predicted proteins bear no sequence similarity with any known protein, are conserved among several higher plant mtDNAs (Schuster and Brennicke, 1994). It remains for future studies to ascertain what proteins are associated with these putative genes as well as to determine how many of the >50 additional ORFs in the liverwort mtDNA are present in the mtDNA of higher plants.

Another feature of plant mtDNA that appears to be unique to plants is the ability to detect the ongoing transfer of genetic information from the organellar to the nuclear genome. This phenomenon was first observed with the gene encoding subunit II of cytochrome *c* oxidase (*coxII*) in legumes. The *coxII* gene had been thought to be universally encoded by mtDNA, but *coxII* has now been shown to be a nuclear-encoded gene in the legumes cowpea, soybean, and mung bean (Corvello and Gray, 1992; Nugent and Palmer, 1992; Mackenzie et al., 1994). In cowpea and mung bean, the mitochondrial gene is absent, whereas in soybean, the mitochondrial gene is present but inactive. Pea also contains both nuclear and mitochondrial versions of the gene, but only the mitochondrial gene is active. More recently, disrupted versions of additional mitochondrially encoded genes have been found in the mtDNA from several different species, including genes for three small ribosomal subunits in *Arabidopsis* (Schuster and Brennicke, 1994). It seems likely that actively expressed copies of these genes will be found within the nuclear genome (Schuster and Brennicke, 1994). Why such gene transfer might appear among higher plant mitochondrial genes, whereas it is essentially nonexistent outside of higher plants, is not clear. However, it is intriguing that the phenomenon of RNA editing is also associated with higher plant mitochondrially encoded genes (see the following discussion).

RNA editing, although not unique to the plant mitochondrial genetic system, appears with a very high frequency among higher plants (Wissinger et al., 1992). The nucleotide sequence directly encoded by the mtDNA usually does not translate into the primary amino acid sequence found in the mature protein,

but editing alters the mRNA sequence so that it does encode the correct amino acid sequence. Plant mitochondria found in pteridophytes through angiosperms show evidence of RNA editing, but it is not found in mosses, liverworts, or eukaryotic algae (Schuster and Brennicke, 1994). Although the exact relationship between the two processes is not obvious, it is clear that the transfer of mitochondrially encoded genes to the nucleus takes place through mRNA that has been fully edited.

Among higher plants, most RNA editing involves a deamination reaction that converts a C to a U, although the reciprocal U to C conversion is observed occasionally. The extent of editing is high, with many hundreds of sites reported in some species, such as wheat (Bonnard et al., 1992). Although most editing sites are found within ORFs, and the editing therefore alters amino acid-encoding codons, editing that produces viable start codons and stop codons has also been reported (Wissinger et al., 1992). Studies of the editing mechanisms in plant mitochondria are limited, but the editing process appears to involve modification of a nucleotide already present in the mRNA. Almost nothing is known about how the fidelity of the editing process is maintained, at least in plant mitochondria, and this important area requires additional study in the future. Furthermore, although it is clear that transcripts from the mtDNA sequence must be edited if a viable mRNA is to be produced, it is not clear why RNA editing should have evolved in the first place. This topic also requires further study.

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