Plant Physiol. (1997) 113: 903–911

The Regulation of Electron Partitioning between the Cytochrome and Alternative Pathways in Soybean Cotyledon and Root Mitochondria[1]

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The regulation of electron partitioning between the cytochrome (Cyt) and alternative pathways in soybean (*Glycine max* L. cv Ransom) mitochondria in the absence of added inhibitors has been studied using the oxygen isotope fractionation technique. This regulation can depend on several factors, including the amount of alternative oxidase protein, the redox status of the alternative oxidase regulatory sulfhydryl-disulfide system, the degree of activation by α-keto acids, and the concentration and redox state of the ubiquinone pool. We studied electron partitioning onto the alternative pathway in mitochondria isolated from etiolated and light-grown cotyledons and roots to ascertain how these factors interact in different tissues. In light-grown cotyledon mitochondria there is some partitioning to the alternative pathway in state 4, which is increased dramatically by either pyruvate or dithiothreitol. In etiolated cotyledon mitochondria, the alternative pathway shows little ability to compete for electrons with the Cyt pathway under any circumstances. In root mitochondria, control of alternative pathway activity is exercised by both the ubiquinone pool and the regulatory sulfhydryl-disulfide system. In addition, oxygen isotope fractionation by the Cyt and alternative pathways in mitochondria were identical to the fractionation for the respective pathways seen in intact tissue, suggesting that residual respiration is not present in the absence of inhibitors.

The regulation of electron partitioning between the cyanide-sensitive Cyt pathway and the cyanide-resistant alternative pathway in plant mitochondria has long been of interest because of the nonphosphorylating nature of the latter pathway and its implications for ATP yield during plant respiration (Moore et al., 1978; Lambers, 1982; Moore and Siedow, 1991). Both pathways obtain electrons from reduced ubiquinone and reduce oxygen to water. Early inhibitor studies indicated that the Cyt pathway was kinetically favored and that the alternative pathway was engaged only when electron flow through the Cyt pathway was either saturated or inhibited (Bahr and Bonner, 1973). These observations led to the development of the concept that the alternative pathway acted in an "energy overflow" capacity (Lambers, 1980) and led to the widespread use of specific inhibitors to ascertain the electron partitioning between the two pathways (Theologis and Laties, 1978; Laties, 1982; Møller et al., 1988).

Several factors are now known to potentially affect alternative pathway activity (Siedow and Umbach, 1995). The role of gene expression, as reflected in the level of alternative oxidase protein present in the membrane, has long been recognized as an important feature affecting the amount of alternative pathway activity observed in isolated mitochondria (McIntosh, 1994). Kinetic analyses of the dependence of alternative oxidase activity on the reduction state of the mitochondrial ubiquinone pool initially supported the concept that the alternative pathway only became active when electron flow through the Cyt pathway was at or near saturation (Dry et al., 1989; Siedow and Moore, 1993). However, more recent studies of alternative pathway regulation have revealed that the alternative oxidase is activated by α-keto acids such as pyruvate (Millar et al., 1993), which act to increase the reactivity of the oxidase toward reduced ubiquinone (Umbach et al., 1994). Additional studies have also shown that the alternative oxidase exists as a dimer in the membrane and contains a regulatory sulfhydryl-disulfide system (Umbach and Siedow, 1993). When this regulatory system is reduced, the

[1] This work was supported by the U.S. Department of Agriculture National Research Initiative grant no. CGP 94-37306-0352, National Science Foundation grant no. DEB9112571, Department of Energy grant no. DE-FG05–89ER14005, and MEC-Fulbright Postdoctoral Fellowship (M.R.-C). This is Carnegie Institution of Washington publication no. 1309.

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Abbreviations: Δa, oxygen isotope fractionation by the alternative pathway; τa, the fraction of total electron flow partitioning to the alternative pathway; AOA, alternative oxidase antibodies; Δc, oxygen isotope fractionation by the Cyt pathway; KCN, potassium cyanide; Δn, oxygen isotope fractionation in the absence of inhibitors; Δr, residual oxygen uptake; SHAM, salicylhydroxamic acid.

oxidase is considerably more active than when the system is oxidized and an intermolecular disulfide bond covalently cross-links the two monomeric subunits (Umbach and Siedow, 1993). The potential for regulation of the redox status of the alternative oxidase sulfhydryl-disulfide system by tricarboxylic acid cycle intermediates has been reported (Vanlerberghe et al., 1995). Finally, the possibility that the absolute level of ubiquinone in the mitochondrial inner membrane can affect the amount of alternative pathway activity in isolated mitochondria has been put forward based on differences seen in the ubiquinone levels of mitochondria isolated from soybean roots and cotyledons (Ribas-Carbo et al., 1995b).

The wealth of recent information on the regulation of the alternative pathway has led to a reexamination of whether the alternative pathway acts solely in an electron overflow capacity through the Cyt pathway. Several studies have demonstrated that the alternative oxidase can compete with the Cyt pathway for electrons in isolated mitochondria (Hoefnagel et al., 1995; Ribas-Carbo et al., 1995a), supporting the earlier suggestion of Wilson (1988) that electrons could be diverted from the alternative to an unsaturated Cyt pathway under certain conditions. These results have also called into question the validity of using specific inhibitors to ascertain the electron partitioning between the two pathways (Millar et al., 1995; Ribas-Carbo et al., 1995a; Day et al., 1996).

The observation of a differential oxygen isotope fractionation by the two oxidases, the alternative oxidase having a higher fractionation against the isotope oxygen-18 than Cyt *c* oxidase, has led to the development of a mass spectrometric technique that permits the measurement of electron partitioning between the Cyt and alternative pathways in the absence of inhibitors in intact tissues (Robinson et al., 1992, 1995) and isolated mitochondria (Guy et al., 1989, 1992; Ribas-Carbo et al., 1995a). The oxygen isotope fractionation observed in the absence of inhibitors is a consequence of the fractionation due to each oxidase and, as such, the relative partitioning of electrons between the two pathways can readily be calculated.

Given the large number of factors that are now known to affect the activity of the alternative pathway (Siedow and Umbach, 1995), we were interested to see how they might interact in regulating the partitioning of electrons to the alternative pathway during electron transfer in mitochondria isolated from different soybean tissues. In this study we used an improved aqueous-phase on-line mass spectrometer system to characterize the factors that affect electron partitioning between the Cyt and alternative pathways in mitochondria isolated from soybean roots and etiolated and green cotyledons. Factors that we considered included the effects of pyruvate and isocitrate, the redox state of the regulatory sulfhydryl-disulfide system, the size of the ubiquinone pool, and the total amount of alternative oxidase protein present in the inner mitochondrial membrane. Finally, comparison of the behavior of mitochondria from etiolated and green cotyledons also reflected an attempt to ascertain how regulation of the alternative pathway in these tissues might differ, given the reported differences in oxygen fractionation by the alternative oxidase in green versus nongreen plant tissues (Robinson et al., 1995).

MATERIALS AND METHODS

Soybean (*Glycine max* L. cv Ransom) seeds were treated with 10% Clorox for 10 min and swelled in distilled water for 2 h with continuous bubbling of air. Seeds were planted in a 1:1 mixture of sand and perlite and grown in a growth chamber at 27/23°C on a 14/10 h (light/dark) regime at 1000 μmol photons m^{-2} s^{-1}. Plants were watered twice a day and the cotyledons were harvested for mitochondrial purification 7 d after planting. Root mitochondria were isolated from soybean plants at 10 d after planting. For dark-grown tissue, soybean seeds were planted in vermiculite and grown in the dark at 20°C. They were watered once a day and etiolated cotyledons were harvested for mitochondrial purification 7 d after planting.

Mitochondrial Isolation

Mitochondria were isolated from soybean cotyledons and purified on Percoll gradients as described by Day et al. (1985) with modifications (Umbach and Siedow, 1993). Protein was estimated by the method of Lowry et al. (1951).

Immunoblotting

Samples were run at 25°C on the oxygen electrode using 2 mM NADH as a substrate under the conditions of Ribas-Carbo et al. (1995a). Mitochondria went through an initial state 3, state 4 transition, after which either 10 mM DTT or isocitrate was added. Once a constant rate was established, a 100-μL sample was removed and added to an equal volume of SDS sample buffer either with or without a final concentration of 5% β-mercaptoethanol. The samples were then run on a 10 to 17.5% gradient gel essentially as described by Laemmli (1970) and transferred to nitrocellulose as described by Towbin et al. (1979). Immunoblotting was performed by an initial incubation in 3% BSA, 2% milk powder in 1× PBS. The filters were probed with the AOA monoclonal antibody against the alternative oxidase protein (Elthon et al., 1989) at a dilution of 1:500 for 1 h. Following three washes in 1× PBS, the filters were incubated with horseradish peroxidase-linked secondary antibody at a dilution of 1:20,000 for 1 h. The filters were washed three times in blot rinse buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% v/v Tween 20), and the bound antibodies were detected using the Amersham enhanced chemoluminescence detection system following the manufacturer's instructions. Quantification of the immunoblots was performed as described by Umbach and Siedow (1993).

Ubiquinone Extraction and Determination

The extraction and determination of ubiquinone by HPLC was performed as described by Ribas-Carbo et al. (1995a).

Assays

Whole soybean cotyledons or large segments of the young soybean root system were weighed and either placed directly in an adjustable volume-closed cuvette maintained under total darkness at room temperature (approximately 25°C) or treated with inhibitor. During inhibitor treatments, either 1 mM KCN (in H_2O) or 10 mM SHAM (from a 1.0 M stock in DMSO) were applied by sandwiching tissues between medical wipes saturated with the corresponding inhibitor for 10 min. All stock solutions were prepared just prior to use. To measure Δr, both inhibitors were added. Following inhibitor treatments, the tissue was surface-dried and transferred to the cuvette. No recovery from inhibitor treatments was observed during the course of the oxygen fractionation measurements because all linear regressions had r^2 values greater than 0.995. It should be noted that excision of roots can lead to changes in respiration activity of the tissue (Bloom and Caldwell, 1988). However, the respiration rates of the tissue and the oxygen fractionation values remained constant throughout the 60- to 90- min time course of the fractionation experiments because all linear regressions obtained had $r^2 > 0.995$.

Isolated mitochondria were placed in a closed cuvette. Respiration was initiated with 2 mM NADH, and pyruvate (5 mM) was used to activate the alternative oxidase when required (Millar et al., 1993). SHAM (2 mM) was used to inhibit the alternative pathway and either 2 mM KCN or 2 μM myxothiazol was used to inhibit the Cyt pathway. For isocitrate and DTT treatments, 10 mM isocitrate and 10 mM DTT were added, respectively.

Oxygen Extraction and Isotope Analysis

Oxygen extraction and isotope analysis in intact tissues were carried out as described in Robinson et al. (1995). For measurements in isolated mitochondria, an aqueous phase oxygen isotope system was modified from that described in Ribas-Carbo et al. (1995a). In this system we separated the flow to the GC-MS from the sampling flow and accelerated the sampling time because it allowed extraction of one sample from the reaction cuvette at the same time the GC-MS was measuring the previous sample. We also switched to Valco (Houston, TX) valves in all cases to lessen the chances for leaks.

Fractionation and Partitioning Calculations

Calculations of oxygen isotope fractionation were made as described in Ribas-Carbo et al. (1995a) for mitochondria and as described in Robinson et al. (1995) for intact tissues. Electron partitioning between the two pathways in the absence of inhibitors was calculated as described by Guy et al. (1989):

$$\tau a = \frac{\Delta n - \Delta c}{\Delta a - \Delta c}$$

RESULTS

Alternative Oxidase Protein Levels and the Redox Status of the Regulatory Disulfide Bond

The relative content of the alternative oxidase protein was measured by immunoblotting using the AOA monoclonal antibody (Elthon et al., 1989) in the presence of β-mercaptoethanol in order to fully reduce the regulatory disulfide bond (Fig. 1A). The level of blottable alternative oxidase protein in root mitochondria (lane 3) is 1.6 times higher than that found in light-grown (green) cotyledons (lane 1) and eight times the level found in etiolated cotyledons (lane 2).

Figure 1B shows the same mitochondrial samples run in the absence of β-mercaptoethanol. In green cotyledon mitochondria most of the alternative oxidase protein is in the reduced form, with only 14% of the protein being in the oxidized state (lane 1). In etiolated cotyledon mitochondria, 40% of the protein is in the oxidized form (lane 2), whereas in root mitochondria 50% of the protein is oxidized (lane 3).

The effect of isocitrate and DTT on the reduction state of the alternative oxidase redox-active regulatory disulfide bond was also determined by immunoblotting in the absence of β-mercaptoethanol (Fig. 2). The redox state of the alternative oxidase regulatory disulfide bond was not affected by added isocitrate (10 mM) in any of the mitochondria (Fig. 2A, lanes 1–3). These results differ from those of Vanlerberghe et al. (1995), but their experiments were carried out using tobacco leaf mitochondria and involved a longer incubation time with isocitrate than was used in the present study. The addition of DTT (10 mM) resulted in the reduction of the regulatory disulfide bond (Fig. 2, lanes 3) and, hence, throughout this study DTT was used to reduce the disulfide bond. It should be noted that the reduction level of the alternative oxidase regula-

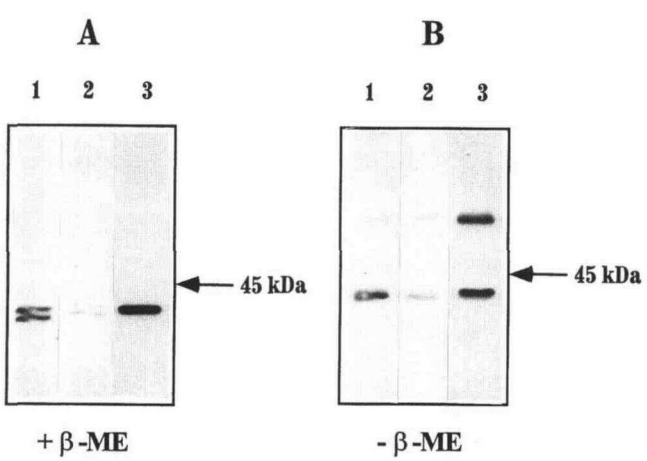

Figure 1. Immunoblots of the alternative oxidase protein in mitochondria isolated from soybean roots and etiolated and green cotyledons in the presence (A) and absence (B) of β-mercaptoethanol. Equal amounts (15 μg) of mitochondrial protein were loaded in each lane and blotted with the AOA monoclonal antibody. Lanes 1, Green cotyledon mitochondria; lanes 2, etiolated cotyledon mitochondria; lanes 3, root mitochondria.

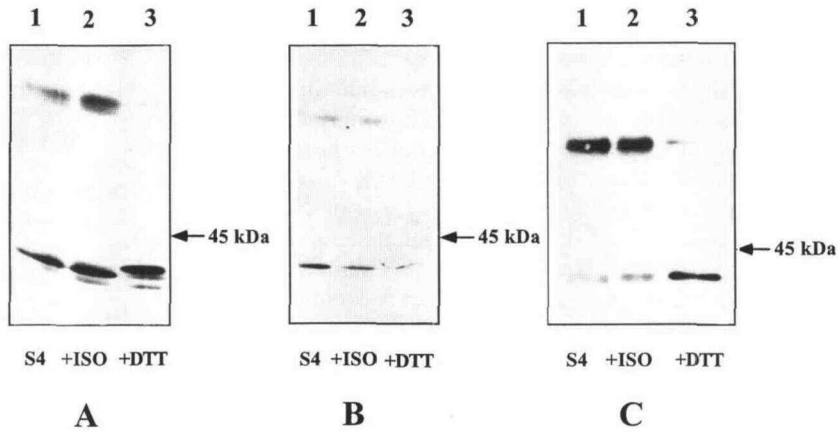

Figure 2. Immunoblots of the alternative oxidase protein in soybean mitochondria isolated from green cotyledons (A), etiolated cotyledons (B), and roots (C). Mitochondrial protein equivalent to 30 μg was loaded in each lane in the absence of β-mercaptoethanol. Blots were developed with the AOA monoclonal antibody. Lanes 1, state 4; lanes 2, state 4 + 10 mM isocitrate; lanes 3, state 4 + 10 mM DTT.

tory disulfide bond varies between mitochondrial preparations and does not necessarily represent the in vivo reduction state of the protein (A.L. Umbach and J.N. Siedow, unpublished observation).

Ubiquinone Levels

The ubiquinone content per milligram of mitochondrial protein was quantified with HPLC. Analogous to the results of Ribas-Carbo et al. (1995b), there was a marked difference in the amount of ubiquinone-10 found in the membranes of mitochondria isolated from soybean roots (0.86 ± 0.02 nmol mg^1 protein) and green cotyledons (3.37 ± 0.06 nmol mg^{-1} protein). The greater amount of ubiquinone-10 in mitochondria from green cotyledons was not a function of the greening of the tissue; mitochondria isolated from etiolated cotyledons showed levels of ubiquinone-10 (3.21 ± 0.12 nmol mg^{-1} protein) comparable to those seen in mitochondria from green cotyledons. The reduction levels of the ubiquinone pools in state 4, measured voltammetrically, of the green and etiolated cotyledons and root mitochondria were 76, 60, and 70%, respectively.

Δc, Δa, and Δr

The Δa was measured in both intact tissues and isolated mitochondria in the presence of 1.0 mM KCN as described in "Materials and Methods." The fractionation values for the alternative pathway were 31.5 and 30.9‰ in green soybean cotyledons, 25.5 and 25.4‰ in etiolated cotyledons, and 25.1 and 25.0‰ in roots for intact tissue and isolated mitochondria, respectively (Table I). Δc was measured in the presence of 2.0 mM (isolated mitochondria) or 10 mM (intact tissue) SHAM, as described in "Materials and Methods." Green soybean cotyledons gave fractionation values of 20.0 and 19.9‰ for intact tissue and isolated mitochondria, respectively. With etiolated cotyledons, the values for intact tissue and isolated mitochondria were 20.6 and 21.1‰ respectively, whereas both root tissue and isolated mitochondria gave a value of 20.8‰ (Table I). These values were used subsequently to calculate electron partitioning between the Cyt and alternative pathways in the absence of inhibitors. With green cotyledon tissue, in the presence of both KCN and SHAM, the oxygen isotope fractionation associated with Δr was 21.0‰, whereas roots gave a value of 19.6‰ for residual respiration (Table I).

Effects of Pyruvate

The effect of pyruvate on electron partitioning between the Cyt and alternative pathways in isolated mitochondria under state 4 conditions was studied using 2 mM NADH as a substrate to avoid pyruvate formation during succinate oxidation (Miller et al., 1996). In green cotyledon mitochon-

Table I. *Δa, Δc, and Δr in intact tissues and isolated mitochondria from soybean cotyledons and roots*

Oxygen isotope fractionation and oxygen uptake rates are expressed in ‰ and μmol O_2 g^{-1} fresh weight min^{-1}, respectively. Results and SEM were calculated from 3 to 14 repetitions of 3 to 7 different mitochondrial preparations.

Pathway/Source	Δ/O_2 Uptake	Cotyledons		Roots
		Green	Etiolated	
Alternative pathway				
Tissue	Δa	31.5 ± 0.3	25.5 ± 0.3	25.1 ± 0.6
	O_2 uptake	0.341	0.126	0.142
Mitochondria	Δa	30.9 ± 0.6	25.4 ± 0.3	25.0 ± 0.6
Cyt pathway				
Tissue	Δc	20.0 ± 0.4	20.6 ± 0.6	20.8 ± 0.5
Mitochondria	Δc	19.9 ± 1.1	21.1 ± 0.5	20.8 ± 0.3
Residual oxygen uptake				
Tissue	Δr	21.0		19.6
	O_2 uptake	0.046		0.026

dria, the addition of pyruvate increased the Δn from 21.7 to 25.3‰ (Table II). This represented a change in the τa from 0.16 to 0.49. A small stimulation of oxygen uptake during the oxidation of NADH in state 4 (from 93 to 110 nmol O_2 mg^{-1} protein min^{-1}) upon addition of pyruvate has been seen previously in soybean cotyledon mitochondria (Ribas-Carbo et al., 1995a).

In mitochondria isolated from etiolated soybean cotyledons, there was very little partitioning of electrons to the alternative pathway in either the presence or absence of pyruvate, and the addition of pyruvate had no effect on the total rate of oxygen uptake (Table II). In root mitochondria, electron partitioning to the alternative pathway was essentially zero, as the fractionation was 20.8‰ in the presence or absence of pyruvate (Table II). Total oxygen uptake showed a slight decrease from 180 nmol O_2 mg^{-1} protein min^{-1} in the absence of pyruvate to 162 nmol O_2 mg^{-1} protein min^{-1} in its presence (Table II), which is within the range of normal sample variation.

Effects of DTT

The effect of adding 10 mM DTT to reduce the alternative oxidase's regulatory sulfhydryl-disulfide bond on electron partitioning between the two pathways was also studied. In green cotyledon mitochondria, adding DTT increased the oxygen isotope fractionation from 21.7 to 23.3‰, and increased electron partitioning through the alternative pathway from 0.16 to 0.31 (Table II). Unlike pyruvate, DTT did not increase the total oxygen uptake rate but rather showed a small inhibitory effect on the Cyt pathway (Table II). In etiolated cotyledon mitochondria, adding DTT had no significant effect on either oxygen isotope fractionation or total oxygen uptake (Table II). With root mitochondria, DTT alone had no significant effect on the electron partitioning between the Cyt and alternative pathways, as in etiolated cotyledon mitochondria, but did reduce the total rate of oxygen uptake, as in green cotyledon mitochondria (Table II).

Combined Effects of DTT and Pyruvate

The combined effects of adding pyruvate and DTT on electron transport through the alternative pathway was also studied (Table II). With green cotyledon mitochondria, oxygen isotope fractionation was 25.5‰ and electron partitioning through the alternative pathway was 0.51 (Table II), an increase from the values seen in the absence of DTT and pyruvate, but almost the same as that seen with pyruvate alone. The combination of DTT and pyruvate had an unexpected effect on mitochondria isolated from etiolated cotyledons. No partitioning of electrons to the alternative pathway remained, but a marked stimulation of oxygen uptake from roughly 80 to 130 nmol O_2 mg^{-1} protein min^{-1} was observed. In root mitochondria, although neither DTT nor pyruvate alone was able to engage the alternative pathway to any significant extent, the combination of the two increased oxygen isotope fractionation to 22.4‰, giving $\tau a = 0.36$ (Table II).

DISCUSSION

Differential Oxygen Isotope Fractionation by the Alternative Oxidase in Green and Nongreen Tissues

Guy et al. (1989) initially determined that Δa was 25‰. However, Robinson et al. (1992, 1995) found that there was an apparent differential in the Δa in green and nongreen tissues. We confirm that in soybean, Δa is different between green cotyledons and nongreen tissues, with intact green cotyledons showing a fractionation value of around 31‰, and roots and etiolated cotyledons showing fractionation values of 25 to 26‰. Fractionation by Cyt *c* oxidase remains roughly constant in all soybean tissues, having a value of 19.5 to 21‰. The origin of the differential fractionation by the alternative oxidase from green versus nongreen tissues is not known, but the differential was maintained in mitochondria isolated from each of these tissue types, suggesting that the differences are inherent to the alternative oxidase species present in each tissue and not an artifact

Table II. *Effect of pyruvate (5 mM) and DTT (5 mM) on Δn, alternative pathway activity (v_{alt}), Cyt pathway activity (v_{cyt}), and τa in mitochondria isolated from soybean roots and etiolated and green cotyledons using NADH (2 mM) as a substrate under state 4 conditions*

Values and SEM are calculated from 3 to 11 repetitions of 3 to 7 different mitochondrial preparations.

Tissue	Pyruvate	DTT	Δn	v_{alt}	v_{cyt}	τa
	mM	mM	‰	nmol $O_2 mg^{-1}$ protein min^{-1}		
Green cotyledons	None	None	21.7 ± 0.4	15	78	0.16
	5	None	25.3 ± 0.3	54	56	0.49
	None	10	23.3 ± 0.5	24	53	0.31
	5	10	25.5 ± 0.5	56	54	0.51
Etiolated cotyledons						
	None	None	21.3 ± 0.3	4	79	0.05
	5 mM	None	21.4 ± 0.2	6	82	0.07
	None	10	20.8 ± 0.2	0	88	0.00
	5	10	20.6 ± 0.2	0	130	0.00
Roots						
	None	None	20.8 ± 0.3	0	180	0.00
	5	None	20.8 ± 0.5	0	162	0.00
	None	10	21.0 ± 0.3	7	134	0.05
	5	10	22.4 ± 0.4	59	106	0.36

associated with tissue differences. The finding that three distinct alternative oxidase isozymes exist in soybeans, which appear to be differentially expressed in various plant tissues (Whelan et al., 1996), suggests the possibility that different isozymes could be the source of the differential oxygen isotope fractionation. It will be important in the future to understand how the catalytic and regulatory properties of different alternative oxidase isozymes relate to the observed oxygen isotope fractionation values.

Residual Respiration in Intact Tissues

Studies of respiration in intact tissues have often reported a level of oxygen uptake in the presence of inhibitors of both the Cyt (KCN) and alternative (SHAM) pathways. Such oxygen consumption has been called "residual respiration" (Bahr and Bonner, 1973; Møller et al., 1988), and this residual oxygen uptake is usually negligible in isolated, purified mitochondria. Guy et al. (1989) reported a fractionation value of 19.8‰ for the residual oxygen uptake in alfalfa sprouts. Oxygen isotope fractionation in the presence of both KCN and SHAM has Δs of 21.0‰ in intact green soybean cotyledons and 19.6‰ in intact roots. Consequently, if residual respiration makes any significant contribution to oxygen uptake in intact tissues in the absence of both inhibitors, Δa (i.e. in the presence of KCN) should give a higher value in isolated mitochondria, where residual respiration is absent, than in intact tissues.

The oxygen uptake rate in intact green cotyledons in the presence of both KCN and SHAM was 0.046 μmol O_2 g^{-1} fresh weight min^{-1} (Table I). Δa in green cotyledon mitochondria was 30.9‰ (Table I). Consequently, if Δr was taking place in intact cotyledons in the presence of added KCN, we would expect the following oxygen isotope fractionation:

$$\Delta(‰) = \frac{0.295 \times 30.9 + 0.046 \times 21.0}{0.341} = 29.6‰$$

In fact, an oxygen fractionation of 31.5‰ was observed in green cotyledons (Table I), which suggests that no significant residual respiration takes place when KCN is present, and, by inference, that in the absence of any inhibitor no residual respiration takes place in green cotyledons. Similarly, calculations using root mitochondria indicate that residual respiration does not make a significant contribution to oxygen uptake in the presence of KCN. A value of 24.0‰ would be expected if all the observed residual oxygen uptake was present. However, a value of 25.1‰ is observed with intact root tissue (Table I), which is identical to the Δa in isolated mitochondria. Only when both KCN and SHAM are added does residual respiration appear to make a significant contribution to oxygen uptake in these intact soybean tissues. Oxygen isotope fractionation can therefore be used to measure the potential contribution of Δr in intact tissues in the absence of KCN and SHAM. Although this result does not obviate the presence of significant levels of residual oxygen uptake in other plants and tissues, questions raised about the problems that residual respiration may cause when measuring electron partitioning between the Cyt and alternative pathways in the absence of inhibitors using oxygen isotope fractionation (Wagner and Krab, 1995) can at least be addressed experimentally in intact tissues.

Regulation of Soybean Mitochondrial Electron Transport

Pyruvate, which acts as an activator of the alternative pathway (Millar et al., 1993), increases electron partitioning through the alternative pathway, in the absence of inhibitors, in green soybean cotyledon mitochondria under both state 3 and state 4 conditions (Ribas-Carbo, 1995a). The redox state of the alternative oxidase regulatory disulfide bond can also affect alternative oxidase activity, with the reduced form having a higher activity and being more activated by α-keto acids than the oxidized form (Umbach and Siedow, 1993; Umbach et al., 1994; Lennon et al., 1995). The alternative oxidase activity seen in isolated mitochondria has long been known to be a function of the amount of alternative oxidase protein present in the mitochondrial membrane (McIntosh, 1994). Finally, the possibility that the size of the ubiquinone pool can play a role in the regulation of the electron partitioning between the Cyt and alternative pathways has been suggested (Ribas-Carbo et al., 1994, 1995b). The regulation of electron transport between the Cyt and alternative pathways will be affected by each of these variables to a certain degree. In this study we used the oxygen isotope fractionation technique, combined with HPLC and immunoblotting, to ascertain the relative importance of each factor for controlling alternative pathway activity in mitochondria isolated from different soybean tissues.

Mitochondria isolated from soybean roots and etiolated and green cotyledons differ with respect to the size of their ubiquinone pools, the amount of alternative oxidase protein present, and the reduction state of the regulatory disulfide bond. Mitochondria isolated from green or etiolated cotyledons have a three- to four-fold larger ubiquinone pool than root mitochondria. The amount of alternative oxidase protein also differs among the three types of mitochondria, as described previously (Ribas-Carbo et al., 1994), with root mitochondria having the largest content of blottable protein, roughly twice the amount found in green cotyledons and eight times that found in etiolated cotyledons (Fig. 1A). In both green and etiolated cotyledon mitochondria two forms of the alternative oxidase were observed on immunoblots, but only a single band, aligned with the upper band in cotyledons, was observed in root mitochondria. The reduction state of the alternative oxidase regulatory disulfide bond also differs in the three mitochondrial types (Fig. 1B). A scheme representing the characteristics of these three mitochondrial types is presented in Figure 3.

In addition, added pyruvate and DTT can affect the control exerted by the ubiquinone pool and the redox state of the alternative oxidase regulatory disulfide bond, respectively. Any stimulation by pyruvate would support the ubiquinone pool controlling the mitochondrial flow to the alternative pathway to some extent. Likewise, because DTT reduces the regulatory disulfide bond (Fig. 2), any stimu-

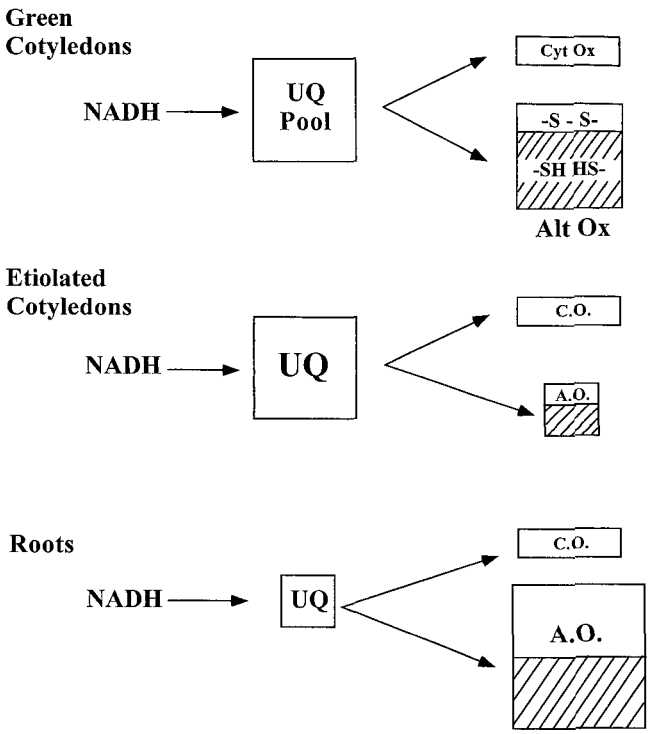

Figure 3. Diagrammatic representation of the size of the ubiquinone pool, amount of alternative oxidase protein, and the redox status of the regulatory disulfide bond system of each soybean mitochondrial type as isolated. Areas of the ubiquinone pool blocks represent the relative amount of total ubiquinone per milligram of mitochondrial protein measured by HPLC. Areas of the alternative oxidase blocks represent the relative amounts of alternative oxidase protein as measured by immunoblotting (Fig. 1). Dashed areas represent the percentage reduction of the alternative oxidase regulatory disulfide bond (Fig. 2). The areas of the Cyt *c* oxidase blocks have been arbitrarily set equal in the three mitochondrial types and are not meant to imply any quantitative relationship. Alt Ox, A.O., Alternative oxidase; Cyt Ox, C.O., Cyt *c* oxidase; UQ, ubiquinone.

lation of alternative oxidase activity by DTT will indicate that the original amount of alternative oxidase activity was exerting some control on electron flow.

Regulation of Electron Transport in Green Cotyledon Mitochondria

Under state 4 conditions and in the absence of pyruvate and DTT, only about 16% of the electrons go through the alternative pathway in green cotyledon mitochondria. Adding pyruvate increases this value to 49%, which is not enhanced by the subsequent addition of DTT. DTT alone also shows enhancement of electron flow to the alternative pathway (to 31%). Consequently, in green cotyledon mitochondria under state 4 conditions, the alternative pathway must be just poised to compete with the Cyt pathway, as any increase in alternative oxidase activity, either by enhanced reduction of the regulatory disulfide or increased reactivity with ubiquinol following pyruvate addition, shifts electrons to the alternative pathway (Fig. 3). Following modulation of alternative oxidase activity, the kinetics of each oxidizing pathway combined with those of the external NADH dehydrogenase (Wagner and Krab, 1995) will establish the actual electron partitioning and lead to a new steady-state redox poise for the ubiquinone pool. It is known that ubiquinone reduction decreases after addition of pyruvate either in the presence (Umbach et al., 1994) or absence (M. Ribas-Carbo, unpublished results) of cyanide. This decrease in reduced ubiquinone would lead to the observed decrease in Cyt pathway activity (Table II).

Regulation of Electron Transport in Etiolated Cotyledon Mitochondria

The major distinguishing feature of etiolated cotyledon mitochondria is the relatively low amount of blottable alternative oxidase protein present (Obenland et al., 1990; Ribas-Carbo et al., 1994; Fig. 1). Neither pyruvate nor DTT had any significant effect on alternative pathway activity (Table II), suggesting that the low total amount of alternative oxidase protein significantly limits the possibility of much competition between the alternative and Cyt pathways in these mitochondria (Fig. 3). A stimulation of the total respiration rate was observed with addition of both pyruvate and DTT, but its origin remains unknown.

Regulation of Electron Transport in Root Mitochondria

Mitochondria isolated from soybean roots have a level of blottable alternative oxidase protein that is significantly greater than that in mitochondria isolated from green cotyledons (Ribas-Carbo et al., 1994). However, this protein is present with a significant fraction of the regulatory disulfide bond in the oxidized state (Fig. 2). In addition, the ubiquinone pool is three times smaller in root mitochondria than in cotyledon mitochondria (per milligram mitochondrial protein). As with etiolated cotyledon mitochondria in state 4, root mitochondria in the absence of pyruvate and DTT showed no alternative pathway activity.

Pyruvate addition should modify the control that ubiquinone exerts on the alternative pathway because of its ability to reduce the apparent reactivity of the alternative oxidase for ubiquinol (Umbach et al., 1994). DTT addition will reduce the alternative oxidase regulatory disulfide-bond (Fig. 2), which should facilitate alternative pathway competition with the Cyt pathway. Adding either pyruvate or DTT alone did not allow the alternative pathway to compete with the Cyt pathway. However, with both DTT and pyruvate present, alternative pathway activity was markedly stimulated (Table II), suggesting two limiting features to alternative pathway activity in isolated root mitochondria. The low amount of ubiquinone limits the availability of substrate, and only in the presence of pyruvate is the affinity of the alternative oxidase for ubiquinol sufficient to overcome this limitation. However, the highly oxidized state of the regulatory disulfide bond limits the activity of the alternative oxidase even when pyruvate is present. Only when the regulatory sulfhydryl/disulfide system is poised primarily in

the reduced state by added DTT does the potential activity of the large amount of alternative oxidase protein present manifest itself, but even this requires the presence of pyruvate to overcome limitations inherent in the small ubiquinone pool.

The results of this study support the concept that electron partitioning between the Cyt and alternative pathways is subject to regulation by a number of different factors. In the three types of soybean mitochondria studied here, the total oxidase protein, the redox status of the alternative oxidase redox-active regulatory sulfhydryl/disulfide system, activation by α-keto acids, and the size of the endogenous ubiquinone pool all interact to different extents in different mitochondrial types to affect alternative pathway activity and its ability to compete for electrons with the Cyt pathway.

ACKNOWLEDGMENTS

We wish to thank Dr. T.E. Elthon for his generous gift of the AOA monoclonal antibodies. We also thank Dr. Xavier Aranda and Dr. Miquel Gonzalez-Meler for their helpful discussions during this work.

Received August 12, 1996; accepted December 4, 1996.

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