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Regulation of Alternative Oxidase Activity by Pyruvate in Soybean Mitochondria[1]

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The regulation of alternative oxidase activity by the effector pyruvate was investigated in soybean (*Glycine max* L.) mitochondria using developmental changes in roots and cotyledons to vary the respiratory capacity of the mitochondria. Rates of cyanide-insensitive oxygen uptake by soybean root mitochondria declined with seedling age. Immunologically detectable protein levels increased slightly with age, and mitochondria from younger, more active roots had less of the protein in the reduced form. Addition of pyruvate stimulated cyanide-insensitive respiration in root mitochondria, up to the same rate, regardless of seedling age. This stimulation was reversed rapidly upon removal of pyruvate, either by pelleting mitochondria (with succinate as substrate) or by adding lactate dehydrogenase with NADH as substrate. In mitochondria from cotyledons of the same seedlings, cyanide-insensitive NADH oxidation was less dependent on added pyruvate, partly due to intramitochondrial generation of pyruvate from endogenous substrates. Cyanide-insensitive oxygen uptake with succinate as substrate was greater than that with NADH, in both root and cotyledon mitochondria, but this difference became much less when an increase in external pH was used to inhibit intramitochondrial pyruvate production via malic enzyme. Malic enzyme activity in root mitochondria declined with seedling age. The results indicate that the activity of the alternative oxidase in soybean mitochondria is very dependent on the presence of pyruvate: differences in the generation of intramitochondrial pyruvate can explain differences in alternative oxidase activity between tissues and substrates, and some of the changes that occur during seedling development.

Plant mitochondria possess a branched electron transport chain with two terminal oxidases: Cyt oxidase and the alternative oxidase. The latter catalyzes the well-known phenomenon of cyanide-insensitive respiration, branching from the Cyt path at ubiquinone and consisting of one to three proteins between 32 and 39 kD, depending on the tissue (see Moore and Siedow, 1991, for a recent review).

The alternative oxidase is encoded by nuclear gene(s) (Rhoads and McIntosh, 1993) and has been extensively studied over the last decade. We now know that gene and/or protein expression can be induced by a number of stress conditions, such as slicing and aging in potato tubers (Hiser and McIntosh, 1990), cycloheximide treatment of cucumber cotyledons (Morohashi et al., 1991), cold treatment in tobacco and wheat (McCaig and Hill,, 1977; Vanlerberghe and McIntosh, 1992a), and poisoning of the Cyt chain in yeast (Minagawa and Yoshimoto, 1987) and tobacco (Vanlerberghe and McIntosh, 1992b; Wagner et al., 1992) cells. Ethylene treatment of fruits and storage tissues (Laties, 1982) and salicylic acid treatment of *Sauromatum gutattum* floral spadices (Rhoads and McIntosh, 1993) also induce expression of the oxidase. In some species, tissue-specific expression of the alternative oxidase precursor is seen (Kearns et al., 1992; Conley and Hanson, 1994). Apart from a role in thermogenesis of certain plant organs (Meusse, 1975), the general function of the alternative oxidase in plant metabolism remains unclear.

In mitochondria from nonthermogenic tissues, large variations are seen in alternative oxidase activity as substrate is varied (Lance et al., 1985). Maximum rates are usually seen with succinate, whereas NAD-linked substrates often give slower rates that correlate with a lower level of Q reduction (Day et al., 1991). Exogenous NADH, on the other hand, is poorly oxidized via the alternative oxidase even though it maintains QH_2 at levels seen with succinate, and exogenous quinols are barely oxidized at all (Day et al., 1991; Moore and Siedow, 1991). More recently, it has been shown that these differences can be abolished by the addition of certain organic acids: succinate in potato (Wagner et al., 1989; Lidén and Ackerlund, 1993) and pyruvate and its analogs in soybean (*Glycine max* L.) and some other species (Millar et al., 1993). In soybean roots, in particular, alternative oxidase activity is very slow upon isolation of mitochondria, and pyruvate stimulation can be very pronounced, even with succinate as substrate (Millar et al., 1993). The effect of these organic acids on alternative oxidase activity does not involve their metabolism and it was suggested that pyruvate is an allosteric activator of the oxidase (Millar et al., 1993).

Early studies with mitochondria from higher plants indicated that the activity of the alternative oxidase is governed largely by the activity of the Cyt path, so that it operates only when the Cyt chain is either saturated with reducing equivalents or inhibited (as in the absence of ADP; Bahr and Bonner, 1973). Measurements of Q redox state during respiration have shown that the alternative oxidase from most plants requires a high QH_2/Q ratio to become active (Moore et al., 1988; Dry et al., 1989; Day et al., 1991), and this response has been extensively modeled (Moore and Siedow,

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Abbreviations: LDH, lactate dehydrogenase; Q, ubiquinone; QH_2, ubiquinol.

1991; Siedow and Moore, 1993). The significance of this in vivo is that the alternative pathway acts as an overflow of the Cyt path and will be active only when substrate concentrations and/or adenylate energy charge are high (Lambers, 1985). However, more recent measurements of Q redox state have indicated that pyruvate changes the response of the alternative oxidase to QH_2/Q such that the oxidase is active at a lower ratio (Umbach et al., 1994). Furthermore, it has been shown that the redox state of the alternative oxidase protein itself influences its activity and its sensitivity to pyruvate (the oxidized, covalently linked dimer being inactive and unresponsive to pyruvate; Umbach and Siedow, 1993; Umbach et al., 1994).

In the present paper, the effect of pyruvate on alternative oxidase activity and the relationship between protein levels and activity is investigated further in soybean tissues. We demonstrate that intramitochondrial production of pyruvate during succinate and malate oxidation enhances alternative oxidase activity with these substrates and that pyruvate activation is readily reversible.

MATERIALS AND METHODS

Reagents

Percoll and low molecular mass standards for SDS-PAGE were purchased from Pharmacia Biochemicals, Inc. (Uppsala, Sweden). Other electrophoresis reagents were purchased from Bio-Rad and Sigma.

Plants

Soybean (*Glycine max* [L.] Merr. cv Bragg or Stevens) seeds were used. For cotyledons and younger roots (up to 12 d after planting), plants were propagated in a growth cabinet in trays of vermiculite; where possible, cotyledons and roots were harvested from the same tray. For older roots, plants were propagated in pots of sand and provided with Herridge's nutrient solution (Delves et al., 1986).

Mitochondrial Isolation

Mitochondria were isolated from cotyledons and roots by the method of Day et al. (1985). At all plant ages, entire roots were used and the material was partly homogenized with a mortar and pestle before using a Polytron blender to more fully disrupt the tissue.

Assays

Oxygen consumption was measured in a Rank Bros. (Cambridge, UK) electrode at 25°C in 2 mL of reaction medium (0.3 M Suc, 10 mM Tes buffer [pH 7.2 unless otherwise indicated], 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% [w/v] BSA). Medium pH was adjusted by adding HCl or KOH. NADH oxidation was measured spectrophotometrically as described by Day et al. (1991). The protein content of samples was estimated by the method of Lowry et al. (1951).

Electrophoresis and Immunological Probing

Up to 60 μg of mitochondrial proteins were solubilized in sample buffer (2% [w/v] SDS, 62.5 mM Tris [pH 6.8], 10% [v/v] glycerol, 0.002% [w/v] bromphenol blue, and 20 mM mercaptoethanol) and were boiled for 1 to 2 min. For visualization of the oxidized dimer of the alternative oxidase, mercaptoethanol was omitted from the sample preparation buffer. Electrophoresis (SDS-PAGE) was then carried out as described by Kearns et al. (1992). Antibodies against alternative oxidase proteins of *Sauromatum guttatum* were generously supplied by T.E. Elthon (University of Nebraska) and L. McIntosh (Michigan State University). A modified version of the method of Towbin et al. (1979) was employed for western blotting. Alternative oxidase bands were visualized using the Amersham ECL system.

RESULTS

Alternative Oxidase Protein versus Activity in Root Mitochondria

Alternative oxidase activity and protein levels in root mitochondria were measured during seedling development in soybean. Alternative path activity (Fig. 1: oxygen consumption in the presence of KCN) declined with root age, yet the quantity of immunologically detectable protein increased slightly (Fig. 2A). Cyt path activity (oxygen consumption in the presence of ADP) also declined with age in roots (Fig. 1), as was noted previously in bean and pea leaves (Azcon-Bieto et al., 1983) and barley roots (McDonnell and Farrar, 1992).

The relationship between activity and protein quantity was investigated further by examining the level of reduction of the alternative oxidase proteins. The alternative oxidase appears to exist in the membrane as a dimer that can be either noncovalently linked (reduced state) or covalently linked (oxidized state), the latter being much less active than the former (Umbach and Siedow, 1993). When reductant is omitted from electrophoretic sample buffer, higher molecular mass forms of the oxidase can be seen, corresponding to its oxidized state (Umbach and Siedow, 1993). In root mitochondria, the portion of oxidized alternative oxidase (about 70 kD) relative to reduced enzyme (36 kD) changed significantly with seedling age, but in an unexpected manner (Fig. 2A). Mitochondria from the youngest roots, which were the most active, had more of the oxidase protein in the oxidized than in the reduced form, whereas the older roots had about equal portions reduced and oxidized (Fig. 2A). Thus, the decline in alternative oxidase activity in roots shown in Figure 1 could not be attributed to increased oxidation of the enzyme itself.

At all ages, when reductant was included in SDS-PAGE sample buffer, only a single polypeptide was detected in root mitochondria by antibodies against alternative oxidase proteins, whereas two bands were consistently observed with cotyledon (and leaf) mitochondria (Fig. 2; see also Kearns et al., 1992). As in root mitochondria, some of the alternative oxidase protein in cotyledons was in the oxidized (higher molecular mass) form when reductant was omitted from the gel sample buffer (Fig. 2B). However, we have been unable to find a clear correlation between rates of cyanide-insensitive

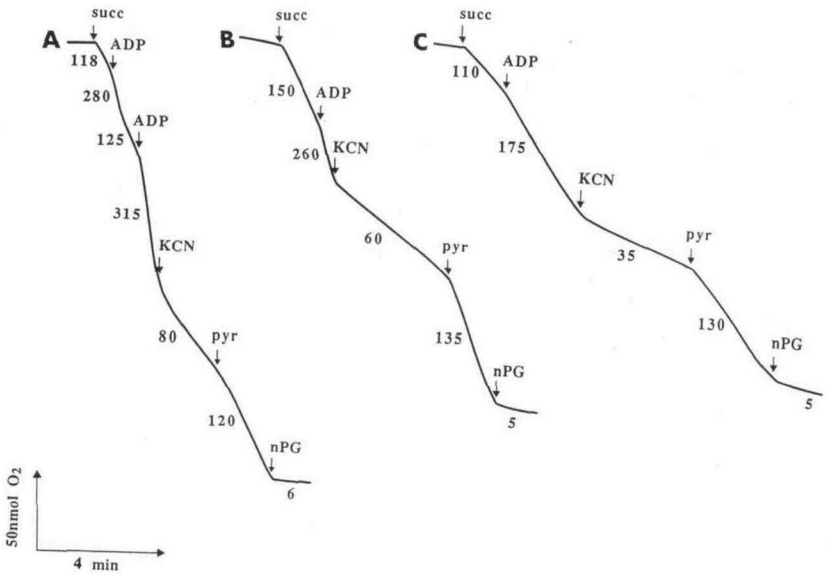

Figure 1. Effect of seedling age on respiration by isolated soybean root mitochondria. Oxygen consumption was measured as described in "Materials and Methods" with 0.1 mM ATP in standard reaction medium. As indicated, 10 mM succinate (succ), 0.5 mM KCN, 0.5 mM pyruvate (pyr), 1 mM ADP, and 0.2 mM n-propyl gallate (nPG) were added. Numbers on traces represent nmol oxygen min^{-1} mg^{-1} protein. A, Three-day-old plants (0.35 mg of mitochondrial protein); B, 5-d-old plants (0.30 mg of mitochondrial protein); C, 7-d-old plants (0.20 mg of mitochondrial protein).

oxygen uptake and the proportion of oxidase protein in the reduced or oxidized state in a large number of preparations from seedlings of different ages (results not shown).

Our results suggest that either the activity of the oxidase in the reduced form varies with development of a given tissue or that the endogenously oxidized form of the oxidase, unlike that generated by diamide treatment (Umbach and Siedow, 1993), has some activity. For example, it is possible that endogenous oxidation involves fewer disulfide linkages than does oxidation by external reagents. Variation in the activity of the reduced enzyme could be caused by variations in other electron transport parameters, such as succinate dehydrogenase activity, amount of quinol in the membrane, and the ratio of quinol to quinone. Consequently, younger roots may need to have less alternative oxidase in the reduced form to achieve the same activity.

In the absence of reductant, an additional band became evident in root mitochondria at 34 kD (Fig. 2A), a size similar to that of the lower band in cotyledon mitochondria. This "extra band" was also observed by Umbach and Siedow (1993), who attributed it to an electrophoresis artifact induced by omitting reductant from the sample preparation buffer, and in this context it should be noted that the dimerized protein in root mitochondria shows only a single band compared to two bands in the cotyledon organelles (Fig. 2B; Umbach and Siedow 1993).

Pyruvate Activation of the Alternative Oxidase

Alternative oxidase activity in root mitochondria was stimulated to approximately the same value at all ages by addition of pyruvate (Fig. 1). That is, the capacity of the alternative oxidase did not change but its dependency on pyruvate became more pronounced as the plants became older. This was also seen with NADH as substrate. In even older roots (up to 60 d after planting), cyanide-insensitive oxygen uptake was almost completely dependent on pyruvate addition (not shown). The stimulation by pyruvate has been described previously and attributed to an allosteric activation of the oxidase, since it does not involve pyruvate metabolism by mitochondria (Millar et al., 1993).

Adding LDH to the reaction medium during NADH oxidation reversed the stimulation by removing the pyruvate (reducing it to lactate); adding more pyruvate stimulated it again (Fig. 3A). Depending on the amount of NADH and pyruvate added, this cycle of stimulation-inhibition-stimulation could be repeated (not shown). Control assays showed that lactate had no effect on the alternative oxidase, nor did LDH prevent the stimulation of cyanide-insensitive NADH oxidation by glyoxylate (Fig. 3B; see Umbach et al., 1994).

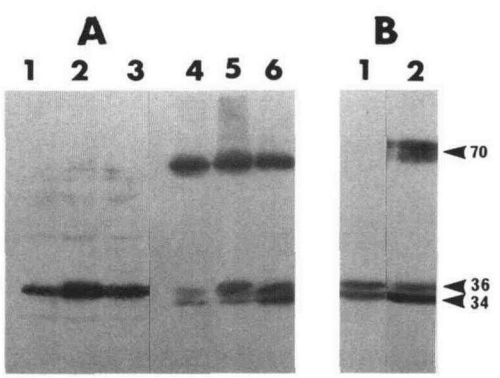

Figure 2. Immunoblots of soybean root and cotyledon proteins using antibodies raised against alternative oxidase proteins. Arrows indicate estimated molecular masses of the bands in kD. A, Root mitochondria; lanes 1 to 3, mitochondria from 3-, 5-, and 7-d-old roots, respectively (samples reduced with mercaptoethanol); lanes 4 to 6, mitochondria from 3-, 5-, and 7-d-old roots, respectively (reductant omitted from sample preparation). B, Cotyledon mitochondria; lane 1, mitochondria from 7-d-old cotyledons (samples reduced with mercaptoethanol); lane 2, mitochondria from 7-d-old cotyledons (reductant omitted from sample preparation). In all cases, 50 μg of protein was loaded per lane.

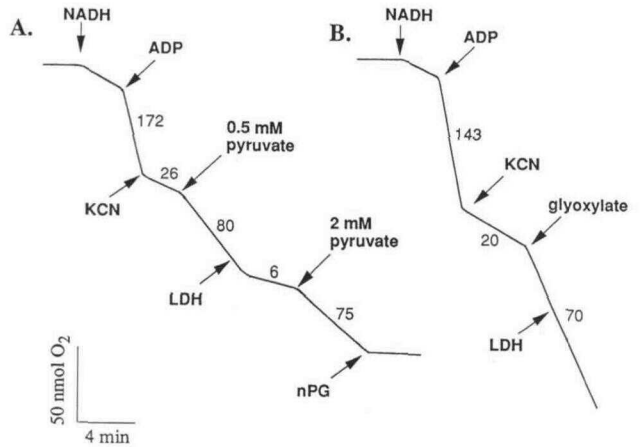

Figure 3. Effect of pyruvate on NADH oxidation by soybean root mitochondria. Mitochondria were purified from the roots of 14-d-old plants and oxygen consumption was measured as described in "Materials and Methods." Where indicated, the following additions were made: 1 mM NADH, 0.5 mM ADP, 1 mM KCN, 10 units of LDH, 100 μM *n*-propyl gallate (nPG), and 0.5 mM glyoxylate. Numbers on traces represent nmol oxygen min^{-1} mg^{-1} protein. A, 0.4 mg of mitochondrial protein was used; B, 0.8 mg of mitochondrial protein was used.

Table I. *Effect of pyruvate on cyanide-insensitive NADH and succinate oxidation by isolated soybean cotyledon mitochondria*

Oxygen uptake was measured as described in "Materials and Methods" in the presence of 2 mM NADH, 1 mM ADP, 1 mM KCN, 0.5 mM pyruvate, 10 units of LDH, and 50 μM *n*-propyl gallate, as indicated.

Sequential Additions	Oxygen Uptake		
	5-d-old cotyledons		9-d-old cotyledons
	Succinate	NADH	NADH
	nmol min^{-1} mg^{-1} protein		
Experiment A			
Substrate + ADP	100	122	140
KCN	55	33	70
Pyruvate	55	56	90
LDH	55	24	35
Pyruvate		48	85
n-Propyl gallate	0	0	0
Experiment B			
Substrate + ADP	95	130	135
KCN	55	42	85
LDH	55	28	40
Pyruvate	55	50	80
n-Propyl gallate	0	0	0

Similar results were obtained with mitochondria from younger roots. These experiments demonstrate that inactivation can occur while the oxidase is functioning and under conditions in which the redox state of Q is highly reduced (Day et al., 1991). Other experiments, in which mitochondria activated by pyruvate were "washed" by centrifugation, confirmed that the pyruvate effect was readily reversible with succinate as substrate in root mitochondria (Fig. 4).

In mitochondria from cotyledons, cyanide-insensitive succinate oxidation was rapid in the absence of added pyruvate and was not stimulated by its addition (Table I). Cyanide-insensitive NADH oxidation by mitochondria from cotyledons increases with seedling age, becoming substantial at about 1 week after planting (Azcon-Bieto et al., 1989); nonetheless, this NADH oxidation was less than that with succinate and was stimulated by pyruvate (Table I). Adding LDH to these mitochondria reversed the pyruvate stimulation, but the final rate of oxygen uptake was slower than that prior to pyruvate addition (Table I). Addition of LDH alone to these cotyledon mitochondria inhibited cyanide-insensitve oxygen uptake (with NADH as substrate) and subsequent addition of pyruvate stimulated it (Table I). It should be noted that LDH alone had no effect on succinate oxidation (Table I) and lactate did not affect oxygen uptake with any substrate (results not shown), suggesting that LDH acted to remove endogenous pyruvate. However, even in the presence of LDH significant cyanide-insensitive oxygen uptake occurred with NADH as substrate, and this activity increased with seedling age (Table I).

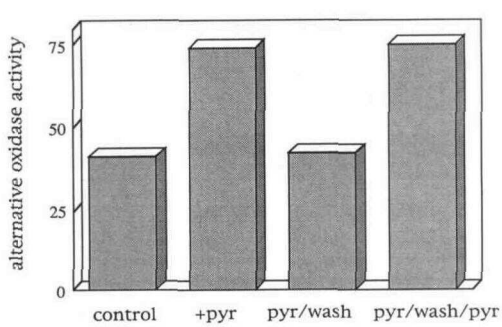

Figure 4. Reversal of pyruvate stimulation of cyanide-insensitive succinate oxidation by soybean root mitochondria. Mitochondria were isolated from 1-week-old seedlings. Oxygen uptake was measured in the presence of 10 mM succinate, 0.1 mM ATP, and 1 mM KCN. Control, No other treatment; +pyr, mitochondria were incubated with 0.5 mM pyruvate; pyr/wash, mitochondria were incubated with pyruvate and then pelleted and resuspended in reaction medium, and oxygen uptake was measured as above; pyr/wash/pyr, 0.5 mM pyruvate was added to washed mitochondria. Rates shown are in nmol min^{-1}.

In root mitochondria, on the other hand, LDH inhibition was much more severe, even when young roots were used as the mitochondrial source (Fig. 3), suggesting that the alternative oxidase in root mitochondria is almost completely dependent on pyruvate. In cotyledons the oxidase appears to be partly activated in the absence of pyruvate, assuming that LDH is as effective at removing endogenous pyruvate in cotyledon mitochondria as it is in roots. It should also be noted that oxoglutarate can activate the alternative oxidase (Millar et al., 1993), and it is possible that cotyledon mitochondria contain significant quantities of this acid.

Pyruvate Generated Intramitochondrially Can Activate Alternative Oxidase

The above results indicate that pyruvate produced inside the mitochondria can activate the alternative oxidase. This

was confirmed by the effect of pH on alternative oxidase activity (Table II). Malate oxidation at pH 6.5, when malic enzyme is active and producing pyruvate (Macrae, 1971; Tobin et al., 1980), was quite resistant to KCN, and adding pyruvate had little effect. At pH 7.5, when malate oxidation occurs largely via malate dehydrogenase and little or no pyruvate is produced (Macrae, 1971; Tobin et al., 1980), oxygen uptake was severely inhibited by KCN, and adding pyruvate relieved the inhibition (Table II). Changing the pH of the reaction medium had a small effect on external NADH and very little effect on quinol oxidation by the mitochondria (Table II), suggesting that in intact mitochondria the alternative oxidase per se was not very sensitive to pH over the range tested. Cyanide-insensitive succinate oxidation, on the other hand, was faster at pH 6.5 than at 7.5 (Table II), suggesting that some pyruvate can be produced intramitochondrially during succinate oxidation when malic enzyme is active (see Douce et al., 1986). This intramitochondrial pyruvate production may account for the differences observed in alternative oxidase activity between succinate and NADH as substrates at neutral pH. At alkaline pH, cyanide-insensitive succinate oxidation was much less and closer to rates seen with NADH as substrate (Table II). These results were seen consistently in root mitochondria. Similar results were seen with cotyledon mitochondria, although the effect of alkaline pH was less marked (not shown). This is consistent with the lower dependence of alternative oxidase on pyruvate in cotyledons.

DISCUSSION

Dependency of Alternative Oxidase on Pyruvate

The results presented show that in soybean, alternative oxidase activity is dependent largely on the provision of pyruvate, either intra- or extramitochondrially. The ability of soybean mitochondria to produce pyruvate endogenously varies with seedling age and the tissue studied. In roots, alternative oxidase activity becomes increasingly dependent on pyruvate added with age, suggesting that intramitochondrial pyruvate production decreases. In this context, it should be noted that malic enzyme activity also declined with age (e.g. in the mitochondria used in the experiment shown in Fig. 1, in the youngest roots malic enzyme activity was 90 nmol NADH min^{-1} mg^{-1} protein, whereas in the oldest it was 40 nmol NADH min^{-1} mg^{-1} protein). Malic enzyme activity is also much higher in cotyledons than in roots (Day and Mannix, 1988), and in cotyledon mitochondria alternative oxidase activity is less dependent on added pyruvate. The inhibition by LDH (Table I) indicates that even when NADH is substrate, some endogenous pyruvate production occurs in cotyledon mitochondria. In this context, it should be noted that significant, albeit slow, endogenous oxygen uptake was observed in cotyledon mitochondria, especially when NAD^+ was added (which also occurs when NADH is oxidized), indicating that a pool of organic acids may be maintained in the matrix in these mitochondria.

NADH oxidation was able to support cyanide-insensitive oxygen uptake in cotyledon mitochondria, even when LDH was added to remove any endogenous pyruvate (Table I). The root alternative oxidase, on the other hand, appeared to be completely dependent on pyruvate, since LDH inhibited cyanide-insensitive respiration severely in mitochondria isolated from this tissue (Fig. 3). There are several possible explanations for this difference.

(a) It may reflect a difference in mitochondrial permeability to pyruvate between the two organs, such that in cotyledon mitochondria external LDH cannot remove all of the matrix pyruvate.

(b) Since some other organic acids, such as oxoglutarate, are also able to activate the alternative oxidase (Millar et al., 1993), the difference could simply be due to greater endogenous tricarboxylic acid cycle turnover in isolated cotyledon mitochondria (see above).

(c) Alternatively, it is possible that differences between tissues reflect differences in the alternative oxidase enzyme itself. For example, the lower molecular mass alternative oxidase protein seen on immunoblots of cotyledon mitochondria is absent in root mitochondria and may behave differently from the higher molecular mass protein. However, although we have observed variation in the relative intensity of the two bands between different preparations from cotyledons (see Obenland et al., 1990), these have not been correlated with the degree of alternative oxidase activity in the absence of pyruvate (results not shown).

(d) It is also possible that differences in substrate dehydrogenase activity, Q redox status, and Q concentration exist between roots and cotyledons, such that alternative oxidase in the latter is less dependent on organic acid activators. In this context, it should be noted that pyruvate affects the interaction between quinone and the alternative oxidase so that the oxidase is active at a lower QH_2/Q ratio (Umbach et al., 1994), and the kinetic model of Siedow and Moore (1993) suggests that this is due to an increased affinity of the oxidase for quinol.

Whether these differences observed with isolated mito-

Table II. *Effect of external pH on the rate of cyanide-insensitive respiration by soybean root mitochondria*

Oxygen uptake was measured as described in "Materials and Methods" in the presence of 1 mM ADP, 1 mM KCN, and 0.5 mM pyruvate, as indicated. Malate and succinate were added at 10 mM, NADH at 1 mM, and duroquinol at 0.1 mM. For malate oxidation, 0.1 mM thiamine pyrophosphate and 5 mM glutamate were present to prevent accumulation of oxaloacetate. Oxygen uptake in the presence of KCN was completely inhibited by 50 μM *n*-propyl gallate in all cases. The nonenzymic rate of duroquinol oxidation at alkaline pH was subtracted from rates of oxygen uptake.

Sequential Additions	Oxygen Uptake			
	Malate	Succinate	NADH	Duroquinol
	nmol min^{-1} mg^{-1} protein			
A, pH 6.5				
ADP	125	184	257	117
KCN	52	60	18	0
Pyruvate	60	60	77	30
B, pH 7.5				
ADP	105	240	294	147
KCN	13	15	6	5
Pyruvate	65	60	60	50

chondria occur in vivo is not known, but it is obvious that care must be taken when assessing alternative oxidase capacity in mitochondria isolated from different tissues.

Site of Pyruvate Activation

The ability of pyruvate produced intramitochondrially to stimulate cyanide-insensitive respiration raises the question of pyruvate's site of action. The results presented here suggest that the site of action may be within the matrix. In a typical experiment measuring succinate oxidation by cyanide-resistant cotyledon mitochondria, it was estimated that about 10 nmol of pyruvate was produced over the first 5 min of oxidation. If this was diluted into the external medium of 2 mL, the concentration would have been 5 μM, too low to cause significant stimulation of the oxidase (Millar et al., 1993); however, if retained within the matrix, the concentration would have been about 5 mM (assuming a mitochondrial volume of 2 μL mg^{-1}; Hanson and Day, 1980). In further experiments, the half-time of pyruvate stimulation of cyanide-insensitive NADH oxidation by root mitochondria, measured spectrophotometrically at fast chart speeds, was estimated to be about 40 s. This rather slow rate of activation is also consistent with an intramitochondrial site of pyruvate action. The inability of the moncarboxylate transport inhibitor hydroxy-cyanocinnamic acid to prevent stimulation of alternative oxidase by added pyruvate in root mitochondria led us previously to postulate that pyruvate acted allosterically from the outside of the inner membrane (Millar et al., 1993). However, this inhibitor does not prevent entry of pyruvate completely into plant mitochondria (Day and Hanson, 1977), and the experiments of Millar et al. (1993) were conducted under steady-state conditions. Thus, small quantities of pyruvate may still have penetrated the inner membrane.

Differences between Substrates

One of the most important conclusions from the results presented is that the often observed differences between substrates in their ability to support alternative oxidase activity in plant mitochondria are due largely to their different abilities to produce pyruvate endogenously. Thus, malate and succinate, which can produce pyruvate by their oxidation in the mitochondrial matrix, can support rapid rates of cyanide-insensitive respiration, but external NADH and quinols cannot.

Implications for In Vivo Regulation

The rapid reversal of the pyruvate stimulation of alternative oxidase, especially in root mitochondria, indicates that continual pyruvate production is required in vivo to keep the alternative oxidase active, at least in some tissues. This provides the plant cell with a powerful feed-forward regulatory mechanism to control the diversion of electrons to the nonphosphorylating alternative pathway and adds a new dimension to the "overflow" nature of the pathway (Lambers, 1985). Only when pyruvate supply from glycolysis is generous will the pathway be activated (Millar et al., 1993). On the other hand, intramitochondrial generation of even small quantities of pyruvate from malate will activate the oxidase, perhaps explaining previous observations of a correlation between cyanide-insensitive respiration and malic enzyme activity (Rustin et al., 1980).

The effects of pyruvate are superimposed on the effects of protein modification (oxidation/reduction) of the oxidase itself (Umbach and Siedow, 1993), and both need to be considered when investigating alternative oxidase capacity and activity in intact tissues. In isolated organelles, intramitochondrial production of pyruvate must also be considered.

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