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Organic acid activation of the alternative oxidase of plant mitochondria

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Alternative oxidase activity (oxygen uptake in the presence of KCN, antimycin or myxothiazol) in mitochondria isolated from the roots of soybean seedlings was very slow, even with succinate as substrate. This activity was stimulated substantially (100–400%) by the addition of pyruvate, with half maximal stimulation occurring at 0.1 mM pyruvate. Mitochondria from soybean shoots displayed high alternative oxidase activity with succinate and malate as substrates but lower activity with exogenous NADH; addition of pyruvate stimulated the activity with NADH up to that seen with succinate. This stimulation of cyanide-insensitive NADH oxidation was seen also with mitochondria from other species. Hydroxypyruvate and oxoglutarate could substitute for pyruvate, although higher concentrations were required to achieve maximum stimulation. Pyruvate stimulation of cyanide-insensitive oxygen uptake was observed with exogenous quinols as substrates, with sub-mitochondrial particles, and in the presence of the pyruvate transport inhibitor, cyanohydroxycinnamic acid, but was not observed with detergent-solubilised mitochondria. It is suggested that pyruvate acts allosterically on the alternative oxidase to stimulate its activity. The implications of these findings for respiration in vivo are discussed.

Alternative oxidase; Plant mitochondrion; Cyanide-insensitive respiration; Pyruvate

1. INTRODUCTION

The alternative oxidase of the plant respiratory chain catalyses cyanide-insensitive oxygen consumption, branching from the main electron transport chain at the level of ubiquinone [1]. The partially purified oxidase from thermogenic floral appendages acts as a cyanide-insensitive, hydroxamic acid-sensitive quinol oxidase [2]. In intact mitochondria, its activity depends on a high level of reduction of the ubiquinone pool [3], and as such it acts as an overflow of the cytochrome chain, only operating when the cytochromes are either inhibited or saturated with reducing equivalents [4].

Expression and activity of the alternative oxidase within a plant is often tissue specific and, in non-thermogenic tissues, its activity depends on the substrate being oxidised. For example, in most tissues, activity is substantially greater with succinate or malate as substrate than with external NADH as substrate [5], even though NADH oxidation via the cytochrome path is rapid and reduces the Q pool very strongly [6]. Furthermore, exogenous quinols are poor substrates for the alternative oxidase in non-thermogenic plants [1,6]. Explanations for these observations have included compartmentation of ubiquinone [5], different reaction path lengths [7] and segregation of respiratory complexes [6]. However, it has recently been demonstrated that reaction conditions can influence alternative oxidase activity greatly, with succinate able to stimulate activity with NADH (even in the presence of malonate) and low temperature stimulating QH_2 oxidation [8].

Here we report that alternative oxidase activity in various plant tissues is stimulated or activated by certain organic acids, principally pyruvate. This activation is very pronounced in soybean root mitochondria where the oxidase shows little activity with all substrates, particularly when older roots are used. It is also seen in other plant tissues, such as soybean cotyledons, where external NADH oxidation via the alternative oxidase is stimulated by pyruvate. These results point to a hitherto unknown regulatory mechanism for the alternative oxidase which may have important implications for its regulation in vivo.

2. MATERIALS AND METHODS

Soybeans (*Glycine max* [L.] Merr., *cv.* Bragg or Stevens) were grown in trays of vermiculite in glasshouses or growth cabinets, as described previously [9–11]. Mitochondria were isolated and purified from cotyledons by the method of [10], and from roots as described by [11]. Inside-out submitochondrial particles were generated by the method of [12]. Membranes were solubilised with 0.5% BIG-CHAPS detergent and alternative oxidase assayed as described in [2].

Oxygen consumption was measured using a Rank Bros (Cambridge, UK) electrode at 25°C in 2 ml of standard reaction medium (0.3 M sucrose, 10 mM TES buffer, 5 mM KH_2PO_4, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% (w/v) BSA, pH 7.2). The redox state of ubiquinone was measured voltametrically with glassy carbon and platinum electrodes in a specially constructed oxygen electrode vessel containing standard reaction supplemented with 1 μM Q-1, as described in [13]. NADH oxidation was followed spectrophotometrically at 340 nm [6] and protein was measured according to [14].

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3. RESULTS

The level of alternative oxidase in soybean varies dramatically from tissue to tissue, with the enzyme absent from the mitochondria of infected nodule cells but prominent in cotyledons as two distinct polypeptides [9]. In isolated cotyledon mitochondria, alternative oxidase activity may be up to 70% of that of the cytochrome path with succinate as substrate [10]. Mitochondria from very young roots (2–3 cm long) also possess an active alternative oxidase [11], although only a single protein can be detected immunologically [9].

Mitochondria from older roots, on the other hand, showed very little cyanide-insensitive oxygen uptake with succinate, malate + glutamate and NADH as substrates (Table IA), despite having relatively large quantities of the alternative oxidase protein as judged by Western blotting (not shown, see [9]). Yet with malate + pyruvate as substrates, cyanide-insensitive oxygen uptake by root mitochondria was similar to that seen with cotyledon mitochondria (Table I). Addition of pyruvate to root mitochondria after KCN, antimycin or myxothiazol, rapidly stimulated oxygen uptake with all substrates (Table IA). Likewise, addition of pyruvate to cotyledon mitochondria oxidising NADH in the presence of cytochrome inhibitors, stimulated oxygen uptake up to the rate usually seen with succinate as substrate (Table IB). Absorbance measurements at 340 nm confirmed that the stimulation of oxygen uptake seen upon addition of pyruvate in the presence of KCN was due to a stimulation of NADH oxidation (Table II).

Table I

Effect of pyruvate on alternative oxidase activity in soybean root and cotyledon mitochondria

Substrate	Oxygen consumption (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein)[a]			
	Control	+KCN[b]	+KCN and pyr	+KCN, pyr and nPG[c]
A. Root mitochondria				
Succinate	210	8	50	8
Malate + glu	100	8	30	8
NADH	235	10	45	–
Malate + pyr	110	58	58	5
Duroquinol	115	13	57	–
B. Cotyledon mitochondria				
Succinate	120	40	42	5
NADH	150	28	45	5
$Q\text{-}1H_2$	64	6	23	–

[a] Oxygen uptake was measured as described in section 2 in the presence of ADP, with substrates added at the following concentrations: malate, glutamate (glu), succinate all at 10 mM; 1 mM NADH; 0.25 mM duroquinol; 50 μM $Q\text{-}1H_2$. Pyruvate (pyr) was added at 5 mM. Representative experiments from among many are shown.

[b] KCN added at 0.5 mM, but similar results were seen with 5 μM myxothiazol or antimycin A.

[c] *n*-Propyl gallate added at 50 μM.

Concurrent measurements of quinone reduction state and oxygen uptake indicated that the stimulation of respiration in the presence of myxothiazol, by pyruvate, was accompanied by a small but reproducible oxidation of the Q pool (Fig. 1A), consistent with activation of the alternative oxidase. There was very little oxygen uptake observed when pyruvate was added alone to either root or cotyledon mitochondria, in the presence or absence of myxothiazol (Fig. 1B).

Fig. 2 shows the concentration dependence of pyruvate-induced alternative oxidase stimulation: the concentration of pyruvate required for half-maximal stimulation was about 0.1 mM, in both the presence and absence of the pyruvate transport inhibitor cyanocinnamic acid. This suggests that the site of pyruvate action may be external to the inner membrane. Hydroxy-pyruvate also stimulated alternative oxidase activity, although a higher concentration was required to see a maximal effect (not shown). The only other compound tested which was found to stimulate the alternative oxidase in soybean mitochondria was oxoglutarate, which was less effective and required 5 mM concentrations to stimulate maximally (data not shown). Compounds tested without effect included glutamate, citrate, phosphoenolpyruvate, mercaptoethanol and dithiothreitol.

Pyruvate stimulation of cyanide-insensitive oxygen uptake was observed with $Q\text{-}1H_2$ or duroquinol as substrates in intact mitochondria (Table I) but not with detergent-solubilised membranes (not shown). Pyruvate stimulation of succinate oxidation was also seen in submitochondrial particles but with a slight lag after pyruvate addition; in intact mitochondria, pyruvate stimulated within seconds. These results are consistent with a site of action on the outside of the inner membrane.

4. DISCUSSION

Differences between the degree of alternative oxidase activity with different substrates have been observed often in the past in a wide range of plant tissues [1,5,6,8]. In some instances, the difference can be explained by the ability of substrate dehydrogenases to keep the Q pool reduced enough to fully engage the alternative oxidase

Table II

NADH oxidation by soybean root mitochondria[a]

Sequential additions	nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$	nmol NADH $\cdot min^{-1} \cdot mg^{-1}$
NADH	235	
Myxothiazol	12	20
Pyruvate	45	85

Measured as described in section 2. NADH was added at 0.1 mM, pyruvate at 5 mM and myxothiazol at 5 μM; ADP was present at all times.

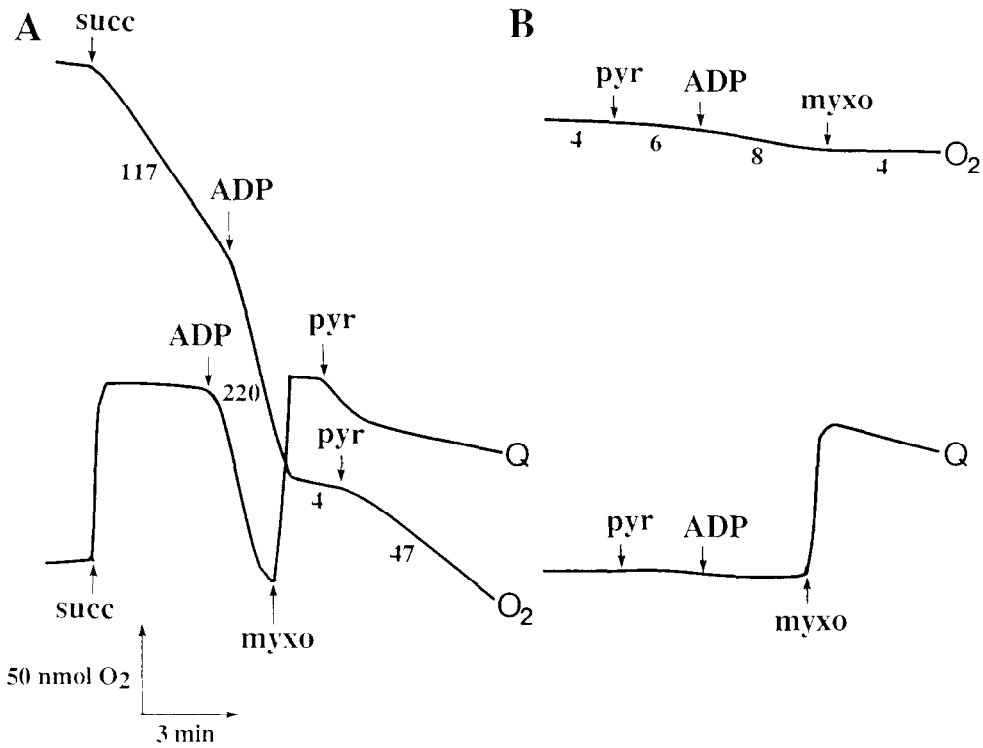

Fig. 1. Alternative oxidase stimulation by pyruvate in isolated soybean root mitochondria. Oxygen consumption and ubiquinone reduction by mitochondria isolated from 1 week-old roots were measured simultaneously as described in section 2. Where indicated, 10 mM succinate (succ), 5 mM pyruvate (pyr), 5 μM myxothiazol (myxo), and 0.1 mM ADP were added. ATP (0.1 mM) was included in the reaction medium to activate succinate dehydrogenase. Q redox level is represented by the bottom recording, with an upward deflection indicating reduction. Numbers on traces are nmol O_2 $min^{-1} \cdot mg^{-1}$ protein.

[3,6]. External NADH oxidation, on the other hand, is very rapid and maintains a highly reduced Q pool [6], yet NADH oxidation is often substantially more severely inhibited by cyanide (and other cytochrome chain inhibitors) than is succinate oxidation. We show here that this difference between NADH and succinate can be eliminated if pyruvate is included in the reaction medium, even under conditions where the pyruvate is not significantly metabolised. Likewise, non-thermogenic plant tissues show virtually no alternative oxidase activity with exogenous quinols under standard reaction conditions [1,6], unless pyruvate is added (Table I). Changes in reaction temperature can stimulate quinol oxidation in potato mitochondria, and succinate also stimulates cyanide-insensitive NADH oxidation under conditions which prevent succinate metabolism [8]. These results suggest that the differences in the rates of cyanide-insensitive respiration between substrates are due to a difference in activation state of the enzyme. Apparently the oxidase is more highly activated with succinate and malate than with exogenous NADH and quinols in soybean cotyledon and potato mitochondria. We have also confirmed this in mitochondria from the leaves of broad bean, which have high levels of cyanide-insensitive respiration (Millar and Day, unpublished results). However, this clearly is not the case in soybean root mitochondria where the alternative oxidase was inactive with all substrates until pyruvate was added (Table IA).

The reason for this difference between root and shoot mitochondria in soybean is not obvious. In roots, the degree of activity seen with isolated mitochondria depended on the physiological age of the plants, being much greater in younger roots, even though the level of immunologically detectable protein did not vary much (results not shown). This suggests that the activation state of the alternative oxidase may reflect the metabolic activity of the tissue prior to mitochondrial isolation, at least in soybean. Some other species which show little or no cyanide-insensitive respiration lack alternative oxidase protein(s) [1,9,15]. For example, mitochondria from pea leaves, which have little cyanide-insensitive respiration, showed only a small stimulation of succinate oxidation by pyruvate in the presence of KCN (results not shown). Soybean roots may therefore be an exception. Nonetheless, it is important that the activation state of the alternative oxidase is taken into account when assessing the enzyme's capacity in any given tissue.

The mechanism by which pyruvate and its analogues stimulate alternative oxidase activity is not clear, but the results indicate that the effect does not involve pyruvate

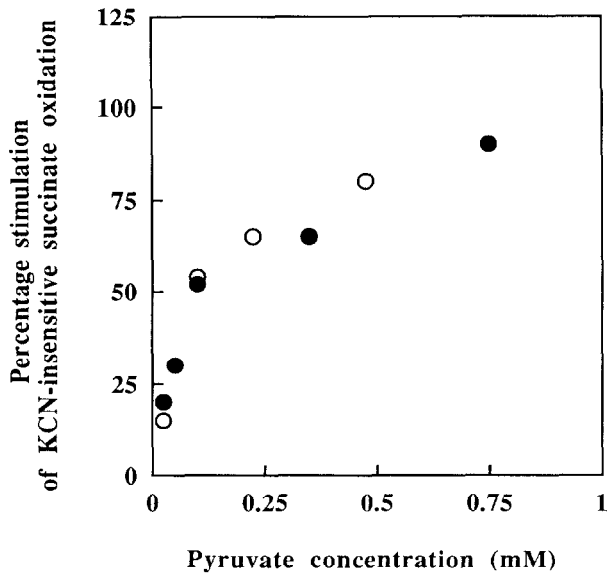

Fig. 2. Effect of pyruvate concentration on the stimulation of alternative oxidase activity in soybean root mitochondria. Oxygen uptake was measured as described in section 2, with 10 mM succinate as substrate in the presence of ADP, 0.5 mM KCN was added to inhibit cytochrome oxidase, followed by 5 mM pyruvate a few minutes later. The stimulated rate was inhibited by *n*-propyl gallate (50 μM). ●, control; ○, plus 0.2 mM cyanohydroxycinnamic acid in the reaction medium.

or oxoglutarate oxidation, or indeed other soluble factors. The effect may occur on the outside of the inner membrane, and in this context it should be noted that the predicted alternative oxidase structure indicates that it is a membrane-spanning protein with a small portion exposed to the intermembrane space [16,17]. It is possible that activation may involve binding of the effectors to this part of the protein, resulting in a conformational change in, and consequent stimulation of, activity. On the basis of the observed stimulation of QH_2 and NADH oxidation, pyruvate seems to alter the manner in which the oxidase interacts with the Q pool of the respiratory chain, and it is essential that the relationship between QH_2/Q ratio and alternative oxidase activity [1,3] is re-assessed with a fully activated system.

Whatever the mechanism, the results may have significance for the regulation of respiration in vivo. Since the alternative oxidase acts essentially as an overflow of the cytochrome path, it needs to be most active when substrate flux to the mitochondrion is greatest, and accumulation of pyruvate, either from glycolytic activity or the operation of malic enzyme within the mitochondrion, would feed-forward to ensure coordination of alternative oxidase activity with the rate of organic acid production.

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