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Regulation of Alternative Pathway Activity in Plant Mitochondria[1,2]

Deviations from Q-Pool Behavior during Oxidation of NADH and Quinols

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ABSTRACT

External NADH and succinate were oxidized at similar rates by soybean (*Glycine max*) cotyledon and leaf mitochondria when the cytochrome chain was operating, but the rate of NADH oxidation via the alternative oxidase was only half that of succinate. However, measurements of the redox poise of the endogenous quinone pool and reduction of added quinones revealed that external NADH reduced them to the same, or greater, extent than did succinate. A kinetic analysis of the relationship between alternative oxidase activity and the redox state of ubiquinone indicated that the degree of ubiquinone reduction during external NADH oxidation was sufficient to fully engage the alternative oxidase. Measurements of NADH oxidation in the presence of succinate showed that the two substrates competed for cytochrome chain activity but not for alternative oxidase activity. Both reduced Q-1 and duroquinone were readily oxidized by the cytochrome oxidase pathway but only slowly by the alternative oxidase pathway in soybean mitochondria. In mitochondria isolated from the thermogenic spadix of *Philodendron selloum*, on the other hand, quinol oxidation via the alternative oxidase was relatively rapid; in these mitochondria, external NADH was also oxidized readily by the alternative oxidase. Antibodies raised against alternative oxidase proteins from *Sauromatum guttatum* cross-reacted with proteins of similar molecular size from soybean mitochondria, indicating similarities between the two alternative oxidases. However, it appears that the organization of the respiratory chain in soybean is different, and we suggest that some segregation of electron transport chain components may exist in mitochondria from nonthermogenic plant tissues.

Plant mitochondria possess a branched respiratory chain with two terminal oxidases: Cyt oxidase and the cyanide-insensitive, alternative oxidase. The latter is a major component of the respiratory chain of thermogenic plant tissues in which it is implicated in heat production, and mitochondria isolated from these tissues show little or no respiratory control (18). However, alternative oxidase activity has been found in one or other tissues of all plants so far examined, although it varies and the oxidase may be a relatively minor respiratory chain component. Its function in nonthermogenic tissues is unknown, but, because it is nonphosphorylating, it has the potential to influence plant performance (17).

The Cyt and alternative paths branch from each other at the ubiquinone (UQ[4]) step of the respiratory chain, but display very different kinetic behavior in response to UQH_2 levels. In particular, while Cyt chain activity varies linearly with Q_r/Q_t, that of the alternative oxidase does not become detectable until the Q pool is almost 50% reduced (7), explaining why the alternative path is to a large extent an "overflow" of the Cyt chain (2); UQH_2 levels only become high enough to activate the alternative oxidase when substrate input to the respiratory chain is high and Cyt path activity approaches its capacity (7). This behavior of the alternative oxidase can be accommodated by a modification of the Q pool hypothesis of electron partitioning (27). However, some of its other properties are more difficult to reconcile with the Q pool model, particularly the variation of its activity with different substrates in the same batch of mitochondria (7, 18, 24). For example, it is now well documented that in some tissues external NADH oxidation is more severely inhibited by cyanide and antimycin than is succinate oxidation even though their uninhibited rates of oxidation may be similar.

We have investigated in more detail the partitioning of electrons between the Cyt and alternative paths during oxidation of different substrates using soybean cotyledon mitochondria and the Q electrode (23). We show that despite its poor access to the alternative oxidase, external NADH reduces the UQ pool to the same extent as succinate. Furthermore, certain added quinols also have poor access to the alternative oxidase in soybean mitochondria. This is in contrast to mi-

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[4] Abbreviations: UQ, the natural ubiquinone; Q, quinone; Q_r, reduced quinone; Q_t, total reducible quinone; Q-1, ubiquinone 5; Q-2, ubiquinone 10.

tochondria from the thermogenic tissue of *Philodendron*. We suggest that some form of nonrandom arrangement of succinate dehydrogenase, UQ, and the alternative oxidase occurs in soybean mitochondria.

MATERIALS AND METHODS

Materials

Seeds of soybean (*Glycine max* [L.] Merr cv Bragg) were planted in trays of vermiculite and grown in an illuminated growth cabinet or glasshouse. The seedlings were watered daily and cotyledons were harvested between 7 and 15 d after planting, when they were dark green, and leaves harvested 3 weeks after planting. Spadices of *Philodendron selloum* were kindly provided by Dr. R. Seymour (Zoology Department, The University of Adelaide). Q-1 and Q-2 were prepared by Dr. A. D. Ward (Organic Chemistry Department, The University of Adelaide) while duroquinone was purchased from Sigma Chemical Co. (St. Louis, MO). Quinones were reduced by adding sodium borohydride and the quinols were kept under an N_2 atmosphere at 0°C until use. Monoclonal antibodies raised against alternative oxidase proteins from *Sauromatum guttatum* mitochondria were donated by Dr. Tom Elthon, University of Nebraska, Lincoln, Nebraska.

Mitochondrial Isolation

Mitochondria were isolated from soybean cotyledons and leaves and *Ph. selloum* spadices and purified on Percoll gradients as previously described (1, 5).

Assay Procedures

O_2 consumption was measured polarographically in 2 mL of standard reaction medium containing 0.3 M sucrose, 10 mM KH_2PO_4, 10 mM Tes buffer, 2 mM $MgCl_2$, and 0.1% (w/v) BSA, adjusted to pH 7.2, either in a specially constructed reaction vessel (University of Sussex Workshops) housing a Rank oxygen electrode, a glassy carbon, and a platinum electrode (*i.e.* a combined O_2 and Q electrode assembly) or in a standard Rank oxygen electrode vessel. When quinols were used as substrates, the BSA was omitted from the reaction medium.

The redox state of ubiquinone was estimated by measuring the redox state of exogenous Q-1 or Q-2 voltametrically using a glassy carbon working electrode and a platinum electrode (Anachem Ltd., London) connected to an $Ag/HgCl_2$ reference electrode. The working electrode was poised at −360 mV with respect to the reference electrode as described previously (6). The outputs of the electrodes were connected to a twin channel Rikadenki recorder. Mitochondria (0.3–0.5 mg protein) in standard reaction medium (see above) were incubated with substrate and 0.5 to 1.0 μM Q-1 or Q-2; the steady-state level of Q_r and O_2 consumption were measured as described previously (23). Fully oxidized Q was taken as the base of the recording following addition of Q and mitochondrial protein. When succinate was substrate, mitochondria were preincubated with 0.1 mM ATP to activate succinic dehydrogenase and succinate subsequently added to begin the reaction. Unless otherwise indicated, when the substrate was NADH, it was added to the vessel prior to the mitochondria and the pen readjusted to zero; mitochondria were then added to initiate the reaction. When malate was substrate, 1 mM thiamine pyrophosphate was also added. Q_t was estimated as the amount reduced under anaerobic conditions in the presence of succinate. The concentrations of added Q used in these experiments had no detectable effect on the rate of O_2 uptake, respiratory control, or ADP/O ratios (22).

Oxidation of Q-1H_2 and reduction of Q-1 were measured spectrophotometrically at 280 nm, using an extinction coefficient of 12 mM^{-1} cm^{-1}, in an Aminco DW-2, spectrophotometer. Mitochondria (0.1–0.2 mg protein) were preincubated with 0.1 mM ATP and 30 to 50 μM Q-1 or Q-1H_2, and the reaction was initiated by adding substrate. For Q-1 reduction, Q_t was estimated by adding sodium borohydride to the cuvette at the completion of the reaction.

NADH oxidation in the presence of succinate was measured spectrophotometrically at 340 nm, in 3 mL of standard reaction medium containing 0.1 mM ATP. Protein was estimated by the method of Lowry *et al.* (20) using bovine serum albumin as standard.

SDS-PAGE and Immunological Analysis of the Alternative Oxidase

Mitochondrial proteins were separated by SDS-PAGE essentially by the technique of Laemmli (16), using a 15% acrylamide resolving gel and a 7% stacking gel. Sample preparation was as described by Azcon-Bieto *et al.* (1). The separated polypeptides were transferred to nitrocellulose and probed with an antiserum raised against *Sauromatum guttatum* alternative oxidase (9) as described by Harlow and Lane (11).

RESULTS AND DISCUSSION

Q-Electrode Response to NADH

Previous studies with the Q electrode have used succinate as substrate and have shown that the electrode is a reliable technique for monitoring the steady-state redox level of UQ in the respiratory chain (7, 23). NAD-linked substrates such as malate respond in a similar fashion (7). Complications can arise with NADH as substrate, however. Figure 1 shows a typical response of O_2 and Q electrodes to NADH oxidation by soybean mitochondria. Addition of NADH by itself resulted in a signal from the Q electrode (Fig. 1A), presumably because of a direct interaction between NADH and the electrode. This is a potential source of error in measurements of steady state UQ reduction by this substrate and experiments were conducted to ascertain whether the electrode can be used with NADH.

Addition of mitochondria after NADH initiated O_2 uptake and caused a large reduction of Q (Fig. 1A). ADP addition resulted in state 3-state 4 transitions similar to those seen with succinate (Fig. 1A) (*cf.* ref. 23). Inclusion of the external NADH dehydrogenase inhibitor, EGTA (21), in the reaction medium prevented O_2 uptake and Q reduction by NADH and mitochondria (Fig. 1B): this inhibition was relieved by subsequent addition of $CaCl_2$ (an activator of NADH dehydrogenase), causing a large reduction of Q (Fig. 1B) although

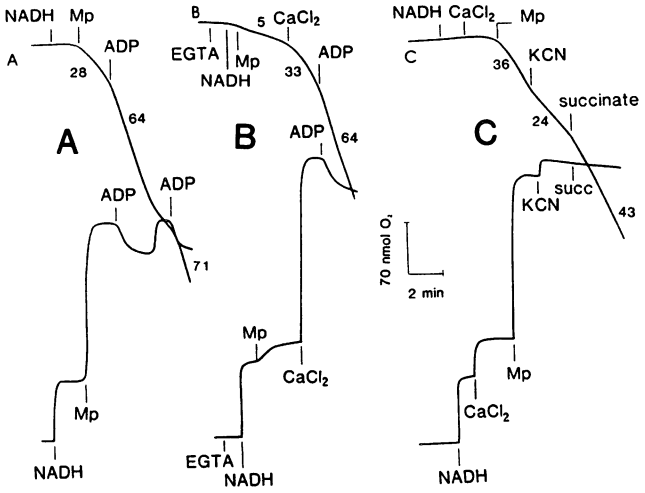

Figure 1. Oxygen uptake and quinone reduction during oxidation of added NADH by soybean mitochondria. Where indicated 1 mM NADH, 0.15 mM ADP, 2 mM $CaCl_2$, 1 mM EGTA, 0.5 mM KCN, 10 mM succinate, and 0.3 mg mitochondrial protein were added. The upper traces are the O_2 electrode recordings, and the lower traces are the Q electrode recordings. Numbers on traces refer to nmol O_2 min^{-1}.

some of the latter response was due to an interaction between $CaCl_2$ and the Q electrode (Fig. 1C). The response of the Q electrode to ADP, EGTA, and $CaCl_2$ during NADH oxidation gives us confidence that steady-state ubiqininone redox levels can be followed accurately with the Q electrode providing suitable allowances are made for direct interaction with NADH. In subsequent experiments, NADH was added by itself to the electrode and the appropriate subtraction made from any other Q electrode recordings.

To confirm the reliability of the Q electrode further, reduction of Q-1 by succinate and NADH was measured spectrophotometrically (data not shown). In these experiments, the respiratory chain was blocked with KCN and *n*-propylgallate, relatively large concentrations of Q-1 were added, and the reaction was allowed to proceed to equilibrium. Although the kinetics of Q-1 reduction under these conditions were different from the steady-state measurements with the Q electrode, the NADH reaction being faster than that with succinate (13), the final degree of reduction of Q-1 was very nearly the same with the two substrates. These results were observed consistently with a number of preparations.

Alternative Oxidase Activity with NADH and Succinate

The experiments described in Table I show that external NADH can reduce both added Q-1 and the endogenous quinone pool (as sensed by the Q electrode) to at least the same extent as succinate and that state 3 rates of O_2 uptake (largely mediated by the Cyt path) (1, 7) are similar with the two substrates. However, alternative oxidase activity with the two substrates was markedly different, being much (approximately twofold) faster with succinate than with NADH (Table I). Further, adding succinate to soybean cotyledon mitochondria oxidizing NADH in the presence of Cyt path inhibitors, stimulated O_2 uptake by approximately 100% but had no effect on Q-redox levels (Fig. 1C and Table I). Results with soybean leaf mitochondria were similar (not shown); in this tissue, state 3 rates of O_2 uptake with NADH in the absence of inhibitors were faster than that with succinate and Q reduction in the presence of NADH and myxothiazol was greater than that observed with succinate. Yet the rate of O_2 uptake in the presence of myxothiazol was much less with NADH than with succinate. Differences between NADH and succinate oxidation via the alternative oxidase have been noted in other tissues (18, 25, 30).

Malate oxidation via the alternative oxidase was also slower than that with succinate, but in this case Q reduction was also less; when succinate was added after malate, O_2 uptake increased but Q reduction also increased (Table I, experiment 2). The relationship between O_2 uptake via the alternative oxidase and the Q reduction level was markedly nonlinear in these mitochondria (not shown), as observed previously (7). Alternative oxidase activity was observed only when the Q pool was more than 60% reduced and thereafter activity increased dramatically. Thus, the difference in alternative oxidase activity between malate and succinate can be explained by the difference in Q reduction by the two substrates. However, the difference between NADH and succinate cannot be explained in this way; according to the data in Dry *et al.* (7), NADH oxidation should have been able to support maximum alternative oxidase activity, because it led to a Q_r/Q_t ratio of 1:0 (Table I). That it did not suggests that some form of segregation of respiratory chain components exists in soybean cotyledon mitochondria.

Table I. *Q Reduction Levels and Alternative Pathway Activity During Oxidation of Various Substrates by Soybean Cotyledon Mitochondria*

Oxygen uptake and Q redox state were measured with 0.5 μM Q-2 in a combined O_2/Q electrode apparatus, as described under "Materials and Methods." Concentrations of reactants were: succinate and malate, 10 mM; NADH 1 mM; myxothiazol, 2 μM; ATP and ADP, 0.5 mM. Q_t was set at 100% with succinate as substrate under anaerobic conditions.

Sequential Additions	Oxygen Uptake	Q_r
	nmol min^{-1}· (mg protein)$^{-1}$	% Q_t
Expt. 1		
Succinate, ATP	74	86
ADP	112	57
Myxothiazol	64	96
NADH	72	111
Expt. 2		
Malate	40	62
ADP	80	31
Myxothiazol	38	78
Succinate	68	94
Expt. 3		
NADH	50	86
ADP	112	73
Myxothiazol	31	94
Succinate	70	94

Table II. *Effects of Succinate on NADH Oxidation by Soybean Cotyledon Mitochondria*

Oxygen uptake was measured polarographically and NADH oxidation spectrophotometrically in parallel experiments with 0.54 mg mitochondrial protein and 1 mM ADP in 3 mL of standard reaction medium. Where indicated, NADH (1 mM), succinate (10 mM), myxothiazol (2 μM), and *n*-propylgallate (100 μM) were added.

Sequential Additions	NADH Oxidation	Oxygen Uptake
	nmol·min^{-1} *(mg protein)*$^{-1}$	*n atoms·min*$^{-1}$ *(mg protein)*$^{-1}$
Expt. 1		
NADH	206	211
Succinate	167	264
Expt. 2		
NADH	206	215
n-Propylgallate	206	215
Succinate	135	252
Expt. 3		
NADH	198	209
Myxothiazol	43	56
Succinate	43	104
Expt. 4		
Succinate		181
Myxothiazol		104
Expt. 5		
Succinate		144
n-Propylgallate		144
NADH		256

Interactions between NADH and Succinate

NADH oxidation and O_2 uptake were measured in parallel experiments in order to investigate interactions between substrates (Table II). Experiments 1 and 4 of Table II show that succinate and NADH compete for a common respiratory chain component when they are oxidized concommitantly, since the O_2 uptake with both substrates was less than the sum of their individual rates and NADH oxidation was inhibited by succinate. Other studies have reached the same conclusion (3, 14). When *n*-propylgallate was added to block the alternative oxidase (which is active when both substrates are oxidized together [4]), the result was similar (experiments 2 and 5, Table II). This behavior is expected when two substrates share a common electron transport pathway and studies with Jerusalem artichoke mitochondria have indicated that the common component is UQ (14). Yet when myxothiazol was present so that all electron flow was to the alternative oxidase, NADH oxidation did not sense that of succinate (experiment 3, Table II). Since in the presence of myxothiazol, O_2 uptake with succinate alone was the same as that with succinate plus NADH (with measureable NADH oxidation, experiments 3 and 4, Table II), it is clear that succinate oxidation via the alternative oxidase is inhibited by concommitant NADH oxidation. Again, these results are indicative of some form of segmentation of the respiratory chain in soybean mitochondria. It is as if two populations of alternative oxidase exist, one of which is readily available to electrons from both external NADH and succinate, and another available only to succinate. It is unlikely that these alternative oxidase populations reside in different populations of mitochondria, since succinate oxidation inhibits NADH oxidation via the Cyt chain relatively severely (experiment 2, Table II).

Quinol Oxidation

In the light of these indications that respiratory chain components are not uniformly interconnected in soybean mitochondria, oxidation of added quinols was investigated. The strong inhibition by KCN of both Q-1H_2 and duroquinone (Table III) oxidation was surprising since KCN-insensitive quinol oxidation is considered an assay for the alternative oxidase (8, 12, 13, 28). Most measurements of quinol oxidation have been carried out with mitochondria from thermogenic tissues which have large quantities of the alternative oxidase. Our results suggest that in soybean, which is not thermogenic, the alternative oxidase does not react readily with added reduced quinols. Because *Philodendron* was flowering at this time in Adelaide, we isolated mitochondria from the thermogenic spadix and measured alternative oxidase activity (Table III). Reduced Q-1 oxidation by these mitochondria was approximately 50% resistant to KCN, and this resistant rate was inhibited about 50 to 60% by *n*-propylgallate (see Table IV). *Philodendron* mitochondria also oxidized NADH readily via the alternative oxidase with rates being similar to those with succinate (Table III). Similar results have been observed with mitochondria from other thermogenic tissues (18, 23), and it appears that there are fundamental differences between spadix and nonspadix tissues in the partitioning of electrons to the alternative oxidase. There is little doubt that the same enzyme is responsible for

Table III. *Oxygen Uptake by Philodendron and Soybean Mitochondria*

Q-1 and duroquinone were reduced with borohydride and kept on ice under nitrogen. The quinol (0.5 mL) was warmed to assay temperature and added to 2.4 mL reaction medium in the electrode vessel followed by 0.39 mg mitochondrial protein and 0.5 mM ADP. After a steady rate was obtained, 0.5 mM KCN was added. This was followed by 50 μM *n*-propylgallate (which completely inhibits cyanide-insensitive succinate oxidation). The residual rates (*Philodendron*, Q-1 = 47, duroquinone = 35; soybean, Q-1 = 3, duroquinone = 9 nmol O_2 min^{-1} mg^{-1} protein) were assumed to be due to auto-oxidation of quinol and were subtracted from the other rates. Under the conditions of these experiments the Q-1 appeared to remain in solution but not the duroquinone, which was added to the oxygen electrode vessel as a suspension. KCN-insensitive succinate and NADH oxidation was completely inhibited by *n*-propylgallate.

Substrates	Oxygen Consumption	
	Control	+KCN
	nmol·min^{-1} *(mg protein)*$^{-1}$	
Philodendron mitochondria		
Q-1H_2	147	67
Duroquinol	80	37
Succinate	117	93
NADH	140	80
Soybean mitochondria		
Q-1H_2	79	13
Duroquniol	34	1

cyanide-insensitive respiration in the two tissues since antibodies raised against voodoo lily (spadix) alternative oxidase (10) react with similar mol wt proteins from soybean cotyledon mitochondria (DA Day, unpublished results). Thus, it appears that organization of the alternative oxidase is different in soybean than in thermogenic spadix tissues.

DISCUSSION

The results presented here and in previous publications (14, 18, 22, 24, 25) are difficult to reconcile with a model of electron transfer in which all respiratory components are randomly distributed within the mitochondrial inner membrane and interact via diffusional processes (15, 19). This model is able to explain most results obtained with mammalian mitochondria (29) but appears to be inadequate for the more complicated, branched respiratory chain of plant mitochondria with two terminal oxidases and several pathways of NADH oxidation. Even in plant mitochondria, oxidation of a single substrate via a single oxidase yields kinetics largely explained by a general Q-pool model, but concurrent oxidation of multiple substrates (especially with respect to external NADH) or operation of both oxidases simultaneously is more complicated (14, 22, 25).

In general, three hypotheses have been formulated to explain the difference between NADH and succinate oxidation via the alternative oxidase (14, 18, 22): a longer diffusion-path length between external NADH dehydrogenase (compared to succinate dehydrogenase) and the alternative oxidase could lead to slower NADH oxidation; some sort of direct interaction ("patching" [29]) between succinate dehydrogenase and the alternative oxidase could lead to faster succinate oxidation and exclude interactions with the external NADH dehydrogenase; or, discrete compartments or pools of UQ may be associated with different dehydrogenases or oxidases. Since NADH and succinate both reduce Q to a similar extent (and reduce most of the available Q) and compete with one another during oxidation by the Cyt chain, and because exogenous quinols would not reduce respiratory chain UQ, we consider the third of the above hypotheses unlikely. The interactions between NADH and succinate in the presence of a Cyt pathway inhibitor (Table III) are most compatible with an association between succinate dehydrogenase and some of the alternative oxidase molecules of the inner membrane. Such an association could involve a smaller number of "trapped" quinones which do not readily communicate with the general Q pool. Different diffusion-path lengths may also be involved, since both succinate dehydrogenase and the alternative oxidase are located on the inside of the inner membrane (26). However, this must also be the case in thermogenic, mitochondria (*e.g. Arum*) which do not show these differences between NADH and succinate oxidation.

The explanation for the different observations with thermogenic and nonthermogenic tissues may lie in the large differences in the alternative oxidase complement of the two tissue types and its distribution within each type. The suggested compartmentation of respiratory chain components may not be significant when an excess of the alternative oxidase is present. However, the mitochondria isolated from *Philodendron* spadices do not quite fall into this category. Their rates of succinate, quinol, and NADH oxidation, in the presence and absence of KCN, were not very different from those of soybean mitochondria. The *Philodendron* rates may have been underestimated due to contaminating nonmitochondrial protein (the yield of mitochondria was too small to ascertain purity) but even allowing for that, rates were much less than those reported for other thermogenic spadices. (To our knowledge, this is the first report of isolated *P. selloum* mitochondria.) Despite this, *Philodendron* mitochondria behaved as did other spadix mitochondria with respect to substrate access to the alternative oxidase. These differences between spadix and nonspadix organelles require further investigation.

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