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Regulation of Nonphosphorylating Electron Transport Pathways in Soybean Cotyledon Mitochondria and Its Implications for Fat Metabolism[1]

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ABSTRACT

The respiration of mitochondria isolated from germinating soybean cotyledons was strongly resistant to antimycin and KCN. This oxygen uptake was not related to lipoxygenase which was not detectable in purified mitochondria. The antimycin-resistant rate of O_2 uptake was greatest with succinate as substrate and least with exogenous NADH. Succinate was the only single substrate whose oxidation was inhibited by salicyl hydroxamic acid alone, indicating engagement of the alternative oxidase. Concurrent oxidation of two or three substrates led to greater involvement of the alternative oxidase. Despite substantial rotenone-resistant O_2 uptake with NAD-linked substrates, respiratory control was observed in the presence of antimycin, indicating restriction of electron flow through complex I. Addition of succinate to mitochondria oxidizing NAD-linked substrates in state four stimulated O_2 uptake substantially, largely by engaging the alternative oxidase. We suggest that these properties of soybean cotyledon mitochondria would enable succinate received from the glyoxysome during lipid metabolism to be rapidly oxidized, even under a high cytosolic energy charge.

Plant mitochondria possess a branched electron transport chain containing bypasses of energy transducing sites, which can be distinguished by their insensitivity to respiratory poisons. In addition to the well documented alternative oxidase which is responsible for cyanide and antimycin resistant respiration (14), there is also a bypass of complex I (the internal NADH dehydrogenase) which is involved in the oxidation of matrix NADH, is insensitive to rotenone and which probably involves a distinct dehydrogenase on the inside of the inner membrane (22). Recent results suggest that these pathways may play key roles in the carbon economy of the plant (11, 26).

Despite a great deal of work in a number of laboratories, the nature and regulation of these nonphosphorylating respiratory pathways remains controversial (13, 14, 18). Yet it is important to understand these processes if we are to place them in the context of plant metabolism and growth. One of the problems associated with experimentation in this field is the lack of highly inhibitor-resistant plant material which is suitable for purification of intact mitochondria with very active bypasses. Recently, however, it has been shown that cotyledons of germinating soybeans yield large quantities of relatively pure and highly cyanide- and antimycin-resistant mitochondria (6, 8). In this paper we describe their electron transport pathways in detail and discuss these pathways in relation to cotyledon metabolism.

MATERIALS AND METHODS

Seeds of soybean (*Glycine max* [L.] Merr.) cv Bragg were generously provided by Dr. B. J. Carroll (Department of Botany, Australian National University) and planted in trays of vermiculite. Germination took place in the dark at 25°C and plants were harvested 5 d after sowing, at which time the cotyledons had just emerged from the vermiculite, except where indicated otherwise. Reagents were purchased mainly from Sigma Chemical Co.

Mitochondrial Isolation. The method used was adapted from Neuburger (19). Cotyledons (100–200 g) were harvested, rinsed thoroughly in distilled water, and chilled. They were homogenized for 1 to 2 s using a Polytron blendor (Kinematica, Switzerland) set on speed 7, in 300 to 500 ml of grinding medium (0.3 M sucrose, 25 mM pyrophosphate buffer, 10 mM KH_2PO_4, 2 mM EDTA, 2 mM $MgCl_2$, 30 mM ascorbate, 1% (w/v) PVP-40, and 1% (w/v) BSA; final pH 7.6). The homogenate was filtered through four layers of Miracloth and centrifuged at 1,000*g* for 5 min. The supernatant was centrifuged for 20 min at 12,000*g* and the crude mitochondrial pellet resuspended in about 10 ml of wash buffer (0.3 M sucrose, 10 mM TES buffer [pH 7.2], 0.1% [w/v] BSA). The crude mitochondria were layered onto 30 ml of a solution containing 0.3 M sucrose, 10 mM TES buffer (pH 7.2), 0.1% (w/v) BSA, and 30% (v/v) Percoll (usually two tubes were used) and centrifuged at 40,000*g* for 45 min in a Sorvall SS34 rotor. The mitochondria were found in a broadband in the bottom half of the centrifuge tube and were extracted from the gradient by pipette, diluted fivefold with wash buffer, and pelleted by centrifuging at 12,000*g* for 15 min. The latter procedure was repeated once and the mitochondria resuspended in 1 to 2 ml of wash buffer.

Mitochondria isolated by this method were shown to be largely intact (at least 90% by the method in Ref. 19) and only slightly contaminated by other membrane fractions, as judged by electron microscopy (5) and measurements of the enzymes catalase (a marker for microbodies) and glucose 6-P dehydrogenase (a marker for plastids) (5). The mitochondria retained full functionality for at least 5 h when stored on ice.

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Table I. *Lipoxygenase and Alternative Oxidase Activities in Soybean Cotyledon Mitochondria*

Lipoxygenase was measured as linoleic acid oxidation and alternative oxidase as O_2 uptake with succinate plus KCN.

Mitochondrial Preparation	Lipoxygenase Activity	Alternative Oxidase Activity
	nmol O_2 min^{-1} · mg^{-1} protein	
A. *1.5 d after planting*		
Crude mitochondria	15	0.5
Purified mitochondria	0	1
B. *5 d after planting, etiolated cotyledons*		
Crude mitochondria	40	15
One-purified mitochondria	5	41
Twice-purified mitochondria	0	41
C. *21 d after planting, green cotyledons*		
Crude mitochondria	10	60
Purified mitochondria	0	50

Assays. Oxygen consumption was measured using a Rank Bros. (Cambridge, U.K.) electrode in 2 ml of standard reaction medium (0.3 M sucrose, 10 mM KH_2PO_4, 10 mM TES buffer, 2 mM $MgSO_4$, and 0.1% [w/v] BSA) with 0.5 to 1.5 mg mitochondrial protein. The pH was 7.0 unless stated otherwise. The pH was varied when necessary by titrating with KOH or HCl. For other details, see the legends to figures and tables. Unless specified otherwise, data shown are from a single, representative experiment. Protein was estimated by the method of Lowry *et al.* (15) using BSA fraction V as standard. Lipoxygenase (25), ME[3] (4), MDH (29), GDH (21), and aspartate aminotransferase (27) were assayed according to published methods.

RESULTS AND DISCUSSION

Lipoxygenase and Alternative Oxidase Activity. In some tissues, lipoxygenase, which catalyses O_2 uptake upon oxidation of linoleic acid, may confuse measurements of alternative oxidase activity; both enzymes are SHAM-sensitive and KCN-insensitive (25). Although the suggestion that lipoxygenase is generally involved in alternative oxidase activity in plant mitochondria has been refuted (18), care must still be exercised when dealing with fatty tissues such as soybean cotyledons. This has been emphasized by a recent report (24) that KCN-insensitive O_2 uptake by mitochondria from soybean axes was associated with lipoxygenase which could be removed by purification on Percoll gradients. These authors suggested that cotyledons might present a similar problem.

In the light of this warning, we undertook a developmental study of lipoxygenase activity and alternative oxidase activity in cotyledons from soybean seedlings. Care was taken to prepare linoleic acid solutions immediately prior to assay, using deoxygenated ethanol (25). With this precaution, at 5 mM linoleic acid appreciable O_2 uptake was observed in crude mitochondrial preparations from 5 d old cotyledons (Table I). Purification removed most of this activity but had no effect on alternative oxidase activity (Table I). A second purification step using 25% (v/v) Percoll eliminated lipoxygenase activity without affecting that of the alternative oxidase (Table IB). Alternative oxidase activity varied with the age of cotyledons, being very low soon after imbibition and reaching a maximum at 21 d after planting; lipoxygenase activity, on the other hand, was only detected in crude preparations and was very low when alternative oxidase activity was greatest (Table I). We therefore conclude that the alternative oxidase activity that we measure is a constitutive property of the respiratory chain and is not related to contaminating lipoxygenase.

Hereafter, attention is focused on cotyledons from 5 d old, etiolated seedlings. At 5 d after sowing, lipid metabolizing enzyme activity in the cotyledon is maximal (3).

Antimycin and Rotenone Resistance. The mitochondria isolated from the cotyledons of 5 d old, etiolated soybean seedlings are capable of oxidizing a wide range of tricarboxylic acid cycle intermediates, as well as external NADH (Table II). Rates of O_2 uptake with any single substrate were relatively slow compared to mitochondria purified from other sources (see Ref. 9 for examples) and were very resistant to antimycin (similar results were observed with KCN). The rate of oxidation of the NAD-linked substrates malate, pyruvate, and α-ketoglutarate were barely affected by addition of antimycin (Table I). Adding a cocktail of NAD-linked substrates together did not stimulate O_2 uptake to a rate faster than that observed with malate and glutamate (not shown), suggesting that electron flow from internal NADH to ubiquinone was rate limiting. Addition of NAD^+ to these mitochondria had no effect on O_2 uptake, indicating that matrix NAD^+ levels were adequate (10). Addition of succinate and NADH to mitochondria oxidizing malate plus glutamate stimulated O_2 uptake dramatically, the rate with the cocktail of substrates being approximately equal to the sum of the rates with the individual substrates (Table II). Under these conditions (i.e. multiple substrates) O_2 uptake rates were rapid and comparable to those observed in other systems. The relatively slow rates with individual substrates (8) were evidently due to the low activity of substrate dehydrogenases. Although total O_2 uptake with multiple substrates was very fast, the rate resistant to antimycin was no faster than that with succinate alone (Table II). If we assume that the rate with malate plus succinate plus NADH was equivalent to the capacity of the electron transport chain, then the alternative oxidase capacity was only 20% of total.

It is noteworthy that antimycin resistance with external NADH as substrate was less than that with succinate even though state 3 rates were identical (Table II). Similar observations have been made previously (13, 18).

The oxidation of NAD-linked substrates was also quite resistant to the inhibitor rotenone; 20 μM rotenone inhibited malate

Table II. *Antimycin-Resistant Oxygen Uptake with Various Substrates*

All substrates were provided at 10 mM, except NADH (1 mM) and malate (0.5 mM) when provided with pyruvate. Thiamine pyrophosphate (1 mM) was provided when pyruvate and α-ketoglutarate were substrates and when malate was added alone; malonate (2.5 mM) was provided with α-ketoglutarate. State 3 refers to oxygen uptake in the presence of ADP. The reaction medium pH was 7.2

Substrate	O_2 Consumption		Resistance
	State 3	+ Antimycin	
	nmol · min^{-1} · mg^{-1} protein		%
Malate[a]	27	24	89
Malate + glutamate	55	27	49
Pyruvate + malate	27	24	89
α-Ketoglutarate	22	22	100
Succinate	73	41	56
NADH	77	19	25
Malate[b] + NADH	134	41	31
Malate[b] + succinate + NADH	216	45	21

[a] Thiamine pyrophosphate (1 mM) added. [b] Glutamate (10 mM) also present.

[3] Abbreviations: ME, NAD-linked malic enzyme; MDH, malate dehydrogenase; SHAM, salicyl hydroxamic acid; GDH, glutamate dehydrogenase.

oxidation by only 12% and that of α-ketoglutarate by 15% (Fig. 1). Similar effects were observed with malate plus glutamate and with pyruvate (not shown).

Malate Oxidation. It has been suggested that ME in plant mitochondria is either structurally (23) or functionally (12) linked to the alternative oxidase. The results in Tables II and III indicate that there is no specific link between these enzymes since all NAD-linked substrates could support substantial antimycin-resistant O_2 uptake (Table II). At pH 7.6, ME is inactive and the NADH generated during malate oxidation comes from the MDH reaction (9). In the absence of an oxaloacetate removal system, matrix NADH levels are low and O_2 uptake is slow; under these conditions alternative oxidase activity was limited (Table III). When glutamate is present, oxaloacetate can be removed by transamination (glutamate-oxaloacetate transaminase activity was substantial: Table IV) and under those conditions O_2 uptake was faster both in the presence and absence of KCN (Table III). At pH 6.8 when ME is active and MDH inhibited, oxaloacetate levels are low and NADH levels are high (20); therefore cyanide resistance was pronounced at pH 6.8 (Table III). The high ME activity of these mitochondria and their ability to oxidize malate readily in the absence of glutamate contrasts to the situation in castor bean endosperm mitochondria (29).

It should be noted that glutamate oxidation via GDH is relatively rapid in these mitochondria (Table IV); nonetheless, the fact that alternative oxidase activity was identical when either ME or MDH plus GDH were operating shows that there is no specific link between ME and the alternative oxidase. The same is true for the rotenone-resistant bypass (4, 29). We believe that

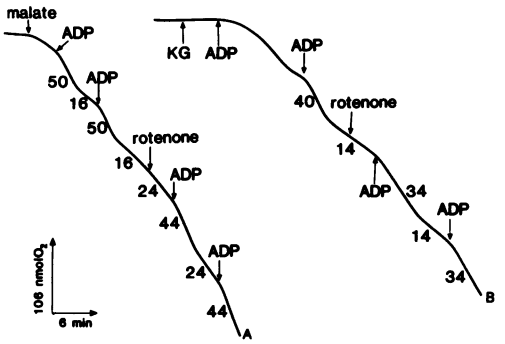

FIG. 1. Rotenone-resistant oxygen consumption. Oxygen consumption was measured in 2.1 ml of reaction medium (pH 7.2 with 1.1 mg mitochondrial protein. Thiamine pyrophosphate (1 mM) was included in the medium and malonate (2.5 mM) was also added with α-ketoglutarate. Other additions as indicated were: 10 mM malate, 200 (with malate) or 400 (with α-ketoglutarate) nmol ADP, 10 mM α-ketoglutarate and 20 μM rotenone. Note that glutamate was not present. Numbers on traces are nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein.

the activity of the nonphosphorylating segments of the plant respiratory chain is dependent on a high steady state concentration of NADH in the matrix (29). *In vivo* the poise between oxaloacetate production and utilization will be critical during operation of the tricarboxylic acid cycle and hence pyruvate provision must be maintained. In that context it may well be that ME activity is necessary to maintain NADH levels adequate to engage the bypasses (22).

Engagement of the Alternative Oxidase. The contribution of the alternative oxidase to O_2 uptake with various substrates in the absence of antimycin or KCN was estimated by using the selective inhibitor SHAM. When NAD-linked substrates were used either singly or together, SHAM had no effect on either O_2 uptake or ADP/O ratios (Tables V and VI). It appears that electron flow through the internal NADH dehydrogenases was not rapid enough to engage the alternative oxidase. Likewise, SHAM had no effect on external NADH oxidation although this substrate was more rapidly oxidized. It should be noted, however, that alternative oxidase activity with different substrates varies depending on the age of the soybean seedling and the conditions of growth (J Azcon-Bieto, DA Day, unpublished results).

In contrast to the other substrates, succinate was able to engage the alternative oxidase in both state 3 and state 4 as evidenced by SHAM inhibition (Table V). The ADP/O ratio was slightly higher in the presence of SHAM, which is also consistent with alternative oxidase involvement. Alternative oxidase activity increased when succinate and malate were oxidized concurrently and became fully engaged when NADH was also included (Table VI). As noted previously, the rates of oxidation of the three substrates were additive in the absence of inhibitors; but when SHAM was present, the rate with the mixture of substrates was less than the sum of the individual rates (Table V). This suggests that the cytochrome path was saturated with the three substrates.

Since adding NADH to mitochondria oxidizing succinate plus malate in the presence of SHAM stimulated O_2 uptake (Table V), it is clear that the cytochrome path was not fully engaged with just the latter two substrates. Yet equally clearly, the alternative oxidase was active when succinate plus malate were oxidized. Furthermore, alternative oxidase activity was more or less identical in both state 3 and state 4 (Table VI). These results contradict the previously held assumption, based on experiments with a single substrate (1), that the alternative oxidase is only expressed when the cytochrome chain is either saturated or inhibited (see Ref. 14 for a review). We are continuing to investigate this problem and that of the poor access of external NADH to the alternative oxidase in these mitochondria.

Control of Alternative Oxidase Activity by ADP. Since the alternative oxidase accepts electrons from ubiquinone (28), H^+ translocation at complex I during oxidation of NAD-linked substrates can occur in the presence of antimycin or cyanide. Indeed direct measurements of Pi esterification (see Ref. 13 for a review)

Table III. *Effect of pH on Malate Oxidation*

The concentrations used were: malate and glutamate, 10 mM; KCN, 0.5 mM; SHAM, 2 mM. KCN and SHAM were added in state 3. The enzymes listed are those considered to contribute to generation of matrix NADH under the conditions imposed.

Substrates	Enzymes Active	O_2 Consumption			
		State 3	State 4	+KCN	+KCN and SHAM
pH 7.6		*nmol · min⁻¹ · mg⁻¹ protein*			
Malate	MDH	18	8	8	3
Malate + glutamate	MDH + GDH	49	20	23	3
pH 6.8					
Malate	ME	46	20	26	3

Table IV. *Activities of Enzymes Associated with Malate Oxidation*

Enzyme assays were performed on solubilized mitochondria, as described in "Materials and Methods." Rates shown are means ± SE of 4 separate experiments.

Enzyme	Rate
	μmol NADH · min⁻¹ · mg⁻¹ protein
MDH	30.97 ± 6.40
ME	0.55 ± 0.05
GDH	0.32 ± 0.02
Glutamate-oxaloacetate transaminase	0.27 ± 0.04

and proton motive force (17) have confirmed this. To our knowledge, however, there have been no reports of observed respiratory control in the presence of cytochrome chain inhibitors. Such control is regularly observed in soybean cotyledon mitochondria oxidizing a wide range of NAD-linked substrates and examples are shown in Figure 2. Antimycin (or KCN) had little effect on either the state 3 or state 4 rates of O_2 uptake (Fig. 2) but ADP/O values were decreased by two-thirds with malate and pyruvate and by one-half with α-ketoglutarate as substrate (Table VII).

Obviously in these mitochondria control of electron flow through complex I, by the energy status of the mitochondria, is tight enough to regulate flow through the entire electron transport system, despite the presence of the rotenone-insensitive bypass (Fig.1); regulation of substrate level phosphorylation with α-ketoglutarate imposes a further restriction by adenylates. Analysis of the data in Table VII indicates that the ADP/O ratios associated with each of the three sites of energy transduction are approximately equivalent (and equal to 0.7–0.8). Since the alternative oxidase is not engaged with NAD-linked substrates (Tables V and VI), this indicates that the rotenone-insensitive bypass is also not operative in state 3 (otherwise the ADP/O ratio with malate as substrate in the presence of antimycin should have been lower than observed).

The observation of respiratory control in the presence of antimycin with malate and pyruvate as substrates (it was also evident when malate + glutamate were used) raises the question of whether the rotenone-insensitive bypass was active in state 4 in these mitochondria. State 4 rates with malate as substrate varied depending on the conditions of assay (Table V), and we have suggested previously (7) that such variability might reflect a greater or lesser degree of engagement of the rotenone-bypass. Clearly, even if the bypass did contribute to O_2 uptake in state 4, this contribution was minor and electron flow through complex I was still the major controlling factor resulting in regulation by availability of ADP.

Significance for Cotyledon Metabolism. The soybean cotyledon at 5 d after sowing is a degradative tissue where starch, protein, and lipid are metabolized to provide sucrose and amino acids to the developing plant (3). Mitochondria may play a role in amino acid metabolism (GDH and transaminase activities are high: Table V) and are certainly involved in lipid metabolism (2).

During conversion of fats to sugars, succinate and NADH produced in the glyoxysome are postulated to be oxidized in the mitochondria via a complicated metabolic exchange system involving the malate-aspartate shuttle in addition to succinate uptake and oxidation (2). This scheme is based on studies with castor bean endosperm mitochondria (16) which oxidize external NADH and malate in the absence of glutamate very poorly, and appear to have the transport systems for organic and amino acids that are required by the scheme.

The transport systems of soybean cotyledon mitochondria have not yet been examined but these mitochondria differ from their castor bean endosperm counterparts in at least two functions:

Table V. *Effect of SHAM on Substrate Oxidation*

Assay conditions were as in Table I; SHAM was added to give a final concentration of 1 mM. Similar results to those shown with malate + glutamate were obtained with α-ketoglutarate and pyruvate as substrates (*i.e.* SHAM had no effect).

Substrate	O_2 Consumption				ADP/O	
	Control		+ SHAM		Control	+ SHAM
	State 3	State 4	State 3	State 4		
	nmol · min⁻¹ · mg⁻¹ protein				*ratio*	
Malate + glutamate	53	24	53	24	2.01	2.01
Succinate	73	43	58	24	1.30	1.40
NADH	77	33	77	33	1.31	1.31
Malate[a] + succinate	121	61	96	38	1.56	1.80
Malate[a] + succinate + NADH	216	102	175	60	1.10	1.50

[a] Glutamate (10 mM) was also present.

Table VI. *Alternative Oxidase Activity with Different Substrates*

The activity of the alternative oxidase (v_{alt}) was calculated from the data in Table V and represents the rate of oxygen uptake inhibited by SHAM in the absence of antimycin. The capacity (V_{alt}) of the alternative oxidase (from Table I) = 41 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein; ρ represents the proportion of the capacity which was active with any given substrate(s). Glutamate (10 mM) was present with malate.

Substrates	v_{alt}		ρ (v_{alt}/V_{alt})	
	State 3	State 4	State 3	State 4
	nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein			
NAD-linked substrates[a]	0	0	0	0
NADH	0	0	0	0
Succinate	15	19	0.37	0.46
Malate + succinate	25	23	0.61	0.54
Malate + succinate + NADH	41	42	1	1

[a] Or any combination thereof.

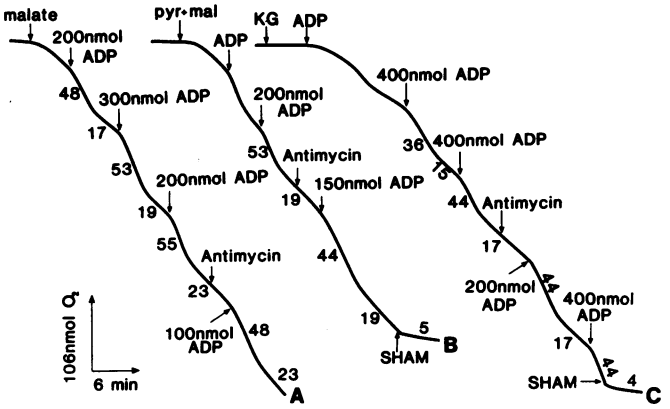

FIG. 2. Respiratory control in the presence of antimycin A. Reaction conditions were as described in Figure 1 and Table 1. The concentration of antimycin was 5 μM and that of SHAM 2 mM. Numbers on traces are nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein.

Table VII. *ADP/O Ratios with NAD-Linked Substrates in the Presence of Inhibitors*

All substrates were added at 10 mM; thiamine pyrophosphate (1 mM) was included with all substrates, 0.5 mM malate with pyruvate, and 2 mM malonate with α-ketoglutarate. Data are means ± SE.

Substrate	Control	+ Antimycin	+ Rotenone
Malate	2.16 ± 0.04 (n = 6)	0.71 ± 0.03 (n = 7)	1.57 ± 0.11 (n = 5)
Pyruvate	2.36 ± 0.05 (n = 5)	0.76 ± 0.13 (n = 3)	
α-Ketoglutarate	3.47 ± 0.13 (n = 5)	1.56 ± 0.06 (n = 4)	2.43 ± 0.02 (n = 3)

they can readily oxidize external NADH and have substantial alternative oxidase activity. Succinate in particular can be oxidized rapidly via the alternative oxidase. Gluconeogenesis requires a high cytosolic ATP/ADP ratio and our results indicate that under these conditions in the soybean cotyledon, turnover of the tricarboxylic acid cycle will be inhibited due to limitations at the α-ketoglutarate dehydrogenase step and respiratory control of electron flow via complex I. This would be desirable to avoid complete oxidation of organic acids supplied by the glyoxysome. Succinate, however, can be oxidized via the alternative oxidase and its metabolism is less restricted by adenylates.

It should be pointed out that only when succinate is supplied in sufficient quantity is the alternative oxidase engaged. The effects of malonate on O_2 uptake and ADP/O ratios associated with α-ketoglutarate metabolism (Table VIII) clearly demonstrate that succinate was being produced and oxidized. Yet SHAM had no effect on α-ketoglutarate oxidation (Table VIII). When succinate was added on top of an NAD-linked substrate (including α-ketoglutarate), electron flow to ubiquinone became great enough to engage the alternative oxidase (see Table V).

Gluconeogenesis also requires a high cytosolic NADH/NAD^+ ratio. External NADH was readily oxidized by soybean cotyledon mitochondria but this oxidation was poorly connected to the alternative oxidase. *In vivo*, therefore, cytosolic NADH oxidation would produce ATP and be subject to adenylate control. This would ensure that both ATP/ADP and NADH/NAD^+ ratios were maintained at a high value in the cytosol.

Soybean cotyledon mitochondria therefore seem to be equipped for the direct oxidation of succinate and NADH produced during lipid metabolism. This plus the high GDH and ME activity of these mitochondria, which would tend to interfere with the transamination reactions involved in the malate-aspartate shuttle, suggests that glyoxysomal-mitochondrial interactions in soybean cotyledons may be different from those thought to occur in castor bean endosperm.

Table VIII. *α-Ketoglutarate Oxidation*

Assay conditions are described in Table I; α-ketoglutarate was added at 10 mM, malonate 2 mM, and SHAM 1.5 mM.

Substrate	O_2 Consumption		ADP/O	Inhibition by SHAM
	State 3	State 4		
	nmol · min⁻¹ · mg⁻¹ protein		*ratio*	*%*
α-Ketoglutarate	30	11	2.52	0
α-Ketoglutarate + malonate	21	6	3.25	0

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