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FACTORS LIMITING RESPIRATION BY ISOLATED CAULIFLOWER MITOCHONDRIA

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Abstract—Oxygen consumption by isolated cauliflower mitochondria oxidising malate, succinate or NADH was less than when a combination of any two substrates was supplied. The rates obtained with any two were less than the aggregate of the individual rates. Only with the combination of malate plus succinate did the presence of the NADH result in faster rates of oxygen uptake. Conversely, malate and succinate separately, or in combination, inhibited the oxidation of exogenous NADH. The rate-limiting step for the oxidation of these substrates lies within the respiratory chain but on that part used exclusively by each substrate. The addition of NADH with either malate or succinate, or both, saturates the chain and makes the rate limiting step a component common to all pathways. Malate and isocitrate oxidation were considerably greater in disrupted, than in intact mitochondria, eliminating substrate dehydrogenases as rate-limiting factors. Substrate entry was also eliminated in the case of malate oxidation. It is suggested that the tricarboxylate transporter may restrict the rate of isocitrate oxidation.

INTRODUCTION

The study of factors limiting respiration by isolated plant mitochondria has focused on the role of adenine nucleotides (respiratory control and ADP/O ratios; see ref. [1]) and regulation by product formation [2–7]. Hence oxaloacetic acid (OAA) accumulation within the matrix is recognized as being particularly important in the control of malate and succinate oxidation [2, 5, 7], while the intramitochondrial NAD/NADH ratio may be an important regulator of pyruvate and isocitrate dehydrogenases [8, 9]. However, the NAD/NADH ratio reflects the intramitochondrial OAA concentration. More recently, substrate transport processes have been implicated in control of malate, citrate and pyruvate oxidation [10, 11], in isolated mitochondria.

Little attention has been paid to the regulation of respiration under conditions of adequate product removal and substrate supply. To establish conditions under which the cytochrome chain is saturated is of some importance, particularly in relation to the control of electron flow along alternate pathways [12]. This paper reports on attempts to saturate the electron transport chain, in terms of the factors that limit the rate of substrate oxidation, and the interactions among substrates during concurrent oxidation of more than one substrate. It also examines the effect of compartmentation on the oxidation of substrates linked to NAD.

RESULTS AND DISCUSSION

NAD-linked substrates

State 3 rates of O_2 consumption by intact cauliflower mitochondria were consistently higher with malate than with isocitrate (Table 1), as substrate. Similar observations have been reported for other plant mitochondria [7]. This difference in rate was maintained when the mitochondria were disrupted with detergent and substrate oxidation was coupled to O_2 consumption via PMS and NAD (Table 1). Since this treatment allows measurement of substrate dehydrogenase activity [10], it suggests lower levels of or lower activity of isocitrate dehydrogenase. However both substrates were oxidised faster with disrupted mitochondria, than with intact mitochondria. Thus, in intact cauliflower mitochondria, the oxidation of malate and of isocitrate may be restricted by electron transfer, by substrate transport or by compartmentation of the enzymes and their substrates or products, but not by the enzymes themselves.

Table 1. Oxidation of NAD-linked substrates by cauliflower bud mitochondria

Substrate	Intact mitochondria state 3 rate	Disrupted mitochondria NAD + PMS	NADP + PMS
Malate	86	160	19
Isocitrate	27	74	7
α-Ketoglutarate	65.0	12.5	—

O_2 uptake was measured as described in the Experimental section. The mitochondria were disrupted by adding 30 μl of 'Decon 90' detergent concentrate. Final concentrations were 10 mM malate, 10 mM isocitrate, 10 mM α ketoglutarate, 0.5 mM NAD and 30 μM PMS. Rates shown are the averages of 3 experiments, and are expressed as nmol O_2 consumed/min/mg protein. A lag of 8 min was observed before the state 3 rate with α-ketoglutarate was obtained and detergent was added during this lag phase.

It has been shown previously [11] that malate oxidation by cauliflower mitochondria was confined to the matrix space where either malate dehydrogenase or NAD-linked malic enzyme (or both) could be involved [4–6]. Under these conditions, externally added NAD (which is considered not to penetrate through the inner membrane into the mitochondrial matrix) stimulated O_2 uptake with malate (Table 1 in ref. [11]). This stimulation occurs because another pathway of NADH

oxidation (initiated on the outside of the inner membrane) is brought into operation [13, 14]. The salient point of this is the stimulation itself, which shows that neither malate transport nor malate dehydrogenase (or malic enzyme) was limiting malate oxidation. Therefore, malate oxidation, in state 3, is limited by the respiratory chain and specifically that region associated with internal NADH oxidation and the energy conservation site 1. However, this region of the chain can not be restricting the lower rate of isocitrate oxidation, which may be limited by transport via the tricarboxylate carrier. This is suggested by its greater (compared to malate) dependence on added phosphate [10] and the slower rate of mitochondrial swelling observed in ammonium citrate [15, 16].

External isocitrate may be a poor substrate because of the exchange nature of the tricarboxylate transporter. Isocitrate entering the mitochondria will, on equilibration by aconitase, yield predominatly (greater than 90%; [17]) citrate. If the transporter then begins to operate as a $citrate_{out}/isocitrate_{in}$ exchange–diffusion system, as is likely, the internal tricarboxylic acid pool will not be increased and the internal isocitrate concentration will be less than 1% of that added externally. Thus, although the rate of operation of the transporter may not appear to be limiting, its mode of operation could be controlling the effective internal substrate concentration. Unlike its mammalian counterpart, the plant mitochondrial isocitrate dehydrogenase does not appear to be allosterically regulated by adenine nucleotides, but its activity may be restricted by NADH [9]. High NADH levels within the matrix of isolated mitochondria, under state 3 conditions and in the absence of other substrates, are unlikely. Furthermore, their existence and restriction of isocitrate oxidation would imply a physical separation and compartmentation between isocitrate dehydrogenase and its NAD/NADH pool, and the other NAD/NADH pool(s) of the mitochondrial matrix and inner membrane respiratory chain. Such a unique arrangement is most improbable [18] leaving transport as the step rate-limiting isocitrate oxidation in isolated cauliflower mitochondria.

Very little O_2 was consumed when NADP replaced NAD in disrupted preparations (Table 1) indicating that both malate and isocitrate dehydrogenases of cauliflower mitochondria are NAD-specific, as observed by others [9, 19]. This suggests that isocitrate oxidation by plant mitochondria is not involved in producing reducing power for biosynthetic pathways (e.g. fatty acid biosynthesis).

α-Ketoglutarate (αKG) was readily oxidized by intact mitochondria (Table 1) providing TPP was supplied, but only after a period of incubation in the presence of substrate and co-factor. This lag period varied from 1–10 min (apparently with the season) and was observed with both cauliflower and beetroot mitochondria. Disruption of the mitochondria and addition of NAD and PMS during the lag period caused only a slight stimulation of O_2 uptake (Table 1) suggesting that penetration of substrate and co-factor was not rate limiting. The requirement for an incubation period prior to α-KG oxidation may simply represent deactivation of the αKG dehydrogenase complex during isolation of the mitochondria, perhaps by depletion of substrate and/or co-factors.

Concurrent oxidation of more than one substrate

Regulation of O_2 uptake in intact mitochondria was further investigated by measuring the oxidation of 2 or 3 substrates together (Table 2). When a combination of any two of malate, succinate and NADH was used, O_2 uptake rates were greater than those associated with the oxidation of a single substrate (Table 2). Upon addition of malonate to mitochondria respiring NADH + succinate, or malate + succinate, O_2 consumption reverted to a rate equal to that found with malate or NADH alone (Table 2). Similarly the addition of rotenone during oxidation of NADH + malate resulted in O_2 uptake rates similar to those observed when external NADH was the only substrate (Table 2). That is, electrons entered the respiratory chain at different sites during the oxidation of more than one substrate. ADP/O values associated with the oxidation of malate + succinate and malate + NADH were intermediate between values usually indicative of 2 and 3 sites of phosphorylation (Table 2). These results suggest that the rate limiting steps involved in O_2 uptake by cauliflower mitochondria are in that area of the respiratory chain close to the NADH and succinate de-

Table 2. Concurrent oxidation of two or more different substrates by cauliflower bud mitochondria

Substrate	State 3 rate	State 4 rate	+ rotenone	+ malonate	ADP/O
Malate	69	26	27	—	2.74
Succinate	90	43	—	19	1.9
Malate + succinate	120	41	—	60	2.2
NADH	118	51	118	—	1.25
NADH + succinate	167	85	—	112	1.6
NADH + malate	160	62	100	—	1.7
Citrate	28	—	—	—	—
Malate + citrate	61	26	—	—	2.4
Malate + NADH + succinate	173	64	—	—	1.45

O_2 uptake was measured as described in the Experimental section. Final concentrations used were: 10 mM malate, 10 mM succinate, 10 mM citrate, 5 mM malonate, 15 μM rotenone, 1 mM NADH and 0.25 mM ADP. When malate was used, 10 mM glutamate was included in the reaction medium and when succinate was used alone as substrate, 15 μM rotenone was included. Otherwise rotenone was added during State 3 respiration. To ensure activation of succinate dehydrogenase, NADH or malate were added when a steady rate with succinate + ADP had been obtained. 2–4 mg mitochondrial protein was used. Rates shown are averages of two experiments and are expressed as nmol O_2 consumed/min/mg protein.

hydrogenases, and may be the dehydrogenases themselves. Since isocitrate oxidation was slower than that of malate (Table 1), the internal NADH dehydrogenase obviously did not limit its oxidation (see above). The presence of citrate did not lead to faster rates of O_2 uptake with malate (Table 2) suggesting that reducing equivalents from these two substrates enter the chain via a common NADH dehydrogenase.

Although O_2 uptake with malate and succinate was faster than with either alone, the rate was less than the sum of the rates obtained with single substrates (Table 2: 120 nmol/min/mg vs 60 plus 90 nmol/min/mg), suggesting that malate and succinate compete for the dicarboxylate carrier when added simultaneously. (Obviously electron transport was not limiting here since NADH + succinate, NADH + malate, and NADH + succinate + malate all yielded rates in excess of that with malate + succinate). When exogenous NADH and succinate or malate are supplied together, however, the rate of electron transfer down the respiratory chain may become limiting because the supply of all 3 causes no further increase in rate. When exogenous NADH oxidation was monitored spectrophotometrically, it was found that the addition of malate or succinate caused an inhibition (Table 3). This inhibition was more pronounced when both malate and succinate were present (Table 3). These results imply competition for a component common to all 3 oxidation pathways, possibly ubiquinone, in the light of recent work by Storey [20, 21], since oxidation of added NADH does not involve transport of substrate across the membrane [11, 22]. Interaction between NADH and succinate has been observed also with mammalian mitochondria [23–25] and with mitochondria from Jerusalem artichoke [13].

It should be noted that external NAD is reduced upon oxidation of malate by these mitochondria [11, 14] and hence the apparent inhibition of NADH oxidation by malate (Table 3) may have been due in part to re-reduction of external NAD (which would have masked the drop in A_{340} nm upon NADH oxidation). This explanation seems unlikely in view of the O_2 uptake data in Table 2 and the inhibition of NADH oxidation by succinate (Table 3). It has also been shown (Day and Wiskich, unpublished) that small quantities of external NADH drastically inhibit transfer of reducing equivalents from within the matrix to external NAD; even in the absence of NADH, the rate of NAD reduction rarely exceeded 50 nmol/min/mg (data not shown). It is therefore unlikely that re-reduction of external NAD contributed significantly to inhibition of the decrease in A_{340} nm, under these conditions.

Table 3. Exogenous NADH oxidation by cauliflower bud mitochondria in the presence of malate and succinate

Substrate	NADH oxidation + ADP	NADH oxidation ADP and succinate
NADH	258	208
NADH + malate	209	177

NADH oxidation was followed spectrophotometrically as described in the Experimental section. Final concentrations were, 0.5 mM NADH, 10 mM succinate, 10 mM malate, 0.2 mM ADP and 0.6 mg protein. Glutamate (20 mM) was present when malate was used. When succinate was present, it was added prior to NADH and the mitochondria were incubated for 1 min at room temp. (with ADP also present), to activate succinate dehydrogenase. The reaction was initiated by adding NADH and ADP. Rates are expressed as nmol NADH oxidized/min/mg protein.

CONCLUSIONS

The data presented here suggest that, in isolated cauliflower mitochondria (which contain an active glutamate-oxaloacetate transaminase) the state 3 oxidation of malate (in the presence of glutamate), succinate and exogenous NADH is restricted by the respiratory chain at a point on the substrate side of the cytochrome components. The respective respiratory-linked dehydrogenases may be such a point. Isocitrate oxidation on the other hand, appears to be limited by the tricarboxylate transporter. Saturation of the cytochrome chain did not occur with any single substrate, nor necessarily with the combination of any two.

EXPERIMENTAL

Mitochondria were isolated from fresh cauliflower (*Brassica oleracea* L.) buds as described previously [14]. O_2 uptake was measured with a Clark O_2 electrode in a sealed Perspex vessel with a circulating-water bath at 25°. The standard reaction medium consisted of 0.25 M sucrose, 10 mM TES buffer (pH 7.2), 10 mM KH_2PO_4 and 5 mM $MgCl_2$. *ca* 1.5 mg of mitochondrial protein was used in a vol. of 3 ml. Protein was estimated by the method of ref. [26] with BSA (fraction V) as standard. ADP/O values were obtained from the O_2 electrode tracings [27]. Dehydrogenase activity in detergent-disrupted mitochondria was measured polarographically as described above; NAD and PMS were added to link dehydrogenase activity to O_2 consumption. That is, upon oxidation of substrate, NAD is reduced and is subsequently oxidized by PMS, which in turn reduces O_2 to H_2O_2. Catalase was present to ensure complete reduction to H_2O. Exogenous NADH oxidation was measured by following the decrease in A at 340 nm (1 cm light path); 3 ml of standard reaction medium were used.

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