**Succinate-driven reverse electron transport in the respiratory chain of plant mitochondria**

**The effects of rotenone and adenylates in relation to malate and oxaloacetate metabolism**

Pierre RUSTIN*† and Claude LANCE
Laboratoire de Biologie Végétale IV (C.N.R.S., U.A. 1180), Université Pierre et Marie Curie, 12 rue Cuvier, 75005 Paris, France

The effects of rotenone on the succinate-driven reduction of matrix nicotinamide nucleotides were investigated in Percoll-purified mitochondria from potato (*Solanum tuberosum*) tubers. Depending on the presence of ADP or ATP, rotenone caused an increase or a decrease in the level of reduction of the matrix nicotinamide nucleotides. The increase in the reduction induced by rotenone in the presence of ADP was linked to the oxidation of the malate resulting from the oxidation of succinate. Depending on the experimental conditions, malic enzyme (at pH 6.6 or in the presence of added CoA) or malate dehydrogenase (at pH 7.9) were involved in this oxidation. At pH 7.9, the oxaloacetate produced progressively inhibited the succinate dehydrogenase. In the presence of ATP the production of oxaloacetate was stopped, and succinate dehydrogenase was protected from inhibition by oxaloacetate. However, previously accumulated oxaloacetate transitorily decreased the level of the reduction of the NAD⁺ driven by succinate, by causing the reversal of the malate dehydrogenase reaction. Under these conditions (i.e. presence of ATP), rotenone strongly inhibited the reduction of NAD⁺ by succinate-driven reverse electron flow. No evidence for an active reverse electron transport through a rotenone-insensitive path could be obtained. The inhibitory effect of rotenone was masked if malate had previously accumulated, owing to the malate-oxidizing enzymes which reduced part or all of the matrix NAD⁺.

**INTRODUCTION**

The oxidation of matrix NADH by plant mitochondria is only partially sensitive to rotenone, a specific inhibitor of the Complex I of the respiratory chain, and is associated with a reduced ADP/O ratio in the presence of rotenone [1]. This pointed to the existence of an additional path for electron transport beside the rotenone-sensitive Complex I of the respiratory chain [1,2]. This additional dehydrogenase would have a $K_m$ for NADH 10-fold higher than the dehydrogenase (Complex I) involved in the rotenone-sensitive path [3]. However, this electron path has only been characterized by kinetic studies carried out on the whole respiratory chain [3]. There is at present no experimental support for this postulated pathway in published papers on the isolation of NADH dehydrogenases of plant mitochondria [4,5]. Therefore, a bypass of the rotenone-sensitive site of Complex I, triggered by conditions of high reduction of the complex (i.e. in the presence of rotenone), could alternatively be invoked to account for the kinetic behaviour of internal NADH oxidation. In support for this hypothesis, it should also be mentioned that the presence of such a pathway is hardly detectable in the absence of rotenone [2].

In animal mitochondria, no rotenone-insensitive oxidation of the internal NADH is observed, and rotenone has been shown to inhibit fully the reduction of NAD⁺ by the succinate-driven reverse electron transport [6]. The occurrence of a reverse electron transport has also been documented in the respiratory chain of plant mitochondria [7–10]. However, its study has been limited and obscured by the complex and various mechanisms of the oxidation of the malate resulting from succinate oxidation [11]. As a result, very few studies have been really focused on this aspect of electron transport in plant mitochondria.

The progress in the understanding of the mechanisms of malate oxidation in plant mitochondria was an incentive to investigate the mechanisms of NAD⁺ reduction by succinate, with particular attention paid to the effects of rotenone.

**MATERIALS AND METHODS**

**Preparation of mitochondria**

Mitochondria were isolated from potato tubers (*Solanum tuberosum L.* ) by differential centrifugation, and further purified on two successive Percoll gradients [12]. Purified mitochondria were stored at 0 °C at a concentration of 50–80 mg of protein/ml. Mitochondrial protein was measured by the method of Bradford [13]. The percentage of intact mitochondria exceeded 95% when assayed by the measurement of the permeability of the outer membrane to cytochrome $c$ [14].

**Reduction level of nicotinamide nucleotides and measurement of oxygen uptake**

The redox state of matrix nicotinamide nucleotides was measured fluorimetrically [15] in a reaction medium (medium A) containing 0.3 mM-mannitol, 10 mM-phosphate buffer, 10 mM-Mops (pH 7.2, unless otherwise indicated), 5 mM-MgCl₂, 10 mM-KCl and 0.1% (w/v) defatted BSA. Oxygen uptake was measured in medium A in a 1.5 ml cell thermostatically maintained at 25 °C, with a Clark oxygen electrode. NADH content of purified mitochondria was determined by bioluminescent assay after

---

Abbreviations used: m-Cl-CCP, carbonyl cyanide m-chlorophenylhydrazone; OAA, oxaloacetic acid; State 3 and State 4, oxygen uptake in the presence and after phosphorylation of ADP respectively.

* Present address: Unité de Recherches de Génétique Médicale (I.N.S.E.R.M., U. 12), Hôpital des Enfants Malades, Tour Technique Lavoisier, 149 rue de Sèvres, 75743 Paris Cedex 15, France.

† To whom requests for reprints should be addressed.
extraction by ethanol. Full reduction of the NAD⁺ was obtained by a subsequent addition of alcohol dehydrogenase [16].

**Oxaloacetate (OAA) and malate determination**

OAA determination during succinate oxidation was performed enzymically with 10 nkat of purified pig heart malate dehydrogenase (EC 1.1.1.37; Sigma)/ml as previously described [17]. Oxidation of succinate was stopped by addition of an excess of HClO₄. After centrifugation at 3000 g for 15 min, the supernatant was neutralized by K₂CO₃. KClO₄ was then spun down at 3000 g for 10 min, and the supernatant used for OAA assays. Malate was determined enzymically with 1 mm-NADP⁺ and 1.67 nkat of chicken liver NADP⁺-dependent malic enzyme (EC 1.1.1.40; Sigma)/ml [11]. Alternatively oxaloacetate production linked to malate oxidation was measured spectrophotometrically as previously described [18].

**Membrane potential determination**

This was done by the safranin method [19,20]. Calibration was performed by K⁺ addition in the presence of valinomycin, indicating a linear response of the probe from 50 to 170 mV. The linearity of the response of the safranin method at higher membrane-potential values (230 mV) was established by comparison with results obtained with a tetraphenylphosphonium electrode [21].

**RESULTS**

**Effect of rotenone on NAD⁺ reduction**

The effect of rotenone on NAD⁺ reduction linked to succinate oxidation under State-3 conditions (presence of ADP) was studied at three different pH values (Fig. 1). At pH 6.6, known to favour the activity of malic enzyme [22], adding rotenone under State-3 conditions caused a rapid reduction of NAD⁺ (Fig. 1, trace a) that amounted to about 75% (notch on the trace) of the NAD⁺ reduced under State-4 conditions in the absence of rotenone (Fig. 1, trace a, dotted line). Then, in the presence of rotenone the State-3/State-4 transition corresponded to a further 25% NAD⁺ reduction (second phase of NAD⁺ reduction after rotenone addition). In the presence of ADP, no reverse electron transport driven by succinate occurs [6,7], and it has been shown that succinate oxidation is associated with a significant production of malate [10,11]. Under our experimental conditions, this amounted to 130 nmol of malate produced/min per mg of protein (average value of three replicates) corresponding to a succinate oxidation rate of 380 nmol of O₂ consumed/min per mg of protein. One can therefore hypothesize that, at pH 6.6, the reduction of NAD⁺ upon rotenone addition (Fig. 1, trace a) was actually linked to the oxidation of malate by malic enzyme. Accordingly, an increase in the pH of the assay medium should strongly affect the reduction of NAD⁺ brought about by addition of rotenone as a consequence of turning off the malic enzyme, the malate dehydrogenase being then the only active malate-oxidizing enzyme [18,23].

As expected, at pH 7.9 the NAD⁺ reduction upon rotenone addition was much decreased (Fig. 1, trace b). This has to be ascribed to a rapid poisoning of the malate dehydrogenase by the accumulated NADH [24]. Finally, at pH 7.2 an intermediate level of reduction was obtained, which could be increased by stimulating malic enzyme activity by addition of CoA, a well-known activator of this enzyme [25]. In all cases, adding OAA caused a full re-oxidation of NADH. But, whereas at pH 6.6 this effect was only transitory, its duration was strongly increased at pH 7.9. Again, at pH 7.2, an intermediate situation was observed, and the activation of malic enzyme by CoA brought about a faster reduction of NAD⁺. Altogether, the effects of pH and CoA suggest that malic enzyme was largely responsible for OAA elimination, as it was for the reduction of NAD⁺ in the presence of rotenone.

The effect of rotenone was further investigated under State-4 conditions (i.e. presence of ATP) (Fig. 2). As malate caused NAD⁺ reduction as soon as it accumulated in sufficient amounts (see Fig. 1, upon addition of rotenone), the effect of rotenone was tested before any significant accumulation of malate occurred. When added before succinate, rotenone caused a major inhibition of the rate of reduction of NAD⁺ (Fig. 2, lower trace). The initial rate measured in the presence of rotenone (0.25 nmol/min per

![Fig. 1. Effect of rotenone on NAD⁺ reduction during the oxidation of succinate at different pH values under State-3 conditions](image-url)
mg of protein) represented about 4% of the rate (6.32 nmol/min per mg of protein) measured in the absence of rotenone (Fig. 2, upper trace). Very similar effects of rotenone were observed at pH 6.6 or 7.9 (results not shown). The slow residual reduction observed in the presence of rotenone could represent a reduction of the NAD+ by the malate still produced along with the oxidation of succinate, although such a malate-dependent reduction should be affected by the pH of the medium. Alternatively, this could also represent a reverse electron flow through the rotenone-insensitive pathway present in plant mitochondria.

In plant mitochondria, as well as in their animal counterparts, the rotenone-sensitive reverse electron transport is known to be largely dependent on the presence of ATP [6-9,26]. In animal mitochondria ATP was shown to be hydrolysed, allowing the energetically unfavourable reverse electron transport from ubiquinol to NAD+ to take place [6]. However, we did not detect any ATP hydrolysis under our experimental conditions when using intact potato tuber mitochondria, and ATP alone did not allow the establishment of a measurable membrane potential (results not shown); rather, the energy required for the unfavourable reduction of NAD+ would solely originate from succinate oxidation. Accordingly, even in the presence of ATP the reverse electron transport in intact potato tuber mitochondria was fully inhibited by antimycin or KCN (results not shown). By inhibiting electron flow through the cytochrome pathway, these latter compounds prevented the establishment of the membrane potential required for the occurrence of a reverse electron flow. The mechanism of the ATP effect was therefore next studied.

Three different parameters associated with succinate oxidation, i.e. oxygen uptake, NAD+ reduction and membrane potential, were measured under various conditions (Table 1). Comparison of values measured with mitochondria alone or with mitochondria supplemented with ATP indicates that ATP simultaneously decreased the rate of oxygen uptake and increased both the rate of NAD+ reduction and the rate of membrane-potential increase. The rate of oxygen uptake measured in the presence of ATP upon a subsequent ADP addition (449 nmol of O2/min per mg of protein, compared with 280 nmol/min per mg in the absence of ATP) indicated that the well-known stimulation of the succinate dehydrogenase by ATP [27,28] did indeed take place. The high oxidation rate and the low membrane potential initially measured in the absence of ATP suggested that in its resting state the ATPase was partially leaky to protons. The addition of ATP eliminated this proton conductance, and a decreased rate of oxygen uptake and a higher rate of membrane-potential increase were thereupon measured. Accordingly, oligomycin, which blocks proton conductance through the F, subunit of the ATPase, mimicked most of the ATP effects, raising the rates of membrane-potential increase and decreasing the rate of O2 uptake, as compared with the rates measured with succinate alone. However, the activity of the succinate dehydrogenase (377 nmol/min per mg of protein), as measured on subsequent addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (m-Cl-CCP), was lower than in the presence of ATP upon subsequent ADP addition (449 nmol/min per mg of protein). This should be ascribed to the lower rate of succinate dehydrogenase activity in the absence of the activator ATP [27,28].

The experiment of Fig. 1 showed that malate produced during the oxidation of succinate by potato tuber mitochondria was actively metabolized, and actually participated in the reduction

---

**Table 1. Effects of ATP and oligomycin on various parameters associated with succinate oxidation**

All measurements were performed as described in the Materials and methods section. NAD+ content of the mitochondrial preparation, estimated by bioluminescence assay, was 1.21 nmol/mg of protein, of which 95% was reduced by succinate under steady State-4 conditions. The value measured for membrane potential under steady-state conditions (State-4 conditions) was 132 mV. Oxygen uptake was determined with 0.26 mg of protein/ml, NAD+ reduction with 0.34 mg of protein/ml, and membrane potential with 0.28 mg of protein/ml. Concentrations used: succinate, 5 mM; ATP, 100 μM; oligomycin, 50 ng/mg of protein; ADP, 40 μM; m-Cl-CCP, 10 μM; safranin, 25 μM. Values in parentheses indicate percentages of the values measured in the presence of ATP.

<table>
<thead>
<tr>
<th>Rate (nmol/min per mg of protein)</th>
<th>Membrane-potential increase (mV/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxygen uptake</strong></td>
<td><strong>NAD+ reduction</strong></td>
</tr>
<tr>
<td>Succinate + ATP then ADP</td>
<td>140 (100%)</td>
</tr>
<tr>
<td>Succinate then ADP</td>
<td>220 (157%)</td>
</tr>
<tr>
<td>Succinate + oligomycin then m-Cl-CCP</td>
<td>120 (86%)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
of NAD⁺. This was in good agreement with previous polarographic studies that have established that OAA resulting from malate oxidation could be produced in sufficient amounts to cause the progressive inhibition of succinate oxidation at alkaline pH values and under State-3 conditions [10]. The next experiment was therefore focused on the potential effect of this OAA on the reduction of NAD⁺ observed in the presence of succinate.

**Effect of OAA on the reduction of NAD⁺**

The effect of varying the amounts of ADP added to mitochondria oxidizing succinate at a low concentration (0.8 mM) on the kinetics of NAD⁺ reduction is shown in Fig. 3. Increasing the duration of the State-3 condition by increasing the concentration of added ADP caused the appearance of a two-step reduction of the NAD⁺ upon establishment of the State-4 condition. Only the first step of the reduction of NAD⁺ was found to be dependent on the duration of the preceding State 3. A two-step reduction could also be obtained by decreasing the concentration of succinate in the medium (at a constant ADP concentration, 40 μM) (Fig. 4). The decrease in the concentration of succinate added to mitochondria was associated with a decrease in the rate of oxidation. This latter decrease was indicated by the increase in the duration of the time required to consume the ADP (see the legend of Fig. 3). This decrease in the rate of succinate oxidation should be ascribed to a decrease in the reducing activity of the...
succinate dehydrogenase caused by substrate limitation. At the lowest concentration of succinate used (0.5 mM), the activity of succinate dehydrogenase was not sufficient to overcome the effect of OAA (Fig. 4, trace d). Under these conditions, the addition of glutamate, allowing the transamination of OAA, was required to bring about a complete and rapid reduction of the matrix NADH, indicative again of the involvement of OAA in the control of the level of NADH reduction. Under these experimental conditions (pH 7.9, presence of ADP) the rate of OAA production was measured to be about 9 nmol/min per mg of protein, whereas no accumulation of OAA was detected under State-4 conditions.

**Relationship between adenylates and malate metabolism**

The above experiments have shown that addition of ADP, by causing re-oxidation of the matrix NADH, led to an OAA accumulation, followed by the biphasic transitions of NADH reduction observed under subsequent State-4 conditions. However, Fig. 5 shows that the effect of ADP was not limited to an activation of electron transport. In trace (a), the constraint on electron flow exerted by the phosphorylation process was released by initially adding both an ATPase inhibitor (oligomycin) and an uncoupler (m-Cl-CCP) to mitochondria oxidizing succinate at a low concentration (0.5 mM). Under these conditions, no inhibition of succinate oxidation was observed. Only the additional presence of ADP induced a progressive inhibition of the oxidation (trace b). Such an inhibition was not observed in the presence of rotenone (trace c), suggesting the involvement of a rotenone-insensitive enzyme, i.e. malate dehydrogenase at pH 7.9, in this ADP-dependent inhibitory process [10]. Again, ATP caused a stimulation of succinate oxidation, despite the presence of the uncoupler and of the ATPase inhibitor (trace d).

The complex relationships between the effect of OAA and adenylates were further studied in the last two experiments (Fig. 5, trace e, and Fig. 6). In the first experiment, it was observed that the inhibitory effect of OAA on the oxidation of 5 mM-succinate could be fully reversed by ATP, as is known for the isolated enzyme [28]. As no phosphoenolpyruvate carboxykinase activity, which decarboxylates OAA to phosphoenolpyruvate in the presence of ATP, as found in some mitochondria [29], could be detected in purified potato tuber mitochondria, metabolism of OAA triggered by ATP could be ruled out. Moreover, adding hexokinase plus glucose, which converts all the ATP present into ADP, restored its full inhibitory potential to OAA and re-established the inhibitory oxidation rate.

Beside the control exerted on the modulation of the succinate dehydrogenase by OAA, the adenylates also interfered directly with the production of OAA (Fig. 6). After the equilibrium of the external malate dehydrogenase reaction had been reached, whose displacement is used to monitor continuously the OAA concentration in the medium [18], ADP was required to measure an active OAA production (Fig. 6, trace a). After the accumulation of about 35 nmol of OAA, which corresponded to the phosphorylation of about 90% of the added ADP (assuming an ADP/O ratio of 2.5 for malate oxidation), adding succinate led to a gradual disappearance of OAA. At pH 7.9 in the presence of rotenone, such a disappearance of OAA triggered by succinate was not observed (results not shown). This elimination of OAA under State-4 conditions was mediated through reverse electron transport, and accounted for the biphasic transitions observed in Figs. 3 and 4.

When using mitochondria initially supplemented with ATP, oligomycin and m-Cl-CCP, no significant OAA production could be measured (Fig. 6, trace b). Despite the presence of the uncoupler, ADP was still required to observe an active OAA accumulation, which gradually ceased (Fig. 6, trace c). Owing to the absence of reverse electron transport in the presence of the uncoupler, this OAA was not eliminated on addition of succinate. This effect of ADP observed with uncoupled mitochondria should be ascribed to the direct regulation of the mitochondrial malate dehydrogenase by the matrix ATP/ADP ratio, which has been demonstrated with plant mitochondria [12,30,31].

**DISCUSSION**

The above results establish that, as in animal mitochondria [6], the ATP-dependent reduction of NADH driven by reverse electron transport from succinate is largely sensitive to rotenone. Only a minor fraction of this electron flow to NADH would make use of the rotenone-insensitive pathway (less than 5%) (Fig. 2). Taking into account the lack of proton translocation associated with the rotenone-insensitive pathway [1,2,17], it can be suggested that the energetically unfavourable NADH reduction cannot be mediated through this pathway, for simple thermodynamic considerations.

As previously shown [7,26], it appears that as soon as malate (arising from succinate oxidation) accumulates, it plays a significant role in the reduction of NADH. Moreover, these confusing relationships between malate (and OAA) metabolism and true reverse electron transport cannot be dissociated from the effect of adenylates on this latter process. These interactions are illustrated in detail in Scheme 1, featuring a portion of the internal membrane of the mitochondria, and the matrix enzymes that are involved in the succinate-driven NADH reduction.

Concerning the effect of adenylates, it was recently proposed to use measurement of reverse electron transport to detect eventual defects in ATPase activity in human pathology, on the basis of the ATP-dependence of this process in the presence of antimycin [32]. However, our data establish that the role of ATP in plant mitochondria is not to sustain a sufficient membrane.
ATP plays ATPase by preventing products of succinate oxidation. Chondria This ATP hydrolysis [20]. Rather, chondria reverse electron activity the succinate-driven metabolism, potentially and, for example, this situation is one subunit of the inner membrane, which potentially interferes in vivo with the metabolism of malate arising from succinate (see [11]). * refers to a negative effect; + refers to a positive effect.

Interestingly, in contrast with animal mitochondria [6], no ATP hydrolysis and no membrane energization triggered by ATP alone can be detected in intact potato tuber mitochondria. This has been ascribed to the presence in potato tuber mito-
chondria of an active ATPase inhibitor (IF), which binds to the F, subunit of complex V and acts as a directional regulator of the ATPase by preventing the back-flow of energy from the ATP [2,33]. This situation is not encountered in all plant mitochondria, and, for example, ATP hydrolysis was shown to sustain potentially a membrane potential in isolated mung-bean mito-
chondria [20]. Rather in potato tuber mitochondria, ATP appears to block the natural proton conductance of the F, subunit of the ATPase in the resting state (negative arrow on Complex V, Scheme 1). Accordingly, oligomycin produced very similar effects (Table 1).

Two other additional effects of ATP, related to malate metabolism, affect the succinate-driven reduction of NAD*. On the one hand, ATP counteracts the inhibitory effect of OAA on the activity of succinate dehydrogenase (see Fig. 4), as previously described for the isolated enzyme [27,28]. This long-known inhibitory effect of OAA on succinate oxidation is dependent on the ratio of succinate to oxaloacetate [28], and is only seen in the absence of ATP (Fig. 5). On the other hand, ATP also tightly controls the production of OAA from the malate arising from succinate oxidation. It has been previously established that the plant mitochondrial malate dehydrogenase is inhibited by ATP competitively with NAD* [31]. These three interfering mechanisms accounting for the dependence on ATP of the reverse electron transport, i.e. effect on (a) the ATPase, (b) the succinate dehydrogenase and (c) the malate dehydrogenase, are indicated in Scheme 1. All these effects concur to increase the reduction of NAD* by succinate under State-4 conditions.

By depleting the matrix ATP, ADP will have opposite effects in terms of enzyme (succinate dehydrogenase and malate dehydrogenase) regulation: increased sensitivity of the succinate dehydrogenase to OAA, and more OAA produced from malate. The presence of this OAA ultimately causes a two-step reduction of the NAD* under State-4 conditions following ADP phosphorylation (Figs. 3 and 4). This two-step reduction appears more pronounced when using higher ADP concentrations (more OAA produced) or lower succinate concentrations (succinate dehydrogenase more sensitive to OAA) (Figs. 3 and 4).
Effect of rotenone on NAD\textsuperscript{+} reduction by succinate

In vivo, the situation would potentially be even more complex, since the cytosolic malate dehydrogenase, in association with the EGTA-sensitive external NADH dehydrogenase (see Scheme 1), can also metabolize the malate excreted during succinate oxidation, producing OAA, which enters the mitochondrial matrix and gives rise to a new set of interactions which were previously studied [11].

Finally, the presence of an NAD\textsuperscript{+}-dependent malic enzyme in plant mitochondria is an additional source of further confusion. Whereas the malate dehydrogenase is essentially inactive under State-4 conditions [2,12,24], malic enzyme is still able to operate. As a consequence, at acidic pH or in the presence of added CoA, a known activator of malic enzyme [22], this enzyme participates to a large extent in the NAD\textsuperscript{+} reduction (Fig. 1). Under conditions of full activation of malic enzyme, and as soon as malate has accumulated in sufficient amounts, it is impossible really to discriminate between an eventual reverse electron transport through a rotenone-insensitive pathway and a reduction of NAD\textsuperscript{+} brought about by the oxidation of malate by malic enzyme.

As a conclusion, it appears that the balance between ADP and ATP tightly controls both the enzyme activities (succinate dehydrogenase, malate dehydrogenase, ATPase) and the direction of electron transport (to oxygen or to NAD\textsuperscript{+}). Despite possible confusion with NAD\textsuperscript{+}-reducing activities of malate dehydrogenase and malic enzyme, the choice of particular conditions (i.e. high pH values, no previous accumulation of malate) allows one to measure a true rotenone-sensitive reverse electron transport. No evidence for such an active reverse electron transport through a rotenone-insensitive path was obtained throughout this study.

We are deeply indebted to Mrs. M. Pouget for technical assistance and to Professor R. Douce and Dr. M. Neuburger for sustained interest in this work and stimulating discussions.

REFERENCES


Received 16 July 1990/11 September 1990; accepted 19 September 1990