Regulation of Electron Transport in Plant Mitochondria under State 4 Conditions

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ABSTRACT

The regulation of electron transport in pea (Pisum sativum L.) leaf mitochondria under state 4 conditions has been investigated by simultaneously monitoring oxygen uptake, the steady-state reduction level of ubiquinone, and membrane potential. Membrane potentials were measured using a methyltriphenylphosphonium electrode while a voltammetric technique was used to monitor changes in the steady-state reduction levels of quinone. It was found that the addition of glycerol to mitochondria oxidising malate in state 4 led to a marked increase in the rate of O2 uptake and increased both the membrane potential and reduction level of the quinone pool. Increases in the state 4 respiratory rate were attributed to both an increase in driving flux, due to increased Q-pool reduction, and in membrane potential. Due to the nonohmic behavior of the inner membrane, under these conditions, an increase in potential would result in a considerable rise in proton conductance. Measurement of dual substrate oxidation, in the presence of n-propylgallate, revealed that the increase in respiratory activity was not mediated by the alternative oxidase. Similar increases in membrane potential and the level of Q-pool reduction were observed even in the presence of rotenone suggesting that the rotenone-insensitive pathway is a constitutive feature of plant mitochondria and may play a role in facilitating rapid state 4 rates even in the presence of a high energy charge.

Within the framework of the chemiosmotic hypothesis (18), respiratory control is considered to be due to the disequilibrium between the respiratory chain and the protonmotive force. The transition to state 4 conditions increases the protonmotive force which exerts a back pressure on the respiratory chain restricting the rate of electron flow and hence overall oxygen uptake. The control of mitochondrial respiration has been most widely studied in mammalian tissues where it has been concluded that in the absence of ADP the major point of regulation resides in the proton leak pathway (2, 13). Using the flux control summation and connectivity theorems of the metabolic control theory, however, Brand et al. (3) reassessed control under nonphosphorylating conditions and calculated that although H+-conductance does have a large flux control co-efficient, there is also a significant contribution to control exerted by the respiratory chain. Although the analysis did not indicate which steps in the chain exerted control, it was suggested that it may be distributed in some or all of succinate dehydrogenase, QO, the bc, complex, Cyt c diffusion, and Cyt oxidase.

The control of respiration in plant mitochondria is somewhat more complicated than in mammalian tissues since the majority of plant mitochondria possess, albeit to varying extents, a cyanide-insensitive alternative oxidase, and a rotenone-insensitive bypass of complex I (10). Since electron flux via these pathways is not linked to proton extrusion (21) their engagement could make a considerable contribution to the overall respiratory rate under state 4 conditions. The degree to which the antimycin-insensitive alternative oxidase contributes to ADP-limited respiration has been generally assessed from the effect of inhibitors of this pathway on respiratory control indices (16). Inhibition of the pathway results in a marked increase in control suggesting it is engaged under state 4 conditions. More recently it has been demonstrated that the degree to which this pathway is engaged is dependent, in a nonlinear fashion, upon the level of reduction of the quinone pool (12). Significant engagement of the alternative oxidase is not apparent until Q-pool reduction levels reached 35 to 40% and was maximal under state 4 conditions (12). The degree to which the rotenone-insensitive bypass of complex I is a constitutive feature of plant mitochondrial respiratory activity is uncertain since there are no inhibitors of this pathway. It is generally considered to be due to the presence of a dehydrogenase located on the inner surface of the inner membrane (19) which has a lower affinity for NADH than complex I (20) although any specific details regarding its nature are lacking. The possibility that rotenone binds to complex I in such a manner that it prevents proton pumping but does not effectively inhibit electron flow is an alternative explanation, but to our knowledge is yet to be explored. Nevertheless, whatever the explanation, treatments which result in an increased intramitochondrial NADH levels stimulate respiration (4–7, 24) even in the presence of rotenone,

Abbreviations: Q, ubiquinone; TPMP+, methyltriphenylphosphonium; FCCP, carbonyl cyanide p-trifluoro-methoxy-phenylhydrazone; TPP+, tetraphenylphosphonium; SHAM, salicylhydroxamic acid; Δκ, protonmotive force; ΔE0, difference in redox potential between two carriers of reducing equivalents; n, number of H+ translocated across the inner membrane; Δφm, membrane potential; QH2, ubiquinol.

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suggesting its operation does contribute to state 4 respiratory rates.

In the present study we have examined the effect of dual substrate oxidation, both in the presence and absence of rotenone, on oxygen consumption, steady-state membrane potential, and the redox state of the quinone pool in an attempt to determine if the rotenone insensitive bypass is a major pathway of electron flow to the Q-pool under ADP-limited conditions. We show, for the first time, that the well-documented respiratory increase observed upon second substrate addition under state 4 conditions is accompanied by increases in both membrane potential and the steady-state reduction levels of the Q-pool. Such increases were observed even in the presence of rotenone, suggesting that the rotenone-insensitive bypass of complex I is a constitutive feature of the plant mitochondrial respiratory chain.

EXPERIMENTAL

Material

Pea (Pisum sativum L., cv Massey Gem) seedlings were grown in trays of vermiculite or soil in a glasshouse for 12 to 15 d. TPMP bromide and FCCP were purchased from Aldrich Chemical Co. (Gillingham, Kent, U.K.). Q-2 was prepared by Dr. A. D. Ward (Organic Chemistry Dept., University of Adelaide). All other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Mitochondrial Isolation

Pea leaves were disrupted with a Polytron PTA-3S probe for 2 to 3 s in 200 mL of ice-cold medium containing 0.3 M sorbitol, 50 mM Mops, 1 mM EDTA, 1 mM MgCl₂, 10 mM isosorbate, 1% (w/v) PVP-40, and 0.4% BSA, all adjusted to pH 7.5. The homogenate was filtered through three layers of cheesecloth and centrifuged for 5 min at 2,000 g. The supernatant was centrifuged at 10,000 g for 20 min and the pellet washed by resuspending in 60 mL of 0.3 M sorbitol containing 20 mM Mops (pH 7.5) and 0.1% BSA, and centrifuged at 10,000 g for 20 min. Final resuspension was in 2 to 3 mL of wash medium. Purified mitochondria were obtained using either a Percoll/PVP gradient as described by Day et al. (5) or using a discontinuous gradient as described by Dry et al. (11).

Assay Procedures

O₂ consumption was measured polarographically in 2.1 mL of reaction medium containing 0.3 M mannitol, 10 mM KH₂PO₄, 1 mM MgCl₂, 10 mM KCl, and 10 mM Mops (pH 7.5) in a specially constructed cell housing a Rank oxygen electrode, a TPMP⁺ sensitive electrode, a glassy carbon, and a platinum electrode. Continuous monitoring of the membrane potential was achieved using a TPMP⁺ sensitive electrode, a modification of the TPP⁺ electrode described by Kamo et al. (15). The electrode consisted of a platinum wire immersed in 5 mM TPMP⁺ bromide, in a 400 μL Eppendorf tube, separated from the assay medium by a polyvinyl chloride membrane impregnated with tetraphenylboron, and was coupled via a salt bridge to a reference electrode. The TPMP⁺ electrode response was calibrated for each incubation with additions of TPMP⁺ up to 2 μM and corrected for binding and response time of the electrode as previously described (30). It should be noted that due to the presence of an endogenous KC⁺/H⁺ antiporter (21), the contribution of pH component to the protonmotive force is negligible and this was further ensured by the composition of the reaction medium so that Δp = ΔV. Membrane potentials were calculated using the Nernst equation on the basis of a matrix volume of 1.4 μL/mg protein (26).

The redox state of Q-2 was measured voltametrically using a glassy carbon working electrode and a platinum electrode connected to Ag/AgCl reference electrode. The working electrode was poised at 360 mV with respect to the reference as previously described (22). Fully oxidized Q was taken as the base of the trace following addition of 1 μM Q-2 and the appropriate quantity of mitochondrial protein, and fully reduced as that in the presence of a bcc inhibitor or upon anaerobiosis. It should be noted that plant mitochondria are normally deenergized upon isolation and possess negligible amounts of endogenous substrates (21). Q-2 at 1 μM had no detectable effect upon either the state 4 respiratory rate or membrane potential (22).

Protein and Chl Determination

Protein was determined by Lowry et al. (17), with BSA as standard. Chl was determined by the method of Arnon (1). Mitochondrial protein was corrected for the contribution by broken thylakoids by assuming a thylakoid protein/Chl ratio of 6.9:1 (23).

RESULTS

Figure 1 shows typical trace depicting changes in oxygen consumption, membrane potential and the redox poise of the quinone pool by pea leaf mitochondria whilst oxidizing malate + glutamate as a respiratory substrate and illustrates the degree of respiratory activity observed under state 4 conditions. Substrate addition resulted in a considerable respiratory rate, the rapid generation of a membrane potential, and partial reduction of the Q-pool. The initial ADP-induced state 3 phase caused an oxidation of the Q-pool to 14% of that observed under anaerobiosis and the expected transient depolarization of the membrane potential but only a small stimulation in respiratory rate. It can be seen from Figure 1 that, following transition to state 4, the reduction of the Q-pool was more pronounced than in state 2 (41% versus 31%) but the oxygen uptake rate was slower (68 versus 74 nmol/min/mg). This difference in rate has been attributed to the ATPase acting as an ion-influx channel, because state 2 conditions favor the release of the inhibitor protein IF, from its inhibitory site on F₁, facilitating increased H⁺-conductance (30). True state 4 conditions favor rebinding of IF₁ to F₁, and, hence, prevent the ATPase from acting as a route for H⁺-reentry (30). The subsequent addition of ADP stimulated the respiratory rate much more markedly than that observed under state 2 conditions which was accompanied by an oxidation of the Q-pool and decrease in membrane potential. The addition of glycinic, which increases intramitochondrial
NADH levels due to the activity of glycine decarboxylase (5), markedly stimulated the state 4 respiratory rate, increased the membrane potential, and further reduced the quinone pool from 41 to 70%. Similar results were obtained when malate + glutamate was added to mitochondria already oxidizing glycine (not shown).

Previous observations on the respiratory stimulation caused by the addition of a second substrate have suggested that, under certain conditions, the stimulation is the result of engagement of the alternative oxidase (30). Because degree of engagement of alternative oxidase activity is dependent upon the level of reduction of the quinone pool (10, 22) and this is increased considerably by second substrate addition, it is conceivable that this may be the case in the results depicted in Figure 1. In the results summarized in Table I we have investigated the effect of n-propylgallate, an alternative oxidase inhibitor, on the glycine or malate dependent state 4 respiratory rate and membrane potential. When n-propylgallate was added during state 4, but prior to the addition of a second substrate, it partially depolarized the membrane potential resulting in stimulation of respiratory activity. This was observed with either malate or glycine as substrates. Second substrate addition, however, still resulted in respiratory stimulation and an increase in membrane potential as observed in Figure 1. In the presence of dual substrates, n-propylgallate had only a minor effect upon the respiratory rate and negligible effect on membrane potential, suggesting that the observed increases in respiratory rate were not due to engagement of the alternative oxidase. It should be noted that since n-propylgallate (and, indeed, SHAM and disulfiram)

Figure 1. Simultaneous measurement of O₂ uptake, steady-state membrane potential, and quinone reduction during malate oxidation by pea leaf mitochondria. O₂ uptake, membrane potential, and quinone reduction were measured, in the presence of 1 μM Q-2 and 2 μM TPMP⁺ as described in "Materials and Methods." Where indicated 10 mM malate, 10 mM glutamate, 10 mM glycine, and 0.15 mM ADP were added. Numbers on the traces refer to: O₂, nmol/min/mg protein; Δψ, mV; Q, % reduction.
Table 1. Effect of n-Propylgallate on Dual Substrate Oxidation by Pea Leaf Mitochondria

Oxygen uptake and steady-state membrane potentials were measured in the presence of 2 μM TPMP⁺ as described in "Materials and Methods." Respiration was initiated by the addition of 10 mM malate + 10 mM glutamate or 10 mM glycine and state 4 measured following a state 3/4 transition. 50 μM n-propylgallate was added where indicated.

<table>
<thead>
<tr>
<th>Sequential Additions</th>
<th>Respiratory Rate nmol/min/mg</th>
<th>Membrane Potential mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate—State 4</td>
<td>46</td>
<td>181</td>
</tr>
<tr>
<td>+ Glycine</td>
<td>85</td>
<td>191</td>
</tr>
<tr>
<td>+ Glycine + npg</td>
<td>79</td>
<td>190</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate—State 4</td>
<td>47</td>
<td>180</td>
</tr>
<tr>
<td>+ npg</td>
<td>59</td>
<td>170</td>
</tr>
<tr>
<td>+ npg + Glycine</td>
<td>79</td>
<td>186</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine—State 4</td>
<td>30</td>
<td>180</td>
</tr>
<tr>
<td>+ malate</td>
<td>88</td>
<td>198</td>
</tr>
<tr>
<td>+ malate + npg</td>
<td>82</td>
<td>195</td>
</tr>
<tr>
<td>Experiment 4</td>
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<td></td>
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<tr>
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<td>180</td>
</tr>
<tr>
<td>+ npg</td>
<td>42</td>
<td>169</td>
</tr>
<tr>
<td>+ npg + malate</td>
<td>82</td>
<td>192</td>
</tr>
</tbody>
</table>

interact with the quinone electrode, it was not possible to simultaneously monitor steady-state redox levels of the Q-pool.

The increase in oxygen uptake observed upon second substrate addition would initially appear contradictory to our current understanding of state 4, a condition in which it is assumed that the protonmotive force is maximal and, therefore, restricting electron flow. This is obviously not the case since in all cases respiratory stimulation is accompanied by a large reduction in the quinone pool and an increase in membrane potential. The increase in the redox poise of the Q-pool will stimulate electron flow to oxygen by raising ΔEₚ. This will be partially negated by the rise in membrane potential since the rate of electron transport is a function of 2 ΔEₚ - nΔp (2). To determine if electron transport between quinone and oxygen has the capacity to handle increased electron flux and is not restricted by the protonmotive force, succinate was used as a substrate. The addition of succinate (Fig. 2) resulted in a small but measurable increase in oxygen consumption, generated a membrane potential comparable to that observed in Figure 1, and the Q-pool was reduced to 54% of that observed under anaerobiosis. The ADP-induced state 3 phase caused the expected transient decrease in membrane potential and a rapid oxidation of the quinone pool. On return to state 4 the reduction of quinone was more rapid and pronounced. This reflects the activation of succinate dehydrogenase either by reduced Q or by ATP (not shown) and explains the absence of a definite state 3/4 transition in the initial part of the oxygen electrode trace. The subsequent addition of ADP shows no such anomaly as succinate dehydrogenase is fully activated. It should be noted from Figure 2 that the state 4...
respiratory rates are considerably higher than those observed in Figure 1 even though the membrane potentials are very similar, suggesting that the protonmotive force is not the principal determinant of electron flux on this segment of the respiratory chain. Presumably, the faster respiratory rate observed under these conditions is due to the increased redox poise of the quinone pool and an altered H⁺/2e⁻ ratio. Such elevated levels of Q-pool reduction would normally be expected to engage the alternative oxidase (12, 22); however, it is apparent from Figure 2, that the degree of cyanide resistance of these mitochondria is low (approximately 15%) (and, hence, fully engaged even with malate under state 4 conditions) and, furthermore, rates of oxygen uptake are considerably higher than those observed with dual substrates. Such a result confirms the notion that the increased respiratory rate observed upon addition of a second substrate is not due to the engagement of the alternative oxidase.

It is clear from the results presented so far that the $QH_2$ – $O_2$ segment of the respiratory chain has the capacity to sustain elevated respiratory rates, suggesting that it is perhaps the reduction of quinone that controls overall electron flux. This would be consistent with our previous observations that the respiratory rate is linearly dependent upon the degree of reduction of the $Q$-pool (22). The experiments illustrated in Figures 1 and 2 suggest that a comparable membrane potential is generated by either NAD⁺-linked substrates or by succinate, under state 4 conditions, and yet they maintain differing levels of $Q$-pool reduction, confirming the idea that electron flux is limited by the quinone redox poise. It is also apparent that elevated state 4 rates with NAD-linked substrates are associated with increased $Q$-reduction levels but this could be due to either the engagement of the rotenone-insensitive dehydrogenase, located on the inner surface of the inner membrane (19) or, alternatively, to an increased flux through complex I. The experiment illustrated in Figure 3, however, indicates that a similar rise in membrane potential is observed upon second substrate addition even in the presence of rotenone, suggesting that the stimulated respiratory rates depicted in Figure 1 are a consequence of increased electron flux, due to elevated $Q$-pool levels, through the bc complex and not complex I. Figure 3 also illustrates that rotenone addition results in the net oxidation of the $Q$-pool and, hence, reduction in the respiratory rate. It is suggested that the level of $Q$-pool reduction observed under these conditions reflects the contribution of the rotenone-insensitive bypass to the overall respiratory rate and, furthermore, is consistent with the pro-

Figure 3. Simultaneous measurement of $O_2$ uptake, steady-state membrane potential, and quinone reduction during malate oxidation, in the presence of rotenone, by pea leaf mitochondria. Assay conditions as in Figure 1. Where indicated 10 mM malate, 10 mM glutamate, 10 mM glycine, 0.15 mM ADP, and 1 μM rotenone (Rot) were added. Numbers on the traces refer to: $O_2$, nmol/min/mg protein; $\Delta V$, mV; $Q$, % reduction.
posal that the bypass is engaged under state 4 conditions even in the absence of inhibitor. Interestingly, there is a slight rereduction of the pool, presumably due to rotenone increasing intramitochondrial NADH levels, which results in an increased rate of \( H^+ \)-pumping through the bc\(_1\) complex as indicated by a rise in membrane potential, and close scrutiny of the oxygen uptake trace reveals a concomitant slight increase in rate. Note that the respiratory rates on these traces were calculated once the uptake rates had become linear.

In summary, the close similarity of Figures 1 and 3 suggests that electron flux mediated by the rotenone-insensitive bypass can reduce the \( Q \)-pool and, hence, is a significant contributor to the overall state 4 respiratory rate even in the absence of rotenone and that, furthermore, its engagement may underlie rapid respiratory rates even when the respiratory chain may be under adeny late control.

**DISCUSSION**

In this report we have investigated the regulation of electron transport in plant mitochondria under state 4 conditions while oxidizing dual substrates, a situation that probably exists in vivo. Pea leaf mitochondria were chosen because not only do they possess nonphosphorylating pathways but they are also capable of oxidizing glycine as a respiratory substrate, an important metabolite of the photorespiratory pathway. We have taken as our system the electron transport chain and have investigated changes in oxygen consumption, steady-state membrane potential, and reduction level of \( Q \) upon perturbation with a second NAD\(^+-\)linked substrate. Changes in the steady-state reduction level of \( Q \) were considered to be a very important parameter to monitor because not only does its redox level affect electron flux through the bc\(_1\) complex but it plays a central role in the distribution of electron flux in a branched respiratory chain (27). Indeed, the continuous monitoring of the redox poise of the quinone pool has previously revealed that, in the absence of alternative oxidase activity, electron transfer is linearly dependent upon the degree of reduction of the pool (22) and, in its presence nonlinear, because engagement of the alternative oxidase is not apparent until a considerable proportion of the pool has been reduced (12). Although there have been numerous reports describing the effects of dual substrate addition under state 4 conditions (4–6, 8, 9) the majority have merely monitored oxygen consumption with some parallel measurements on NAD reduction or changes in membrane potential. While these have provided considerable information on the characteristics of stimulation of the state 4 rate (4, 6, 8) and the possibility of preferential oxidation by some substrates (5, 9, 29), details at the level of the respiratory chain components have been lacking. It was therefore of importance to ascertain whether increased respiratory rates even under ADP-limited situations are accompanied by changes in the redox poise of the quinone pool and membrane potentials. The data presented in Figures 1 and 3 indicate quite clearly that changes in respiratory rate are a reflection of the degree of reduction of the quinone pool. Thus succinate, for instance, reduces the quinone pool substantially more than malate, under state 4 conditions, and this results in a faster respiratory rate (cf. Figs. 2 and 1). The reason this substrate reduces the Q-pool more than malate is because succinate has a higher donor activity (\( V_{\text{red}} = 733 \); for details see ref. 27) than malate (\( V_{\text{red}} = 115 \)) (AL Moore, unpublished observations). In the presence of dual substrates, however, the pool becomes substantially reduced with a concomitant increase in the respiratory rate and this appears to be the case even in the presence of rotenone (Fig. 3). A further point arising from the dual substrate experiments is that the input of electron flux from the dehydrogenases is so large that the \( Q \)-pool is still maintained relatively reduced (39%, Fig. 1) even when restrictions on the bc\(_1\) have been reduced (i.e. depolarization of the membrane potential due to ADP addition). Again this larger reduction level, in comparison to that observed with succinate as substrate (22% under state 3 conditions), is reflected by a higher respiratory rate (180 nmol/min/mg in comparison with 141 nmol/min/mg, Fig. 1 versus Fig. 2) adding further confirmation to the notion that steady-state redox poise of the quinone pool regulates electron transfer under both state 3 and state 4 conditions.

There are obviously many different points which can regulate respiratory activity but, in general in isolated mitochondria, the rate of respiration is considered to be controlled by the kinetic properties of the respiratory chain mainly at the level of Cyt oxidase and by \( \Delta E \) and \( \Delta p \) (2). Other parameters which affect its activity include the \( H^+ \)-ATPase, the adenine nucleotide carrier, the intramitochondrial adenine nucleotide and phosphate pools, the level of matrix enzymes, and the proton permeability of the mitochondrial inner membrane (2). Recent application of the control theory (14) has demonstrated that in state 4, respiration is both controlled by the proton leakage across the inner membrane (13) and the respiratory chain (3). As respiration is increased by increasing ADP supply then control shifts, in mammalian tissues at least, away from proton leakage to other reactions such as the adenine nucleotide and dicarboxylate carrier and Cyt oxidase (28). Interestingly, in plant mitochondria under state 3 conditions control was found to be distributed between Cyt oxidase, the bc\(_1\) complex, and the ATPase with no control at the level of the adenine nucleotide carrier (25). Under the conditions we have chosen to examine the regulation of respiration, however, control will be limited to the proton conductance pathway, the redox level of the \( Q \)-pool, and complexes III and IV. Thus, any increase in electron flux through the respiratory chain will require either an increase in the proton conductance of the inner membrane or the engagement of nonphosphorylating pathways. We have previously demonstrated that in potato mitochondria, which lack an alternative oxidase, proton conductance is a reflection of the magnitude of the protonmotive force which itself is a function of the redox driving force (30). Thus, any increases in respiration under state 4 conditions will necessitate increases in both of these parameters. The data shown in Figures 1 and 3 confirm that such a situation does occur when a second respiratory substrate is added to mitochondria under state 4 conditions. The increase in membrane potential will have a dual effect for, not only will it induce a considerable increase in membrane conductance (30) and therefore allow a faster rate of respiration, but it will also oppose some of the increase in driving force caused by the additional substrate. The dramatic rise in the level of steady-state reduction of
Such data suggest that the bypass is operative and to rate MOORE even in electron flow is apparent presence I and, because electron transfer is also be achieved and, because electron transfer is a function of 2 \( \Delta E_s - n\Delta p \), the overall driving force will be increased. This obviously appears to be the case because rotenone addition only causes a partial reoxidation of the quinone pool, suggesting that the bypass is operative and is a major pathway of electron flow to the Q-pool.

It is also apparent from a comparison of Figure 1 with Figure 3 that a considerable increase in quinone reduction is observed upon addition of a second NAD-linked substrate even in the presence of rotenone, suggesting that the increased electron flux primarily occurs via the rotenone-insensitive bypass. Such data demonstrate the important role of the rotenone-insensitive pathway in facilitating rapid rates of respiration even under ADP-limited conditions and suggest it is a constitutive feature of the plant mitochondrial respiratory chain.

**LITERATURE CITED**