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Measurement of the redox state of the ubiquinone pool in plant mitochondria

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We have investigated the dependence of the respiratory rate on the redox poise of the quinone pool in isolated turnip and pea leaf mitochondria. A linear relationship has been found between these two parameters during succinate oxidation under both state 3 and 4 conditions. When succinate is oxidised by the alternative oxidase the dependence of oxygen uptake on the steady-state reduction level of quinone is markedly non-linear. These results are discussed within the framework of a homogeneous quinone pool.

Quinone pool; Force-flow relationship; Mitochondria; Alternative oxidase

1. INTRODUCTION

It is generally accepted that the functional linkage between the mitochondrial dehydrogenases and oxidases is a mobile pool of quinone [1–5]. According to the model of Kroger and Klingenberg [6,7] the redox behaviour of the quinone pool can be quantitatively described by a simple kinetic model in which the rate of oxygen uptake is directly proportional to the redox poise of the quinone pool. Although there is a considerable amount of evidence for such a pool (see [5]) there are a number of examples in which deviations from this simple kinetic model occur [8–12]. Recently, Ragan and Cottingham [5] devised a kinetic scheme, based on detailed consideration of reactions at the quinone reductase and quinone-oxidase sites, to account for such deviations and have shown that these can reduce to simple Q-pool kinetics under certain conditions. To substantiate this scheme evidence was later presented that in systems which were cytochrome *c* limited [13], the rate of overall electron transport is largely insensitive to changes in the quinone redox state, i.e. first-order behaviour was lost.

Although there have been relatively few studies reported on quinone pool behaviour in plant mitochondria (see [14]), it is generally considered to be an area rich in deviations (reviews [5,15]). Particular examples of such deviations are mitochondria isolated from cassava [10] and soybeans [16] where the distribution of electron flux between the cyanide-sensitive and -insensitive pathways is strongly dependent on the nature of the substrate. On the basis of such data, it has been concluded that quinone is compartmentalised in plant mitochondria [17]. A major difficulty with such studies, however, is that they have merely relied on oxygen uptake rates and no independent measurements of the redox state of quinone have been performed. It is thus difficult to distinguish effects exerted at the level of the quinone pool from transport phenomena or possible control of

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Abbreviations: Q, ubiquinone; Q-1, ubiquinone-1; Qt, fully reduced ubiquinone; Q_r, ubiquinone reduced under steady-state conditions

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maximum fluxes by substrate or product concentration.

Here, use has been made of a novel technique to measure the redox state of quinone in situ. We have used this technique to measure directly the redox state of quinone in a variety of plant mitochondria under both state 3 and 4 conditions and have found that, in agreement with the model of Kroger and Klingenberg [6,7], the level of reduction of the quinone pool varies linearly with electron flux to the quinone pool. However, when pea leaf mitochondria were used, under ADP-limited conditions, the dependence of electron flux on the redox state of quinone is markedly non-linear. We describe the implications of this type of behaviour for the regulation of electron transport in systems possessing a branched respiratory chain.

2. MATERIALS AND METHODS

2.1. *Materials*

Pea (*Pisum sativum* L., cv. Massey Gem) seedlings were grown in trays of vermiculite or soil in a glasshouse for 12–15 days. Fresh turnip (*Brassica rapa* L.) tissue was obtained from local sources. Q-1 was generously provided by Hoffman La Roche (Basel, Switzerland). Myxothiazol was obtained from Boehringer (Lewes, England) and all other chemicals were of the highest quality commercially available and were purchased mainly from Sigma (MO, USA).

2.2. *Isolation of mitochondria*

Mitochondria were isolated and purified on a 0–4% PVP/Percoll gradient essentially as described by Day et al. [18] and from turnip according to Soole et al. [19].

2.3. *Assay procedures*

Oxygen consumption was measured polarographically in 1.8 ml reaction medium containing 0.3 M sorbitol, 10 mM KH_2PO_4, 10 mM Tes, 2 mM $MgCl_2$ and 0.1% (w/v) BSA, all adjusted to pH 7.2, in a specially constructed cell (University of Sussex Workshops) housing a Rank oxygen electrode, a glassy carbon and platinum electrode.

The redox state of exogenously added (1 μM) Q-1 was measured polarographically (a technique devised by Dr P.R. Rich, Glynn Res. Plc., Bodmin; European Patent no. 85900699.1/) using a glassy carbon working electrode and a platinum electrode (Anachem, Luton) connected to an $Ag/AgCl_2$ reference electrode. The working electrode was poised at -360 mV with respect to the reference electrode with a specially constructed voltammeter (University of Sussex Workshops) as in [20]. The outputs of the electrodes were connected to a 2-pen Rikadenki recorder. Q-1 was taken to be fully oxidised on addition of Q-1 and mitochondrial protein (in the absence of substrate) and fully reduced upon anaerobiosis. 1 μM Q-1 had no detectable effect upon either the state 4 respiratory rate, respiratory control, ADP/O ratios or protonmotive force (see [20]).

2.4. *Protein and chlorophyll determination*

Protein was determined according to Lowry et al. [21], with BSA as standard. Chlorophyll was determined by the method of Arnon [22]. Mitochondrial protein was corrected for the contribution by broken thylakoids by assuming a thylakoid protein/chlorophyll ratio of 6.9:1 [23].

3. RESULTS

Fig.1 shows the simultaneous measurement of O_2 uptake and steady-state level of reduction of quinone by purified turnip mitochondria whilst oxidising succinate under state 4 conditions. Mitochondria were titrated with malonate to provide progressive inhibition of succinate oxidase activity. In the absence of an oxidisable substrate, there is negligible respiratory activity and the quinone pool is essentially oxidised. Note that under these conditions the addition of malonate or ADP, in the absence of substrate, did not result in any further oxidation of the pool confirming this suggestion (not shown). The addition of succinate resulted in a rapid reduction of the quinone pool (to 80% of the level achieved upon anaerobiosis) and initiated O_2 uptake. An aliquot of ADP was added to ensure a true state 4 was achieved. In the presence of ADP the quinone pool is rapidly re-oxidised returning to a level of reduction slightly greater than that observed under state 2 conditions upon ADP exhaustion. As the malonate concentration was increased (up to 12 mM), the steady-state level of reduction of quinone by succinate showed a progressive oxidation until a final reduction level of 4% was reached.

Fig.2 shows the results of a number of these experiments with both purified turnip and pea leaf mitochondria. Turnip mitochondria possessed negligible alternative oxidase activity in comparison to antimycin A-insensitive rates of approx. 18% in pea leaf mitochondria. The data are presented as the ratio of v/V_0 (where v is the initial rate of oxygen uptake in the presence of inhibitor and V_0 the uninhibited rate) plotted vs the proportion of quinone in the reduced state (Q_r/Q_t). It can be seen with turnip mitochondria that an apparent linear relationship exists between these two parameters (fig.2A), whereas pea leaf mitochondria show a distinctly non-linear relationship between the state 4 respiratory rate and Q_r/Q_t

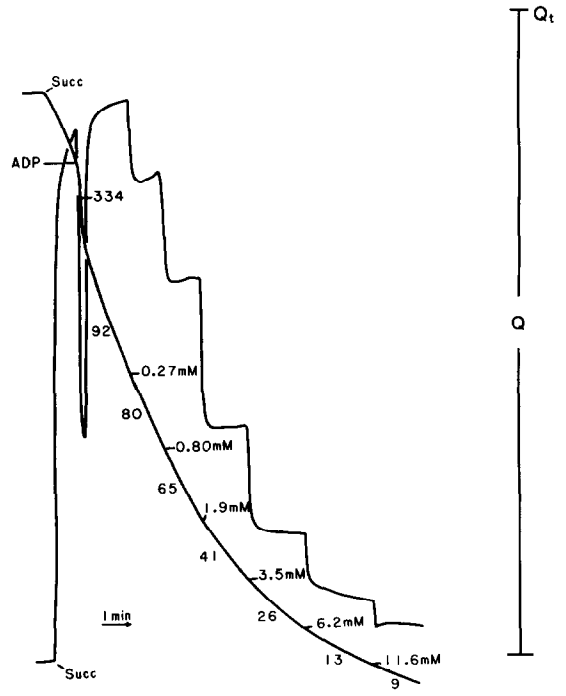

Fig.1. Simultaneous measurement of O_2 uptake and steady-state reduction level of Q-1 with purified turnip mitochondria. Oxygen uptake and steady-state level of quinone reduction were measured in 1.8 ml reaction medium containing 0.496 mg mitochondrial protein and 1 μM Q-1. Respiration was initiated by the addition of 5 mM succinate in the presence of 100 μM ATP and 50 μM ADP. Malonate additions were as indicated up to a final concentration of 11.6 mM. Numbers on the respiratory trace are nmol O_2/min per mg protein. Fully oxidised Q-1 was taken as the base of the trace prior to addition of substrate and fully reduced upon anaerobiosis (Q_t).

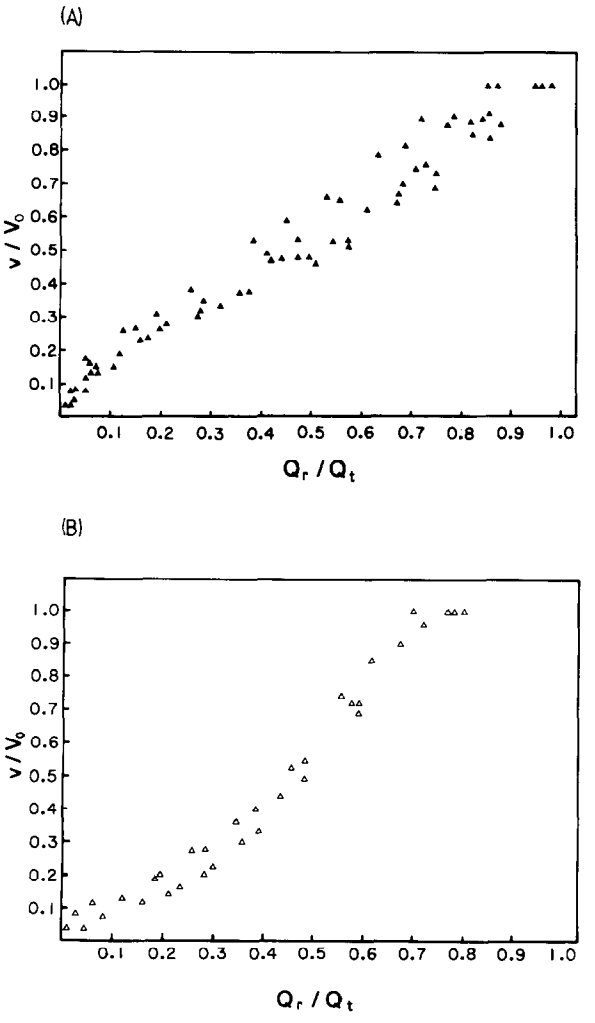

Fig.2. The dependence of the respiratory rate on the quinone redox state in purified turnip and pea leaf mitochondria under state 4 conditions. Assays were performed as described in fig.1 following the attainment of state 4 using either 0.44 mg (A-turnip) or 0.8 mg (B-pea leaf) mitochondrial protein. v is the initial respiratory rate in the presence of malonate and V_0 the uninhibited rate. Q_r refers to Q-1 reduced under steady-state conditions and Q_t to fully reduced Q-1. Results represent the summation of results with four separate turnip and two separate pea leaf preparations. Average uninhibited state 4 rates: turnip, 93; pea leaf, 71 nmol O_2/min per mg protein.

(fig.2B). In the presence of excess ADP, however, the redox state of the quinone pool varied linearly with the rate of electron donation to the pool (fig.3). The slope of the line was, however, significantly increased in comparison with that observed under state 4 conditions. This was anticipated, since ADP induces state 3 by providing a P_i acceptor for the ATP synthase and decreasing Δp thereby allowing the cytochrome *bc* complex to achieve maximum velocity. Fig.4 shows the dependence of the rate of respiration on the redox state of the quinone pool as the state 3 rate is progressively decreased by additions of malonate in the presence of varying amounts of myxothiazol to partially inhibit the cytochrome *bc* complex. As observed in fig.3 a linear relationship still exists even in the partially inhibited state. Myxothiazol treatment merely decreases the slope of the line without affecting its shape.

The effect of malonate additions, in the presence of myxothiazol, is depicted in fig.5. It is apparent from these data that as the amount of myxothiazol is increased more malonate is required to achieve

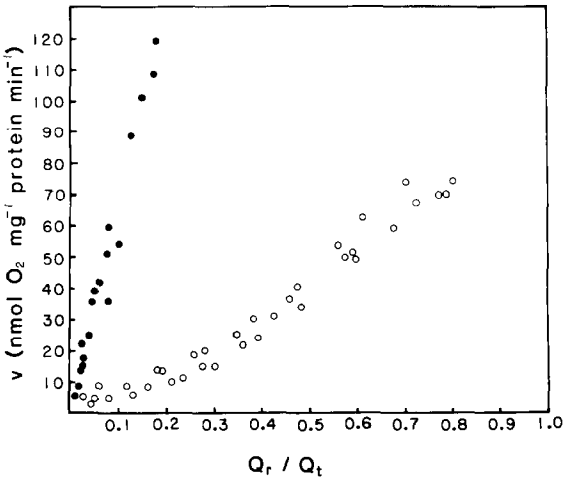

Fig.3. The dependence of the respiratory rate on the quinone redox state in pea leaf mitochondria under state 3 and 4 conditions. Assays were performed as described in fig.1 using 0.8 mg pea leaf mitochondrial protein either under state 4 conditions (○) or in the presence of 0.75 mM ADP to initiate state 3 (●).

a given degree of inhibition when the activity of the cytochrome *bc* complex is severely curtailed. This result is similar to the recent observations of Reed and Ragan [13] using reconstituted bovine heart NADH:cytochrome *c* oxidoreductase.

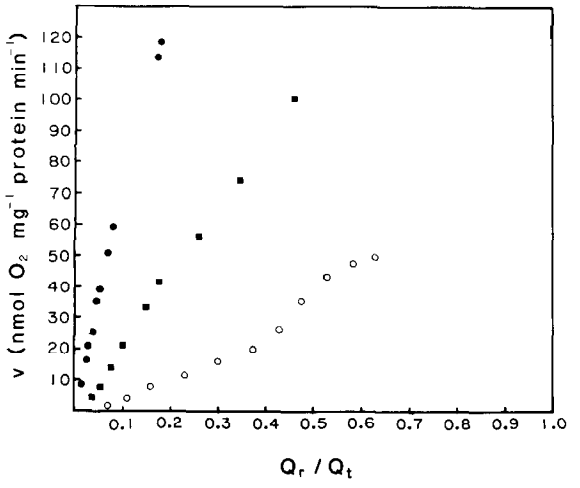

Fig.4. The dependence of the respiratory rate in pea leaf mitochondria on the quinone redox state under state 3 conditions and in the presence of myxothiazol. Assays were performed as described in fig.1 using 0.84 mg pea leaf mitochondrial protein and in the presence of 0.75 mM ADP (●) and additionally either 0.05 μM (■) or 0.07 μM (○) myxothiazol.

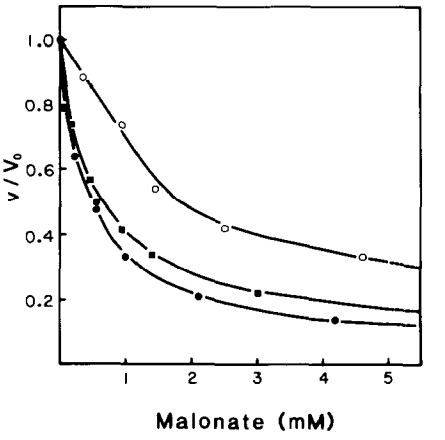

Fig.5. Malonate inhibition of succinate oxidation under state 3 conditions in the presence of myxothiazol. Respiratory activity was measured in the presence of 5 mM succinate and varying amounts of malonate as indicated (●) and either 0.05 μM (■) or 0.07 μM (○) myxothiazol. Dotted lines represent extrapolations to data points beyond 5 mM malonate.

4. DISCUSSION

Here, use has been made of a voltametric technique to monitor changes in the steady-state reduction level of quinone following perturbation of the respiratory rate in plant mitochondria. The advantage of this technique over solvent extraction methods is that it allows continuous monitoring of changes in the reduction level of quinone alongside the simultaneous measurement of oxygen consumption and has enabled us to investigate Q-pool behaviour in isolated mitochondria. Plant mitochondria were chosen (as the ideal system for such a study) because they possess a branched respiratory chain and have been widely reported to deviate considerably from ideal quinone pool behaviour (see [5,15]). Such deviations have led to the suggestion of the presence of multiple quinone pools in plant mitochondria [17,24]. To date, however, no direct measurements of the quinone redox state have been reported and the compartmentation of quinone in the membrane has been based solely upon oxygen consumption measurements.

It can be seen from fig.1 that in the presence of succinate as an oxidisable substrate, under state 4 conditions, the quinone pool is 85% reduced and that progressive inhibition of the input of reducing

equivalents from complex 2 results in the net oxidation of quinone. Figs 2 and 3 confirm the original suggestions of Kroger and Klingenberg [6,7] that the respiratory rate, under state 3 and 4 conditions, is directly proportional to the redox poise of the quinone pool. It is interesting to note, however, that when pea leaf mitochondria, under ADP-limited conditions, were used the plot is markedly non-linear (see figs 2B,3). The deviation from linearity may be a reflection of either the control of respiration by the proton leak of the inner membrane [25] or the engagement of the alternative oxidase. It is generally considered that the alternative oxidase, which branches from the main respiratory chain at the level of quinone and is non-protonmotive (see [15]), operates mainly under state 4 conditions, i.e. when the Q-pool is significantly reduced. The data presented in fig.2 would tend to suggest that it is not the proton conductance that is causing the deviation from linearity, since similar deviations are not observed with turnip mitochondria (fig.2A) or with pea leaf mitochondria under state 3 conditions (fig.3). It is therefore interesting to speculate that it may be engagement of the alternative oxidase, under state 4 conditions, that causes marked deviations from the expected behaviour of a homogeneous quinone pool. Indeed, the kinetic model of Ragan and Cottingham [5] accounts for such types of deviations by suggesting that if the reoxidation of reduced quinone by an oxidase is slow (in this case the alternative oxidase) then overall electron transfer will become largely insensitive to the quinone redox state [13]. In fact, the close similarity of the curve obtained under state 4 conditions (fig.3) with that predicted by the theoretical equation of Reed and Ragan (see fig.5 [13]) suggests that such a situation does exist when only the alternative oxidase is operating.

Thus, deviations from the simple kinetic model of Kroger and Klingenberg [6,7] can still be accommodated within the framework of a homogeneous quinone pool without invoking the existence of multiple pools [17].

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