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Review

Control of plant mitochondrial respiration

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Abstract

Plant mitochondria are characterised by the presence of both phosphorylating (cytochrome) and non-phosphorylating (alternative) respiratory pathways, the relative activities of which directly affect the efficiency of mitochondrial energy conservation. Different approaches to study the regulation of the partitioning of reducing equivalents between these routes are critically reviewed. Furthermore, an updated view is provided regarding the understanding of plant mitochondrial respiration in terms of metabolic control. We emphasise the extent to which kinetic modelling and 'top-down' metabolic control analysis improve the insight in phenomena related to plant mitochondrial respiration. This is illustrated with an example regarding the affinity of the plant alternative oxidase for oxygen.

Keywords: Plant mitochondrion; Respiration; Alternative oxidase; Ubiquinone-pool; Kinetic modelling; Metabolic control analysis

1. Introduction

Like all living organisms, plants require energy for their growth, development, reproduction and maintenance. This energy is generally conserved in the form of ATP which, in plants, mainly occurs via two mechanisms: chloroplastic photophosphorylation and mitochondrial oxidative phosphorylation. Energy transduction in plants is hence a complex interplay between chloroplastic and mitochondrial metabolism which is effected by compounds such as ATP, NAD(P)H and carboxylic acids (recently reviewed by Hoefnagel et al. [1]). The degree to which each organelle contributes to ATP production is subject to developmental, spatial and diurnal regulation. In mature green leaf tissues for example, mitochondria account for virtually all ATP production in the dark, whereas chloroplasts are the main source of ATP formation in the light. Even under illuminated conditions, however, mitochondria in these tissues are believed to at least partly contribute to the total ATP required for sucrose synthesis, optimum CO_2 fixation, metabolite transport and protein synthesis [1,2].

Apart from playing a catabolic role in metabolism, plant mitochondria also fulfil an anabolic function, particularly in the light [2]. Plants are autotrophic and therefore need to assimilate nitrogen for amino acid synthesis. The carbon skeletons required for this process are derived from tricarboxylic acid (TCA) cycle intermediates (cf. [1]). Consequently, this metabolic cycle has to turn over continuously during bio-

Abbreviations: BHAM, benzhydroxamic acid; Q, ubiquinone; QH_2, reduced ubiquinone; SHAM, salicyl hydroxamic acid; TCA, tricarboxylic acid; UCP, uncoupling protein

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synthesis, in a manner independent of the energy charge of the cell. The plant mitochondrial respiratory chain contains several non-protonmotive protein complexes (cf. Section 2.1) that enable electron transfer to proceed without concomitant ATP production and thus allow TCA cycle turnover even when the cytosolic ATP:ADP ratio is high [3,4].

The presence of phosphorylating as well as non-phosphorylating pathways complicates plant mitochondrial respiration in so much that reducing equivalents are potentially transferred from respiratory substrate to molecular oxygen via more than one route. It is therefore clear that plant mitochondrial respiratory activity has to be tightly regulated in order to satisfy different (i.e. catabolic and anabolic) metabolic cellular demands in a flexible fashion. In this minireview we aim to discuss current understanding of the control of electron transfer within plant mitochondrial respiratory networks.

2. Plant mitochondrial respiration

2.1. The electron transfer network

Plant mitochondrial respiratory chains differ in several aspects from their mammalian counterparts (Fig. 1). In addition to Complex I, they contain at least four other substrate dehydrogenases that enable the oxidation of matrix and cytoplasmic NAD(P)H [5]. The activity of these enzymes is readily distinguishable from Complex I activity because of its insensitivity to inhibitors such as rotenone. Furthermore, plant mitochondria are characterised by the presence of an alternative respiratory pathway, in addition to the orthodox cytochrome pathway, through which reducing equivalents can be transferred to molecular oxygen. This pathway branches at the level of the ubiquinone (Q)-pool and comprises a single enzyme, the alternative oxidase (see [4,6–10] for reviews). This enzyme, which enables plants to respire in the presence of toxic compounds such as cyanide and carbon monoxide, is functionally a ubiquinol:oxygen oxidoreductase [11] and, importantly, is non-protonmotive [12]. Similarly, neither the rotenone-insensitive NAD(P)H dehydrogenases [13,14] nor Complex II [15] are proton translocating. It is therefore clear that plant mitochondria can theoretically oxidise both NAD(P)H and succinate in a manner that is not energy conserving and therefore is beyond the control of the protonmotive force.

2.2. Plant mitochondrial uncoupling protein (UCP)

Relatively recently, it has become clear that plant mitochondria possess, in addition to the non-protonmotive respiratory enzymes, an extra protein (UCP)

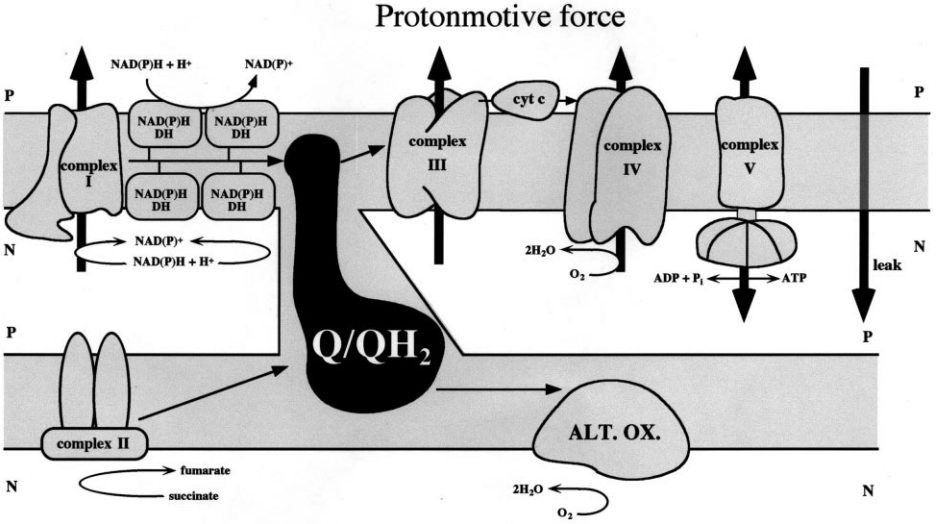


Fig. 1. Schematic representation of the plant mitochondrial respiratory chain. Thin and thick arrows represent transfer of electrons and protons, respectively, along and across the inner mitochondrial membrane. DH, dehydrogenase; cyt *c*, cytochrome *c*; ALT. OX., alternative oxidase; P and N refer to the positively and negatively charged sides of the inner-mitochondrial membrane, respectively.

that also effectively uncouples electron transfer from phosphorylation [16]. Jezek and colleagues have shown, in isolated mitochondria [17] and in proteoliposomes [18], that the plant UCP translocates protons via a fatty acid cycling mechanism, similarly to the mammalian UCPs. This UCP has been identified in several plant species [16,19,20] as well as in protozoa [21]. It is clear that activity of both the plant alternative oxidase and UCP have the same apparent effect: dissipation of free energy as heat. An interesting question therefore arises as to the physiological need for two distinct enzymes. An answer might be related to the fact that the mechanisms by which both enzymes uncouple electron transfer from the protonmotive force are fundamentally different: the plant UCP directly lowers the energy status of the inner mitochondrial membrane, whilst the alternative oxidase is a priori not controlled by the protonmotive force. In other words, both UCP and the alternative oxidase allow electron transfer to occur when the energy charge of the cell is high, but whilst activity of the former will decrease this charge, activity of the latter will not affect it. On the other hand, the alternative oxidase, due to its kinetic characteristics (cf. Section 3.1), can only be engaged in electron transfer at relatively high Q-reduction levels and is merely able to sustain a relatively low respiratory rate, whereas the UCP-stimulated electron transfer rate can be significantly higher and can occur at Q-reduction levels that are relatively low.

3. Electron partitioning between reduced ubiquinone (QH_2)-oxidising pathways

The relative distribution of reducing equivalents between phosphorylating and non-phosphorylating respiratory pathways determines to a great extent the efficiency of plant mitochondrial energy conservation. A considerable number of studies, reviewed in this section, has been performed to investigate the regulation of electron partitioning between QH_2-oxidising pathways, particularly to determine the extent to which and the physiological conditions under which the 'energy-wasting' alternative oxidase is engaged in the overall respiratory activity (cf. [10]).

3.1. Predictions from Q-kinetic in vitro experiments

Bahr and Bonner [22,23] were the first to study the partitioning of electrons between the cytochrome and alternative pathways. Hydroxamic acids (e.g. salicyl hydroxamic acid (SHAM)) were used to titrate alternative pathway activity in plant mitochondria and from these experiments it was concluded that this alternative oxidase activity only occurs upon saturation of the cytochrome pathway when the Q-pool becomes highly reduced. In other words, Bahr and Bonner suggested from their experiments a non-proportional kinetic dependence of alternative oxidase activity upon the Q-redox poise. The introduction of the Q-electrode ([24]; European patent no.

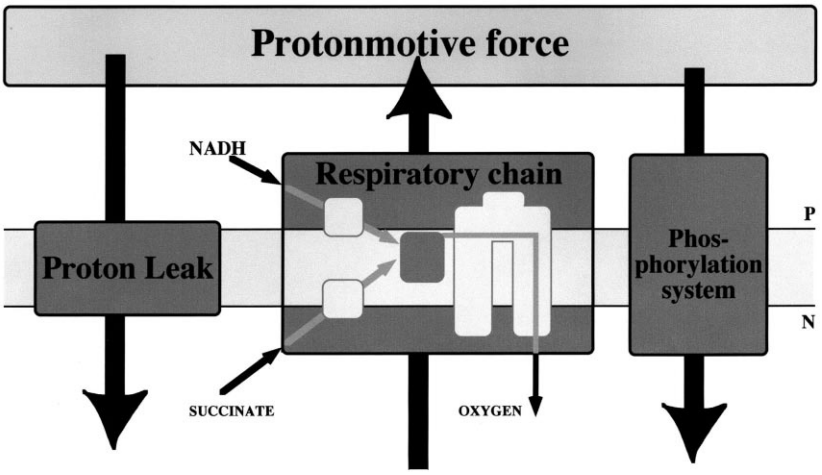


Fig. 2. Schematic representation of respiration in potato tuber mitochondria. Respiration is considered from the 'top-down' perspective defined by Kesseler et al. [70] and divided in processes that either produce or consume the protonmotive force (as indicated by the thick arrows). P and N, as defined in Fig. 1.

85900699.1, P.R. Rich) enabled simultaneous measurements of oxygen uptake rates and Q-reduction levels. Such measurements revealed that cytochrome pathway activity was linearly dependent upon the Q-redox poise [24]. The alternative oxidase, in contrast, did not show any activity at low Q-reduction levels, whilst its activity increased more than proportionally at high levels [24,25], which indeed agreed well with the original prediction of Bahr and Bonner [22,23].

Several kinetic models were subsequently developed to describe the experimentally determined behaviour of the alternative oxidase with respect to the Q-redox poise [6,26,27]. Moore and Siedow [6,26] postulated a reaction mechanism in which the enzyme is sequentially reduced by two QH_2 molecules before it completely reduces dioxygen. The derived rate equation for such a mechanism was used successfully to describe alternative oxidase kinetics in mitochondria from a range of plant species at various developmental stages [26]. To account for changing affinities of the alternative oxidase for oxygen upon changes in the Q-reduction level, this model was adapted to include an extra activation step prior to the reaction of the enzyme with dioxygen ([27]; cf. Section 4.3).

Krab and colleagues [28–30] reasoned that an enzyme that is kinetically dependent upon the reduction level of the Q-pool would also be dependent, albeit indirectly, upon any other enzyme that interacts with this pool. They therefore modelled the behaviour of the alternative oxidase as an integral part of the kinetic interplay between Q-reducing and QH_2-oxidising enzymes. Each enzyme in this model is assumed to exhibit reversible Michaelis–Menten kinetics with respect to the Q-reduction level, but with the restriction that the size of the Q-pool is constant [28–30]. The model provided theoretical ground to explain the apparent preference of the alternative oxidase for particular respiratory substrates, without the need to invoke multiple Q-pools [30]. Furthermore, the frequently observed 'reversed respiratory control' when studying succinate oxidation, could be readily explained by predicting a dual effect of ADP on both cytochrome pathway (stimulation) and succinate dehydrogenase (inhibition) activity [30]. Subsequent studies with mitochondria isolated from *Arum maculatum* spadices provided experimental proof for such an explanation [31].

Arguably this model's most far-reaching implication was that it seriously challenged the early notion of Bahr and Bonner that the alternative oxidase only becomes active upon saturation of the cytochrome pathway [22,23]. Based on this notion, the contribution of the alternative oxidase to the overall mitochondrial respiratory activity had been generally estimated as the fraction of activity that is sensitive to hydroxamic acids. Krab's model, however, predicted that inhibition of the alternative oxidase by SHAM would induce an increase in the reduction level of the Q-pool. This would in turn result in altered activity of the cytochrome pathway, typically dependent upon the kinetics (with respect to the Q-redox poise) of this path as well as those of the substrate dehydrogenase(s). This could theoretically result in a considerable underestimation of engagement of the alternative oxidase in respiration [30] which, indeed, has been substantiated experimentally [28].

Underestimation of alternative pathway activity would only be a problem when the cytochrome pathway indeed operates in a non-saturated fashion. Studies to directly address this matter have revealed that reducing equivalents can be readily diverted from the alternative to the cytochrome pathway and vice versa, suggesting that neither path operates at its maximum capacity [32–34]. It has furthermore been shown that the alternative oxidase is able, in the presence of pyruvate, to actively compete with the cytochrome pathway for reducing equivalents [34]. From these studies it became clear that approaches other than Bahr-and-Bonner-like inhibitor titrations were required to adequately determine the relative contributions of the oxidative pathways to the overall respiratory activity.

The modular Q-kinetic approach developed by Krab and colleagues (see [28,35–37] for practical examples) aims to explain the behaviour of the overall plant electron transfer system in terms of the kinetics of its component parts. By measuring, in isolated mitochondria, the kinetics with respect to the Q-redox poise of both Q-reducing and QH_2-oxidising enzymes, steady state reduction levels of the Q-pool can be predicted. The electron transfer rates through each of the enzymes and, consequently, the distribution of reducing equivalents between the alternative and cytochrome pathways in these steady states, can then readily be calculated. That such approxima-

tions of the alternative oxidase's in vitro engagement could be a useful indicator for its in vivo participation in respiration, is illustrated by studies performed with the fission yeast *Schizosaccharomyces pombe* [35].

Work by Albury and colleagues [38] has resulted in the functional heterologous expression of the plant alternative oxidase (from *Sauromatum guttatum*) in *S. pombe*. In this system, the oxidase is cloned on an extra-chromosomal element under the control of a thiamine-repressible promoter and its presence or absence can therefore be modulated by simply omitting or including thiamine in the growth medium, respectively [38]. From kinetic studies using mitochondria isolated from *S. pombe* cells expressing the alternative oxidase, it was predicted that the non-protonmotive oxidase would contribute considerably (up to 24%) to the total respiratory activity, thereby lowering the efficiency of energy conservation [35]. Growth studies showed that expression of the alternative oxidase had a profound negative effect on *S. pombe* growth (yield decreased $\sim$20% when cells were batch-cultured with glycerol as the sole carbon source) which is, most likely, a reflection of a high engagement of the oxidase in respiration [35].

3.2. Predictions from oxygen uptake in vitro experiments

More recently, Sluse and co-workers [10,39] have developed an alternative approach to assess, in isolated mitochondria, electron partitioning between the alternative and cytochrome pathways. Their ADP/O method is based on a concept that has been suggested a number of years ago [40]. Previously, general potential difficulties associated with ADP/O measurements have been critically evaluated by Hinkle et al. [41]. The method relies on the fact that cytochrome pathway activity is linked to phosphorylation of ADP, whereas alternative oxidase activity is not, provided that the alternative respiration is not dependent upon Complex I activity. The ADP/O ratio relates the amount of ADP phosphorylated to the amount of oxygen consumed and, therefore, indicates the relative contribution of the cytochrome and alternative pathways to the overall rate of oxygen uptake. By measuring the ADP/O ratios in the presence and absence of benzhydroxamic acid (BHAM), an alternative oxidase inhibitor [42], the relative contributions of the two QH_2-oxidising pathways have been successfully determined in amoeba mitochondria [39]. It should be noted, however, that this method is restricted to estimations of alternative oxidase engagement under state 3 conditions and is furthermore restricted to the use of succinate or external NADH as respiratory substrates. Additionally, every mitochondrial system under investigation, has to meet a set of criteria (cf. [10,39]): the ADP/O ratio should be zero when the cytochrome pathway is fully inhibited; complete inhibition of the alternative pathway (with e.g. BHAM), should not induce proton leak; the ADP/O ratio in the presence of BHAM should be independent of the state 3 respiratory rates; the isolated mitochondria should be tightly-coupled and stable. It is therefore clear that the ADP/O method is only useful if all these requirements have been satisfied.

3.3. Non-invasive in vivo measurements of electron partitioning

The most serious limitation of the modular Q-kinetic and ADP/O approaches is that they are restricted to in vitro measurements and, therefore, only allow the in vivo activity of the alternative oxidase to be predicted. The extent to which the alternative oxidase is engaged during whole plant respiration, can be determined directly by oxygen discrimination measurements [43,44]. Such non-invasive measurements rely on the differential fractionation by the alternative and cytochrome pathways of ^{16}O and ^{18}O isotopes and have been successfully performed to establish, in vivo as well as in vitro, the relative contributions of the respective paths to the overall respiratory activity [43–49]. The obvious advantage of the technique is that in planta alternative oxidase engagement can be studied under a range of physiologically interesting conditions, although it has to be ensured, as pointed out by Guy et al. [43], that respiration is not limited by O_2-diffusion.

It is anticipated that oxygen-isotope discrimination factors (*D*-values) of neither the alternative oxidase nor cytochrome *c* oxidase would differ much within the plant kingdom [43]. If this were the case then the

mechanism by which oxygen is reduced by either oxidase would be species-specific which, intuitively, is unlikely. In this respect, it is surprising that the *D*-value of the alternative oxidase in green soybean leaves is higher than the value observed in roots from the same plant [45]. Similarly, the *D*-value measured in soybean cotyledon mitochondria [47] is high compared to that determined in mitochondria isolated from non-green *Symplocarpus foetidus* spadices [43]. Intriguingly, the relatively high *D*-value of the alternative oxidase in soybean cotyledon mitochondria [47] is not observed when these mitochondria are isolated from cotyledons taken from etiolated soybean plants [48]. In this case, the *D*-value is similar to that measured in mitochondria isolated from non-green tissues [43].

Oxygen-fractionation measurements in whole plants could be complicated by potential oxygen-consuming processes other than alternative and cytochrome pathway activity. The *D*-value of this 'residual respiration' can be determined by measuring oxygen-fractionation in the presence of both SHAM and cyanide. The relative contribution of the individual oxygen-consuming processes to the overall respiratory activity, however, cannot be calculated in the case that more than two of such processes exist. Fortunately, it has been demonstrated that, in soybean cotyledons or roots, residual respiration does not significantly contribute to the overall oxygen uptake [48]. We would like to emphasise, however, that the extent of extra oxygen-consuming (and/or -producing) activities is experimentally difficult to assess, particularly under conditions where the oxygen-discrimination method would be of most use, i.e. in intact plant cells and tissues. Related to this, the use of the method is restricted to dark-respiration, since photosynthetic release of oxygen currently prevents a conclusive interpretation of the measured *D*-values.

Nevertheless, the non-invasive oxygen discrimination is the only method of those discussed that allows the physiological conditions (both developmental and environmental) under which the alternative oxidase engages in plant respiration, to be revealed directly. The technique, however, requires specialised and expensive equipment which hampers a routine and broad application. In this respect, it would be of interest to establish whether or not the predictions made using the modular Q-kinetic or ADP/O methods can be confirmed by oxygen fractionation measurements.

4. Control of plant mitochondrial respiration

4.1. Control exerted by mitochondria on plant cellular metabolism

Alterations in plant mitochondrial respiration, notably alternative pathway and UCP activity, have been correlated with many physiological phenomena. Changes in the expression and/or activity of the alternative oxidase have been observed (to cite but a few of many reports) during temporal events such as leaf development [50,51], thermogenesis [31,52] and fruit ripening [53]. Furthermore, the alternative oxidase has been implicated to play a role during conditions such as, amongst others, oxidative stress [54–56] and plant pathogenic attack [57–60]. It has been reported that expression of the plant UCP is cold-induced [61]. Similar to the alternative oxidase, this UCP has also been suggested to prevent generation of reactive oxygen species by the respiratory chain [62] and to be important during fruit ripening [63]. Qualitatively, all of these observations indicate physiological circumstances where mitochondrial respiration exerts a certain degree of control on plant metabolism.

To make any quantitative statements with respect to control of mitochondrial respiration on plant cell physiology, studies should ideally be performed in terms of metabolic control analysis (see [64,65] for general reviews). Although whole plant metabolism has been subjected to several quantitative studies (see [66] for review and [67,68] for recent examples), to our knowledge no such investigations have been carried out, to date, to specifically address the role of plant mitochondrial respiration. It is in the authors' opinion that this lack of information might be related to the practical difficulty of defining an appropriate system with distinct boundaries. Metabolic control theory aims to analyse flux–force relations between enzymes and the metabolic intermediates through which these enzymes communicate. It is therefore required to define a system with appropriate fluxes and intermediates that can experimentally

be manipulated. Of importance in such a definition is that the system indeed has a constant boundary, in other words that the chemistry peripheral to it can be kept constant under experimental conditions. Although the manipulation of enzyme levels in whole cells has been greatly facilitated by the advent of molecular genetic techniques, it is still not straightforward to design a system in which the edges can be kept solid during experimentation.

4.2. Internal control of plant mitochondrial respiration

When respiration is studied in isolated mitochondria, it becomes less difficult to define a tight experimental system that is suitable for the application of metabolic control analysis. During the last decade, several quantitative investigations have been performed in an attempt to obtain a better insight in the way plant mitochondrial respiration is controlled [29,35,69–77]. Most of the studies referred to, have been reviewed fairly recently in detail by Krab [30] who drew particular attention to the structural similarity between two experimental approaches that were developed independently by Kesseler et al. [70], who performed studies in potato tuber mitochondria (alternative oxidase absent), and by Krab et al. [29,73], who performed studies in mitochondria isolated from potato callus (alternative oxidase present). In both methods, mitochondrial respiration was considered from a 'top-down' perspective [65,78,79], which simplifies the respective systems under consideration in so much that certain enzymes are considered to behave as single units that kinetically interact with each other via a (significantly reduced) number of intermediates. Furthermore, in both approaches only one common intermediate was defined through which three enzymatic branches communicated. Additionally, control coefficients were in either case derived from elasticity coefficients that were experimentally determined by means of inhibitor titrations (cf. [30]).

Kesseler and colleagues [70] designed a system (Fig. 2) where the protonmotive force – measured as the electrical potential across the mitochondrial inner membrane – was chosen as the central intermediate. The phosphorylation system as a whole and the proton leak across the inner membrane, were defined to 'consume' this intermediate, whereas the respiratory chain as a whole was defined to 'produce' it. It was found that control on the respiratory rate (with either NADH or succinate as reducing substrate) was mainly exerted by the respiratory system, except for rates close to state 4 where the proton leak had equal or more control. Control on phosphorylation also lay predominantly with the respiratory chain, although at very low rates the phosphorylation system itself exerted control. The rate of proton leak was positively controlled by respiration as well as by the leak process itself and negatively controlled by phosphorylation. Relatively little control was exerted, by any of the processes, on the protonmotive force [70]. The power of this approach was clearly further demonstrated in subsequent studies which were designed to probe the effect of cadmium on respiration in potato tuber mitochondria [74–77]. The main conclusions from these studies were that cadmium inhibits substrate oxidation, increases the proton leak, but does not affect phosphorylation. The effects on all processes studied were dependent on the cadmium concentration as well as the energy demand.

The approach of Kesseler et al. [70], in which the enzymes that constitute the respiratory network are grouped into a single kinetic unit (Fig. 2), does not distinguish between control exerted on the respiratory flux by the cytochrome and alternative pathways, respectively. The method applied by Krab and co-workers [29,30,73] does enable such a distinction to be made. As can be observed from Fig. 3, the defined system centres around the mitochondrial Q-pool via which four enzyme groups interact: two Q-reducing units (succinate dehydrogenase linked to the dicarboxylate carrier (from this point simply referred to as succinate dehydrogenase) and the external NADH dehydrogenase) and two QH_2-oxidising ones (the cytochrome pathway as a whole and the alternative oxidase). The total electron flux through the system was determined as the rate of oxygen uptake (at saturating O_2 concentrations), whereas the common intermediate (defined as the QH_2 concentration) was measured as the reduction level of the Q-pool. In typical experiments, one respiratory substrate (at a saturating concentration) was applied at the time, which reduces the system to three branches linked by a single intermediate. Under experimental conditions, the magnitude of the proton-

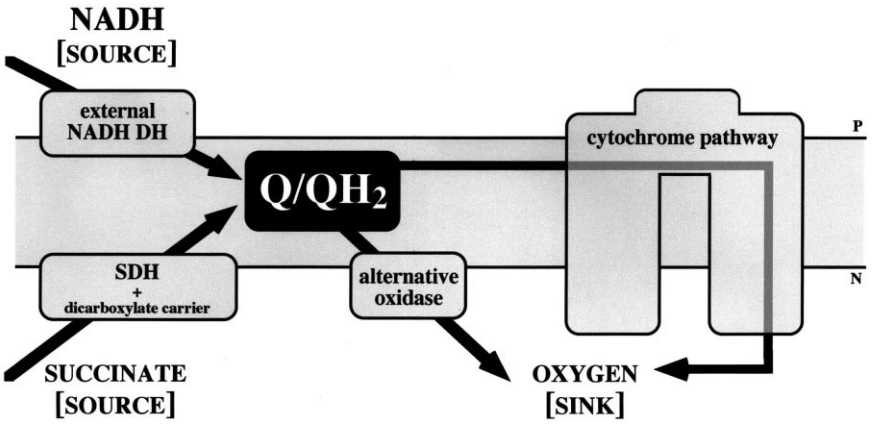


Fig. 3. Schematic representation of plant mitochondrial electron transfer. Electron transfer is considered from the 'top-down' perspective defined by Krab et al. [29,30,73] and divided in Q-reducing and QH_2-oxidising processes. NADH DH, NADH dehydrogenase; SDH, succinate dehydrogenase; P and N, as defined in Fig. 1.

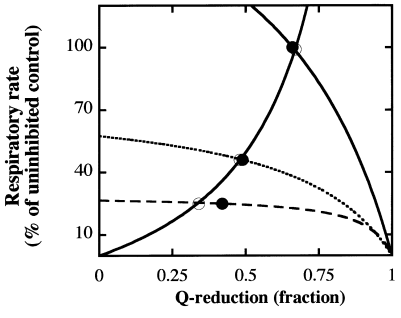
motive force was assumed to be constant [30] which may not always be the case (cf. Section 5).

Application of the method to data obtained from experiments with potato callus mitochondria [28], revealed that in this system very little control on respiratory flux was exerted by the alternative oxidase, either under state 3 [73] or under state 4 conditions [29,73]. Both the flux control coefficient of the alternative oxidase and that of the cytochrome pathway were (somewhat) higher in state 4 than in state 3 [73]. The increase in control exerted by the QH_2-oxidising enzymes was obviously at the expense of control exerted by succinate dehydrogenase [73]. We speculate that this re-distribution of flux control is related to the dual effect that ADP potentially has on succinate dehydrogenase (inhibitory) and cytochrome pathway (stimulatory) activity, which has been experimentally observed in *Arum* mitochondria ([31]; cf. Section 3.1). It should be emphasised, however, that in potato callus mitochondria, the difference in cytochrome pathway kinetics between state 4 and state 3 was indeed evident from the experimental data [28], whereas the change in succinate dehydrogenase kinetics was inferred from mathematical modelling of the results (which yielded the control coefficients reported in [73]). The range of experimental data was not sufficient to determine the significance of the apparent difference in succinate dehydrogenase kinetics between state 4 and state 3 in this system [28].

The functional expression of the alternative oxidase in *S. pombe* confers a mitochondrial respiratory system to this yeast, which closely resembles those found in plants [35,38]. The all-or-nothing character of this expression was exploited to investigate the effect of the presence of the alternative oxidase on the distribution of control within the electron transfer system [35]. Previous modelling predicted that a differential presence of the alternative oxidase could result in significant changes in the way control is distributed [29], which was indeed confirmed by experiments performed with mitochondria isolated from transformed *S. pombe* cells [35]. From the modelled data, it was expected that the presence of an additional QH_2-oxidising pathway would increase the degree of flux control exerted by the Q-reducing enzymes [29]. Interestingly, however, control by the reducing side of the system did not change significantly upon expression of the alternative oxidase in *S. pombe*. Under state 4 conditions, the alternative oxidase itself claimed approximately 22% of the control on overall electron transfer, fully at the expense of the control formerly exerted by the cytochrome pathway [35]. From the kinetic data it appeared that, in *S. pombe* mitochondria, the alternative oxidase was able to actively compete with the cytochrome path for reducing equivalents, which was reflected in a similar degree of control (in absolute terms) exerted by the respective enzyme units on the ratio of electron fluxes through the QH_2-oxidising pathways [35].

4.3. The predictive value of kinetic modelling and metabolic control analysis

As may be clear from the preceding discussion, a considerable degree of insight into the regulation of plant mitochondrial electron transfer can be obtained from kinetic modelling and metabolic control analysis. To illustrate this further, an example concerning determinations of apparent oxygen affinities of the alternative oxidase, is presented in this section. Ideally, the oxygen affinity of an oxidase is measured by monitoring spectral changes during the deoxygenation of haem-based oxygen carrier proteins (cf. [80]). It should be noted, however, that the following discussion concerns affinity measurements that were made with an oxygen electrode. From the kinetic model developed by Siedow and Moore [26] it was predicted that the apparent K_m for O_2 of the alternative oxidase would decrease upon an increase in Q-reduction. To test this notion, Ribas-Carbo et al. [27] performed experiments in mitochondria isolated from soybean and mungbean and found rather the opposite: the apparent K_m for O_2 increased upon a rise in Q-reduction, which led these authors to adapt the kinetic model by introducing an extra step in the reaction mechanism.



[malonate]	steady state parameters experimental ● and [**modelled**] ○				Flux control values C^J	
mM	Q-reduction (fraction)		Rate (% of control)		SDH	AOX
0	0.66	[**0.67**]	100	[**99**]	0.72	0.28
1	0.49	[**0.48**]	46	[**46**]	0.84	0.16
4	0.42	[**0.34**]	25	[**25**]	0.96	0.04

Fig. 4. Kinetic modelling of mitochondrial Q-pool kinetics. Experimental steady states (●) were taken from [27] and modelled (○) according to [28]: solid, dotted and dashed lines represent SDH kinetics in the presence of 0, 1 and 4 mM malonate, respectively. Rates are expressed as a fraction of the uninhibited control which was ~50 nmol O_2 min^{-1} mg^{-1} [27]. Flux control coefficients (C^J) were calculated as described in [29]. SDH, succinate dehydrogenase, AOX, alternative oxidase.

Based on modelling predictions (Fig. 4), we suggest that the observed correlation between the apparent $K_{m\,(O_2)}$ of the alternative oxidase and the Q-reduction level is not necessarily a dependency intrinsic to the alternative oxidase enzyme, but is, at least partly, a reflection of the different control distributions that prevailed under the different experimentally steady states created to probe this potential correlation. The oxygen affinities were experimentally determined in mitochondria isolated from etiolated soybean cotyledons that were oxidising succinate in the presence of myxothiazol; to vary the Q-redox poise succinate dehydrogenase was inhibited to different extents with malonate [27]. It is clear that the measured respiratory activity under such conditions is not the sole result of alternative oxidase activity, but rather of the kinetic interplay of this enzyme with succinate dehydrogenase. An inhibitory effect of a limiting oxygen concentration on alternative oxidase activity would only be noticeable as a decrease of the oxygen uptake rate, if the overall rate of electron transfer was fully controlled by the oxidase.

We have modelled the different steady states measured by Ribas-Carbo et al. [27] in terms of Q-pool kinetics (Fig. 4) with the assumption that they should all be described by a single curve reflecting the kinetic behaviour of the alternative oxidase. From Fig. 4 it is clear that the steady states are reasonably well modelled assuming reversible Michaelis–Menten kinetics [28]. Curves describing succinate dehydrogenase kinetics in the presence of the different malonate concentrations, were modelled such that they described the respective experimental steady states as well as the theoretical inevitable steady state (i.e. Q-pool fully reduced, no Q-reducing activity). Flux control coefficients, tabulated in Fig. 4, were calculated for the three modelled steady states; note the proximity of these steady states to the experimentally determined ones (Fig. 4). An intuitively anticipated relationship indeed appears to exist between the malonate concentration and the way control is distributed: the more succinate dehydrogenase is inhibited, the more it controls the respiratory activity, obviously at the expense of the alternative oxidase. Based on these model-derived data, we suggest that the observed decrease in the apparent $K_{m\,(O_2)}$ of the alter-

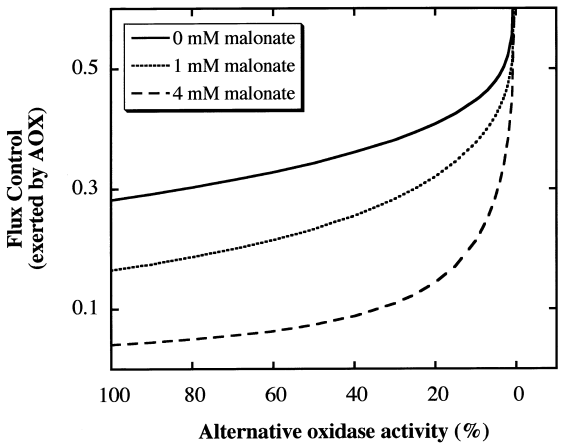


Fig. 5. Modelled relation between flux control exerted by the alternative oxidase and the enzyme's relative activity. Flux control coefficients were calculated as described in [29]. AOX, alternative oxidase.

native oxidase upon a decrease in Q-reduction [27] is, at least partly, due to the fact that an inhibitory effect of oxygen limitation is masked by the oxidase's relative lack of flux control.

From Fig. 4 it can be seen that the maximum flux control exerted by the alternative oxidase in any of the experimentally created steady states, does not exceed 28%. To determine at which point during oxygen limitation the alternative oxidase would commence, due to the fact the enzyme is inhibited, to exert more control on the flux, we have modelled (for the different malonate concentrations) the relation between the flux control coefficient of the alternative oxidase and its relative activity. From Fig. 5 it can be predicted that the oxidase does not claim more than 50% of the flux control until its activity is inhibited by more than 97%. Therefore, if our modelling predictions are correct, then the maximum apparent $K_{m\,(O_2)}$ value observed by Ribas-Carbo et al. in mitochondria from young aetiolated soybean cotyledons ($\sim$18 μM O_2; [27]) would still be an underestimation. Experiments in *S. pombe* mitochondria containing the alternative oxidase [38], suggest this is indeed the case. When *S. pombe* mitochondria oxidise NADH in the presence of antimycin A, the alternative oxidase exerts 94% of the control on the overall respiratory activity (cf. the kinetic data in [35]). The determination of NADH-dependent, antimycin-insensitive respiratory activity as a function of the dissolved oxygen concentration, indicates that apparent $K_{m\,(O_2)}$ values of the alternative oxidase under these conditions exceed 30 μM (Affourtit, C. and Moore, A.L., unpublished).

5. Concluding remarks

The preceding discussion illustrates how kinetic studies implementing metabolic control analysis, improve the understanding of phenomena relating to plant respiration. It becomes increasingly apparent that kinetic modelling of plant mitochondrial electron transfer provides useful insight into the regulation and control of the relative activities of phosphorylating and non-phosphorylating pathways. From this, clues may be obtained as to the metabolic circumstances under which either activity is important and hence as to the exact physiological role of the 'energy-wasting' enzymes. It should be stressed that such in vitro studies merely have predictive power. In future investigations, it could be attempted to expand the experimental plant systems under consideration. Quantitative studies e.g. into the interaction between the respiratory chain and the TCA cycle, might provide relevant experimental information as to the way in which electron transfer activity reacts to changes in TCA cycle function in response to different catabolic and anabolic cellular demands. In this light, quantitative studies into the interplay between the different plant organelles, particularly mitochondria and chloroplasts, would also be of interest. As discussed, the challenge of such experimental approaches is to define a system that can be experimentally manipulated easily, yet has solid boundaries.

Finally, it should be mentioned that the edges of the systems defined to quantitatively study mitochondrial respiration, are also not always as hard as desired. For example, the boundary of the Q-kinetic system defined by Krab and colleagues [29], is assumed to include the magnitude of the protonmotive force. This force is unlikely to be constant, particularly during the antimycin A titrations required to determine Q-reducing kinetics. An experimental approach that combines measurements of respiratory activity, the Q-reduction level, phosphorylation and the protonmotive force, should clarify the extent to which these concerns are justified. In other words, a

synthesis of the approaches of Kesseler et al. [70] and Krab et al. [29] seems a logical way to obtain a more complete understanding of the activity of the separate mitochondrial respiratory enzymes, the phosphorylation system and proton leak, processes which are all kinetically dependent, directly or indirectly, upon both the Q-redox poise and the protonmotive force.

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