Kinetic and Regulatory Aspects of the Function of the Alternative Oxidase in Plant Respiration

Klaas Krab[1]

The kinetic modelling of the respiratory network in plant mitochondria is discussed, with emphasis on the importance of the choice of boundary conditions, and of modelling of both quinol-oxidising and quinone-reducing pathways. This allows quantitative understanding of the interplay between the different pathways, and of the functioning of the plant respiratory network in terms of the kinetic properties of its component parts. The effects of activation of especially succinate dehydrogenase and the cyanide-insensitive alternative oxidase are discussed. Phenomena, such as respiratory control ratios depending on the substrate, shortcomings of the Bahr and Bonner model for electron distribution between the oxidases and reversed respiratory control, are explained. The relation to metabolic control analysis of the respiratory network is discussed in terms of top-down analysis.

KEY WORDS: Plant mitochondria; alternative oxidase; kinetics; regulation.

## THE RESPIRATORY NETWORK IN PLANT MITOCHONDRIA

Plant mitochondria differ in several interesting ways from mitochondria found in a number of well-studied mammals (such as rats and cows). Considering respiratory electron transfer, a striking difference with respect to mammalian mitochondria is the presence of two extra NAD(P)H dehydrogenases that are not conserving energy, are not inhibited by inhibitors of the energy-conserving NADH dehydrogenase, and oxidize matrix NADH and cytoplasmic NAD(P)H, respectively (Møller *et al.*, 1993). In addition to these extra dehydrogenases, it is the presence of the cyanide-insensitive alternative oxidase that gives the plant mitochondrial respiratory chain its special character (see Moore and Siedow, 1991 and McIntosh, 1994 for recent reviews). In this respect plants resemble yeasts and bacteria, organisms that have respiratory networks where generally also more than a single oxidase is present. The alternative oxidase is not a standard fea-

ture of plant mitochondria: in some tissues it is constitutively present, while in others it is induced, e.g., as a function of developmental state (Moreau and Romani, 1982), or as a result of stress (Hiser and McIntosh, 1990).

A schematic view of the plant mitochondrial respiratory network is shown in Fig. 1. Note the central position of ubiquinone (Q) in the scheme; this coenzyme is thought to link the different branches of the network (Moore and Siedow, 1991). The branched nature of the plant mitochondrial respiratory chain makes it very suitable for study of regulation within a metabolic network.

The aim of this paper is to elucidate the role of the alternative oxidase in the functioning of the respiratory network. Not so much the question *when* the enzyme is present, but the question *what* it does, when present, will be discussed, in the hope some insight is gained *why* it is present. The starting point in this discussion will be kinetic modelling, and the emphasis will be on the effects of kinetics and regulation of the individual enzymes on the behavior of the system as a whole. In the literature there is a confusing use of the word "regulation"; here it is used to indicate

---
[1] Department of Molecular and Cellular Biology, Vrije Universiteit, De Boelelaan 1087, NL-1081 HV Amsterdam, The Netherlands.
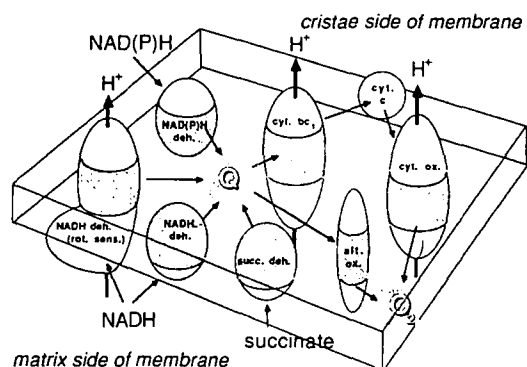
Fig. 1. Schematic view of the respiratory chain enzymes in the inner mitochondrial membrane. The box represents a slice of membrane viewed from above; the inner face of the membrane is shaded light gray. Darker shaded areas: part of the enzymes immersed in the membrane. Thin arrows represent reactions involving transfer of reducing equivalents; fat arrows indicate proton translocation. Q: the ubiquinone pool.

effects on enzymes distinct from the normal relationships between substrate concentrations and rates.

KINETIC MODELLING

Before discussing kinetic modelling of respiration in plant mitochondria, it is necessary to emphasize the importance of the boundaries between which this modelling takes place. This amounts to setting a "hard edge" to the system that has to be modelled, that does not respond to the changes that are considered. Of the factors controlling plant respiration, the energy state of the mitochondrial inner membrane (reflected in the value of $\Delta\bar{\mu}_H^+$) looms largest. Also factors such as oxygen concentration and substrate availability determine activities within the respiratory network. In the last few years much discussion of regulation of respiration has focused on the role of the redox state of the ubiquinone pool.

In view of the central role of the quinone pool (Q pool) in connecting the respiratory enzymes, here the emphasis will be on kinetic models that describe especially the effect of the quinone reduction state on the rate of oxygen uptake (the usual measure for respiration). Energy state, substrate availability, and oxygen concentration in the first approximation will be treated as part of the "hard edge" and their effects on the rate of oxygen uptake will be discussed only where necessary.

The Single Quinone Pool

Kröger and Klingenberg (1973a,b) first demonstrated that the idea of a central Q pool that links dehydrogenases and reductases may be successfully used to model steady-state rates of oxygen uptake in terms of the rate constants of the quinone-reducing and quinol-oxidizing steps. Their kinetic model relied on linear relationships between the redox state of the Q pool and rates of oxidation and reduction, and in the uncoupled mammalian submitochondrial particles that they used to test the model these relationships indeed were observed (Kröger and Klingenberg, 1973a).

Application of this model to the yeast *Saccharomycopsis lipolytica* where, as in plants, alternative oxidase is present in addition to the cytochrome pathway, led De Troostembergh and Nijns (1978) to the conclusion that electron transfer is proportionally shared between the two quinol-oxidizing pathways. However, some years earlier Bahr and Bonner (1973a,b) concluded from inhibitor titrations of the alternative oxidase in plant mitochondria that alternative oxidase "kicks in" only when the cytochrome pathway is saturated, and the Q pool becomes highly reduced. In an attempt to solve this controversy, Cottingham and Moore (1983) showed that indeed the Kröger and Klingenberg model fails to describe respiration in plant mitochondria as soon as both cytochrome pathway and alternative oxidase are active.

The reason for this failure became apparent when Ragan and colleagues first proposed (Ragan and Cottingham, 1985), and then showed (Ragan and Reed, 1986; Reed and Ragan, 1987), that introduction of hyperbolic relationships between rates of individual steps and quinone reduction state leads to a modification of the Kröger and Klingenberg model that explains the deviations observed in plant mitochondria. Although the term "quinone pool behavior" often is reserved for the classic Kröger and Klingenberg model (including the linear relationships), also the modified models have, at the core, functioning of a central, homogeneous Q pool.

Q-Pool Kinetics

Introduction of the Q-electrode technique (Moore *et al.*, 1988) to monitor the reduction state of the Q pool during respiration provided means to test the above kinetics models directly. In plant mitochondria it was

soon found that especially the kinetics of the alternative oxidase in most cases shows nonlinear characteristics (Moore et al., 1988; Dry et al., 1989). In line with the ideas of Bahr and Bonner, appreciable activity of the alternative oxidase was observed only when the Q pool was in a very reduced state. The kinetics of the cytochrome pathway were linear (Moore et al., 1988).

Siedow and Moore (Moore and Siedow, 1991; Siedow and Moore, 1993) proposed a detailed model to explain the dependence of the rate of quinol oxidation catalyzed by the alternative oxidase on Q reduction. This model included reaction of the alternative oxidase with two quinol molecules to reduce a single oxygen, and was able to explain a whole range of kinetic curves observed in different plant mitochondria, or in mitochondria from tissues in different stages of development (Siedow and Moore, 1993). A refinement of this model was the inclusion of an extra activation step of the completely reduced enzyme, which allowed one to predict correctly the changing affinity for oxygen upon changes in Q-pool reduction (Ribas-Carbo et al., 1994). A related approach was followed recently by James et al. (1994). The kinetics of all respiratory pathways (including the alternative oxidase) were modelled with hyperbolic expressions by Van den Bergen et al. (1994). In this approach pathways are treated as Michaelis–Menten enzymes, operating not under initial conditions, but with the restriction that the total amount of ubiquinone + ubiquinol is constant (Van den Bergen et al., 1994). At present no choice can be made yet which kinetic model for the alternative oxidase is best, due to the quality of available kinetic data, and the lack of knowledge about the mechanism of the enzyme.

Focusing on a single intermediate such as quinone reduction makes it relatively easy to describe the behavior of the system as a whole (determined by its hard edge) in terms of the kinetic characteristics of its component parts. Thanks to the availability of methods to monitor the redox state of the Q pool by electrode (Moore et al., 1988; Dry et al., 1989) or extraction (Siedow et al., 1993; Van den Bergen et al., 1994), it is possible to determine these kinetic characteristics directly. The quinol-oxidizing pathways have been characterized in many plant mitochondria by modulation of the rate of quinone reduction (mostly using succinate respiration with malonate as inhibitor), so that oxygen uptake rates can be related with quinone reduction. In a similar way the kinetics of the quinone-reducing pathways (the dehydrogenases) can be determined by titration of respiration with inhibitors of

the oxidizing pathways (Moore et al., 1993; Van den Bergen et al., 1994).

To illustrate the power of analysis in terms of individual enzyme kinetics, the data of Day et al. (1991), which were taken to indicate deviations from Q-pool behavior, may be analyzed. The data of Table I in Day et al. (1991) were plotted in Fig. 2, grouped according to state of the oxidizing pathway (Fig. 2A), and according to substrate (Fig. 2B). Figure 2A gives a view on the kinetics of the cytochrome pathway and alternative pathway (compare Dry et al., 1989). Of interest is that when NADH is exclusively the substrate, the points lie below the curves suggested by the other points (compare closed triangle with open
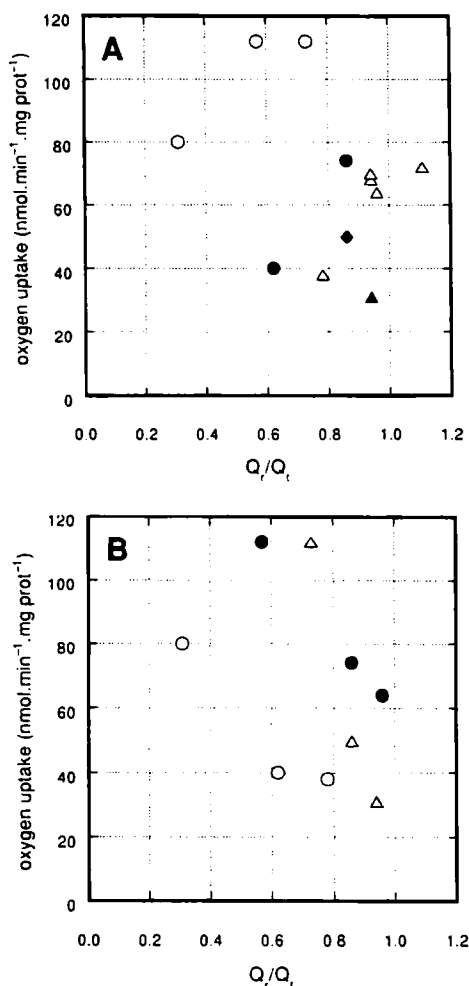
Fig. 2. Kinetics of quinol-oxidizing and quinone-reducing pathways in soybean cotyledon mitochondria. Data from Table I in Day et al., 1991. A. oxidizing pathways: ●, state 4 (succinate or malate); ◆, state 4 (NADH); ○, state 3; △, myxothiazol and succinate or malate present; ▲, myxothiazol present. B. reducing pathways: ●, succinate; ○, malate; △, NADH.

triangles, and filled diamond with filled circles). This is easily explained by activation of the alternative oxidase by succinate or malate (see below). Figure 2B groups the same data with respect to substrate (or reducing pathway). Kinetic curves for succinate dehydrogenase and external NADH dehydrogenase are fairly steep; the points for malate (reflecting the internal dehydrogenases) indicate a less active enzyme; the exception here is the point at low Q-pool reduction (state 3), but this may be explained as an effect of energy state on the rotenone-sensitive dehydrogenase (site 1). The conclusion is that these data are fully compatible with quinone acting as a homogeneous pool.

Interplay Between Pathways

Once the kinetics of the individual pathways are known, steady states and the effects of modulations of these steady states can be calculated (Van den Bergen et al., 1994). What becomes immediately clear is that not only the well-studied kinetics of the cytochrome pathway and alternative oxidase are determining the steady-state properties, but that the often neglected dehydrogenase kinetics are as important.

Let us look at two examples to illustrate the importance of the kinetic characteristics of the quinone-reducing enzyme for interpretation of steady-state data.

First, consider the steady states observed in the absence and presence of inhibitors of the two quinol-oxidizing pathways. In Fig. 3 the kinetic curves of cytochrome pathway and alternative oxidase, as they have been determined for potato callus mitochondria (Van den Bergen et al., 1994), are combined with assumed kinetic curves for a set of dehydrogenases. The dehydrogenase kinetics are modelled such that the uninhibited steady state is identical for the whole set (72.3 nmol O_2 min^{-1}.mg protein^{-1} at $Q_r/Q_t = 0.6$), and the dehydrogenase-catalyzed rate is 0 at $Q_r/Q_t = 1$. This way, in the model of Van den Bergen et al. (1994) there is a single parameter (γ) that may be varied: this parameter is a measure for the affinity difference of the dehydrogenase toward quinone and quinol, respectively. The smaller (or more negative) γ, the larger the preference for quinone. Note that as the uninhibited steady state is identical for these dehydrogenases, the same holds for the use of the two quinol-oxidizing pathways in this state. Figure 3A shows that the value of γ determines the extent in which the dehydrogenase-catalyzed rate varies with quinone reduction.

Using the assumption of Bahr and Bonner (1973a,b) that the cytochrome pathway operates at maximal capacity, the extent of participation of the alternative oxidase in oxygen consumption in the uninhibited state is generally determined by comparison with the rates observed when either one of the two pathways is completely inhibited. Analysis of Fig. 3A shows that this procedure gives varying results, as illustrated in Figs. 3B and 3C. As pointed out before, this widely used method fails because changes in the activity of the noninhibited oxidizing pathway are not taken into account (Van den Bergen et al. 1994). However, the point here is that both the capacity and apparent participation in the uninhibited state of the alternative pathway when determined in this way depend on the kinetics of the dehydrogenase. The value of ρ increases when the dehydrogenase has a lower preference for quinone above quinol (Fig. 3C). This effect may contribute a lot to the often observed substrate dependence of access to the alternative oxidase (Moore and Siedow, 1991).

As a second example, a similar influence of dehydrogenase kinetics may be expected in determinations of the respiratory control ratio. Here the kinetics of the cytochrome pathway change upon addition of ADP (Moore et al., 1988; Dry et al., 1989; Van den Bergen et al., 1994). Again the shape of the kinetic curve of the dehydrogenase determines what the actual increase in steady-state rate is. As an example, let us assume that the dehydrogenases represented in Fig. 1 are not energy coupled (the kinetics in state 3 and state 4 is identical). Using the data for succinate respiration in potato callus mitochondria for the oxidizing pathways in state 3 and state 4 (Van den Bergen et al., 1994), it can be calculated that use of the dehydrogenase from Fig. 3A with $\gamma = 0$ would cause the mitochondria to show a respiratory control ratio of 2.2, while use of the dehydrogenase with $\gamma = -0.99$ would generate a respiratory control ratio of 1 (no control). So a substrate dependence of respiratory control may be observed in the absence of direct energy dependence of the dehydrogenase kinetics itself. A similar difference in respiratory control ratio between the two dehydrogenases would occur when the alternative oxidase is blocked.

Obviously, regulatory phenomena (such as pathway activation) will also affect the outcome of this type of experiment (see below), but to determine their
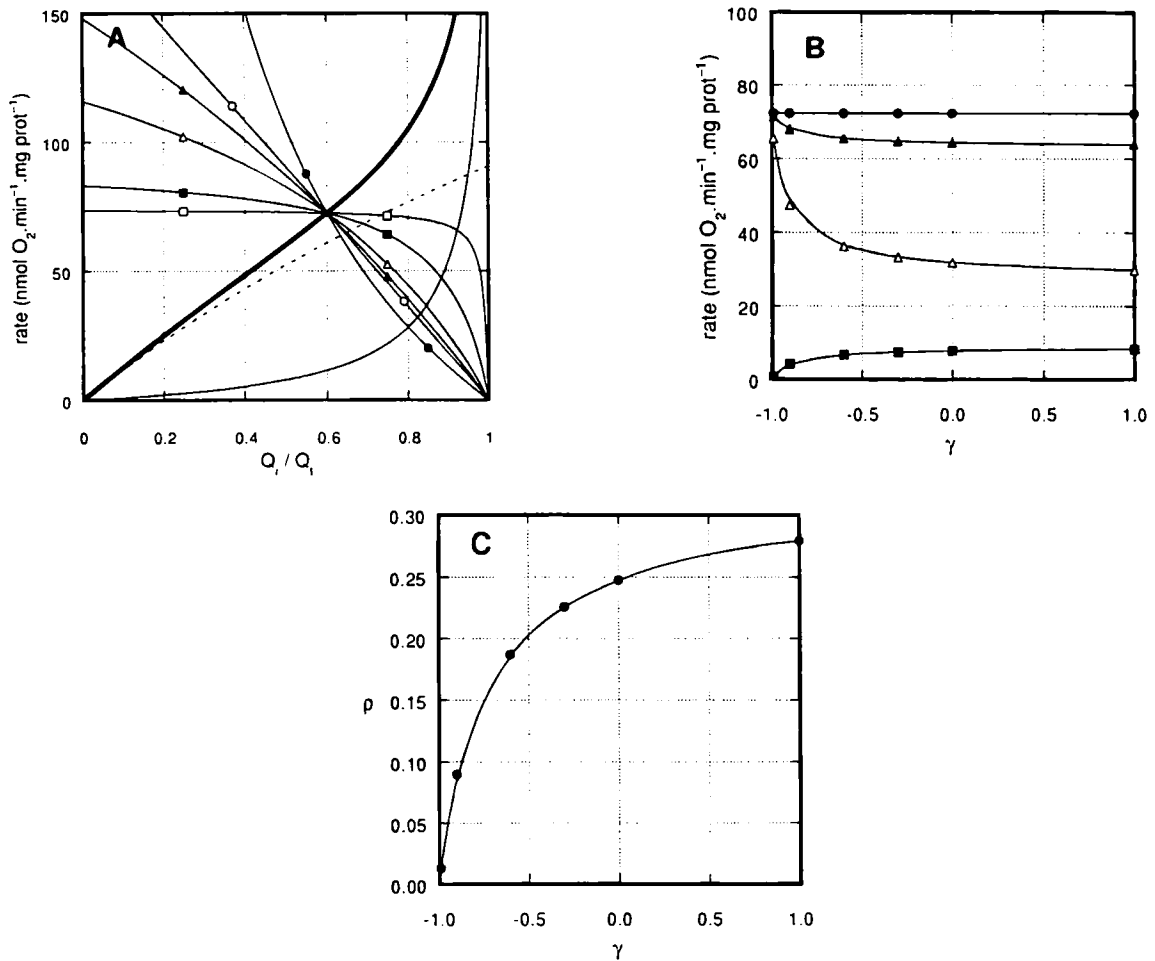
Fig. 3. Dehydrogenase kinetics and the effect of inhibition of quinol oxidation on the steady state. A. Rates as a function of Q pool reduction. Dashed, cytochrome pathway; thin line (no markers), alternative oxidase; thick line, total quinol oxidation; marked lines, dehydrogenase: □ γ = -0.99; ■ γ = -0.9; △ γ = -0.6; ▲ γ = -0.3; ○ γ = 0; ● γ = 10. The lines for oxidizing pathways are those in Fig. 6 of Van den Bergen *et al.*, 1994. See text for further details. B. Calculated rates as function of γ. ● uninhibited; ▲ alternative oxidase blocked; △ cytochrome pathway blocked; ■ apparent activity of alternative oxidase in uninhibited state ("ρ V_{alt};" see Bahr and Bonner 1973a). C. Factor ρ as function of γ.

existence, magnitude, and relevance, a complete kinetic analysis of the system has to be performed first.

APPLICATION OF METABOLIC CONTROL THEORY

To describe the behavior of the respiratory network in a quantitative way, metabolic control analysis is the tool of choice. Application of this theory requires the selection of fluxes (processes) and intermediates, and a similar definition of a "hard edge" (consisting of sources and sinks, in terms of intermediates) as in kinetic modelling. At present a number of different

studies of isolated plant mitochondria have been made, with different processes and intermediates selected.

Padovan *et al.* (1989) have performed inhibitor titrations of ubiquinol:cytochrome *c* oxidoreductase, cytochrome *c* oxidase, adenine nucleotide carrier, and ATP synthase in turnip (*Brassica rapa*) mitochondria respiring on different substrates in state 3 and state 4. They obtained control coefficients from these titrations following the procedures of Groen *et al.* (1982). They found that control of state 3 respiration resides to a large extent in the two respiratory enzymes, while this was not so for state 4 respiration. It is clear from the schematic view of mitochondrial respiration and ATP synthesis in Fig. 4 that this is a fairly incomplete

analysis; in particular, the proton leak was not included. A similar approach was followed by Hill *et al.* (1993) in the study of succinate oxidation in cucumber (*Cucumis sativus*) mitochondria; this time, however, succinate dehydrogenase, ubiquinol:cytochrome c oxidoreductase, cytochrome c oxidase, and the adenine nucleotide carrier were titrated. Different steady states with different ATP demands were studied. It was concluded that much control in this experimental system resides in succinate transport into the mitochondria, a process less likely to be of significance in the functioning cell.

A different approach was followed by Kesseler *et al.* (1992) who followed the "top-down" approach (Hafner *et al.*, 1990; Brown *et al.*, 1990) to study mitochondria isolated from fresh potato (*Solanum tuberosum*). This study concentrated on energy-yielding and energy-using reactions with the electrochemical proton gradient across the inner mitochondrial membrane (characterized by the value of $\Delta\bar{\mu}_H^+$) as common intermediate. The processes that make up the network are then the respiratory chain taken together, the phosphorylating system taken together, and proton leak across the inner mitochondrial membrane (see Fig. 4). Particularly elegant in this study was that a whole range of steady-states between states 3 and 4 were characterized in terms of control by the respiratory system, the phosphorylating system, and the proton leak process on the rates of respiration, ATP synthesis and proton leak, and on the magnitude of $\Delta\bar{\mu}_H^+$. Both respiration on succinate and external NADH were analyzed. Control of respiration was dominated by the respiratory system, except in state 4 where the leak process exerted much control. Control of phosphorylation was also mainly by the respiratory system, except at low rates where the phosphorylating system did contribute significantly. Control of the leak rate was shared by the respiratory process, the leak process (positive contributions) and the phosphorylating system (a negative contribution). Little control was found on $\Delta\bar{\mu}_H^+$, showing that this parameter is effectively kept constant in this system (Kesseler *et al.*, 1992).

The same group has applied this method to study quantitatively the effects of cadmium on succinate respiration in potato mitochondria (Kesseler and Brand, 1994a,b,c). They concluded from their analysis of control that cadmium inhibits substrate oxidation and stimulates proton leak in this system, but has no effect on phosphorylation (Kesseler and Brand, 1994a). Further analysis, in terms of partial response coefficients, of internal regulation (Kesseler and Brand, 1994b), and response to cadmium (Kesseler and Brand, 1994c), showed how cadmium, depending on its concentration and on the energy demand, affects respiration, phosphorylation, proton leak, $\Delta\bar{\mu}_H^+$, and P/O value.

A variant of the top-down approach centered around the Q pool (Q_r/Q_t) as intermediate was developed independently (Krab *et al.*, 1995; Moore *et al.*, 1994) using the kinetic modelling of respiration in potato callus mitochondria (Van den Bergen *et al.*, 1994) as basis. In this case the energetic state of the mitochondria (particularly $\Delta\bar{\mu}_H^+$) was taken to be part of the hard edge. Applying the method to state 4 titrated with malonate or myxothiazol, this is not too bad an assumption (Mandolino *et al.*, 1983; Whitehouse *et al.*, 1989). In this approach control coefficients can be calculated for dehydrogenase, cytochrome path (see Fig. 4), and alternative oxidase (Moore *et al.*, 1994). Also shifts in control caused by changes in alternative oxidase can be predicted (Krab *et al.*, 1995).

The structural similarity between the approaches of Brand and co-workers and of Van den Bergen *et al.* is large; in both cases the system consists of three branches linked by a single intermediate. Titration experiments are used to obtain elasticity coefficients and to determine the position of the steady state; control coefficients are then calculated from the steady-state values of elasticity coefficients, fluxes, and linking intermediate concentration.

Comparing the different attempts to perform metabolic control analysis of plant mitochondrial respiration, the advantages of the top-down approach are clear: the system under consideration is well defined, and its definition (the hard edge) can be verified experimentally.

REGULATORY PHENOMENA

Regulation by Energy State

Strictly speaking, most effects of energy state on respiration should not be classified as regulation. In those parts of the respiratory network where proton translocation is coupled to electron transfer (rotenone-sensitive NADH dehydrogenase and cytochrome pathway, see Fig. 1), protons are substrate or product, depending on at which side of the membrane they are involved. However, when modelling electron transfer it may be efficient to consider only a single energy state at the time. This is particularly useful when study-
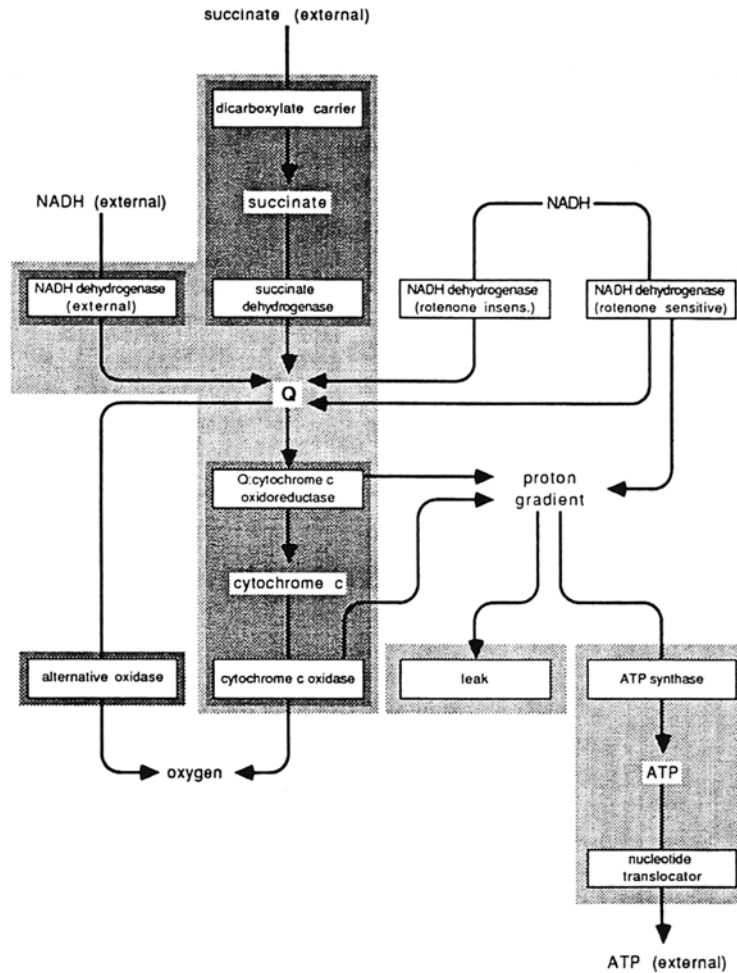
Fig. 4. Comparison of metabolic control theory treatments of plant mitochondrial respiration. The enzymes are boxed; the intermediates are not. Lightly shaded areas: processes grouped together in top-down analysis centered around $\Delta\bar{\mu}_H^+$ by Kesseler et al. (1992). Darkly shaded areas: processes grouped together in treatment centered around Q_r/Q_t by Krab et al. (1995).

ing the role of the Q pool in determining the kinetic performance of the respiratory system, as, e.g., in the approach of Van den Bergen et al. (1994), where state 4 and state 3 are modelled separately. When the effects of energy state are seen as regulation, there are profound effects on kinetics of the cytochrome pathway, as exemplified by the differences between state 3 and state 4 (Moore et al., 1988; Dry et al., 1989; Van den Bergen et al., 1994). No data are available yet about the kinetics of the rotenone-sensitive NADH dehydrogenase in relation to the energy state.

Obviously, there may be effects of energy state that may be of a pure regulatory nature. These effects need not be of $\Delta\bar{\mu}_H^+$ itself, but may be quite indirect:

via ATP concentration (as in the case of succinate dehydrogenase, see below) or via (local) pH changes.

Regulation of Succinate Dehydrogenase

Succinate dehydrogenase is an enzyme that is subject to extensive regulation (for an overview, see Gutman, 1978), also in plant mitochondria (Oestreicher et al., 1973). Inactivation occurs by formation of a complex with oxaloacetate, which results in a downward change in redox potential of the flavin that makes it impossible for succinate to reduce this group (Gutman et al, 1980). A number of activating sub-

stances counteract inactivation by oxaloacetate, in particular some dicarboxylates (succinate, malonate, fumarate), ATP, and QH_2 (Gutman, 1978). The activating effects of succinate, malonate, and fumarate have been nicely quantified in terms of binding to a regulatory site (Gutman, 1978), but this is not the case for the physiologically interesting activators ATP and QH_2. In experiments with isolated mitochondria, succinate dehydrogenase is mostly activated by addition of ATP, and when necessary by addition of glutamate (which removes any oxaloacetate by transamination). In the absence of these precautions, activation might occur during state 3 to state 4 transitions due to generation of ATP, and an increased steady-state level of QH_2 in state 4. In this respect it should be noted that activation by ATP is faster (2–4 min) than by succinate or QH_2 (15–20 min, Gutman et al., 1971a,b; Gutman, 1980). Activation may change the kinetics of succinate dehydrogenase such that even though the kinetics of the cytochrome pathway change in the expected manner (Moore et al., 1988; Dry et al., 1989), the resulting steady-state oxygen-uptake rate is higher in state 4 than in state 3 (inverse respiratory control). This is illustrated in Fig. 5, where 50% inactivation of succinate dehydrogenase in state 3 leads to a reversed respiratory control ratio of 0.8. Such phenomena have been observed in plant mitochondria obtained from different sources (Leach and Moore, unpublished and Wagner, unpublished).

The relevance of these regulatory phenomena for the in vivo function of succinate dehydrogenase at present still is an open question; however, detailed
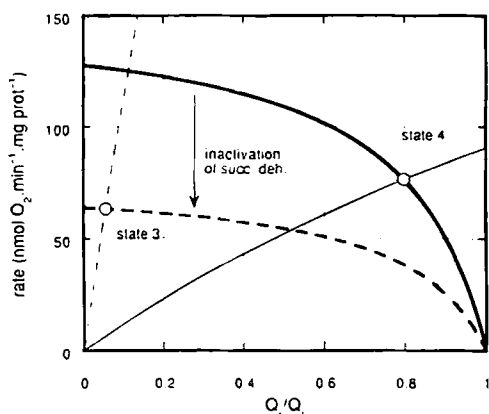
Fig. 5. Activation of succinate dehydrogenase and reversed respiratory control. Thin lines: kinetics of cytochrome pathway in state 3 (dashed) and state 4 (continuous). Thick lines: kinetics of succinate dehydrogenase in activated (continuous) and 50% inactivated (dashed) state. Steady states are indicated by circles, with a respiratory control ratio of 0.82.

characterization of the effects of activation on the kinetics of succinate dehydrogenase is required to allow the prediction of the way activation would affect the steady-state behavior of the respiratory network.

Regulation of Alternative Oxidase

Not only the amount of alternative oxidase varies with tissue conditions, but also the kinetic characteristics. The clearest example is afforded by mitochondria isolated from spadices of Arum maculatum in different developmental stages, where the plot of rate vs. Q-pool reduction changes from nonlinear in stage α to nearly linear in stage γ–δ (Siedow and Moore, 1993; Leach, Krab, and Moore, unpublished). A clue to this behavior is the recent finding that there are a number of regulatory phenomena that affect the activity of the alternative oxidase.

Firstly, stimulating effects of a number of carboxylic acids on the enzyme have been found. Increased activity with NADH as substrate has been observed after treatment of mitochondria isolated from potato callus and from Petunia hybrida cell suspensions (Wagner et al., 1989, 1995) or from apple (ethylene)-treated potatoes (Lidén and Åkerlund, 1993) with succinate or malate. It was demonstrated that this activation occurs at the level of the alternative oxidase itself (see below), and that respiration of succinate or malate is not required for activation (Wagner et al., 1989). It is obvious that this effect cannot be observed with succinate as substrate. In addition there is a stimulating effect of pyruvate (Millar et al., 1993), also on the alternative oxidase, and this time also observed with succinate as substrate. The relation between the effects of pyruvate (and related compounds such as hydroxypyruvate, oxoglutarate, and glyoxylate) and those of succinate, D($+$) malate and L($-$) malate at present is unclear. Half maximal stimulation is found with ca. 0.1 mM pyruvate (Millar et al., 1993), and 3–5 mM succinate (irrespective of the presence of malonate) or malate (Wagner et al. 1995). The results of Wagner et al. (1995) make it highly unlikely that pyruvate generation from succinate is responsible for the observed stimulation. It is possible that all of these activators react at the same site with different affinities.

The absence of stimulatory effects on respiration via the cytochrome path (Wagner et al., 1989) or on duroquinone reduction (Wagner et al., 1995), stimulation of duroquinol oxidation (Lidén and Åkerlund, 1994; Millar et al., 1994), and shift of the ubiquinone pool to a more oxidized state upon stimulation (Millar

et al., 1994; Umbach et al., 1994; Wagner et al., 1995) all point to an effect on the alternative oxidase itself. In the case of pyruvate stimulation, the result is a shift of the kinetic curve to lower reduction levels of the Q pool (Umbach et al., 1994). This means that the affinity of the alternative oxidase for quinol is increased and/or that for quinone is decreased.

As pointed out above, the data of Day et al. (1991) plotted in Fig. 2 also show evidence of activation of alternative oxidase by succinate or malate. Perhaps a redundant warning: a stimulatory treatment need not always result in an increased rate, because the kinetics properties of the quinol-reducing enzyme also have a large influence on this. In a kinetic plot as in Fig. 2 these effects should be detectable, however.

Secondly, reduction of the alternative oxidase by dithiothreitol affects its activity. Much progress in our understanding of the alternative oxidase has been made thanks to the development of first polyclonal (Elthon and McIntosh, 1987) and then monoclonal (Elthon et al., 1989) antibodies to detect the alternative oxidase protein. At first this way several proteins of ca. 35–37 kDa were found, of unsure relationship. In 1993 Umbach and Siedow reported that in the absence of dithiothreitol (present in the until then usual medium for analysis of alternative oxidase protein) the alternative oxidase protein exists also in dimeric form (Umbach and Siedow, 1993). Cross-linking experiments indicated the existence of both covalent and noncovalent dimers of the alternative oxidase protein. Umbach and colleagues showed that reduction of the alternative oxidase protein (breaking of the S–S bond) results in a higher activity of the (pyruvate-stimulated) enzyme, without an additional shift in the quinol kinetic curve (Umbach et al., 1994). This finding shows that activation by pyruvate and by reduction are two different phenomena. The result is that there is a complicated web of sulfur bridge reduction, monomerization, and effects of carboxylic acids to be elucidated. It is clear that such phenomena by their effect on the alternative oxidase kinetics immediately affect the position of the steady state, both in rate and Q-pool reduction. In fact, during titrations ideally it should be checked that the activation state of the alternative oxidase does not change.

CONCLUDING REMARKS

The above represents an attempt to understand the functioning of the plant respiratory network in terms of the kinetic properties of its component parts.

Of special interest is the role that the cyanide-insensitive alternative oxidase has in the network. Changes in kinetics as a function of tissue growth stage (Siedow and Moore, 1993) or as a result of activation (Umbach et al., 1994) will affect the position of the respiratory steady states and, as a consequence, the energetics of cell respiration will change. To be able to say something about this, a number of starting points have to be clear.

Firstly, the system under consideration should be well defined at the beginning, and some care should be taken to verify its underlying assumptions (the hard edge). This holds true both for kinetic modelling in general, and for applications of metabolic control analysis. The top-down approach introduced by the group of Brand "monoclonal" and "polyclonal" refer to "antibodies" (Hafner et al., 1990; Brown et al., 1990; Kesseler et al., 1992; Kesseler and Brand, 1974a,b,c), and the variant developed by Krab et al. (1995) at the moment seem to satisfy this requirement the best. Secondly, in view of the peculiarities of regulation of electron transport in the individual enzymes, the role of Q pool reduction should be studied more intensively. A lot of work remains to be done on kinetics of reducing pathways (Van den Bergen et al., 1994), and on effects of activation on kinetics of the alternative oxidase (Umbach et al., 1994) and succinate dehydrogenase. Thirdly, it pays to make a clear distinction between kinetic effects of substrates (particularly ubiquinol, ubiquinone, oxygen, and components of the proton gradient) occurring at the time scale of oxidations and reductions, and regulatory phenomena (that might occur at longer time scales). Typical regulatory phenomena of the latter kind are the activation of succinate dehydrogenase and alternative oxidase. Another type of regulation, affecting the expression of the respiratory enzymes, is not discussed here. A conclusion that is inescapable is that once the interplay between the different enzymes is accounted for, a lot of observed phenomena become less puzzling. Experiments thought to disprove a simple pool function of ubiquinone suddenly are compatible with such a function, and quite natural explanations arise for phenomena such as a respiratory control that depends on the nature of the substrate. However, these model-derived explanations in many cases still await experimental testing.

A result of immediate importance for work on respiration in plant cells and mitochondria is that the classical Bahr and Bonner view of the function of alternative oxidase requires revision. It is obvious that such revision will affect discussions about the function

of this pathway. The discovery of regulatory effects on the alternative oxidase makes it even more interesting to measure and model the way these effects determine the steady-state properties of the respiratory network.

ACKNOWLEDGMENTS

REFERENCES

Bahr, J. T., and Bonner, W. D. (1973a). *J. Biol. Chem.* **248**, 3441–3445.

Bahr, J. T., and Bonner, W. D. (1973b). *J. Biol. Chem.* **248**, 3446–3450.

Brown, G. C., Hafner, R. P. and Brand, M. D. (1990). *Eur. J. Biochem.* **188**, 321–325.

Cottingham, I. R., and Moore, A. L. (1983). *Biochim. Biophys. Acta* **724**, 191–200.

Day, D. A., Dry, I. B., Soole, K. L., Wiskich, J. T., and Moore, A. L. (1991). *Plant Physiol.* **95**, 948–953.

De Troostembergh, J.-C., and Nijns, E.-J. (1978). *Eur. J. Biochem.* **85**, 423–432.

Dry, I. B., Moore, A. L., Day, D. A., and Wiskich, J. T. (1989). *Arch. Biochem. Biophys.* **273**, 148–157.

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., Van der Meer, R., and Tager, J. M. (1982). *J. Biol. Chem.* **257**, 2754–2757.

Gutman, M. (1978). *Mol. Cell. Biochem.* **20**, 41–60.

Gutman, M., Kearney, E. B. and Singer, T. P. (1971a). *Biochemistry* **10**, 2726–2733.

Gutman, M., Kearney, E. B., and Singer, T. P. (1971b). *Biochemistry* **10**, 4763–4770.

Gutman, M., Bonomi, F., Pagani, S., Cerletti, P., and Kroneck, P. (1980). *Biochim. Biophys. Acta* **591**, 400–408.

Hafner, R. P., Brown, G. C., and Brand, M. D. (1990). *Eur. J. Biochem.* **188**, 313–319.

Hill, S. A., Bryce. J. H. and Leaver, C. J. (1993). *Planta* **190**, 51–57.

Hiser, C., and McIntosh, L. (1990). *Plant Physiol.* **93**, 312–318.

James, A. T., Venables, W. N., Dry, I. B., and Wiskich, J. T. (1994). *Biometrika* **81**, 219–235.

Kesseler, A., and Brand, M. D. (1994a). *Eur. J. Biochem.* **225**, 897–906.

Kesseler, A., and Brand, M. D. (1994b). *Eur. J. Biochem.* **225**, 907–922.

Kesseler, A., and Brand, M. D. (1994c). *Eur. J. Biochem.* **225**, 923–935.

Kesseler, A., Diolez, P., Brinkmann, K., and Brand, M. D. (1992). *Eur. J. Biochem.* **210**, 775–784.

Krab, K., Van den Bergen, C. W. M., and Moore, A. L. (1995). *Biochem. Soc. Trans.* **23**, 289S.

Kröger, A., and Klingenberg, M. (1973a). *Eur. J. Biochem.* **34**, 358–368.

Kröger, A., and Klingenberg, M. (1973b). *Eur. J. Biochem.* **39**, 313–323.

Lidén, A. C., and Åkerlund, H.-E. (1993). *Physiol. Plant.* **59**, 369–374.

Mandolino, G., De Santis, A., and Melandri, B. A. (1983). *Biochim. Biophys. Acta* **723**, 428–439.

McIntosh, L. (1994). *Plant Physiol.* **105**, 781–786.

Millar, A. H., Wiskich, J. T., Whelan, J., and Day, D. A. (1993). *FEBS Lett.* **329**, 259–262.

Møller, I. M. Rasmusson, A. G., and Fredlund, K. M. (1993). *J. Bioenerg. Biomembr.* **25**, 377–384.

Moore, A. L., and Siedow, J. N. (1991). *Biochim. Biophys. Acta* **1059**, 121–140.

Moore, A. L., Dry, I. B., and Wiskich, J. T. (1988). *FEBS Lett.* **235**, 76–80.

Moore, A. L., Leach, G., and Whitehouse, D. G. (1993). *Biochem. Soc. Trans.* **21**, 765–769.

Moore, A. L., Leach, G., Whitehouse, D. G., Van den Bergen, C. W. M., Wagner, A. M., and Krab, K. (1994). *Biochim. Biophys. Acta* **1187**, 145–151.

Moreau, F., and Romani, R. (1982). *Plant Physiol.* **70**, 1385–1390.

Oestreicher, G., Hogue, P., and Singer, T. P. (1973). *Plant Physiol.* **52**, 622–626.

Padovan, A. C., Dry, I. B., and Wiskich, J. T. (1989). *Plant Physiol.* **90**, 928–933.

Ragan, C. I., and Cottingham, I. R. (1985). *Biochim. Biophys. Acta* **811**, 13–31.

Ragan, C. I., and Reed, J. S. (1986). *J. Bioenerg. Biomembr.* **18**, 403–418.

Reed, J. S., and Ragan, C. I. (1987). *Biochem. J.* **247**, 657–662.

Ribas-Carbo, M., Berry, J. A., Azcon-Bieto, J., and Siedow, J. N. (1994). *Biochim. Biophys. Acta* **1188**, 205–212.

Siedow, J. N., and Moore, A. L. (1993). *Biochim. Biophys. Acta* **1142**, 165–174.

Siedow, J. N., Moore, A. L., Umbach, A. L., Van Rotterdam, B., and Ribas-Carbo, M. (1993). In *Plant Mitochondria* (Brennicke, A., and Kuck, U., eds.), VCA, Weinheim, pp. 275–282.

Umbach, A. L., and Siedow, J. N. (1993). *Plant Physiol.* **103**, 845–854.

Umbach, A. L., Wiskich, J. T., and Siedow, J. N. (1994). *FEBS Lett.* **348**, 181–184.

Van den Bergen, C. W. M., Wagner, A. M., Krab, K., and Moore, A. L. (1994). *Eur. J. Biochem.* **226**, 1071–1078.

Wagner, A. M., Kraak, M. H. S., Van Emmerik, W. A. M., and Van der Plas, L. H. W. (1989). *Plant Physiol. Biochem.* **27**, 837–845.

Wagner, A. M., Van den Bergen, C. W. M., and Wincencjusz, H. (1995). *Plant Physiol.*, **108**, 1035–1042.

Whitehouse, D. G., Fricaud, A.-C., and Moore, A. L. (1989). *Plant Physiol.* **91**, 487–492.