Biochimica et Biophysica Acta, 1059 (1991) 121–140

ADONIS 000527289100189H

BBABIO 43463

Review

The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria

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(Received 6 February 1991)

Key words: Alternative oxidase; Cyanide-insensitive respiration; NADH dehydrogenase; Q-pool; Regulation; Respiratory chain; (Plant mitochondria)

Contents

Summary

In addition to possessing multiple NAD(P)H dehydrogenases, most plant mitochondria contain a cyanide- and antimycin-insensitive alternative terminal oxidase. Although the general characterisics of this terminal oxidase have been known for a considerable number of years, the mechanism by which it is regulated is unclear and until recently there has been relatively little information on its exact nature. In the past 5 years, how-

Abbreviations: AOA, monoclonal antibodies that react with the major polypeptides of the alternative oxidase and designated 'alternative oxidase all'; BIGCHAP, *N,N*-bis-3-D-glucoamidopropyl deoxycholamide; DCCD, *N,N'*-dicyclohexylcarbodiimide; E_h, ambient redox potential with respect to standard hydrogen electrode; SHAM, salicylhydroxamic acid; TCA, tricarboxylic acid; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; UQ, ubiquinone; UQH_2, ubiquinol.

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ever, the application of molecular and novel voltametric techniques has advanced our understanding of this oxidase considerably. In this article, we review briefly current understanding on the structure and function of the multiple NADH dehydrogenases and consider, in detail, the nature and regulation of the alternative oxidase. We derive a kinetic model for electron transfer through the ubiquinone pool based on a proposed model for the reduction of the oxidase by quinol and show how this can account for deviations from Q-pool behaviour. We review information on the attempts to isolate and characterise the oxidase and finally consider the molecular aspects of the expression of the alternative oxidase.

I. Introduction

The respiratory components of plant mitochondria are arranged into multiprotein units comparable to those found in other eukaryotic organisms and are responsible for electron transfer between the various TCA cycle substrates and molecular oxygen. The four major complexes are the internal NADH: ubiquinone reductase (complex I), succinate: ubiquinone reductase (complex II), ubiquinol: cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). A currently accepted form of the organisation of the respiratory chain of plant mitochondria is illustrated in Fig. 1. This scheme is based upon oxidation-reduction kinetics, redox-potential studies, sites of action of respiratory inhibitors and inference from studies with mammalian mitochondria. As in mammalian systems, complexes I, III and IV are transmembraneous and involved in the generation of a proton electrochemical gradient across the inner mitochondrial membrane. The proton gradient is subsequently dissipated either by way of the F_0F_1-ATPase, substrate carriers or leaks. Ubiquinone acts as a mobile, laterally diffusing electron-redistributing pool linking the dehydrogenases with the oxidases, whereas cytochrome *c* acts as a mobile electron carrier connecting complex III with complex IV. The overall process of electron transfer can therefore be considered to include lateral diffusion within the membrane and random collisions between the multiprotein complexes.

The characteristic features of the plant mitochondrial respiratory chain that make it distinct from its mammalian counterpart are the presence of multiple NAD(P)H dehydrogenases and the appearance in most plant species (albeit to varying extents) of a cyanide- and antimycin-resistant, alternative oxidase. In this review we will briefly describe current understanding on the structure and function of the multiple NADH dehydrogenases and consider, in detail, the nature and regulation of the alternative oxidase in isolated mitochondria. Literature on the physiological role of the alternative oxidase in intact tissues is vast and considered beyond the scope of this review and the reader is therefore referred to several reviews on this topic [1–6].

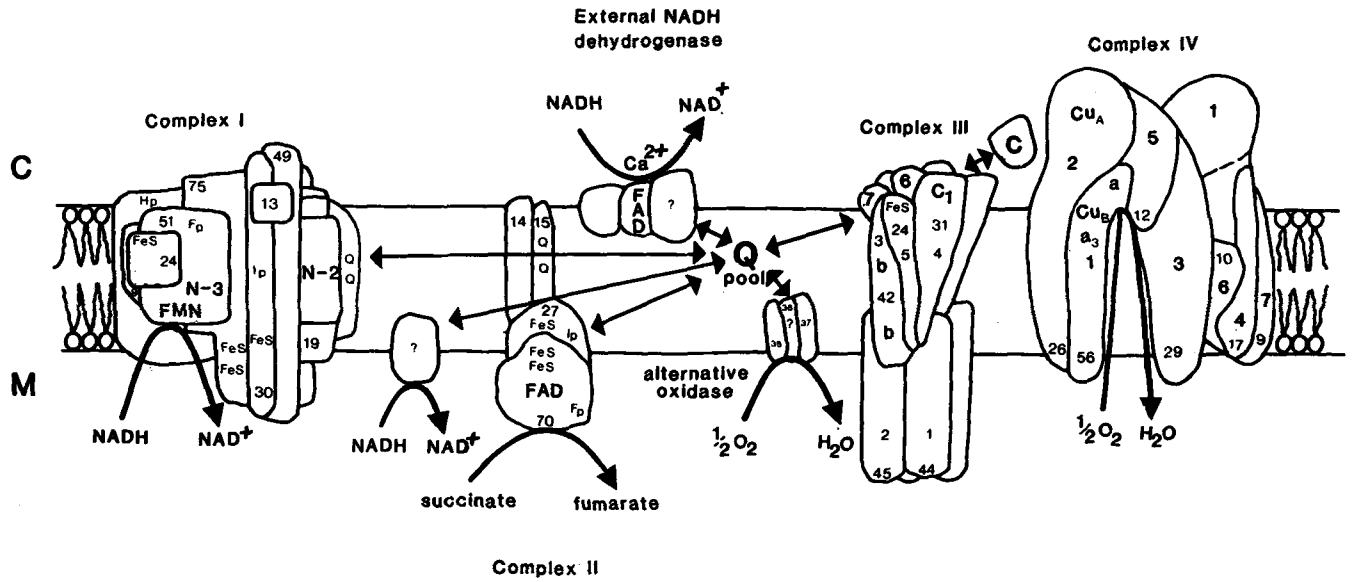

Fig. 1. Organisation of the respiratory chain of plant mitochondria. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; FeS, iron-sulphur centre; C, cytochrome *c*; C_1, cytochrome c_1; b, cytochrome *bs*; Cu_A and Cu_B, the atoms of Cu associated with cytochromes *a* and a_3, respectively; Q, proposed ubiquinone binding site; Q pool, ubiquinone pool; H_p, insoluble hydrophobic fraction; I_p, water-soluble iron-sulphur protein fraction; F_p, water-soluble iron-sulphur flavoprotein fraction; small numbers refer to the molecular weights of the respective polypeptides whereas large numbers refer to the subunit number; C and M refer to the cytosolic and matrix sides of the inner membrane respectively.

II. NAD(P)H dehydrogenases

II-A. External NAD(P)H dehydrogenases

Most plant mitochondria possess the capability of oxidising NAD(P)H by a dehydrogenase located on the outer surface of the inner mitochondrial membrane [7–12] comparable to that found in *Neurospora crassa* [13] and yeast such as *Saccharomyces cerevisiae* and *Candida parapsilosis* [14–17]. This external NAD(P)H dehydrogenase is specific for β-hydrogen of NADH [9] and donates its reducing equivalents non-electrogenically to complex III via the ubiquinone pool [18,19]. Hence, oxidation of external NADH is insensitive to rotenone (and other complex I inhibitors), sensitive to antimycin and myxothiazol and has an ADP/O ratio comparable to that observed with succinate [20]. Although a number of flavonoids inhibit the oxidation of external NADH, none is specific for this dehydrogenase and additionally, in some cases, uncouple oxidative phosphorylation [21].

The oxidation of exogenous NADH is stimulated by a number of cations (see Refs. 11 and 12) and has a specific requirement for micromolar concentrations of free calcium [8,22–25]. Non-specific cation stimulation of NADH oxidation is considered to be due to electrostatic screening of fixed membrane charges on the outer surface of the inner membrane thus reducing the size of the negative surface potential and hence increasing the effective concentration of negatively charged NADH at the active site of the membrane [25,26]. The specific requirement of the dehydrogenase for Ca^{2+} is much more uncertain. It does not involve calmodulin [27,28] and is lost upon solubilisation [29,30]. The oxidation of NADH can be inhibited by chelators such as EDTA, EGTA and citrate [8,22–24]. Interestingly, EGTA added prior to external NADH is much more potent than when added after the NADH, suggesting that actively respiring mitochondria bind Ca^{2+} more tightly than resting mitochondria [23]. The earlier studies of Coleman and Palmer [8] localised Ca^{2+} stimulation to a site prior to complex III on the basis that Ca^{2+} had no effect on NADH-ferricyanide reductase activity. Unfortunately, these experiments were complicated by the presence of a, then unknown, outer membrane NADH-ferricyanide reductase [31], making interpretation of the data somewhat equivocal. More recently, Soole et al. [32] confirmed that Ca^{2+} does not stimulate the NADH dehyrogenase itself, but does markedly increase electron flow to ubiquinone analogues, suggesting that the stimulatory effect is at the ubiquinone reducing site. Whether Ca^{2+} binding induces a conformational change in the enzyme [10], thereby increasing its interaction with ubiquinone, is yet to be determined. Although there have been several reports on the partial solubilisation and purification of the external NADH dehydrogenase [29,30,33], they differ considerably in polypeptide composition possibly due to contamination by complex I. Thus it is not yet possible to draw any firm conclusions as to its molecular mass, polypeptide composition or metal components.

External NADPH can also be oxidised by plant mitochondria showing inhibitor sensitivities similar to those of the externally located NADH dehydrogenase and a comparable ADP/O ratio (< 2.0) [34–37]. However, the oxidation of external NADH and NADPH do differ with respect to sensitivity to added chelators and mersalyl [35,38]. On the basis of these observations it has been suggested [12] that there are two separate dehydrogenases, both of which are localised on the outer surface of the inner mitochondrial membrane.

II-B. Internal NAD(P)H dehydrogenases

The oxidation of endogenous NADH by plant mitochondria is also somewhat more complicated than in mammalian mitochondria in so much as the oxidation of NAD-linked dehydrogenases, in plants, is only partially sensitive to rotenone or piericidin A (see Refs. 1, 10–12). The generally accepted hypothesis attributes this to the presence of two separate NADH dehydrogenases on the inner surface of the inner membrane only one of which is sensitive to rotenone and piericidin A [1,12]. The inhibitor-sensitive dehydrogenase is coupled to three sites of proton extrusion, whereas the inhibitor-insensitive one is only coupled to two sites at complexes III and IV. The key observations which have necessitated the postulation of this additional dehydrogenase are the incomplete rotenone- and piericidin A-sensitivity of the oxidation of NAD-linked substrates and the rotenone-dependent decrease in ATP synthesis from three to two [20,39–41]. Further support for a second internal NADH dehydrogenase has come from temperature-dependency studies of internal substrate oxidation [42] and an analysis of NADH oxidation by submitochondrial particles having a mixed polarity with respect to cytochrome oxidase orientation [43]. The latter study described a rotenone-insensitive dehydrogenase with a K_m for NADH of 8 μM and a rotenone-insensitive one with a K_m for NADH of 80 μM. Apart from the differential affinity for NADH and inhibitor sensitivities no other properties are apparent that distinguish the two dehydrogenases. It is conceivable that the rotenone-insensitive activity merely represents a second ubiquinone reduction site for complex I and not an additional dehydrogenase. In support of this notion, complex I isolated from beef heart [44] and *Neurospora crassa* [45] both display different affinities for ubiquinone analogues in the absence and presence of rotenone, suggesting different binding sites for ubiquinone. Further evidence in support of this

proposal has recently been obtained by Soole et al. [46] during a kinetic analysis of NADH oxidation by complex I in submitochondrial particles prepared from purified beetroot mitochondria (which lack the external NADH dehydrogenase [47]). A similar affinity of NADH-oxygen reductase for NADH was observed when UQ-1 was present in both the absence and presence of rotenone, suggesting that there may be only one NADH binding site involved in the two activites. A quantitative two-stage model for complex I was postulated [46] with one NADH binding site and two sites of UQ-reduction (one of which is insensitive to rotenone) with a common intermediate whose level of reduction can influence the NADH binding site. The poor affinity of the rotenone-insensitive NADH dehydrogenase for NADH was suggested to be due to a restricted access of UQ-10 to the rotenone-insensitive site. Restriction may arise if the rotenone-insensitive site is located towards the outer surface of the lipid bilayer (where only low amounts of UQ-10 may be found) or alternatively is the result of steric hindrance due to the site being located in a cleft in the enzyme [46]. An alternative, more trivial possibility, is that rotenone binds to complex I in such a manner that it prevents proton pumping but does not effectively inhibit electron flow.

It has been postulated that this second dehydrogenase functions to allow the turnover of citric-acid cycle intermediates under conditions of high cytosolic ATP/ADP ratio [43]. Furthermore, it was concluded that it is the absolute concentration of NADH in the mitochondrial matrix and not the NADH/NAD ratio that controls the activity of the rotenone-insensitive route [43]. This conclusion was based on the observation that substantial decreases in the NADH/NAD ratio did not markedly affect rotenone-insensitive respiration in submitochondrial particles and this result has been subsequently confirmed by other workers [46,48]. Under state 3 conditions, the rotenone-insensitive route is presumed to be inoperative [49], since rotenone addition results in severe inhibition of respiratory activity. As rotenone addition has no effect on the state 4 respiratory rate it has been suggested that the rotenone-insensitive pathway may be a major route of electron flow under ADP-limited conditions [50]. An alternative explanation for such data is that if the rotenone-insensitive pathway has a capacity similar to that of the sensitive pathway, under state 4 conditions, then rotenone addition would merely switch electron flux from the inhibitor-sensitive route to the insensitive route without any detectable alteration in the overall rate.

Current information on the structural composition of the plant complex I [51,52] suggests that it is comprised of at least six major polypeptides with molecular masses of 76, 58, 46, 39, 33 and 27 kDa which approximately correlate with the major polypeptides in ox heart complex I (75, 53, 49, 39, 30 and 24 kDa). By analogy with mammalian complex I, the 58 kDa protein is probably the flavin-containing polypeptide [53], whereas the 33 kDa protein may contain the rotenone- and DCCD-binding sites [54,55]. When purified ox heart complex I was immunoblotted with antisera raised to plant mitochondrial submitochondrial particles a cross-reaction occurred with a polypeptide having a molecular mass in excess of 80 kDa and it was suggested that this may represent the transhydrogenase [51]. The presence and location of the transhydrogenase on the matrix side of the inner membrane of plant mitochondria was subsequently confirmed [56] by immunoblotting potato mitochondria with antibodies raised against purified rat liver transhydrogenase. Although its functional role within the plant cell is at present uncertain, there is an increasing amount of evidence as to the presence of other NADP-dependent enzymes within the mitochondrial matrix such as glutathione reductase, isocitrate and malate dehyrogenases [57], suggesting that NADP(H) may have a much more important role in plant mitochondrial respiratory activity than previously recognised.

The immuno-cross-reactivity of the subunits within the plant enzyme with antibodies raised against ox heart complex I shows that complex I is highly conserved across a wide evolutionary range which is presumably related to the highly specialised function of this enzyme as a transmembraneous proton pump.

III. The cyanide-insensitive alternative oxidase

III-A. General characteristics

Perhaps the most unusual characteristic of plant mitochondria is the possession of a finite level of substrate oxidation that is insensitive to inhibition by the conventional cytochrome oxidase inhibitors such as cyanide, azide and carbon monoxide [1–3,58–63]. The degree to which plant mitochondria are resistant to these inhibitors varies from as little as a few percent in the case of potato tubers to as much as 100% in the case of mitochondria isolated from aroid spadix tissues [60]. It should be stressed that the level of cyanide-resistant respiration in the aroid spadices is not trivial, rivalling those found in insect flight muscle [64], with respiratory rates exceeding 2 μmol of oxygen reduced/min per mg protein.

In addition to higher plants, a cyanide-resistant alternative oxidase similar to that seen in higher plants has also been reported in fungi [65,66], a large number of yeasts [17,58,66] and various protista [67–72], including several members of the brucei group of African trypanosomes [73–75]. Thus, while cyanide-resistant respiration is often most associated with higher plant

mitochondria, the phenomenon is quite widespread among aerobic organisms.

Although observations on cyanide-resistant respiration in higher plants can be traced back to the 1920's [76], there is still controversy on its exact nature, its location within the membrane and the mechanism of regulation of electron flux through the pathway. There are, however, certain features of the pathway that are generally accepted. On the basis of P/O ratios, steady-state redox levels of components, rates of oxidation and reduction during stopped-flow oxygen pulse experiments, quinone extraction and inhibition by quinone analogues [77–86], it is well established that the branchpoint of the alternative oxidase from the main respiratory chain is on the substrate side of complex III at the level of the ubiquinone pool. Respiratory activity via the alternative oxidase is potently inhibited by such primary benzohydroxamic acids as SHAM [87], as well as *n*-propyl gallate [88,89] and disulfiram [90]. The ultimate product of the reduction of O_2 is H_2O and not superoxide or H_2O_2 (see subsection III-C.1). The affinity of the alternative oxidase for O_2 is lower (1–2 μM) than that of cytochrome oxidase (0.1–0.6 μM) [91]. In the range (100–250 μM) normally utilised in plant respiration studies, O_2 uptake through the alternative oxidase will be independent of O_2 concentration. Electron flux from reduced ubiquinone to O_2 via the alternative oxidase is not associated with energy conservation (however, see Ref. 92 for an alternative view), the free energy released being lost as heat [93–95]. Hence, the pathway is non-phosphorylating, when either succinate or external NADH are used as respiratory substrates. When NAD-linked substrates are used the pathway is not totally non-phosphorylating, as ATP can be synthesised as electrons flow through complex I [93]. As noted earlier, however, most plant mitochondria possess an internal rotenone-resistant pathway and when the latter is operative the entire pathway becomes non-phosphorylative.

The topological orientation of the alternative oxidase within the inner mitochondrial membrane is still somewhat debatable. Rich and Moore [79] originally suggested that the oxidase was localised toward the matrix side of the inner membrane based upon the postulated close association of the alternative pathway with part of the protonmotive quinone cycle and succinate dehydrogenase [80]. Lance et al. [60] suggested a location more toward the outer surface of the inner membrane based on a differential effect of Triton X-100 on the activities of the cytochrome and alternative pathways. Recently Rasmusson et al. [96] have provided the first direct evidence for the location of the alternative oxidase based on the effects of added trypsin on the cyanide-resistant duroquinol oxidase activity associated with mitoplasts and inside-out submitochondrial particles, respectively. Trypsin had no effect on the quinol oxidase activity in mitoplasts, but inhibited approx. 50% of the activity in submitochondrial particles. Although these results do not rule out that some portion of the alternative oxidase faces the C-side of the inner mitochondrial membrane, they clearly indicate a preferential orientation of the oxidase toward the matrix side of the membrane.

III-B. Regulation of electron flux between the cytochrome and alternative pathways

The mechanism by which electron flux is partitioned between the main respiratory chain and the alternative oxidase has until recently remained unresolved. Although it is clear that the main respiratory chain is always operative, to varying extents dependent upon cellular constraints such as the supply of ADP, and that the alternative oxidase does contribute to the overall respiratory rate in vivo (see Refs. 4 and 5), the mechanism by which it is engaged is uncertain. The uncertainty has given rise mostly to a confusion in terminology. In order to address the question of the regulation of the alternative pathway it is necessary to recognise the distinction between the total capacity of the alternative pathway and the degree to which it is engaged. The capacity of the alternative pathway refers to the total level of the alternative oxidase present in a given plant tissue and experimentally is determined as the component of respiration that is inhibited by the addition of SHAM in the presence of sufficient concentration of cyanide to totally inhibit cytochrome oxidase. Engagement refers to the degree to which alternative oxidase activity contributes to the overall respiratory rate and can be experimentally ascertained from the extent of respiratory inhibition caused by the addition of SHAM in the absence of cyanide. The ratio of these two measurements (namely the alternative pathway engaged/the total capacity of the pathway, which is commonly referred to as rho [97] and has a value between 0 and 1) is frequently used to express the fraction of the total alternative pathway capacity contributing to the respiratory rate in any particular system. When the alternative pathway does not contribute at all to the overall respiratory rate the ratio is zero and increases to one when the pathway is fully engaged.

III-B.1. Kinetic information

The use of a ratio to express the degree of engagement of the alternative pathway stems from the kinetic studies of Bahr and Bonner [98,99]. A model was presented which was based upon the observation that the total respiratory rate measured in the absence of a respiratory inhibitor was always inferior to the mathematical sum of the rates observed via the main cy-

tochrome pathway when the alternative oxidase was fully inhibited and the alternative pathway when cytochrome oxidase was inhibited. It was suggested that the alternative pathway only became engaged when the quinone pool became sufficiently reduced to allow the oxidation of ubiquinol by the alternative oxidase to become thermodynamically favourable and furthermore the latter only occurred when electron flux through the main chain became saturated. In isolated mitochondria such a situation is envisaged under state 4 conditions whereas in plant cells this could be achieved when cellular ADP levels are low and electron transfer becomes limiting. When the Q-pool is oxidised, for instance under state 3 conditions, ubiquinol oxidation via the alternative oxidase becomes thermodynamically unfavourable and, although present, the pathway is no longer engaged.

An alternative model, based on the 'Q-pool' model of Kroger and Klingenberg [100,101], suggests that in *Saccharomycopsis lipolytica* [102] at least, the distribution of reducing equivalents between the two pathways is in direct proportion to their relative kinetic capacities. The model made the assumption that when the two oxidases operate simultaneously they compete for electrons from a single homogeneous Q-pool. As long as neither pathway becomes saturated electron flow will proceed through both in a constant ratio. Using succinate or glycerol phosphate as substrate and after modifying the cytochrome pathway acceptor activity by addition of either ADP, antimycin or cytochrome *c* and/or the alternative oxidase acceptor activity by benzhydroxamic acid, it was found that the stimulatory or inhibitory agents only affected the acceptor activity specifically associated with the site of action of these agents [102]. In other words, the addition of ADP stimulated the acceptor activity of the cytochrome pathway without affecting either the acceptor activity of the alternative pathway or the donor activity of the dehydrogenases. Such data were taken as evidence in favour of a mobile pool of quinone and that reducing equivalents from this pool simply partition between the main and alternative pathways as a function of the relative rate constants for the reaction of UQH_2 with the UQH_2 oxidising sites on the respective pathways. Such a model does not accommodate the well-documented situation where the alternative pathway is present but not operative (i.e., under state 3 conditions) [98,99]. Furthermore, if this model were correct, then partial inhibition of either the cytochrome or the alternative pathway should have no effect on the total observed respiratory rate since electron flux, on the basis of a random distribution model [103–105], should be able to switch between either pathway. A close examination of the data in Ref. 102, however, reveals that this is not the case, as the total respiratory rate decreases immediately upon titration with an inhibitor of either pathway, suggesting there is no switch of electron flux. It should be noted that there are other factors that regulate electron flow such as the kinetic properties of the chain (mainly at cytochrome oxidase), ΔE_h, the protonmotive force and the proton leak (see Refs. 106–108). As the experiments described in Ref. 102 were performed, in the majority of cases, in the absence of ADP or uncoupling agents, such factors could act as considerable constraints in allowing additional respiratory flux. Thus, if the turnover of complex III is already severely restricted (by the protonmotive force for instance), the addition of an inhibitor of the alternative pathway would not necessarily switch electron flux to the cytochrome pathway and does not imply that the cytochrome pathway is saturated. For instance, there is some evidence to suggest that where regulatory constraints have been removed it is possible for electron flux to be diverted from the alternative pathway to the cytochrome pathway and vice-versa [109].

An additional feature that has particularly served to complicate attempts to understand the regulation of electron transport in plant mitochondria is the observation that the distribution of electron flux between the cyanide-sensitive and -insensitive pathways can be strongly dependent upon the nature of the substrate [60,63,85,110–112]. Such a result is not consistent with the notion of a homogeneous quinone pool that is equally accessible to all quinone reductases and oxidases. Substrate dependency is most common in non-aroid tissues which consistently are able to oxidise malate and succinate via the alternative oxidase, while external NADH is oxidised almost exclusively via the main cytochrome pathway, even though absolute rates of NADH oxidation tend to exceed those of the other two substrates [60,63,85]. The possibility that this could be due to a direct interaction between the external NADH dehydrogenase and complex III is unlikely, since it has been demonstrated that the antimycin A sensitivity of the external NADH oxidase activity in sweet potato mitochondria (a tissue displaying a strong substrate dependency on the observed rate of alternative oxidase activity) is dependent upon the turnover of the dehydrogenase [19] and identical to that observed with mung bean mitochondria (which show little substrate dependency). A lack of interaction is furthermore confirmed by the well-documented observation that in aroid spadix mitochondria, such as *A. maculatum*, external NADH is oxidised primarily by the alternative pathway [19,85]. Hence, the reason for the inability of external NADH to feed electrons into the alternative pathway in non-aroid tissues is not readily apparent, particularly in view of the high lateral mobility reported for proteins within the inner mitochondrial membrane [103]. The anomalous behaviour of external NADH oxidation has led to the suggestion that

ubiquinone is compartmentalised into a series of discrete pools in plant mitochondria [60,113]. Thus, complexes I and II are proposed to be associated with a quinone pool that is readily accessible to both pathways, whereas the external NADH dehydrogenase feeds electrons into a quinone pool that has only limited access to the alternative pathway but full access to the cytochrome chain. A third pool, associated with the internal rotenone-insensitive NADH dehydrogenase, has been postulated, to account for cyanide-resistant malate oxidation catalysed by malic enzyme [60]. Other explanations for substrate dependency include the existence of completely separate respiratory chains [114] and heterogeneous distribution of respiratory complexes within the inner mitochondrial membrane [62,115–117]. Separate respiratory chains appear highly unlikely in view of the frequent observation that the respiratory rate measured using combinations of substrates, such as external NADH and succinate, is always significantly lower than the sum of the individual rates [118]. Non-random distribution of respiratory enzymes in the inner mitochondrial membrane seems a more plausible explanation for the behaviour of external NADH oxidation [115]. In such a model it is proposed that complexes I and II are physically more closely associated with the alternative oxidase than is the external NADH dehydrogenase [62]. The heterogeneous distribution of Photosystems I and II between the appressed and non-appressed region of the chloroplast thylakoid membrane [119,120] provides a good example of the non-random distribution of electron transfer protein complexes and, as reviewed extensively by Rich [115] and Ragan and Cottingham [121], it is not difficult to envisage a similar situation occurring in the mitochondrial cristae membrane. As far as the plant system is concerned, if the alternative oxidase is located on the inner surface of the inner mitochondrial membrane [96] and the external NADH dehydrogenase is on the outer surface of this membrane [9,11], it is easy to imagine an uneven distribution of these components and hence preferential channelling of electron flux between the external NADH dehydrogenase and the main cytochrome chain. In this situation the quinone would no longer act kinetically as a single pool. Another possibility is that the transmembraneous transfer of reducing equivalents may become rate-limiting when two competing acceptors are on different sides of the membrane [19].

III-B.2. Ubiquinone pool behaviour

Kroger and Klingenberg [100,101] proposed that functional linking of dehydrogenases and oxidases was via a mobile pool of quinone. Furthermore, it was suggested that the behaviour of quinone as a mobile redox carrier can be described by a simple kinetic model in which the rate of electron transfer is directly proportional to the amount of quinone in the reduced state. This simple kinetic behaviour (called Q-pool behaviour) has been used to derive expressions for the dependence of inhibitor titration curves on the relative activities of of the quinone-reducing and quinol oxidising enzymes [100,101] or for the distribution of electron fluxes in branched respiratory chains [102]. According to this model, the quinone pool is equally accessible to all dehydrogenases and oxidases, its redox state being determined only by the relative reducing and oxidising fluxes and not by their ultimate source. An analysis of mitochondria from a variety of plant tissues revealed that Q-pool kinetics were exhibited by either the main cytochrome pathway or the alternative pathway when one was inhibited [19]. In contrast to the results obtained with *S. lipolytica* [102], however, a deviation from the Q-pool model was observed during the simultaneous oxidation of two substrates [19]. Another apparent deviation from Q-pool behaviour was reported by Beconi et al. [122], who found that the K_i for SHAM inhibition of the alternative pathway was lower in the presence of antimycin-treated mitochondria than the K_i for SHAM observed in untreated controls. However, this result need not be ascribed to a specific synergistic interaction between the two inhibition sites, as suggested [122]. Such a result is completely consistent with ubiquinone behaving in a Q-pool fashion. The change in inhibition properties of SHAM in the presence of antimycin (and vice versa) simply reflects the ability of the alternative pathway to accommodate the increase in electron flux that results when the main pathway is specifically inhibited (Stegink, S.J. and Siedow, J.N., unpublished observations). Such a result is therefore consistent with the observations expressed in Ref. 19 but still only addresses the appearance of Q-pool behaviour in the presence, but not in the absence of inhibitors. Deviations from Q-pool behaviour were addressed in the kinetic model of Ragan and Cottingham [121]. It was shown how deviations from Q-pool theory could be minimised, particularly during dual substrate oxidation, by the appropriate choice of maximum fluxes. In the experiments depicted in Ref. 19, for instance, rate constants are calculated from oxygen electrode measurements which tend to be somewhat unreliable. Such a method is prone to large errors, since small changes in the measured rate lead to very large changes in the calculated rate constants [121]. Ragan and Cottingham [121] found that at the 5% agreement level, there is a range of solutions in which the donor rate constants (using either succinate or NADH as substrates) did not vary and hence simple Q-pool theory was still applicable even in the case of dual substrates. It is also apparent that, in addition to possible errors in oxygen uptake measurements, the method is applicable only to systems in which dehydrogenase or oxidase activities are comparable (Moore,

A.L., unpublished observations). For instance, when the capacity of one oxidase far exceeds that of the other (as is the case with *A. maculatum* mitochondria where alternative oxidase capacity is far larger than the cytochrome pathway or in mung bean mitochondria where the reverse situation occurs), then the calculated rate constants result in values which are inconsistent with simple Q-pool theory (see Table II of Ref. 19). Even in cases where the capacities of the two oxidases are comparable (e.g., *S. lipolytica*) calculated acceptor rate constants can vary markedly (Table I, Ref. 102), suggesting that care should be taken in the interpretation of data obtained with these equations.

An explanation for selective channelling of electrons from a dehydrogenase to a specific oxidase was accounted for by the Ragan and Cottingham model [121] by the suggestion that under conditions when k_{sub} (the pseudo-first-order-rate constant covering binding of substrate, electron transfer to the dehydrogenase and internal electron transfer to the component of the enzyme which reversibly reacts with oxidised ubiquinone) is small, the velocity of reduction of ubiquinone can be independent of the quinone redox state [121]. Such a model provided a mechanism to explain the observed first-order kinetics of oxido-reduction of quinone, even when the ubiquinone concentration is saturating. Furthermore, it was subsequently demonstrated how modification of the homogeneous Q-pool model to incorporate an unfavourable equilibrium associated with the quinol oxidase step [123] may cause marked deviations from first-order behaviour. Under such conditions overall electron transport became largely insensitive to changes in ubiquinone redox state at low Q_r levels but increased disproportionately with increasing Q-pool reduction. It was concluded that marked deviations from first-order behaviour can be explained within the framework of a homogeneous quinone pool without invoking multiple pools [123].

Recently the redox state of the ubiquinone pool has been monitored continuously during electron turnover in isolated plant mitochondria, using a voltametric technique [124–128]. It was found that the respiratory rate of turnip root mitochondria (which are cyanide-sensitive), either under state 3 or state 4 conditions, was directly proportional to the redox poise of the quinone pool [124]. However, when partially cyanide-resistant (pea leaf) mitochondria were used, under ADP-limited conditions, the relationship between the respiratory rate and the redox state of the quinone pool was distinctly non-linear [124]. The non-linearity was postulated to be due to the engagement of the alternative pathway in these mitochondria. When highly cyanide-resistant (soybean cotyledon) mitochondria were used it was found that significant engagement of the alternative pathway was not apparent until Q-pool

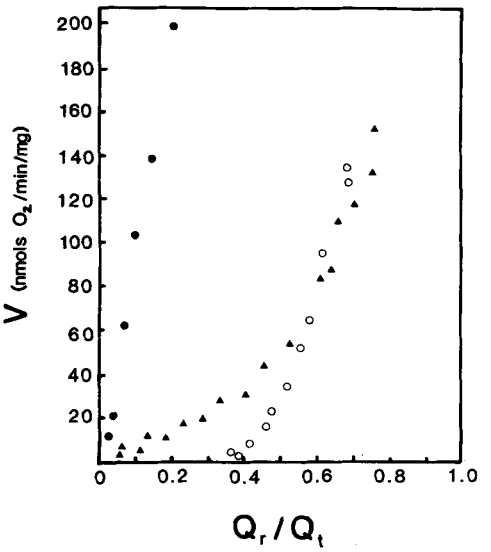

Fig. 2. Dependence of the respiratory rate on the redox poise of the quinone pool in soybean cotyledon mitochondria. Oxygen uptake and steady-state level of quinone reduction were measured voltametrically as described in Refs. 123 and 124 in 1.8 ml of reaction medium containing approx. 0.8 mg of mitochondrial protein, 1 μM Q-2 and 5 mM succinate. Respiratory activity was progressively inhibited by the addition of malonate either in the presence of 0.75 mM ADP to initiate state 3 (●), or under state 4 conditions (▲) or under state 4 conditions in the presence of 2 μM myxothiazol (○). Q_r/Q_t represents the proportion of quinone in the reduced state.

reduction reached 35–40%, and increased disproportionately on further reduction [125]. The dependence of the overall respiratory rate on the redox state of the quinone pool is illustrated in Fig. 2. It compares malonate titration data obtained under state 3, state 4 and in the presence of myxothiazol (to inhibit the bc_1 complex) where the respiratory flux is expressed in terms of absolute rates of oxygen uptake. It clearly illustrates how the non-linearity of the curve observed under state 4 conditions is related to the engagement of the alternative pathway at high Q_r/Q_t levels. Furthermore, it also shows that a significant proportion of the uninhibited state 4 respiratory rate (i.e., in the absence of myxothiazol) is due to electron flow via the alternative pathway consistent with the observations of Bahr and Bonner [98,99] and the estimated contribution of the alternative pathway to total respiratory flux obtained in the presence of SHAM [124]. Under state 3 conditions the Q-pool remains relatively oxidised, even at the maximal respiratory rate, thus preventing any engagement of the alternative pathway to any significant extent. Obviously the degree to which the alternative pathway is engaged, even under state 3 conditions, will be dependent upon the relative electron rates of input and output from the Q-pool.

Such results are analogous to those predicted by Reed and Ragan [123] and at first approximation are supportive of a Bahr and Bonner model [98,99] in which alternative pathway activity is regulated by the

existence of a thermodynamically unfavourable step with a redox potential at least 35 mV more negative than ubiquinone (i.e., reducing equivalents can be donated to the alternative pathway only when ubiquinone becomes sufficiently reduced). When the observed alternative pathway activity, at different quinone redox poises, was compared with the predicted activity based on the Bahr and Bonner model, however, it was found that the experimental data (as illustrated in Fig. 2) did not fit the model [125]. Engagement could therefore not operate along the lines of an 'on/off switch' which was dependent upon the cytochrome chain reaching near or complete saturation. It was apparent, however, that while there was some degree of similarity between the observed and predicted curves, a model based solely on a single, limiting equilibrium between the quinone pool and the alternative oxidase could not entirely account for the observed relationship.

In an attempt to obtain a closer fit to the experimentally observed curves, we have developed a kinetic model based on a theoretical equation in which the reduction of the alternative oxidase by quinol is considered to occur by the following steps:

$$Q_r + E_0 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Q_0 + E_r$$

$$Q_r + E_r \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} Q_0 + E'_r$$

$$E'_r \xrightarrow{k_{sub}} E_0 \qquad (1)$$

where E_0, E_r and E'_r are the oxidised, semi-reduced and fully reduced forms of the oxidase, Q_0 and Q_r the oxidised and reduced forms of quinone, k_{+1} and k_{-1} are rate constants governing the first reversible electron transfer from Q_r to the oxidised oxidase (E_0), k_{+2} and k_{-2} are rate constants governing the second reversible step from Q_r to the semi-reduced oxidase (E_r), and k_{sub} is a pseudo-first-order rate constant covering the reduction of oxygen by the fully reduced enzyme. The steady-state rate through the complex is then given by an equation of the form:

$$\begin{aligned} v = {} & \{Q_r^2 \cdot V_0 \cdot [k_{+1} \cdot (k_{+2} \cdot Q_t + k_{sub}) + k_{sub} \cdot k_{+2}]\} \\ & \times \{Q_t \cdot [Q_r \cdot (k_{+1} \cdot k_{+2} \cdot Q_r + k_{sub} \cdot k_{+2} + k_{+1} \cdot k_{sub} \\ & + k_{-2} \cdot k_{+1} \cdot (Q_t - Q_r)) \\ & + k_{-1}(Q_t - Q_r) \cdot (k_{sub} + k_{-2} \cdot (Q_t - Q_r)]\} \end{aligned} \qquad (2)$$

In this equation, v is the velocity and V_0 is the maximum rate of electron flux when $Q_r = Q_t$. The behaviour of Eqn. 2 is illustrated in Fig. 3 and compared to that obtained experimentally using soybean cotyledon and *Arum maculatum* mitochondria. It can

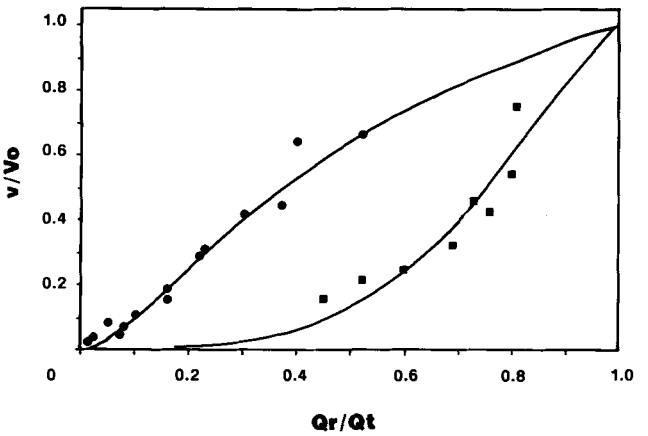

Fig. 3. Comparison of observed alternative pathway activity in soybean cotyledon and *Arum maculatum* mitochondria with predicted activity based on the kinetic model. The dependence of alternative pathway activity on the redox poise of the quinone pool was determined experimentally in soybean cotyledon (■) and *Arum maculatum* (●) mitochondria as described in Refs. 123 and 124 under state 4 conditions in the presence of 2 μM myxothiazol. Solid lines represent that predicted by the kinetic model using Eqn. 2 and the following values for the kinetic constants; for the line of best fit for soybean cotyledon mitochondrial data $k_{+1} = 0.35$; $k_{-1} = 1.2$; $k_{+2} = 0.7$; $k_{-2} = 0.99$; $k_{sub} = 0.14$; $V_0 = 1.0$ and $Q_t = 1.0$. For the line of best fit for *Arum maculatum* data $k_{+1} = 0.35$; $k_{-1} = 0.1$; $k_{+2} = 0.7$; $k_{-2} = 0.1$; k_{sub}, $= 0.14$; $V_0 = 1.0$ and $Q_t = 1.0$. v is the steady-state respiratory rate in the presence of malonate, V_0 is the uninhibited rate and Q_r/Q_t is the proportion of quinone reduced under steady-state conditions relative to total reducible quinone. See the text for further details.

be seen that there is a good degree of similarity between the observed and predicted curves with both sets of mitochondria, even though they vary considerably in their degree of cyanide resistance (from 49 to 89% cyanide-insensitive, respectively). It is apparent that the dependence of v/V_0 on Q_r/Q_t becomes more linear as k_{-1} and k_{-2} are decreased, which is similar to that obtained in the kinetic model of Reed and Ragan [123]. In other words, the overall rate of electron transport via the alternative oxidase is dependent on the magnitude of the equilibrium constants k_{+1}/k_{-1} and k_{+2}/k_{-2}. When this value is low the rate of electron transport becomes largely insensitive to changes in the redox state of the ubiquinone pool at low values of Q_r and as it increases velocity becomes linearly dependent upon the degree of reduction of the ubiquinone pool. Interestingly, the shape of the curve is largely insensitive to variations in k_{sub}, in agreement with the observation [91] that, in vitro, alternative oxidase activity is not rate-limited by the concentration of oxygen.

The kinetic model outlined above quantitatively accounts for deviations from Q-pool behaviour predicted by the Kroger and Klingenberg model [100,101] and furthermore predicts with greater precision than other models (see Ref. 125) the observed dependence of

velocity on quinone reduction state. Clearly, however, the model requires more experimental scrutiny before answers will emerge as to whether it can satisfactorily account for how electron flow is regulated between the main and alternative pathways.

III-C. Nature of the alternative oxidase

III-C.1. Characterisation of the catalytic reaction

Huq and Palmer [85] first demonstrated that the primary product of oxygen reduction by the alternative oxidase during NADH and succinate oxidation in submitochondrial particles isolated from *Arum maculatum* spadices was H_2O and not hydrogen peroxide (H_2O_2) or superoxide anion (O_2^-). One potential complication with these experiments involved the presence of contaminating catalase activity in their submitochondrial particles which could act to mask any H_2O_2 production. The catalase activity was reported to have been completely inhibited by addition of 1 mM KCN, but complete inhibition of plant catalase activity by cyanide is difficult to achieve (Siedow, J.N., unpublished observation). Rich and Bonner [129,130] concluded that NADH oxidation through the alternative pathway did not produce significant quantities of H_2O_2 or O_2^- in mitochondria and submitochondrial particles isolated from a variety of different plant sources. Kay and Palmer [131] later measured the stoichiometry of the cyanide-resistant duroquinol oxidase activity in isolated and detergent-solubilized *Arum maculatum* mitochondria and found it to be consistent with the reduction of oxygen to water. In aggregate, these results establish that oxygen reduction by the alternative oxidase involves the four-electron reduction of oxygen to water rather than the energetically more facile reduction to O_2^- or H_2O_2 [132]. This also suggests that the catalytic centre of the alternative oxidase does not consist of a simple, autoxidizable organic cofactor and, most likely, contains a complex transition metal center of some sort [59].

Attempts to characterise the nature of the, apparently unique, catalytic center associated with the plant mitochondrial alternative oxidase can be traced to the introduction, by Schonbaum et al. [87], of the first specific inhibitors of the alternative pathway, the aryl-substituted hydroxamic acids. Addition of 1.0 mM SHAM to isolated, respiring plant mitochondria specifically inhibited electron flow through the alternative pathway, facilitating the application of such standard techniques as UV-VIS and EPR spectroscopy in attempts to identify spectral transitions linked to the alternative pathway and/or oxidase. Speculation regarding possible candidates for alternative oxidase-associated electron transfer cofactors have included flavoproteins [133], *b*-type cytochromes [134] and iron-sulphur centres [87]. However, no conclusive evidence supporting a role for any of these cofactors in the alternative oxidase has appeared and various arguments have been marshalled against the specific involvement of each, as outlined in previous reviews [59,61,136]. Characterisation of the alternative oxidase active site has also been hindered by the lack any published optical or spectral change that can be specifically attributed to an alternative oxidase-related species.

In 1987 Rich et al. [137] reported that, in addition to its effects on the alternative pathway, SHAM also inhibits tyrosinase and peroxidase in a competitive fashion with respect to their phenolic reducing substrates. This led to the suggestion that, by analogy, SHAM inhibits the alternative oxidase as a competitive inhibitor of reduced ubiquinone, a site of action which may not involve an interaction between SHAM and the oxidase's oxygen-reducing catalytic center [137]. Bonner and Rich subsequently proposed that the alternative oxidase is, functionally, a ubiquinol : oxygen oxidoreductase [138]. Using this theoretical framework, Rich and Bonner [139] and Huq and Palmer [140] demonstrated that isolated aroid spadix mitochondria were capable of catalyzing a cyanide-resistant, SHAM-sensitive quinol oxidase reaction using either duroquinol (2,3,5,6-tetramethyl-*p*-benzoquinol), menaquinol or ubiquinol-1 as water-soluble ubiquinol-10 analogues to donate electrons to the alternative oxidase. Duroquinol oxidase thus provided a direct assay of alternative oxidase activity that was not dependent upon the need to maintain an association between the alternative oxidase and any other mitochondrial electron transfer complex. This assay represented the missing ingredient that had precluded any previous attempt to isolate and purify the alternative oxidase. Taking advantage of the duroquinol oxidase assay, Huq and Palmer [141] and Rich [142] simultaneously described the solubilization of a cyanide-resistant, SHAM-sensitive quinol oxidase activity from *Arum maculatum* spadix mitochondria. While the appearance of the quinol oxidase assay has greatly facilitated further isolation and characterisation of the alternative oxidase from aroid species, it should be noted that the alternative oxidase in all non-spadix tissues shows only very low levels of activity with artificial quinol substrates, relative to the rates seen with standard mitochondrial electron donors such as succinate and malate [1,143]. Attempts to surmount this problem by finding an effective quinol donor to the alternative oxidase from non-spadix mitochondria have met with failure (Siedow, J.N., unpublished observation).

In addition to its application in attempts to isolate and purify the alternative oxidase, the quinol assay has also been utilised in an extensive kinetic analysis of the cyanide-resistant, SHAM-sensitive duroquinol oxidase activity from *Arum maculatum* mitochondria [131]. This

study reported that the duroquinol oxidase activity (in both taurocholate-solubilized and isolated mitochondria) could be stimulated 4- to 5-fold by the addition of high concentrations (0.5–1.0 M) of anions. Multivalent anions (citrate, phosphate, sulfate) showed a greater stimulation than monovalent ones (chloride or acetate), and the order of anion effectiveness loosely followed the Hofmeister lyotrophic series [144]. Kinetically, most of the anion effect could be attributed to a large increase in V_{max} although a small decrease in the apparent K_m for duroquinol (0.58 mM to 0.38 mM) was also observed. At present, the mechanism of stimulation by anions is not well understood. Kay and Palmer [131] suggested that the anions may stabilize a conformation of the oxidase that has an increased catalytic efficiency, but given the limited amount that is currently known about the oxidase, it is difficult to know what this might mean. In addition, the anion effect is not consistently seen, at least with isolated skunk cabbage mitochondria. Levels of stimulation of quinol oxidase activity by 0.7 M citrate ranging from zero to 5-fold have been observed in different skunk cabbage mitochondrial preparations, with the only obvious correlation being the lower the basal rate in the absence of added citrate, the greater the observed anion stimulation (Berthold, D.A. and Siedow, J.N., unpublished observation).

Kay and Palmer (131] also found that inhibition of the solubilized alternative oxidase activity with respect to duroquinol concentration was of a mixed-type for SHAM, was strictly noncompetitive for *n*-propyl gallate and UHDBT and was competitive for inhibition by fatty acids. This suggests that SHAM is capable of binding to both the free oxidase (E) and the oxidase-duroquinol complex (ES); an observation that is inconsistent with the suggestion [137] that SHAM inhibition of the alternative oxidase, analogous to its effects on tyrosinase and peroxidase, is competitive with respect to its phenolic reducing substrate (i.e., duroquinol). Even more surprising is the observed noncompetitive inhibition by UHDBT. UHDBT clearly represents a ubiquinone analogue [145], and it is difficult to understand how it could noncompetitively inhibit the quinol oxidase activity unless it acts to displace a quinone species that normally exists tightly bound to the alternative oxidase active site. However, the duroquinol titrations in this study were carried using 0.15 to 1.0 mM duroquinol in the presence of 0.7 M sodium citrate. This includes the range within which duroquinol becomes insoluble in an aqueous solution of low ionic strength [146]. When using a compound that is not extremely water soluble in a kinetic study such as this, care needs to be taken to ensure that any observed substrate saturation is an intrinsic property of the enzyme under study and not due to some extrinsic limitation such as substrate solubility. Further, determination of kinetic constants for membrane-bound enzymes utilizing hydrophobic substrates requires consideration of both substrate partition coefficients and the total lipid concentration in the assay mixture, in addition to the substrate concentration itself [147]. While the results of Kay and Palmer are interesting, further studies are needed to definitely establish the sites of inhibition of the alternative oxidase by SHAM, *n*-propyl gallate and UHDBT.

III-C.2. Isolation and purification of the alternative oxidase

Huq and Palmer [141] reported the first partial purification of an alternative oxidase fraction from *Arum* spadix mitochondria (Table I). SDS-PAGE of the resulting preparation was reported to show five major, and numerous minor, polypeptides, but no molecular weights were cited for any of the major bands [147]. Spectrally, this preparation contained no cytochrome absorption spectra but did show significant levels of carotenoid, judging from the major transitions seen at 421, 447 and 477 nm in the visible region [147]. A small amount of bleaching was observed at 421 nm upon reduction of the preparation with dithionite. No

TABLE I

Characteristics of aroid spadix quinol oxidase purifications

Preparation	Sol./purification proc.	Purif. (times)	Sp. act. [a]	Metals (nmol/mg)	Ref.
Arum maculatum	Lubrol/2× DEAE cellulose	18	5400	Cu (3–6)	142, 148
Arum maculatum	deoxycholate/NH_4 acetate ppt., lauryl maltoside sucrose gradient centrifugation	5	1550	Fe (13) Cu (3–4)	149
Sauromatum guttatum	deoxyBIGCHAP/CM-Sepharose, phenyl-Sepharose	–	–	–	152, 153
Skunk cabbage	deoxyBIGCHAP/DEAE-cellulose, Extracti-Gel ppt., Sephadex G-200	20–30	4900	–	– [b]

[a] nmol oxygen taken up/min per mg protein.

[b] Berthold, D.A. and Siedow, J.N., unpublished observations.

fluorescence was observed in freshly prepared enzyme, but an emission spectrum centered at 520 nm, indicative of a flavin species, was seen after 2 h at 20°C, coincident with a marked loss of quinol oxidase activity [147]. Metal analysis using plasma atomic emission and neutron activation analyses showed the presence of 3–6 nmol copper/mg protein and essentially no zinc or manganese [141,147]. The copper was found to be EPR-silent [141]. Curiously, no mention was made of the iron content of this preparation. Neutron activation analysis should have given Huq and Palmer an indication of the level of iron present in their preparation. Why the iron content was not reported along with the zinc, which is somewhat less interesting from an electron transport point of view, is not clear.

Bonner et al. [148] also developed a procedure for partial purification of the *Arum* oxidase (Table I). Whilst their partially purified product showed a large number of Coomassie blue-staining bands on SDS-PAGE, a major species was seen at approx. 34 kDa. Spectrally, this oxidase preparation showed no evidence of contaminating cytochrome or carotenoid. Metal analysis using atomic absorption spectroscopy indicated that iron was the only major metal species (among Fe, Cu and Mn) that co-fractionated with the oxidase activity on the lauryl maltoside/sucrose gradient. The iron content of the partially purified fraction was 0.43 Fe/34 kDa protein (Table I). However, activity staining on nondenaturing polyacrylamide gels has indicated that this oxidase preparation contains contaminating succinate and NADH dehydrogenase activities [149], and EPR spectroscopy shows the presence of signals associated with contaminating iron-sulfur centers from succinate:UQ and NADH:UQ reductases (Moore, A.L., unpublished observations); so the exact significance of the iron analysis is somewhat uncertain. For example, 1 mol of intact complex II would contain 9 mol of iron-sulfur iron [150]. The copper content of the purified fraction was roughly 30% that of the iron content (= 3–4 nmol Cu/mg protein). The latter value is similar to that seen in the preparation of Huq and Palmer [147], but Bonner et al. [148] attributed the copper in their purified preparation to contamination by a copper-containing protein that, while concentrated at the top, ran throughout the entirety of the density gradient.

As noted previously, if H_2O, and not H_2O_2 or O_2^-, is the primary product of oxygen reduction [129,130], this requires that a coupled transition metal complex be present in the catalytic site of the alternative oxidase based upon precedents provided by other water-forming oxidases [132]. When the latter point is coupled with the fact that the alternative oxidase displays a number of unusual properties relative to any other known water-producing oxidase, it becomes important to determine conclusively what metals are specifically associated with the purified alternative oxidase. Based upon the two preparations that are, presently, the best characterized, the answer to that question remains essentially unknown.

Following these earlier isolations Elthon and McIntosh [151], as part of the work that led to their successful development of antibodies against an alternative oxidase-associated protein (see subsection III-C.3), reported a solubilization and partial purification of the quinol oxidase activity from *Sauromatum guttatum* spadix mitochondria (Table I). SDS-PAGE of the phenyl-Sepharose fraction showed the presence of 13–20 bands following Kodavue staining, with major species including the important trio at 35, 36 and 37 kDa (See below). The authors reported a 166-fold purification in their final fraction, but this value was calculated by factoring in the loss of activity during purification and there is no indication that any active fraction was ever eluted from the phenyl-Sepharose column. Apparently, the only way that oxidase-associated protein can be released from the phenyl-Sepharose column is by elution in 2% SDS. Such a treatment would be expected to inactivate the oxidase so, not surprisingly, no further quantitative characterizations were reported on this oxidase preparation. However, this fraction was of marked utility in developing antibodies against the alternative oxidase. It is of interest to note that the solubilized *Sauromatum* preparation of Elthon and McIntosh [152] adsorbs to a cation-exchange column (CM-Sepharose), while the preparation of Huq and Palmer from *Arum* spadices [147] bound to an anion exchanger (DEAE-cellulose). Both preparations were solubilized with neutral detergents, but given the many differences associated with the two preparations, it is difficult to know what significance, if any, to attach to this observation.

More recently, a procedure for isolating the alternative oxidase from skunk cabbage mitochondria has been developed by Berthold and Siedow (unpublished observations) (Table I). SDS-PAGE of this oxidase fraction showed only four major Coomassie blue staining bands at 29, 36, 57–59 and 65 kDa, three of which (29, 36 and 65 kDa) react on immunoblots with the AOA monoclonal antibody ('alternative oxidase all' because it reacts with all three bands on immunoblots) raised against the alternative oxidase protein [153]. The oxidase ran with an apparent molecular weight of 160000 on a Sephadex G-200 column, and the purified oxidase showed no absorption in the visible region of the spectrum (i.e., no contaminating cytochromes or carotenoids). Of most interest was the appearance of the first putative spectral change of any kind reported for the alternative oxidase. A borohydride-reduced minus air-oxidized redox difference spectra in the UV showed a broad absorbance increase in the region 290–335 nm and an absorbance decrease below 285 nm

with a minimum centered around 275 nm. Further work is needed to characterize the nature of this spectral species and its role, if any, in the alternative oxidase catalytic cycle.

There are several reasons why purification of the alternative oxidase has proceeded so slowly, since the quinol oxidase concept was first proposed 12 years ago. Most notably, the solubilized oxidase activity is extremely labile [1,147,148]. Even when no purification procedure is applied, the solubilized aroid oxidase shows major losses of activity over the course of 12–24 h at O °C. In addition, the availability of aroid spadix tissue is seasonable. In the U.S., skunk cabbage blooms only in late winter/early spring, depending upon its location, and the same is true of *Arum maculatum* in Europe. While *Sauromatum* can readily be grown in the greenhouse, it is difficult to induce it to flower more than once per year, yielding a limited supply of mitochondria for preparative-scale purposes. Finally, as noted previously, mitochondria isolated from non-spadix sources show only very low levels of quinol oxidase activity, and the lack of any comparable direct assay of the alternative oxidase activity from non-spadix tissues has effectively precluded the use of more readily available plant tissues in attempts to purify it.

III-C.3. Immunological characterization of the alternative oxidase protein

Elthon and McIntosh [152] utilized the partially purified *Sauromatum* oxidase fraction they obtained from the CM-Sepharose column to raise antibodies, first polyclonal and subsequently monoclonal [153], in mice against alternative oxidase-associated proteins. In this work, advantage was taken of the variations in alternative pathway activity associated with the developmental sequence seen in *Sauromatum* spadices prior to, during and following the appearance of thermogenesis to identify antibodies specific for alternative oxidase proteins [151]. This approach led them to focus their attention on antibodies that reacted with three bands on Western immunoblots at 35, 36 and 37 kDa [152]. In support of the contention that these three proteins are associated with the alternative oxidase, Elthon, McIntosh and co-workers showed: (1) the levels of the three antigenic species generally correlated with the appearance of alternative pathway activity in *Sauromatum* spadices during the development of thermogenesis [152,154]. The increase in intensity of these three bands appeared whether thermogenesis was allowed to develop naturally or was prematurely induced by addition of salicylic acid [152]; (2) the monoclonal antibody designated AOA, when incubated with a BIGCHAP-solubilized alternative oxidase fraction, was able to inhibit 50% of the duroquinol oxidase activity [153]. While this result is of interest, the reported rates of the solubilized quinol oxidase activity (30 nmol O_2/min per mg protein) are well below what one would expect of a solubilized spadix fraction [131,141,148], leaving some uncertainty about the exact significance of the observed inhibition; and (3) the monoclonal antibody AOA cross-reacted with two polypeptides at 36.5 and 37 kDa, respectively, on immunoblots of mitochondria from *Neurospora crassa* in which the alternative pathway had been induced to appear by the addition of chloramphenicol to inhibit mitochondrial protein synthesis [155]. No cross-reactivity was observed with mitochondria from uninduced *Neurospora*, which lack alternative oxidase activity, or with mitochondria from a mutant strain (*aod-2*) that is unable to develop the alternative pathway under inducing conditions. Together, these observations serve to establish unambiguously that the three cross-reactive polypeptides in *Sauromatum* at 35, 36 and 37 kDa are associated with the alternative oxidase.

It is still uncertain how the three polypeptides seen on immunoblots of *Sauromatum* are related to the functional holoenzyme. Polyclonal antibodies were generated against each of the individual protein bands, and they each react with every other band on immunoblots [152]. As noted, the monoclonal antibody AOA also reacts with all three bands in *Sauromatum* mitochondria [153]. In *Sauromatum*, the low level of alternative oxidase activity seen in either prethermogenic spadices or nonspadix tissues correlates with the presence of only the 37 kDa polypeptide on immunoblots [152]. Induction of the alternative pathway during thermogenesis leads to an increase of the 37 kDa band along with the appearance of the other two species [152]. The antibodies against the *Sauromatum* alternative oxidase show a broad cross-reactivity with polypeptides from other aroid spadices as well as widely divergent plant species (Ref. 153; Siedow, J.N. and Moore, A.L., unpublished observations); in every case, one or more bands in the range 35–37 kDa are observed to cross-react on immunoblots. Generally, the AOA monoclonal antibody cross-reacts with two or three polypeptide bands in the region of 35–37 kDa in aroid spadix mitochondria. Although there have been few reports on the cross-reactivity of these antibodies with mitochondria isolated from non-aroid sources it is apparent that soybean cotyledon mitochondria, at least, show two distinct bands in this same molecular weight region in immunoblots [156], while other non-green tissues show a single band around 35–37 kDa [153]. As noted, the cross-reactivity of the AOA monoclonal antibody extends to fungi [155] and recently a 35 kDa band associated with cyanide-resistant trypanosome mitochondria was observed to cross-react with the AOA antibody (McIntosh, L., personal communication).

Clearly, the work of Elthon and McIntosh has provided a major impetus to this area of research. Not only have they provided the tools required to isolate

cloned cDNAs associated with the alternative oxidase, but the results from the use of their antibodies demonstrate positively that the alternative oxidase is a legitimate component of the electron transfer chain of cyanide-resistant mitochondria. This research has served to refute irrevocably the ill-founded, but persistent, suggestions that the alternative oxidase in plant mitochondria is due to either contaminating activity associated with the enzyme lipoxygenase [158] or some form of peroxyradical cycle [158].

III-C.4. Additional characterisations of the alternative oxidase

Berthold et al. [159] utilized radiation-inactivation analysis to determine a functional molecular mass for the cyanide-resistant, SHAM-sensitive quinol oxidase activity associated with spadix mitochondria [159,160]. The quinol oxidase activity in both skunk cabbage and *Sauromatum* mitochondria gave a molecular mass of 30–31 kDa. This value suggests that the functional alternative oxidase is not a large multiprotein complex; cytochrome *c* oxidase was inactivated with a target size of 72 kDa in the same system [160]. Further, 30 kDa is similar to the molecular weight of the alternative oxidase-associated polypeptides described by Elthon et al. [152]. More recently, the amino-acid sequence derived from a cloned cDNA of the *Sauromatum* alternative oxidase has been reported (see subsection III-D), and it gave a calculated molecular mass of 32 kDa for the mature alternative oxidase polypeptide [161]. This value is essentially identical to that obtained with radiation-inactivation analysis, given the uncertainties associated with calibrating the ionizing radiation in such studies. The differential between the value of 32 kDa derived from the amino-acid sequence and the 35–37 kDa migration consistently seen on SDS gels can be attributed to the often reported anomolous behaviour of integral membrane proteins on SDS-PAGE [162]. In keeping with precedents in the photosynthetic literature [163], this review will refer to the alternative oxidase polypeptide in terms of its behaviour on SDS gels. Still uncertain from the combination of results presently in the literature is how the 37 kDa protein relates to the estimated molecular weight of the solubilized alternative oxidase derived from Sephadex G-200 chromatography (160 000). While nonspecific protein aggregation cannot be ruled out, another explanation is that the functional alternative oxidase exists as a multimer of several 37 kDa polypeptides within the membrane, analogous to the dimeric complexes postulated for complex III and cytochrome *c* oxidase in the inner mitochondrial membrane [150].

A separate line of investigation implicating an association between a 35–37 kDa protein and the alternative oxidase has been provided by the work of Minagawa and co-workers using the ascomycetous yeast *Hansenula anomala* [164–167]. This yeast normally produces a strictly cyanide-sensitive mitochondrial electron transfer chain. However, when it is grown in the presence of an inhibitor of the cytochrome pathway, such as antimycin·A, a cyanide-resistant pathway is induced to appear [164], analogous to the situation in *Neurospora* [168]. Cyanide-resistant respiration associated with mitochondria isolated from induced cells shows the same general properties as the alternative pathway seen in higher plant mitochondria [167]. Pulse labeling of *H. anomala* cells with [^{35}S]methionine in the presence of antimycin A led to the appearance of a membrane-associated 37 kDa protein that was not seen when cells were labelled in the absence of antimycin A [165]. Further, when the uncoupler CCCP was added to isolated *H. anomala* spheroplasts to inhibit the uptake and processing of cytosolically synthesized polypeptides into the mitochondria, the addition of antimycin A led to the appearance of a band at 39 kDa [166]. The 39 kDa band could subsequently be chased into a 36 kDa species upon removal of the CCCP, and both bands were localized to the mitochondrial fraction. These results indicate that, in *H. anomala*, the alternative oxidase protein is initially synthesized on cytosolic ribosomes as a 39 kDa precursor which is transported into the mitochondria, proteolytically processed to a mature 36 kDa polypeptide and then incorporated into the inner mitochondrial membrane. When this work is combined with the results of Elthon and McIntosh [152–154] and Berthold et al. [160], the role for a 35–37 kDa polypeptide as the major, if not sole, species associated with the functional alternative oxidase seems clearly established. However, it still remains to be determined whether any additional proteins, which are possibly constitutively expressed [1,169], are required by the alternative oxidase to couple the oxidase to the rest of the mitochondrial electron transfer chain. More recently, Minagawa et al. [166] have used *H. anomala* to demonstrate that induction of cyanide-resistant respiration by antimycin A was blocked in the presence of an added chelator such as *o*-phenanthroline. However, the 36 kDa protein described above was still synthesised in the presence of the iron chelator and the addition of Fe(II) to cells led to the rapid appearance of cyanide-resistant respiration, even in the presence of cycloheximide. No activation of the alternative pathway was seen when Fe(III) was used in place of Fe(II). This elegant study strongly implicates a role for iron in the conversion of the inactive 36 kDa protein to an active alternative oxidase. While not necessarily serving to prove such, the simplest explanation of these results is that iron represents the metal species required in the active site of the alternative oxidase. Fe(III) has also been reported to be required to observe induction of the alternative pathway in the yeast *S. lypolytica* [170]. Uncertain from

the existing literature on the alternative oxidase from *H. anomala* is whether it shows any quinol oxidase activity comparable to that of the aroid spadices or whether it behaves more like non-spadix plant mitochondria. However, *H. anomala* clearly provides an interesting system for characterisation of the alternative oxidase, and it should prove useful in future studies.

III-D. Molecular studies of the alternative oxidase

Studies of molecular aspects of expression of the alternative oxidase in higher plants have only recently appeared. This derives from the fact that, prior to development of antibodies against alternative oxidase-related proteins, there were no specific probes available for ascertaining expression of the alternative oxidase beyond simply monitoring the appearance of the operational pathway from oxygen uptake measurements. Such earlier studies provided considerable information for speculation regarding the role of the alternative pathway in plant metabolism [60,61,171], but have not provided much insight into changes associated with the expression of components of the alternative pathway.

More recently, however, Elthon et al. [154] carried out a thorough investigation of the developmental changes taking place in the mitochondrial electron transfer chain of *Sauromatum* spadices between 5 days prior to and 3 days after thermogenesis (D-day). A marked increase in the capacity of the alternative pathway in the appendix region of the spadix (from a rate of 50 nmol O_2/min per mg protein 3 days prior to thermogenesis to 250 two days later) is observed just before the onset of thermogenesis. The alternative pathway reaches a maximum on D-day and decreases over 90% during the 3 days that follow [154]. Consistent with these observations, immunoblots indicated an increase in the three 35–37 kDa alternative oxidase-associated proteins, most notably the two lower bands at 35 and 36 kDa. Interestingly, the capacity of the main cytochrome pathway declined precipitously on the day that thermogenesis occurred. Together, these coordinated developmental changes force electron flux through the alternative pathway on the day of thermogenesis, ensuring a maximum production of heat and the consequent volatilisation of pollinator-attracting compounds. The loss of the cytochrome pathway could be correlated with a loss of cytochrome oxidase activity in the appendix mitochondria with the advent of thermogenesis [151,154]. The latter was related to a marked decrease in mitochondrial mRNA levels as D-day approached which manifest itself as a reduction in the capacity of the mitochondria to carry out protein synthesis [154]. During these studies, Elthon et al. [154] demonstrated that none of the three alternative oxidase-related polypeptides was among the proteins synthesised by the isolated mitochondria, indicating that those proteins were encoded by one (or more) nuclear genes and not the mitochondrial genome. Finally, Elthon et al. [154] showed that induction of thermogenesis in spadix tissue by application of salicylic acid [172] also stimulated expression of the three polypeptides that cross-react with the alternative oxidase polyclonal antibody.

The McIntosh group has also investigated the appearance of alternative pathway activity following the wounding of potato tubers [173]. Induction of the alternative pathway appears in potato tubers over a 24 h 'aging' period following tuber slicing, and the process has long been known to be sensitive to inhibitors of cytosolic protein synthesis [97]. Hiser and McIntosh [173] found that the increase in the alternative pathway activity over this aging period correlated with the appearance of a single band at 36 kDa on immunoblots using the AOA monoclonal antibody [153]. As with development of the alternative pathway in *Sauromatum* spadices [154], the results of Hiser and McIntosh suggest that induction of alternative pathway activity is associated with the de novo synthesis of one or more of the 35–37 kDa polypeptides.

In a third study, Obenland et al. [156] used the monoclonal antibody against the alternative oxidase to correlate changes in alternative pathway capacity during soybean cotyledon development with the appearance of cross-reactive proteins. Soybean cotyledons give two distinct bands on immonoblots with the AOA antibody [156]. In light-grown cotyledons, only the lower-molecular-weight band appeared in 4-day-old cotyledons, while both bands were seen 9 days after imbibition. The observed increase in alternative pathway activity over this time period was generally reflected in increases in the intensity of cross-reactive bands on the immunoblots from days 3 to 9, but significant changes in alternative pathway activity between days 9 and 12 did not correspond with any obvious changes on the immunoblots. Light also had a marked effect upon the appearance of the two bands in the early stages of growth (3–4 days after imbibition), the upper band being dominant in dark-grown ones. By day 9, both bands were present, irrespective of growth conditions. No attempts were made to correlate this difference in band distribution with any distinct activities found in mitochondria isolated from light-versus-dark-grown cotyledons. The latter would be interesting; at present, neither the nature of the modification that leads to the apearance of multiple oxidase bands on SDS-PAGE nor the physiological significance of the individual bands is understood. Systems such as soybean cotyledon, where developmental changes in alternative pathway activity have been well characterised [174,175], should prove useful in helping to establish

the functional significance of the multiple alternative oxidase species seen on SDS gels.

The most thorough understanding of the genetics of the alternative pathway is provided by the fungus *Neurospora crassa*. Under normal growth conditions, *Neurospora* shows no alternative pathway activity, but a SHAM-sensitive, cyanide-resistant respiration can be induced to appear when cells are grown in the presence of agents that interfere with mitochondrial electron transfer or energy conservation or inhibitors of mitochondrial protein synthesis [168,176]. Bertrand et al. [177] reported the isolation of a series of nuclear mutations that failed to show alternative pathway activity under inducing conditions. These mutants could be separated into two complementation groups, *aod-1* and *aod-2*. All but one of the mutants belonging to the former group accummulated a polypeptide around 35 kDa on SDS-PAGE under inducing conditions, while the *aod-2* mutants failed to show the appearance of this protein on SDS gels. Bertrand et al. [177] suggested that *aod-1* represents the structural gene for the alternative oxidase, while *aod-2* encodes a regualtory species that is required for induction of alternative pathway activity. Two *aod-1* mutants were investigated; one showed the two polypeptides on immunoblots under inducing conditions, while the second failed to accumulate either polypeptide. This latter mutant represented the one example of *aod-1* reported by Bertrand et al. [177] not to accumulate the 35 kDa polypeptide on SDS gels. The one *aod-2* mutant investigated by Lambowitz et al. [176] showed the faintest indication of a band at 37 kDa on immunoblots following induction and none of the 36.5 kDa species, again consistent with earlier finding of Bertrand et al. When these data are combined with earlier observations that induction of the alternative pathway can be inhibited by agents that block transcription of nuclear genes [176,177], it would appear that induction of the alternative pathway in *Neurospora* results from the transcriptional activation of nuclear genes encoding the alternative oxidase. The role of *aod-1* encoding an alternative oxidase-associated structural protein seems clear. Less certain is whether the product of the *aod-2* locus encodes a regulatory component, a factor associated with incorporation of the alternative oxidase into the membrane and/or its activation (an iron-incorporating protein, for example), or an additional structural protein required to form an active alternative oxidase complex within the mitochondrial membrane.

The recent studies of Minagawa and co-workers on the induction of the alternative pathway in *H. anomala* [164–166] (subsection III-C.4) provide additional support for the general concept derived from the studies outlined above. Namely, the functional alternative oxidase consists of one or more 35–37 kDa polypeptides that are each derived from a single nuclear-encoded gene, differing by an as yet uncharacterised (post-translational?) modification. The alternative oxidase structural protein is synthesised on cytosolic ribosomes as a larger precursor protein that is then post-translationally transported into the mitochondria and proteolytically processed by removal of the transit peptide. The latter could conceivably represent the source of the observed polypeptide heterogeneity, although no evidence for this exists at present. Induction of increased levels of alternative pathway activity in systems such as *Sauromatum* species, wounded potato tubers or inhibitor-treated fungi appear to involve the transcriptional activation of the oxidase structural gene, although that has yet to be demonstrated directly in any particular case.

As indicated earlier, Rhoads and McIntosh [161] have reported the isolation of a cDNA clone encoding the alternative oxidase protein from *Sauromatum*. This cDNA clone encodes a deduced polypeptide sequence of 349 amino-acids with an estimated M_r of 38900. By comparison with the amino-acid sequence determined from N-terminal sequence analysis of the gel-purified 36 kDa protein eluted from an SDS gel, it was established that the first 60–65 amino-acids deduced from the cDNA clone encode a putative mitochondrial transit peptide. This would appear to make the plant transit peptide ($M_r = 6500$) somewhat larger than the apparent 3 kDa transit peptide on the alternative oxidase protein in *H. anomala* [166], although molecular weights derived from mobilities on SDS gels are notoriously subject to inaccuracies. Given the removal of the transit peptide during import into mitochondria, then the mature *Sauromatum* protein contains 284–289 amino acids having a calculated molecular weight of approx. 32000. This value is remarkably similar to the value obtained for the molecular mass of the functional alternative oxidase using radiation-inactivation analysis [160]. The amino-acid sequence of the mature alternative oxidase protein derived from the cDNA clone [161] is presented in Fig. 4. Immediately above the amino-acid sequence are + and − symbols, which indicate the positions of positively and negatively charged amino-acid residues (at physiological pH), respectively, Below the sequence is the predicted secondary structure (based on the method of Robson et al. [178] as modified by Crofts [179]) using the symbols H for α-helix, E for β-sheet, T for β-turn and C for coil (aperiodic structure). Three regions of the deduced protein are predicted to be strongly α-helical: amino-acids 106 to 137, 142 to 163, and 168 to 197. Within two of these helical regions are spans of relatively high hydrophobicity (namely amino-acids 108 to 126 and 170 to 191) which are likely to produce membrane spanning helices as deduced from a hydropathy analysis using the algorithm of Kyte and Doolittle [180] (Fig. 5). If the alternative oxidase is anchored to the inner mitochon-

```
      - + -+      +     --   -+ -           +  +--   - +    +
LSAPAQDGGK EKAAGTAGKV PPGEDGGAEK EAVVSYWAVP PSKVSKEDGS EWRWTCFRPW  -60
CCCHHHHCCH HHHHHHCTCC CCCCCCHHHH HHHHEEECCC TCCCCCCTTT TTTTTTCCTT

-    -   -  ++++       -+   +  +   +   -     ++   +     -
ETYQADLSID LHKHHVPTTI LDKLALRTVK ALRWPTDIFF QRRYACRAMM LETVAAVPGM  -120
CTTTHHHHHH HHCCCCEEHH HHHHHHHHHE ECTCCTTEEE TTTTTHHHHH HHHHHHHHHH

      + +   ++ -+      +   -- -  -+ +     -     +  -+
VGGVLLHLKS LRRFEHSGGW IRALLEEAEN ERMHLMTFME VAQPRWYERA LVLAVQGVFF  -180
HHHHHHHHHH HHEETTTTTC HHHHHHHHHH HHHHHHHHHH HHCCTHHHHH HHHHHHHHHH

            +  ++      ---  +    -  +- -      -          -  +
NAYFLGYLLS PKFAHRVVGY LEEEAIHSYT EFLKDIDSGA IQDCPAPAIA LDYWRLPQGS  -240
HHHHHHHHHC CTHHEEECCC HHHHHHHHHH HHHHCTTTTT TTTECCCEEE EETTECTTTC

  +-     +  --  +++-   +   - +  -  - +          +
TLRDVVTVVR ADEAHHRDVN HFASDVHYQD LELKTTPAPL GYH                    -283
CEEEEEEEHH HHHHHHHHHC CCCCTHHHHH HHTTCCCCCT TEE
```

Fig. 4. Secondary structure prediction analysis of the mature alternative oxidase amino-acid sequence. The top line represents the amino-acid sequence taken from Rhoads and McIntosh [161]. Below the sequence lies the prediction; H, E, T and C represent α-helix, β-sheet, β-turn and coil (aperiodic structure) respectively. The prediction was generated according to the methods of Robson et al. [178] as modified by Crofts [179]. See the text for further details.

drial membrane by these two proposed membrane-spanning regions, this would put the majority of the mass of the protein on one side of the membrane, presumably the matrix side based upon the data of Rasmusson et al. [96]. Rhoads and McIntosh [161] have suggested that the protein contains three membrane-spanning helices based upon their structural analysis of the amino-acid sequence. This would place the N- and C-terminal regions of the protein on opposite sides of the membrane. Further work is required to ascertain the topological orientation of the alternative oxidase beyond that reported to date [96], but the availability of antibodies against the alternative oxidase protein should facilitate such studies.

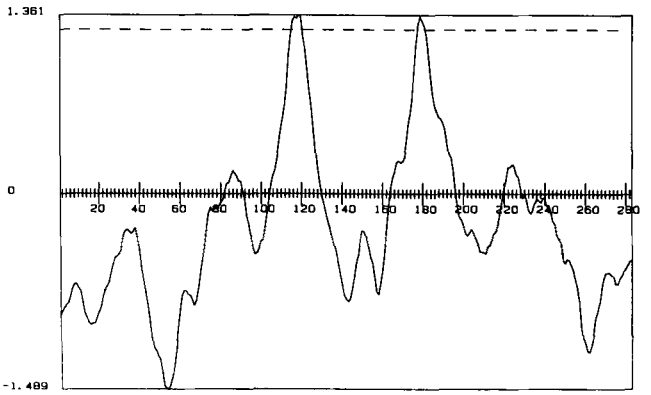

Fig. 5. Hydropathy analysis of the mature alternative oxidase amino-acid sequence. Hydropathy analysis was deduced using the algorithm of Kyte and Doolittle [180] and generated using the Sequence Analysis Package of Crofts [179]. The values on the vertical axis reflect the free-energy involved in displacement of the residue from the lipid to the aqueous phase. A window length of 19 was chosen for calculation of the average hydropathy, followed by smoothing through one cycle with a length of 7 (see Ref. 179).

Unfortunately, the deduced amino-acid sequence does not shed any light on the nature of any cofactors associated with the alternative oxidase, since the sequence does not reveal any motifs common to any known metal-binding sites on other proteins (e.g., Fe/S centres [181,182]). The protein is, however, very hydrophilic, with charged residues distributed over much of its mass (Fig. 4) and it contains significant numbers of Try and His residues and several ionic motifs which possibly may play a role in polypeptide and metal cofactor binding. Obviously, further information obtained on a purified enzyme is required before a fuller understanding of what cofactors are involved in the functioning of the alternative oxidase can be achieved.

IV. Conclusions

In this review we have summarised our understanding of the nature and regulation of the cyanide- and antimycin-resistant alternative oxidase in plant mitochondria. Numerous questions remain to be answered about the alternative oxidase, but there is little question that the past 5 years have seen remarkable advances with respect to the clarification of what molecular species are involved in the operation of this interesting but enigmatic enzyme. It should be evident, however, that for a full understanding of the role and regulation of this oxidase in plant metabolism, we must await further information on the structure and catalytic composition of the purified enzyme.

Acknowledgements

We gratefully acknowledge the support of the Science and Engineering Research Council (A.L.M.), The

Royal Society (A.L.M.) and the National Science Foundation (J.N.S.). We are also indebted to Dr. L. McIntosh (Michigan State University, U.S.A.) for providing us with his manuscript prior to publication.

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