THE INVOLVEMENT OF THE PROTONMOTIVE UBIQUINONE CYCLE IN THE RESPIRATORY CHAIN OF HIGHER PLANTS AND ITS RELATION TO THE BRANCHPOINT OF THE ALTERNATE PATHWAY

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1. Introduction

In recent years much attention has been focused upon the respiratory chain of higher plants and in particular upon the fact that many species possess a pathway of electron transport to oxygen which is not via cytochrome oxidase and which is insensitive to cyanide (for recent reviews, see [1,2]). The exact nature of this alternate oxidase and the branch point at which electrons are taken from the main pathway to it have remained elusive. Various workers have established, however, by the use of P/O ratios [3], steady state redox levels of components and rates of reduction and oxidation during stopped-flow oxygen pulse experiments [4–10], that this branch point is on the substrate side of coupling site 2, i.e., in the flavoprotein–cytochromes *b*–ubiquinone region of the main respiratory pathway.

Although a variety of pathways of electron transport have been proposed [2,11,12], none so far are able satisfactorily to explain the available data on the redox behavior of components under different conditions. The recent hypothesis of Mitchell [13,14], however, of the protonmotive ubiquinone cycle to explain the chemiosmotic properties of mammalian mitochondria, has led us to re-evaluate this data and to propose similar possible schemes for the respiratory chain of higher plant mitochondria. It is proposed that the alternate oxidase pathway of higher plants is closely associated with part of this ubiquinone cycle and that its presence provides a useful tool for investigation of the cycle which is not available with mammalian mitochondria. Considerations of the available data and results of succinate pulse experiments from this laboratory have allowed us to order the reaction sequence of the Q cycle and to propose that succinic dehydrogenase reduces the $QH^{\cdot}/QH_2$ couple, whilst the alternate pathway reverses this step.

Abbreviations: Q, ubiquinone; $QH^{\cdot}$, ubisemiquinone; C-side, cytoplasmic side of inner mitochondrial membrane; M-side, matrix side of inner mitochondrial membrane; NADH, reduced nicotinamide adenine dinucleotide; e.p.r., electron paramagnetic resonance; SHAM, salicylhydroxamic acid; TMPD, *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenylenediamine dihydrochloride.

2. Materials and methods

Mung bean mitochondria were prepared by the method of Bonner [15]. Succinate- and oxygen-pulse experiments were performed on a Johnson Research Foundation double beam spectrophotometer or the triple dualwavelength spectrophotometer of Chance [16].

3. The electron transport components and their relation to the alternate oxidase pathway

Higher plant mitochondria contains cytochromes *a*, *b* and *c*, flavoproteins and ubiquinone and possess sites of energy coupling analogous to those found in other systems. The situation is complicated, however,

by the presence of at least four *b*-type cytochromes the three major of which being *b*-566 (E_m = −70 mV), *b*-560 (E_m = 40–80 mV) and *b*-556 (E_m = 75–100 mV) from their α-band maxima in reduced *minus* oxidized difference spectra at 25°C [17,18]. Cytochrome *b*-566 is considered analogous to cytochrome *b*-566 (b_T) of mammalian mitochondria and cytochrome *b*-560 to mammalian cytochrome *b*-562 (b_K) [16,19,20]. Cytochrome *b*-556 apparently has no counterpart in mammalian mitochondria.

The stopped-flow oxygen pulse experiments of Storey [4–10] performed under conditions of slow input of electrons into the system from substrate, have demonstrated that ubiquinone, the cytochromes *b* and a part of the flavoprotein complement can be oxidized by the alternate pathway when the cytochrome oxidase route is inhibited by cyanide. These experiments have further demonstrated that antimycin A slows down the oxidation of the cytochromes *b*, but not of ubiquinone or the flavoprotein component, by this alternate pathway. The rates of oxidation of the *b*-type cytochromes via the alternate oxidase is too slow for them to be part of the alternate pathway. This conclusion is further substantiated by the fact that antimycin A does not inhibit electron transport through the alternate pathway from succinate or externally added NADH (via an externally facing inner membrane NADH dehydrogenase [21]) to molecular oxygen [2].

4. Proposed schemes of electron transport

Schemes 1 to 4 (fig.1) represent the possible models of electron transport proposed for the higher plant respiratory chain in the ubiquinone–cytochrome

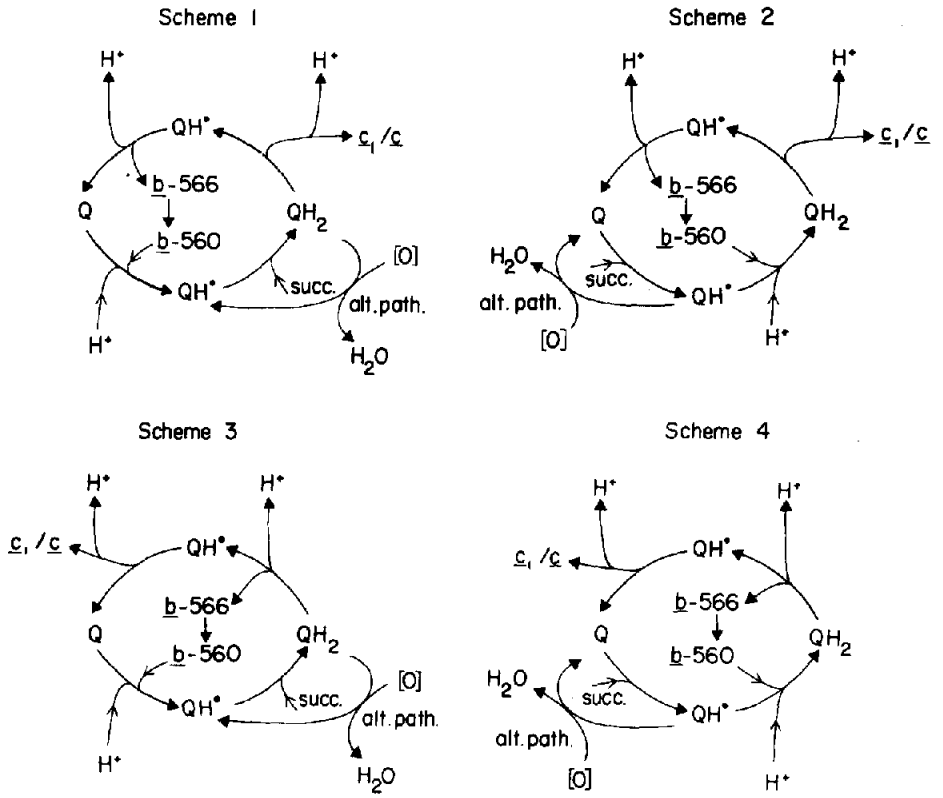

Fig.1. The proposed possible schemes of electron transport involving a protonmotive ubiquinone cycle in higher plant mitochondria. Specific details are discussed in the text, succ., succinate; alt. path., the cyanide-insensitive alternate pathway to molecular oxygen.

b region. The cyclic role of ubiquinone in these schemes closely parallels that described by Mitchell for the mammalian respiratory chain [13,14] and by Dutton et al. for the electron transport system of photosynthetic bacteria [22].

In these schemes: [1] cytochrome *b*-566 is considered analogous to mammalian cytochrome b_T and cytochrome *b*-560 to mammalian cytochrome b_K, and together they transport electrons from the C-side to the M-side of the membrane; [2] antimycin A blocks the oxidation of cytochrome *b*-560 by either the $Q/QH^{\cdot}$ couple (schemes 1, 3) or the $QH^{\cdot}QH_2$ couple (schemes 2, 4); [3] the alternate pathway branchpoint is located at the reversal of the step of $QH^{\cdot}$ reduction (schemes 1, 3) or Q reduction (scheme 2, 4) by the succinic dehydrogenase complex; [4] reducing power from external NADH is donated by a hydrogen carrier into the Q cycle at the same point as donation into the cycle by the succinic dehydrogenase complex.

The schemes differ in that cytochrome *c* reduction may occur before (schemes 1 and 2) or after (schemes 3, 4) cytochrome *b*-566 reduction, and reduction of a Q cycle intermediate by succinate may occur before (schemes 2, 4) or after (schemes 1, 3) reduction by cytochrome *b*-560.

If one assumes that transmembrane diffusion of $QH^{\cdot}$ cannot occur, cf. [13], the alternate pathway branchpoint must be located on the reversal of the succinic dehydrogenase reduction step of the cycle since only in this way can one rationalize the fact that antimycin A inhibits electron transport from substrate to molecular oxygen via cytochrome oxidase but not via the alternate oxidase and yet inhibits the slow oxidation of the cytochromes *b* through the alternate pathway.

It appears unlikely that cytochrome *b*-556 plays a crucial role in these schemes since, although its rate of reduction is very rapid, its rate of re-oxidation by either the alternate or the cytochrome oxidase pathway is much too slow for it to be an integral part of either [5,10]. A fourth *b*-type cytochrome, *b*-558 [17], displays properties analogous to those of cytochrome *b*-566, and hence these may be closely associated.

Although it has been demonstrated that a part of the flavoprotein component may be closely involved with the alternate pathway [10], the numbers and types of these flavoproteins is unclear [23], and further work must be done on this before their possible role in these schemes may be postulated.

5. Experimental evidence for the schemes

These schemes are able to explain most, if not all, of the available data of other workers on the higher plant respiratory pathway. The major points which support the hypotheses are:

(1) the uncoupled (non-electrogenic) oxidation of succinate and external NADH by the alternate pathway;

(2) the observation that neither cyanide, sulphide nor, more importantly, antimycin A inhibit the oxidation of succinate or externally added NADH by the alternate pathway [1];

(3) the cytochromes *b* may be oxidized by the alternate oxidase at a rate too slow for them to be on the alternate pathway directly and the fact that this rate is inhibited by antimycin A [7];

(4) ubiquinone can be oxidized by the alternate pathway and this rate of oxidation through the alternate oxidase is unaffected by antimycin A [7];

(5) antimycin A causes a 2 nm wavelength shift in the α-band of reduced cytochrome *b*-560 [17], indicating that the antimycin A block is located close to this component;

(6) hydroxamic acids, which are specific inhibitors of the alternate pathway [24], do not affect the rates of cytochromes *b* oxidation by the cytochrome oxidase pathway;

(7) in the presence of substrate, antimycin A causes the almost complete reduction of the *b*-type cytochromes in the aerobic steady state. Upon anaerobiosis, a re-oxidation of cytochrome *b*-566 is observed [25], a property analogous to that of cytochrome b_T of mammalian systems [26].

(8) the rate of cytochrome *b*-566 oxidation via the alternate pathway is inhibited in the presence of antimycin A when cytochrome *c* is fully oxidized, although the rate of reduction of cytochrome *b*-566 by substrate is unaffected by antimycin A [27].

6. Further considerations

It is of great interest to determine which of the models proposed is most likely to be operative.

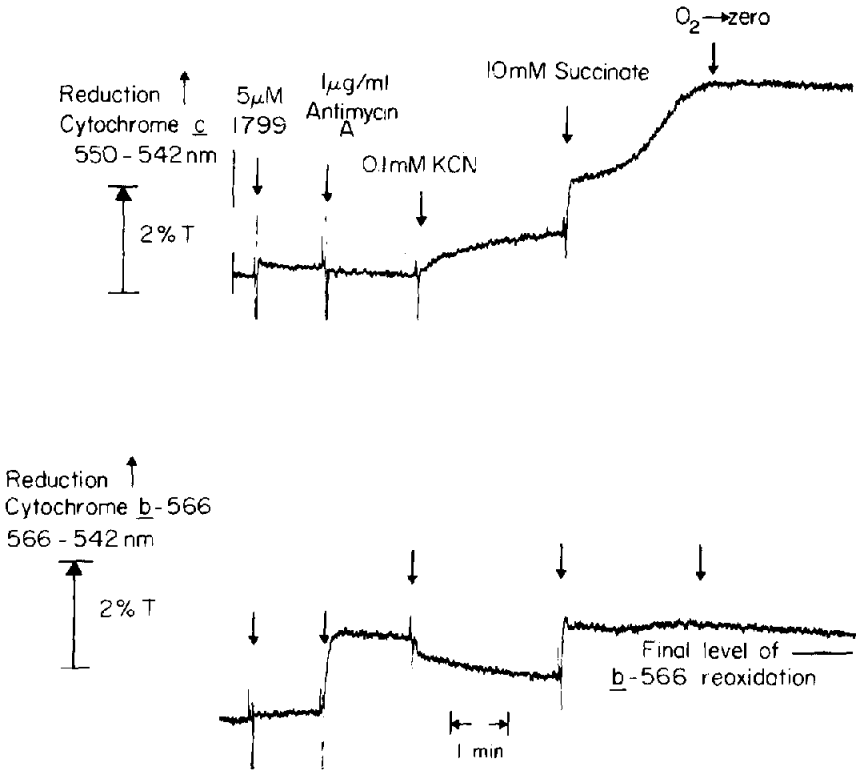

Fig.2. The reduction of mung bean cytochromes *b*-566 and *c* by succinate under conditions of partial inhibition of cytochrome oxidase. The experiments were performed with a Johnson Research Foundation double beam spectrophotometer set at appropriate wavelength pairs. Each run was performed separately, but under identical conditions, and additions were made as shown. Medium: 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM potassium phosphate, pH 7.2; temperature: 25°C; protein: approx. 3 mg/ml.

Succinate-pulse experiments were performed upon mung bean mitochondria in the presence of uncoupler and antimycin A and under conditions in which cytochrome oxidase was partially inhibited by 0.1 mM potassium cyanide. The redox levels of cytochromes *b*-566 and *c*-549 were monitored throughout. The addition of antimycin A after uncoupler caused a partial reduction of cytochrome *b*-566 due to endogenous substrate. The further addition of 0.1 mM cyanide caused a partial reduction of cytochrome *c*-549 and a partial reoxidation of cytochrome *b*-566. A succinate pulse added to the system under these conditions caused a rapid antimycin A-insensitive reduction of both cytochromes (fig.2). Cytochrome *c*-547 (not shown) behaved in a manner similar to that of cytochrome *c*-549, but cytochrome *b*-560 became almost fully reduced upon addition of antimycin A, did not reoxidize upon addition of KCN, and showed almost no further reduction upon succinate addition.

Similar results have been obtained by Dr M. K. F. Wikström with mammalian systems (personal communication). When the same experiment was performed in the presence of SHAM, a specific inhibitor of the alternate pathway [24], a similar result was obtained although the steady-state level of reduction of the cytochromes was higher in the presence of cyanide and antimycin A and hence the amounts of rapidly reducible cytochromes *b*-566 and *c*-549 by succinate were correspondingly lower.

This experiment demonstrates that the succinic dehydrogenase reduction step occurs after the step of cytochrome *b*-560 reduction of Q (i.e., schemes 1 and 3). A kinetic analysis of the rapid rates of

cytochromes *b*-566 and *c*-549 reduction on addition of succinate or NADH under similar conditions to fig.2, but at lower temperatures, demonstrated that the rates of reduction cytochromes *b*-566 and *c*-549 ($t_{1/2}$ (NADH reduction) = 1.5–1.9 s at 13°C; $t_{1/2}$ (succinate reduction) = 1.8–2.2 s at 13°C) were kinetically indistinguishable. However, when a similar experiment was performed, but with cytochrome oxidase fully inhibited and with ascorbate (10 mM) and TMPD (0.1 mM) present so that the cytochromes *c* and *a* were fully reduced even before the succinate pulse, it was found that the rapid reduction of cytochrome *b*-566 still occurred to the same extent. Since TMPD will remain fully reduced and ascorbate oxidation is irreversible at this pH [28] i.e., reduced cytochrome *c* has no electron acceptor, this experiment tends to indicate that reduction of cytochrome *b*-566 is occurring before that of the cytochromes *c* i.e., as illustrated in scheme 3. It is felt that further experiments along these lines must be performed before this conclusion may be substantiated and hence the generalized scheme as we envisage it at present is illustrated in fig.3, with the two one-electron steps of reduction of cytochromes *b*-566 and *c* as yet unordered.

This general scheme is also in agreement with the results of other workers using different approaches with mammalian systems. For example Ingledew and Ohnishi [29] have recently suggested that the reduction catalyzed by succinic dehydrogenase is that of $QH^{\cdot}$ to QH_2 in mammalian mitochondria. E.p.r. work from this laboratory in conjunction with Dr Ingledew suggests that this is also the case for mung bean mitochondria and supports the hypothesis that the alternate pathway reverses this reduction

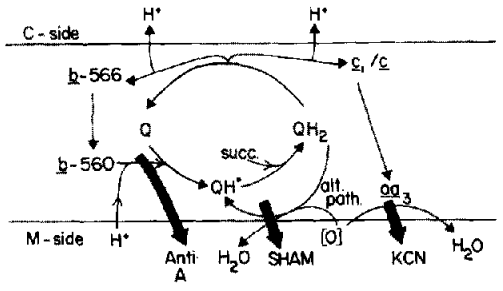

Fig.3. The proposed protonmotive ubiquinone cycle scheme of electron transport in higher plant mitochondria.

(unpublished work). A detailed kinetic analysis [16] and computer simulation (Wikström, M. K. F. and DeVault, D., to be published) of the redox reactions of cytochromes b_K, b_T and c_1 of mammalian systems also fits in with the proposed scheme.

7. Conclusions

We hope to have demonstrated in this communication that the protonmotive ubiquinone cycle is the best model to date which can explain the data which is available on the higher plant respiratory chain. This provides evidence of the general applicability of the Q cycle. Further, the fact that many higher plants possess the alternate oxidase pathway which is closely associated with this cycle may provide an extremely useful extra parameter which may be utilized in investigations of the validity of these possible schemes. The specific location of the branchpoint of electron flow into the alternate pathway at the reversal of the step of succinic dehydrogenase reduction of $QH^{\cdot}$ to QH_2 is of major importance in this respect and we hope that this information will be of use in the detailed elucidation of component ordering in this region of the respiratory chain.

Acknowledgments

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