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THE RESPIRATORY CHAIN OF PLANT MITOCHONDRIA

XV. EQUILIBRATION OF CYTOCHROMES c_{549}, b_{553}, b_{557} AND UBIQUINONE IN MUNG BEAN MITOCHONDRIA: PLACEMENT OF CYTOCHROME b_{557} AND ESTIMATION OF THE MIDPOINT POTENTIAL OF UBIQUINONE

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SUMMARY

1. Cycles of oxidation followed by reduction at pH 7.2 have been induced in uncoupled anaerobic mung bean mitochondria treated with succinate and malonate by addition of oxygen-saturated medium. Under the conditions used, cytochromes b_{557}, b_{553}, c_{549} (corresponding to c_1 in mammalian mitochondria) and ubiquinone are completely oxidized in the aerobic state, but become completely reduced in anaerobiosis.

2. The time course of the transition from fully oxidized to fully reduced in anaerobiosis was measured for cytochromes c_{549}, b_{557}, and b_{553}. The intramitochondrial redox potential (IMP_h) was calculated as a function of time for each of the three cytochromes from the time course of the oxidized-to-reduced transition and the known midpoint potentials of the cytochromes at pH 7.2. The three curves so obtained are superimposable, showing that the three cytochromes are in redox equilibrium under these conditions during the oxidized-to-reduced transition.

3. This result shows that the slow reduction of cytochrome b_{557} under these conditions, heretofore considered anomalous, is merely a consequence of its more negative midpoint potential of +42 mV at pH 7.2, compared to +75 mV for cytochrome b_{553} and +235 mV for cytochrome c_{549}. Cytochrome b_{557} is placed on the low potential side of coupling site II and transfers electrons to cytochrome c_{549} *via* the coupling site.

4. The time course of the transition from fully oxidized to fully reduced was also measured for ubiquinone. Using the change in intramitochondrial potential IMP_h with time obtained from the three cytochromes, the change in redox state of ubiquinone with IMP_h was calculated. When replotted as IMP_h *versus* the logarithm of the ratio (fraction oxidized)/(fraction reduced), two redox components with $n=2$ were found. The major component is ubiquinone with a midpoint potential $E_{m7.2}=+70$ mV. The minor component has a midpoint potential $E_{m7.2}=-12$ mV; its nature is unknown.

Abbreviations: IMP_h, intramitochondrial potential, referred to the normal hydrogen electrode; $E_{m7.2}$, midpoint potential at pH 7.2.

INTRODUCTION

The type and organization of the electron transport carriers of the respiratory chain of plant mitochondria are similar in overall design to those of the carriers of mammalian and avian mitochondria. They differ considerably in detail, however, as discussed in recent reviews[1–3]. With regard to type, it is the cytochromes *b* of plant mitochondria which provide the greatest contrast to mammalian and avian mitochondria. There are three *b* cytochromes[4–6], designated b_{553}, b_{557}, and b_{562}, the subscripts denoting absorbance maxima at 77 °K in the reduced-*minus*-oxidized difference spectrum[7]. The midpoint potentials of these cytochromes, measured at pH 7.2 and designated $E_{m7.2}$ are as follows: b_{553}, 75 mV; b_{557}, 42 mV; and b_{562}, −77 mV[8]. The midpoint potential of cytochrome b_{562} is unaffected by the energy state of the mitochondrion. By contrast, the corresponding long wavelength cytochrome *b* of animal mitochondria, designated b_T[9–11] or b_{566}[12,13], shows an apparent shift of midpoint potential of nearly 300 mV to a more positive value on energization of the mitochondrial membrane[9]. Cytochrome b_{562} and cytochrome b_T also differ in their reduced-*minus*-oxidized difference spectra. At room temperature, cytochrome b_T is reported to have an absorbance maximum at 566 nm with a pronounced shoulder at 558 nm in the α-band region[11,14]; the split into two bands at 555 and 563 nm is evident at 77 °K. Cytochrome b_{562} has but a single absorbance maximum in the α-band region at 565 nm at room temperature and 562 nm at 77 °K. Cytochrome b_{557} corresponds approximately to cytochrome b_K in animal mitochondria, in that they have similar midpoint potentials, but in oxygen pulse experiments, the rate of oxidation of cytochrome b_{557} is considerably more rapid than that of cytochrome b_K[7,15–17]. The presence of a third cytochrome *b* in animal mitochondria, with a shorter wavelength peak corresponding to cytochrome b_{553}, has been reported by Wikström[13,18], implying that the two absorbance maxima reported for cytochrome b_T are actually due to two, rather than one, cytochrome. Agreement has not yet been reached on this point.

The organization of the respiratory chain mitochondria of plant mitochondria is similar to that of animal mitochondria, in that both contain similar complements of *a*, *b*, and *c* type cytochromes, flavoproteins, ubiquinone, and endogenous pyridine nucleotide[19–22]. By use of the crossover theorem developed some years ago[23], the sites of energy conservation have been localized between cytochromes *a* and a_3 (Site III)[8,24], between the cytochromes *b* and cytochromes *c* (Site II)[8,24,25], and between endogenous pyridine nucleotide and ubiquinone–flavoprotein–cytochromes *b* (Site I)[22]. They are thus located in much the same regions as are the energy conservation sites of the mammalian and avian respiratory chain.

One of the most useful tools in elucidating the pathway of electron transport in plant mitochondria has been the study of the oxidation and reduction kinetics during a cyclic perturbation. Such a perturbation is the addition of a pulse of NADH[26] or durohydroquinone[27] to aerobic mitochondria to induce a cycle of reduction followed by reoxidation, or addition of a pulse of oxygenated medium to anaerobic mitochondria to induce a cycle of oxidation followed by reduction. The latter technique, developed by Chance[28], is particularly useful because of the very rapid rate of reaction of cytochrome oxidase with oxygen. It has been used extensively by Chance and coworkers[15,16,29,30] over the past years to characterise in depth the

sequence and interaction of the electron transport carriers of animal mitochondria, and to outline the carrier sequence in mitochondria from skunk cabbage (*Symplocarpus foetidus*) spadices[31,32]. More recently, the technique has been extended to studies of mitochondria from mung bean (*Phaseolus aureus*) seedlings as well as skunk cabbage mitochondria[7,14,20,33–35]. The oxygen pulse experiments with these mitochondria are usually carried out using succinate as substrate in the presence of sufficient malonate to give extensive inhibition of succinate dehydrogenase. Under these conditions, maximal rates of carrier oxidation are observed, and oxidation of the initially reduced carriers is complete in the presence of excess oxygen. An oxidation–reduction cycle induced by an oxygen pulse has three readily discernible phases. There is a rapid "on" phase, corresponding to oxidation of the carrier; an aerobic steady state; and a slower "off" phase, corresponding to re-reduction[28]. The half-time for oxidation is designated $t_{\frac{1}{2}\,on}$, while the time from the point of half oxidation to half reduction is designated $t_{\frac{1}{2}\,off}$.

Higgins[36,37] has established a series of ordering theorems for linear enzyme sequences bases on the relative values of $t_{\frac{1}{2}\,on}$ and $t_{\frac{1}{2}\,off}$, which have been applied to the mitochondrial respiratory chain with considerable success in the $c_1/c/a/a_3$ region[15]. The same theorems have also been applied to the respiratory chain of plant mitochondria[7,33,34]. One contrast in the behavior of cytochromes b_{557} and b_{553} is that the former is rapidly oxidized in oxygen pulse experiments, but is slowly re-reduced, while the latter shows the opposite behavior. In terms of the half-time designations, $t_{\frac{1}{2}\,on}$ for b_{557} is much less than $t_{\frac{1}{2}\,on}$ for b_{553}, while $t_{\frac{1}{2}\,off}$ for b_{557} is much greater under the same set of experimental circumstances. This is contrary to what would be predicted by the ordering theorems if these parameters were strictly controlled by kinetics. The assumption was made in previous papers that this was indeed so. But if these parameters merely reflect rapid equilibration of equivalents between the respiratory chain carriers in accordance with their characteristic midpoint potentials, then the theorems do not apply, and the half-times cannot be used to deduce enzyme sequences.

In an oxygen pulse experiment, cytochrome b_{557} has a characteristic $t_{\frac{1}{2}\,on}$ of 6–8 ms, while b_{553} has a characteristic half-time of 150–200 ms at 24 °C[7,35]. It is evident that these carriers, which differ in midpoint potential by about 30 mV, are not in equilibrium during the oxidation phase of the reaction cycle. The aerobic steady state and reduction phase of the cycle may last many seconds, or even minutes, however, particularly at high malonate concentrations; the reduction phase may be slow enough that equilibrium is established between the carriers. If this be the case, the $t_{\frac{1}{2}\,on}$ values may be useful in establishing carrier sequences, but the $t_{\frac{1}{2}\,off}$ values certainly are not. The latter values should reflect the redox potential within the mitochondria as reducing equivalents are slowly fed to the oxidized carriers through the highly inhibited succinate dehydrogenase. The first object of this study was to establish whether cytochromes b_{557}, b_{553} are in equilibrium with each other and with cytochrome c_{549} during the reduction phase of an oxygen pulse cycle, and to include cytochrome b_{557} in the sequence of the respiratory chain carriers based on this result.

The second object of this study was to estimate the midpoint potential of ubiquinone. One great difficulty with measuring the midpoint potential of ubiquinone by the Dutton method[38] is that external redox mediators are required; all of these

which have midpoint potentials in the range of interest absorb strongly in the ultraviolet region where one measures the redox changes of ubiquinone by spectrophotometry. The problem is bypassed by utilizing the cytochromes as internal indicators of the redox of the mitochondria, provided that the redox states of the cytochromes are in equilibrium, and in equilibrium also with the redox state of ubiquinone. Evidence for this equilibration and an estimate of the midpoint potential of ubiquinone at pH 7.2 are reported in this paper.

METHODS

Mitochondria were prepared from the hypocotyls of 5-day-old etiolated seedlings of mung bean (*Phaseolus aureus*), using the method described by Bonner[39] and Ikuma and Bonner[40] as modified by Storey and Bahr[33]. Mitochondrial preparations made in this manner contain virtually none of the very dense material reported by Douce *et al.*[41]. They yield one major band on the discontinuous sucrose gradient described by Douce *et al.*[41] with a diffuse, less dense minor band comprising mitochondria with damaged outer membranes; they also yield a reduced-*minus*-oxidized difference spectrum[34] characteristic of the gradient-purified mitochondria described by these authors. The mitochondria were assayed for respiratory control with succinate or malate as substrate and ADP as P_i acceptor in a closed curvette equipped with a Clark Electrode (Yellow Springs Instrument Co.) as described by Estabrook[42]. The suspending medium contained 0.3 M mannitol, 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanosulphonic acid, 5 mM P_i, and was adjusted to pH 7.2 with KOH. This medium is designated Medium TP; the same medium with P_i omitted is designated Medium T. Mitochondrial protein content was determined by the method of Miller[43].

Adenine nucleotides were obtained from Boehringer Mannheim Corp; succinic and malonic acid from Aldrich Co., and oligomycin from Sigma Chemical Co; these were used without further purification. The uncoupler 1799 (bis-hexafluoroacetonyl acetone) was generously supplied by Dr Peter Heytler of E. I. duPont de Nemours Co.

Cycles of oxidation and reduction were induced by addition of oxygen-saturated medium to the anaerobic mitochondrial suspension containing succinate as substrate and malonate as inhibitor. The reaction was carried out in Medium T which had been bubbled with argon to reduce the oxygen content. The mitochondria were pretreated with oligomycin, followed by ATP and the uncoupler 1799. Succinate was added to allow the mixture to become anaerobic, followed by addition of malonate. In anaerobic, uncoupled mung bean mitochondria reduced with succinate under these conditions, ubiquinone and cytochromes c_{549} (corresponding to c_1 of mammalian mitochondria), b_{553}, and b_{557} become fully reduced, but the lower potential components, including cytochrome b_{562} remain mostly oxidized[22]. Under these same conditions, cytochromes c_{549}, b_{553}, b_{557} and ubiquinone become completely oxidized in an oxygen pulse experiment[34], so that these respiratory chain carriers cycle between full oxidation in the aerobic steady state and full reduction in anaerobiosis, and the fraction oxidized or reduced can be calculated at any point between these extremes. The absorbance changes characteristic of changes in the redox states of the cytochromes were monitored by means of the dual wavelength spectrophotometer[44], using the wavelength pairs 552–540 nm for c_{549}, 556–540 nm

for b_{553}, and 560–540 nm for b_{557}. The redox state of ubiquinone was monitored at 282–295 nm. Permanent records were obtained with a strip chart recorder. The oxygenation reaction was carried out in the manually driven, rapid mixing, regenerative flow apparatus with 0.1-cm light path, used in previous kinetic experiments[7,20,33]. A pair of reflector arrays which increased the effective path length to 0.35–0.40 cm was used for measurements in the 540–560 nm region of the spectrum. The apparatus is so designed that the oxygen-saturated medium is accurately diluted 1/100 by the anaerobic suspension. It acts as a convenient metering device which delivers the same amount of oxygen to the anaerobic suspension and thus insures that the initial oxygen concentration is the same in each experiment. In order to keep conditions as nearly identical as possible for each cycle, a series of four oxidation–reduction cycles was run sequentially for each experiment with the sets of wavelength pairs for c_{549}, b_{553}, b_{557}, and ubiquinone, although not necessarily in that order. The sequence of four cycles was run after ascertaining that the $t_{\frac{1}{2}\,off}$ observed for cytochrome c_{549} in preliminary experiments had become essentially constant.

In each experiment of four cycles, the fraction reduced as a function of time was calculated for each of the three cytochromes, taking zero time as the point of oxygen addition. The fraction reduced at any time was converted into a value of intramitochondrial redox potential, designated IMP_h, using the previously determined variation of potential (referred to the normal hydrogen electrode) with fraction reduced at 552–540 nm and 556–540 nm reported in ref. 8, and taking the absorbance changes at 560–540 nm to be solely due to cytochrome b_{557} with midpoint potential $E_{m7.2} = +42$ mV. This conversion then yielded three curves, one for each cytochrome, of calculated intramitochondrial potential IMP_h as a function of time. The values of fraction reduced as a function of time were calculated for ubiquinone in a similar manner from the absorbance changes at 282–295 nm. These were then converted to fraction reduced as a function of intramitochondrial potential calculated from the redox state of the cytochromes, as described above. This curve was then replotted with the calculated potential IMP_h as the ordinate and the logarithm of the ratio (fraction oxidized)/(fraction reduced) as abscissa. The midpoint potentials of the components involved were then determined by the method of Wilson and Dutton[45].

RESULTS

The absorbance changes recorded in one experiment comprising four oxidation–reduction cycles in uncoupled mung bean mitochondria are shown in Fig. 1. for the wavelength pairs 552–540 nm (A), 556–540 nm (B), 560–540 nm (C), and 282–295 nm (D). As expected from previous experiments of this kind, there is a fast phase of oxidation, followed by an aerobic steady state, followed in turn by a slow reduction phase. The oxidation step has been the focus of previous work and is not considered here. From the records of Figs 1A, 1B and 1C, the fraction reduced at a given time is calculated for each set of wavelength pairs, is converted to a value of intramitochondrial potential IMP_h at that time, and is plotted as shown in Fig. 2. It is quite evident that the three potential curves calculated from each cytochrome overlap to a remarkable degree over the whole time range of the experiment. The intramitochondrial redox potential is essentially the same when measured by the

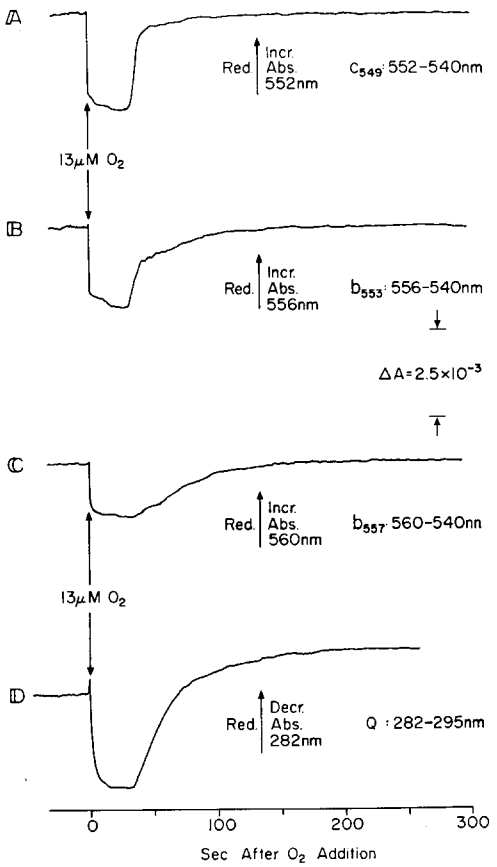

Fig. 1. Cycles of oxidation and reduction of the respiratory chain carriers induced by adding oxygen-saturated medium to uncoupled, anaerobic mung bean mitochondria suspended in Medium T (see Methods) at 2.1 mg protein/ml. The mitochondria are treated sequentially with oligomycin (2 μg/mg), 0.25 mM ATP, 20 μM uncoupler 1799, 5 mM succinate, followed by 40 mM malonate. In Traces A–C, the effective optical path length is 0.35 cm; in Trace D it is 0.1 cm. The deflection of the trace beyond the original baseline in Trace D is routinely observed in measurements of the redox state of ubiquinone; it is a dilution artifact[34]. These records were obtained on the same suspension in sequence, after preliminary experiments had shown that the cycling time, observed at 552–540 nm had become constant. Q= ubiquinone.

redox state of each of the three cytochromes, showing that these three carriers are indeed in redox equilibrium.

The fraction reduced of ubiquinone, measured at 282–295 nm (Fig. 1D), as a function of time calculated from the addition of oxygen, can be converted to a potential axis using the curve of Fig. 2, and the fraction reduced of ubiquinone as a function of the intramitochondrial potential IMP_h is plotted in Fig. 3. The Nernst plot derived from Fig. 3 is shown in Fig. 4A. There is a definite deviation from linearity at low potential, due to a minor component of low midpoint potential. Arithmetic separation of the components according to the method of Wilson and Dutton[45] results in two straight lines with slopes corresponding to $n=2$, as expected

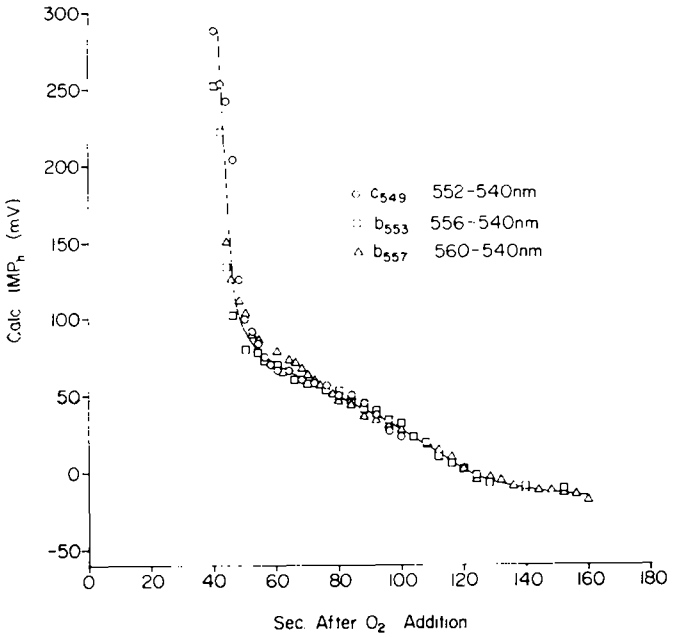

Fig. 2. Plot of intramitochondrial potential referred to the normal hydrogen electrode, IMP_h, as a function of time after addition of oxygen, calculated from the records shown in Fig. 1, using cytochrome c_{549} at 552–540 nm (Fig. 1A), cytochrome b_{553} at 556–540 nm (Fig. 1B), and cytochrome b_{557} at 560–540 nm as internal redox indicators. The method of calculation is described in Methods. ○—○, points wavelength pair 552–540 nm; □—□, 556–540 nm; △—△, 560–540 nm.

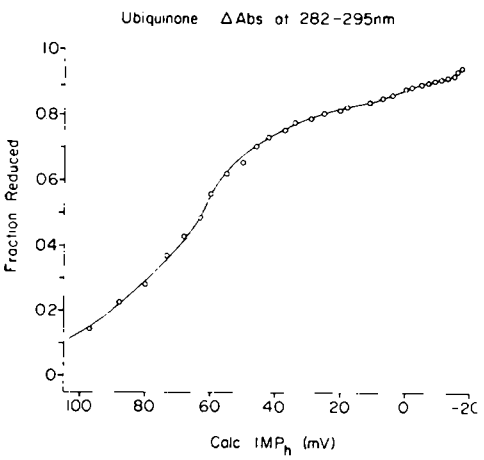

Fig. 3. Plot of fraction of ubiquinone reduced, calculated from the record of Fig. 1D, *versus* intramitochondrial potential, IMP_h, using the curve of Fig. 2 to interconvert time after addition of oxygen and IMP_h, as described in Methods.

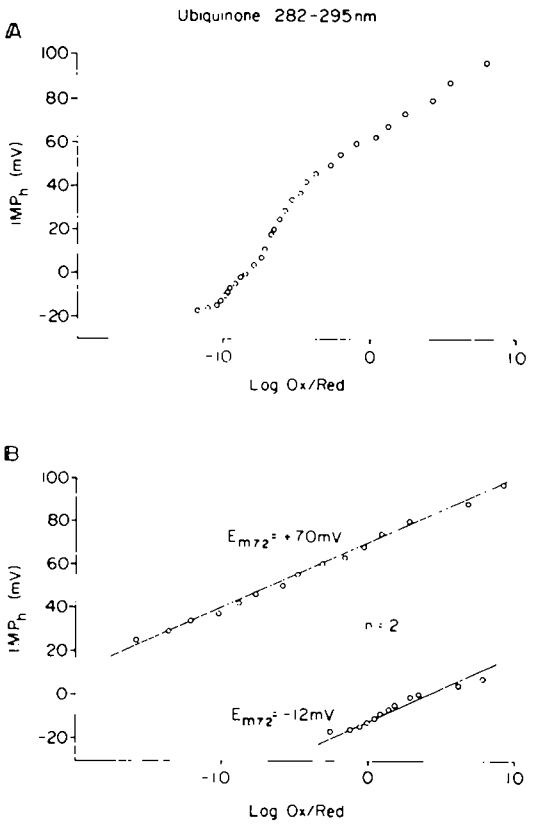

Fig. 4. (A) Plot of intramitochondrial potential IMP_h *versus* the logarithm of the ratio (fraction oxidized)/(fraction reduced) for ubiquinone, using the data points shown in Fig. 3. (B) Resolution of the curve of A into two linear components drawn with slopes for $n = 2$, corresponding to a two-electron redox process.

for the two redox components which are two-electron carriers (Fig. 4B). The major component, representing 83% of the absorbance change at 282–295 nm has a midpoint potential $E_{m7.2} = +70$ mV; this is the two-electron carrier, ubiquinone. The minor component representing 15% of the absorbance change has a midpoint potential $E_{m7.2} = -12$ mV. These midpoint potentials are reproducible to within ± 5 mV when due care is taken to maintain constant conditions for each set of four cycles. The relative amount of minor component varied between 15% and 25% of the total change observed at 282-295 nm; the variation is caused at least in part in measuring the small absorbance changes at low intramitochondrial potentials. The nature of this component is at present unknown.

DISCUSSION

It is evident from Fig. 2 that, in the reduction phase of an oxidation–reduction cycle induced by an oxygen pulse in anaerobic mung bean mitochondria, cytochromes c_{552}, b_{553}, and b_{557} are essentially in equilibrium. The fact that they can be

used as indicators of the intramitochondrial redox potential to yield a linear Nernst plot for ubiquinone is consistent with equilibration of this carrier with the three cytochromes. Further support for equilibration of these four carriers comes from the actual time course of the decrease of the calculated intramitochondrial redox potential shown in Fig. 2. The rapid decrease in this potential at the end of the aerobic state slows markedly at about 100 mV as ubiquinone, which is present in some 6-fold excess over the cytochrome components, starts undergoing reduction and thus acting as an internal redox poise.

Equilibration of the carriers during the reduction phase of the oxidation–reduction cycle renders invalid the previous arguments concerning the sequence of carriers[33,34] which were based on values of $t_{\frac{1}{2}\,off}$. Cytochrome b_{557} may now be included in the sequence of carriers from succinate to oxygen, as shown in Fig. 5. Its rapid rate of oxidation indicates that it transports electrons to cytochrome c_{549} *via* coupling site II. The justification for the rest of the sequence in Fig. 5 is given in the previous paper[34]. The position of cytochrome b_{562} and of the lower potential flavoproteins is as yet uncertain, as is the exact point at which the alternate, cyanide insensitive oxidase interacts with the plant respiratory chain. Proposed points of interaction are shown by the dotted lines in Fig. 5.

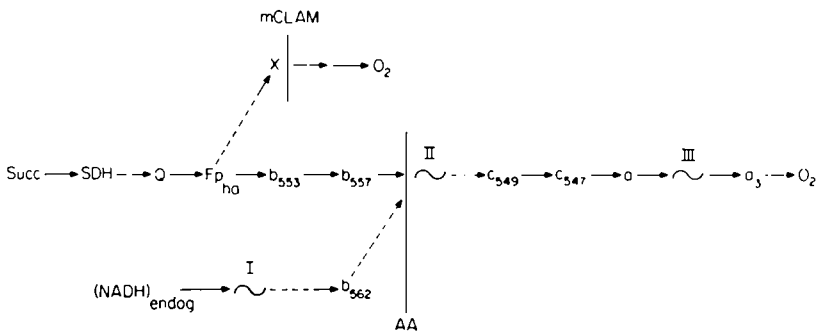

Fig. 5. Proposed sequence of electron transport carriers in the respiratory chain of plant mitochondria. Dotted lines show suggested paths of electron transport for which there is insufficient evidence at present. The site of antimycin A (AA) inhibition is shown to emphasize the fact that all three cytochromes *b* respond to this inhibition. The alternate, cyanide and antimycin A insensitive pathway is shown consisting of at least one, unknown component X, which is presumed to bind *m*-chlorobenzhydroxamic acid (mCLAM), the highly specific inhibitor of this pathway[50]. There has been no evidence reported for plant mitochondria, analogous to that reported by Muraoka and Slater[51] for rat liver mitochondria, which would support the existence of a fourth site of energy conservation between cytochrome a_3 and oxygen; such a site is therefore omitted from the scheme. Succ, succinate; SDH, succinate dehydrogenase; Q, ubiquinone, Fp, flavoprotein.

The midpoint potential $E_{m7.2} = +70$ mV for ubiquinone in intact mung bean mitochondria is in good agreement with the value of $E_{m7.0} = +65$ mV obtained by Urban and Klingenberg[46] in submitochondrial particles from beef heart muscle, using the succinate–fumarate redox couple to poise the system. This finding indicates that the environment of ubiquinone in the mitochondrial membrane is very similar, whether the mitochondria come from plant or animal, or be intact or fragmented. As pointed out by Urban and Klingenberg[46], the midpoint potential of ubiquinone as measured in the mitochondrial membrane is about 40 mV more negative than

those values measured for purified ubiquinone in alcohol systems. Part of this discrepancy may be due to the difference between standard states in alcohols and water. Slater[47] has recalculated the midpoint potential $E_{m7.0}$ of ubiquinone from the data obtained by Morton *et al.*[48] in ethanol, correcting for this difference, and arrived at a value of +89 mV with aqueous solution as the standard state, which is close to the value measured *in situ* in the membrane.

The minor component with apparent $E_{m7.2} = -12$ mV and $n=2$, poses a problem. This midpoint potential does not coincide with that of any electron transport carrier of the plant respiratory chain which has been measured so far. It is not very accessible experimentally, since its characteristic absorbance change with redox state is in that part of the ultraviolet region dominated by the absorbance changes due to ubiquinone. It is even possible that the absorbance change at 282–295 nm seen at low intramitochondrial potential is an artifactual "drift" due to light scattering changes which occur after prolonged anaerobiosis. While such a drift would not be expected to give a linear Nernst plot with $n=2$, the relatively small absorbance changes with accompanying errors in calculation indicate that caution must be exercised in accepting the existence of this component. It is tempting to speculate, however, that this component is ubiquinone itself, either bound to another respiratory chain carrier or sequestered in the membrane, in such a way as to stabilize the quinone over the hydroquinone form. This would constitute a small, separate pool of ubiquinone, functioning at a lower intramitochondrial potential than the main pool; this pool might be associated with cytochrome b_{562} while the main pool is associated with cytochromes b_{553} and b_{557}[34]. Evidence for two such pools and, in fact, a dual respiratory chain in beef heart mitochondrial membranes has recently been put forward by Norling *et al.*[49].

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