Plant Physiol. (1976) 58, 521-525

Respiratory Chain of Plant Mitochondria

XVIII. POINT OF INTERACTION OF THE ALTERNATE OXIDASE WITH THE RESPIRATORY CHAIN[1]

Received for publication April 15, 1976 and in revised form June 17, 1976

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ABSTRACT

Oxidation of the respiratory chain carriers of anaerobic, CO-saturated skunk cabbage (*Symplocarpus foetidus*) mitochondria, by means of an O_2 pulse, proceeds primarily through the cyanide-insensitive alternate oxidase, since the oxidation of cytochromes *a* and a_3 takes place with a half-time of 3 seconds, corresponding to the rate of dissociation of CO from reduced cytochrome a_3. Ubiquinone and part of the flavoprotein are oxidized within 1 second under these conditions, and this rapid rate of oxidation is strongly inhibited by *m*-chlorobenzhydroxamic acid (*m*CLAM), a specific inhibitor of the alternate oxidase of plant mitochondria. The rate of ubiquinone oxidation under these conditions in white potato (*Solanum tuberosum*) mitochondria, which have no alternate oxidase, is the same as that in skunk cabbage mitochondria treated with *m*CLAM. Ubiquinone, thus identified as the carrier common to both the cytochrome and alternate oxidase pathways, is linked to the alternate oxidase by a flavoprotein of midpoint potential 50 millivolts more negative with which it is in equilibrium. This arrangement provides a switch for diverting electron transport primarily through the cytochrome pathway under state 3 conditions and primarily through the alternate oxidase pathway under state 4 conditions.

The cyanide-insensitive respiratory pathway in plants has been shown to be mediated by an alternate oxidase in the mitochondria which interacts directly with the "main" respiratory chain containing the Cyt (3, 11, 16, 18). This alternate oxidase has been shown to be part of the inner mitochondrial membrane (16). Previous work on the mechanism of the alternate pathway with mitochondria isolated from both skunk cabbage (*Symplocarpus foetidus*) spadices and etiolated mung bean (*Phaseolus aureus*) hypocotyls has resulted in the development of a specific inhibitor for the oxidase (20) and has shown that the alternate oxidase interacts with the main respiratory chain in the ubiquinone-flavoprotein-Cyt *b* region (15, 22). But the precise point of interaction was never established. During active oxidative phosphorylation (state 3) in skunk cabbage mitochondria, much of the electron transport proceeds through the energy-conserving main respiratory chain, while in the resting state (state 4), much of the electron transport occurs through the alternate oxidase (28). As a result, respiratory control is generally not observed with skunk cabbage mitochondria. The rate of electron transport through the alternate pathway in state 4 is as rapid as through the main pathway in state 3. In mitochondria with a much less active alternate pathway, such as those isolated from mung bean hypocotyls, or in mitochondria with an inactive alternate pathway, such as those isolated from fresh white potato (*Solanum tuberosum*) tubers, good respiratory control with appropriately low state 4 rates is observed. Inhibition of the alternate pathway in skunk cabbage mitochondria with the specific inhibitor *m*CLAM[3] (20) restores respiratory control by lowering the state 4 rate relative to the state 3 rate.

Bahr and Bonner (1, 2) have examined the characteristics of the control mechanism which partitions the flux of electron transport between the two pathways. They concluded that the branch point which partitions the flux consists of two components whose redox states are in quasi equilibrium, one of which is part of the main respiratory chain, the other of which transfers electrons directly to the alternate oxidase. While this conclusion is useful in defining the nature of the branch point, it does not locate it. The most obvious way to examine electron transport to O_2 in the alternate pathway is to inhibit electron transport to O_2 through the main pathway, and then identify those components which are readily oxidized by added O_2 and re-reduced in anaerobiosis in this inhibited condition. Considerable effort has been expended on this approach, but the results have been unsatisfactory. With cyanide as inhibitor of electron transport in the main pathway, rapid oxidation of the Cyt *c* and Cyt *b*-560[4] still occurs through Cyt oxidase, since the reduced Cyt a_3/CN complex is very rapidly oxidized by O_2, and the rate of rearrangement to the inhibitory form of the complex with oxidized Cyt a_3 is not sufficiently rapid to prevent this oxidation (23). This set of rapid reactions of electron transport completely obscures those occurring via the alternate oxidase. With antimycin A as inhibitor, Cyt *a* and a_3 and the Cyt *c* are rapidly oxidized by Cyt oxidase as expected (21). In addition, however, a flavoprotein component

[1] This research was supported by National Science Foundation Grant GB-23063.

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[3] Abbreviations: *m*CLAM: *m*-chlorobenzhydroxamic acid; $E_{m7.2}$: midpoint potential at pH 7.2 referred to normal hydrogen electrode.

[4] Nomenclature for the Cyt in plant mitochondria. The three Cyt *b* of plant mitochondria were originally resolved by means of difference spectrophotometry at 77 K, at which temperature the α bands of the reduced forms are found at 553, 557, and 562 nm (4, 5). These maxima represent a shift of 3 mm toward shorter wavelength from the maxima at 556, 560, and 565 observed at room temperature. Some years ago, Bonner (5) made the logical suggestion that the plant Cyt *b* be identified as b_{553}, b_{557}, and b_{562}, the subscripts giving the reduced minus oxidized difference absorbance maxima in the α region at 77 K. This convention has been followed in the previous papers of this series for the three *b* and two *c* Cyt. Recently, the Commission on Biochemical Nomenclature (13) has recommended that the absorbance maxima be those determined at room temperature, and these be written as a suffix rather than a subscript. The *b* Cyt are *b*-556, *b*-560, and *b*-565 in this nomenclature; thus, b_{553} and *b*-556 are the same Cyt, as are b_{557} and *b*-560, b_{562} and *b*-565. For the *c* Cyt, c_{547} and *c*-550 are the same component, as are c_{549} and *c*-552. The numerical suffixes referring to the room temperature α bands will be used in this and subsequent papers in accordance with the Commission's recommendation.

is rapidly oxidized in synchrony with partial reduction of Cyt *b*-565 (26). This partial reduction of Cyt *b*-565 in plant mitochondria treated with antimycin A exactly parallels the same reaction of Cyt *b*-566 in animal mitochondria treated with antimycin A (29), and thus has nothing to do with the alternate pathway. This reaction, plus the rapid oxidation of the Cyt, also masks any reactions attributable to the alternate pathway. The use of CO as inhibitor of electron transport to O_2 through the main pathway should avoid these difficulties. The oxidation rate of the reduced Cyt a_3/CO complex depends on the thermal or photolytic dissociation of CO from the complex (8–10). In the presence of the measuring light of the spectrophotometer, the dissociation at room temperature is incomplete and occurs over a period of 3 to 5 sec (9), while the half-times for electron transport through the respiratory chain are characteristically 1 to 500 msec. With Cyt a_3 maintained in the reduced state as the CO complex for a time long enough for the components of the alternate pathway to transfer electrons to O_2 via the alternate oxidase, these electron transport reactions may be examined free of interference from the Cyt reactions. The criteria for identifying the point of interaction of the alternate pathway with the main pathway are rapid oxidation of a reduced electron transport carrier which is part of the main chain on addition of O_2 to CO-saturated anaerobic mitochondria, and sensitivity of this rapid oxidation to inhibition by *m*CLAM. A kinetic study of the oxidation by O_2 of the respiratory chain carriers of plant mitochondria made anaerobic in the presence of CO is reported in this paper. A preliminary report of this work has been presented in abstract form (27).

MATERIALS AND METHODS

Skunk cabbage (*Symplocarpus foetidus*) flowers were collected from selected marshy areas adjacent to the Wissahickon Creek in Whitemarsh Township, Pa., and stored at 4 C. Mitochondria were prepared from the excised spadices by the method of Bonner (6) as modified by Storey and Bahr (28). Mitochondria from the hypocotyls of 6-day-old etiolated mung bean (*Phaseolus aureus*) seedlings and from the tubers of the white potato (*Solanum tuberosum*) were prepared by the same method. Mitochondrial preparations obtained in this way are nearly as devoid of contamination and damaged organelles, as defined by density gradient distribution and by succinate-Cyt *c* reductase activity, as are mitochondria prepared by means of the more complex and time-consuming method of gradient purification (14).

All reagents obtained commercially were of the best grade available and were used without further purification. The uncoupler of oxidative phosphorylation, bishexafluoroacetonyl acetone, designated 1799, was a generous gift from Dr. Peter Heytler of E. I. du Pont de Nemours Co.

Kinetic measurements of respiratory chain carrier oxidation by O_2 pulses as monitored by characteristic absorbance changes were carried out in the manually operated, regenerative rapid-mixing flow apparatus with 0.1-cm optical path attached to a dual wavelength spectrophotometer equipped with a compensation circuit to reduce noise from light source fluctuations (12) by the same technique described in earlier studies (21). A pair of reflector arrays which increased the effective optical path length to 0.4 cm was used for the Cyt and flavoprotein absorbance measurements, while the 0.1 cm optical path was used for the ubiquinone absorbance measurements. Succinate plus malonate was used as reductant as previously described (21). Once the mitochondrial suspension had become anaerobic with succinate, it was saturated with CO by blowing pure CO gas over the surface while gently swirling. The CO-saturated suspension was then immediately transferred into the barrel of a storage syringe, taking care to avoid the introduction of a gas phase. The syringe was then attached to the flow apparatus and equilibrated with the medium in the flow apparatus, which is a completely closed system. In this way, loss of CO was avoided. The wavelength pairs used for monitoring the absorbance changes of the respiratory chain carriers characteristic of their redox state changes were as follows: ubiquinone, 282 and 295 nm; flavoprotein, 468 and 493 nm; Cyt a_3, 445 and 455 nm; Cyt *b*, 424 and 410 nm. The use of the very pronounced absorbance change observed during redox state changes of the Cyt *b* in the Soret region improves the signal-to-noise ratio when measuring rapid rates, and so the wavelength pair 424 and 410 nm, which monitors both Cyt *b*-566 and *b*-560 (21), was chosen, rather than the appropriate wavelength pairs in the region of the α band absorption. Under the conditions of these experiments, Cyt *b*-565 remains oxidized and does not contribute to the observed absorbance changes at 424 and 410 nm.

RESULTS

The time course of oxidation of O_2 of the respiratory chain carriers of skunk cabbage mitochondria presaturated with CO is shown in Figure 1. In contrast to the very short half-time of 0.8 msec for complete oxidation of Cyt a_3 by O_2 observed in the absence of CO (23), the half-time in the presence of CO (Fig. 3A) is 3 sec and the oxidation is only about 25% complete. The extent of oxidation of Cyt a_3 in the aerobic steady state under these conditions is set by the rates of CO dissociation under the influence of the measuring light and thermal recombination in competition with oxidation of uncomplexed a_3 by the limited amount of O_2 introduced into the suspension. The oxidation of the rapidly oxidized components of the Cyt *b* (Fig. 1B), ubiquinone (Fig. 1C), and flavoprotein (Fig. 1D) is, by contrast, complete within 1 sec. Recording of the complete cycle of flavoprotein oxidation and subsequent re-reduction on a slow time scale (record not shown) reveals that the flavoprotein component which is rapidly oxidized comprises 25 to 30% of the total oxidizable flavoprotein pool.

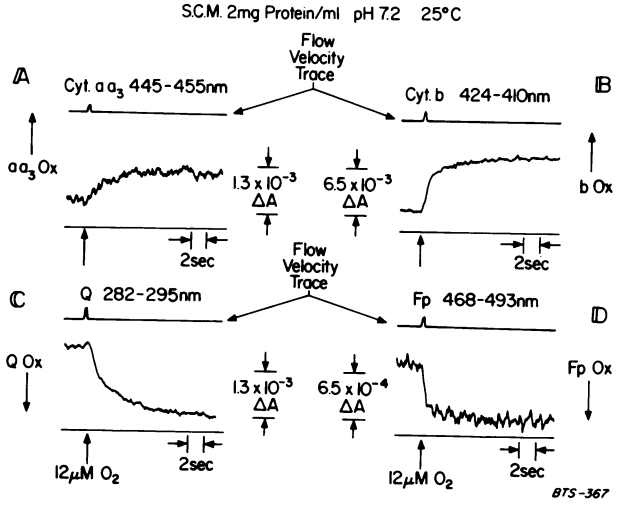

FIG. 1. Oxidation of Cyt *a*, and a_3 (A), the Cyt *b* (B), ubiquinone (C), and flavoprotein (D) in anaerobic, CO-saturated skunk cabbage mitochondria by means of an O_2 pulse delivered at the point shown by the arrow in each trace. The redox changes of the carriers were monitored by means of changes in absorbance using the dual wavelength spectrophotometer set at the wavelengths indicated in each trace. The mitochondria were suspended in 0.3 M mannitol, 10 mM TES, and 5 mM K-phosphate, pH 7.2 (medium TP), containing 0.2 mM ATP, 10 μM 1799, 5 mM succinate, and 60 mM malonate. At the point shown, O_2 was delivered in O_2-saturated medium TP by means of the rapid mixing flow apparatus to the anaerobic suspension to give an initial concentration of 12 μM. Oscilloscope sweep speed was 2 sec/cm for all traces.

The effect of adding *m*CLAM on the oxidation rate of these rapidly oxidized components was examined. The time course traces for ubiquinone and flavoprotein are given in Figures 2 and 3, respectively. The trace in A of each figure is the control in the absence of *m*CLAM, and the trace in B is in the presence of 1 mM *m*CLAM. In each case, those components which are rapidly oxidized in the absence of *m*CLAM are slowly oxidized in its presence. The effect of *m*CLAM on the redox state of ubiquinone in the coupled and uncoupled aerobic steady states is shown in Figure 4. The carrier is more reduced in both states in the presence of *m*CLAM. The time course of the oxidation of the Cyt *b* under these conditions is given by the experimental records in Figure 5. Two sets of records were obtained to show clearly the biphasic nature of the oxidation. Figure 5, A and C, shows the long time scale; Figure 5, B and D, shows the short time scale. Both in the presence and absence of *m*CLAM, one of

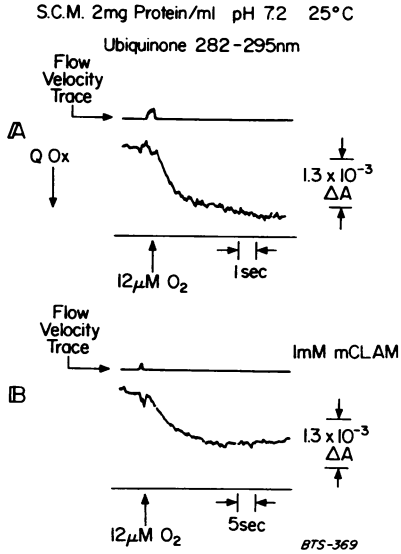

FIG. 2. Oxidation of ubiquinone in anaerobic, CO-saturated skunk cabbage mitochondria by means of an O_2 pulse in the absence (A) and presence (B) of 1 mM *m*CLAM. Experimental conditions were as described for Fig. 1. Note that the oscilloscope sweep speed was 1 sec/cm in A and 5 sec/cm in B.

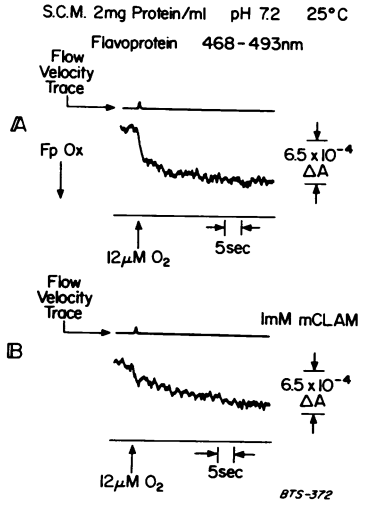

FIG. 3. Oxidation of flavoprotein in anaerobic, CO-saturated skunk cabbage mitochondria by means of an O_2 pulse in the absence (A) and presence (B) of 1 mM *m*CLAM. Experimental conditions were as described for Fig. 1. Oscilloscope sweep speed was 5 cm/sec for both traces.

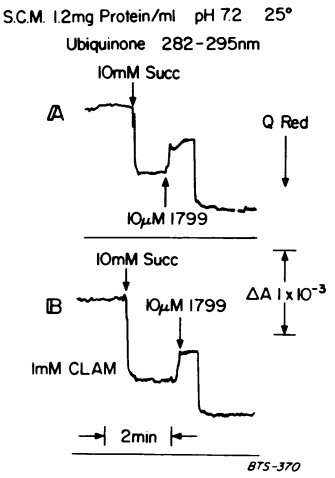

FIG. 4. Redox level of ubiquinone in the aerobic steady state in coupled and uncoupled skunk cabbage mitochondria in the absence (A) and presence (B) of 1 mM *m*CLAM. The mitochondria are suspended in medium TP (see Fig. 1) containing 0.2 mM ATP and oligomycin at 1 μg/mg mitochondrial protein. In A, ubiquinone is 66% reduced in the coupled state and 33% reduced in the uncoupled state induced by 10 μM 1799, taking reduction on anaerobiosis to be 100%. In B, ubiquinone is 71% reduced in the coupled state and 45% reduced in the uncoupled state.

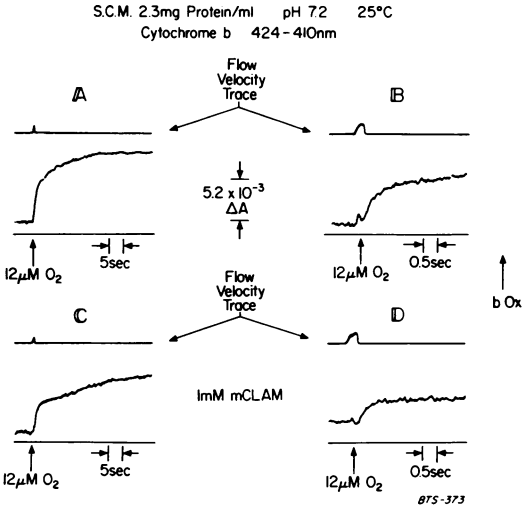

FIG. 5. Oxidation of the Cyt *b* in anaerobic, CO-saturated skunk cabbage mitochondria by means of an O_2 pulse in the absence (A and B) and presence (C and D) of 1 mM *m*CLAM. Experimental conditions were as described for Fig. 1. Note that the oscilloscope sweep speed in A and C is 5 sec/cm, while in B and D it is 0.5 sec/cm.

the Cyt *b* is rapidly and completely oxidized with a half-time of 200 msec. Similar experiments carried out in the wavelength range 555 to 565 nm show that this component is Cyt *b*-560, and that the component more slowly oxidized is Cyt *b*-556. In the absence of *m*CLAM, the rate of oxidation of the Cyt *b*-556 is sufficiently rapid that, on the slower time scale (Fig. 5A), its kinetics are not resolved in time from the Cyt *b*-560. On the faster time scale (Fig. 5B), the two components can be resolved in time, and the rate and extent of oxidation of the rapidly oxidized Cyt *b*-560 is seen to be the same in the absence and presence of *m*CLAM, while the oxidation rate of Cyt *b*-556 is markedly reduced by the inhibitor. Cyt *b*-565 remains oxidized under the conditions of these experiments and so does not interfere with these kinetic measurements (25).

In order to rule out the possibility that the effects of *m*CLAM observed in the kinetic experiments described above with skunk

cabbage mitochondria were due to a direct interaction with ubiquinone or with flavoprotein rather than direct inhibition of the alternate oxidase, the same set of experiments was carried out with mung bean mitochondria, which have low alternate oxidase activity, and with white potato mitochondria, which have essentially none (7). The results with mung bean mitochondria are shown in Figure 6 for ubiquinone and the Cyt *b*. The rate of ubiquinone oxidation (Fig. 6A) lies between that of uninhibited (Fig. 2A) and *m*CLAM-inhibited skunk cabbage mitochondria (Fig. 2B). The rate of ubiquinone oxidation in white potato mitochondria (Fig. 7A) is the same as that in *m*CLAM-inhibited skunk cabbage mitochondria (Fig. 2B). The effect of *m*CLAM must therefore be specifically that of inhibition of the alternate oxidase. In mitochondria from both mung bean seedlings and white potato tubers, the time course of oxidation of the two Cyt *b* is clearly biphasic (Figs. 6B and 7B) and resembles that observed in the trace of Figure 5C obtained with *m*CLAM-treated skunk cabbage mitochondria. In other experiments (records not shown), the more rapidly oxidized component was also found to be Cyto *b*-560 in mitochondria from these two tissues and to have an oxidation half-time of about 200 msec. This half-time is evidently independent of the presence or absence of the alternate oxidase, and so must be the half-time characteristic for the Cyt system with CO as inhibitor. This half-time is about 30 times longer than that observed for Cyt *b*-560 oxidation in the absence of respiratory inhibitors or in the presence of cyanide (22).

DISCUSSION

The time course of the oxidation of ubiquinone and flavoprotein in CO-saturated skunk cabbage mitochondria in O_2 pulse experiments represents a far more rapid rate than that of Cyt a_3, and this rate is sensitive to inhibition by *m*CLAM, the specific inhibitor for the alternate oxidase. The oxidation rate of ubiquinone in CO-treated white potato mitochondria, which contain no alternate oxidase, is the same as that of ubiquinone in CO-treated skunk cabbage mitochondria in the presence of *m*CLAM, and corresponds to the rate mediated through CO-inhibited a_3. The inhibitor *m*CLAM thus reproduces in skunk cabbage mitochondria the effect of the natural absence of alternate oxidase in white potato mitochondria. Ubiquinone is the component of the plant mitochondrial respiratory chain which best fits the criteria for the point of interaction of the alternate pathway with the main pathway. Ubiquinone is part of the main

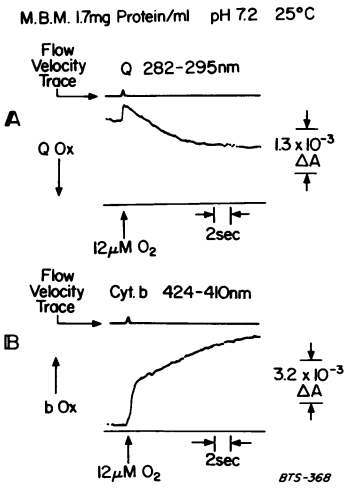

FIG. 6. Oxidation of ubiquinone (A) and the Cyt *b* (B) in anaerobic, CO-saturated mung bean mitochondria by means of an O_2 pulse. Experimental conditions were as described in Fig. 1. Oscilloscope sweep speed was 2 sec/cm for both traces.

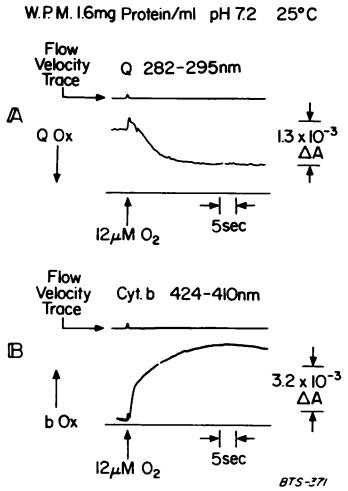

FIG. 7. Oxidation of ubiquinone (A) and the Cyt *b* (B) in anaerobic, CO-saturated white potato mitochondria by means of an O_2 pulse. Experimental conditions were as in Fig. 1. Oscilloscope sweep speed was 5 sec/cm for both traces.

pathway and has been shown to be that component of the plant respiratory chain which transports electrons directly from succinate and exogenous NADH dehydrogenases to the Cyt *b* (25), similar to its function in the respiratory chain of animal mitochondria (17, 19). It is rapidly oxidized on addition of O_2 to CO-saturated anaerobic mitochondria which have the alternate pathway, and this oxidation rate is sensitive to inhibition by *m*CLAM. Ubiquinone can be oxidized through both pathways and, since it is the electron transport carrier reacting directly with succinate and exogenous NADH dehydrogenases, is the logical choice for the point of interaction of the pathways. When the main pathway is blocked, reducing equivalents from succinate or exogenous NADH pass directly from dehydrogenase to ubiquinone to the components of the alternate pathway to O_2.

The flavoproteins of skunk cabbage mitochondria have been differentiated on the basis of their midpoint potentials, $E_{m7.2}$ (24). One of these, Fp_{ma} with $E_{m7.2} = +20$ mv, comprises 25% of the mitochondrial flavoprotein pool reduced under the conditions of these experiments. This flavoprotein component fits perfectly the specifications given by Bahr and Bonner (2) for the carrier of the alternate oxidase pathway which equilibrates rapidly its redox state with the branch point component on the main Cyt pathway: namely, ubiquinone. The flavoprotein Fp_{ma} has $E_{m7.2}$ sufficiently more negative than ubiquinone with $E_{m7.2} = +70$ mv (27) that a small change in the fraction of reduced ubiquinone produces a large change in the fraction of Fp_{ma} reduced. These are precisely the requirements for a switch which can divert the flux of electron transport from the Cyt chain as primary pathway in state 3 to the alternate oxidase as primary pathway in state 4. Bahr and Bonner (2) calculated that, for the switch to operate as observed, their component A interacting with the alternate pathway should have $E_{m7.2}$ at least 35 mv more negative than its partner, their component B, which interacts with the Cyt pathway and equilibrates with A. In this case, Fp_{ma} has $E_{m7.2}$ 50 mv more negative than ubiquinone.

The switch mechanism can be better appreciated by consideration of the steady state redox levels of ubiquinone reduction in skunk cabbage mitochondria seen in the trace of Figure 4A. In coupled, oligomycin-treated mitochondria, a condition comparable to state 4, ubiquinone is 66% reduced, while the uncoupled state, in which electron transport rates and the redox states of the carriers are comparable to those in state 3, it is 33% reduced. If Fp_{ma} is effectively in redox equilibrium with ubiquinone, it is 4% reduced in state 4 and 1% reduced in state 3, corresponding to a 4-fold change in percent reduction compared

to the 2-fold change for ubiquinone. If one makes the reasonable assumption that, at these low percent reductions, the rate of flux through the alternate oxidase is proportional to fraction of reduced Fp_{ma}, which is the identifiable component closest to the alternate oxidase, then the alternate oxidase would carry four times the flux in state 4 than it would in state 3. In the presence of either cyanide or antimycin A, ubiquinone is more than 90% reduced in the aerobic steady state (22). This would further increase the reduction of Fp_{ma}: at 90% and 99% reductions of ubiquinone, the percent reductions of Fp_{ma} would be 16% and 68%, respectively. At the higher percent reduction of Fp_{ma}, the flux would no longer be proportional to reduced Fp_{ma} but would be limited by the electron transport capacity of the alternate oxidase. This mechanism shows how the flux of electron can be diverted completely through the alternate oxidase in the presence of these two inhibitors of the Cyt chain.

The kinetic measurements reported here for the oxidation of the Cyt *b* in CO-saturated anaerobic mitochondria add further support to the concept that it is Cyt *b*-560 which reacts with the Cyt *c* of plant mitochondria, and the electron transport rate at this locus is much greater than that observed with animal mitochondria (21). Further, the rate at which electrons are transferred from Cyt *b*-556 to Cyt *b*-560 is relatively slow, so that, under the conditions of these kinetic experiments, oxidation of Cyt *b*-560 is rapid and complete, even in the presence of respiratory inhibitors. The respiratory chain of plant mitochondria seems to be so organized that Cyt *a*, a_3, *c*-552, *c*-550, and *b*-560 form a kinetic unit of rapidly reacting electron transport components, which is linked to ubiquinone and associated flavoproteins by Cyt *b*-556, which is more slowly oxidized by Cyt *b*-560. The group of components comprising ubiquinone, the flavoproteins Fp_{ma} and Fp_{ha}, and Cyt *b*-556 seem to be in quasi redox equilibrium with each other, and these form another kinetic unit. It is this second kinetic unit which connects to the alternate oxidase.

Acknowledgment—I am grateful to D. Rivers for highly skilled technical assistance.

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