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The Respiratory Chain of Plant Mitochondria

VIII. REDUCTION KINETICS OF THE RESPIRATORY CHAIN CARRIERS OF MUNG BEAN MITOCHONDRIA WITH REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE

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ABSTRACT

Addition of 90 micromolar reduced nicotinamide adenine dinucleotide (NADH) in the presence of cyanide to a suspension of aerobic mung bean (*Phaseolus aureus*) mitochondria depleted with ADP and uncoupler gives a cycle of reduction of electron transport carriers followed by reoxidation, as NADH is oxidized to NAD^+ through the cyanide-insensitive, alternate oxidase by excess oxygen in the reaction medium. Under these conditions, cytochrome b_{553} and the nonfluorescent, high potential flavoprotein Fp_{ha} of the plant respiratory chain become completely reduced with half-times of 2.5 to 2.8 seconds for both components. Reoxidation of flavoprotein Fp_{ha} on exhaustion of NADH is more rapid than that of cytochrome b_{553}. There is a lag of 1.5 seconds after NADH addition before any reduction of ubiquinone can be observed, whereas there is no lag perceptible in the reduction of flavoprotein Fp_{ha} and cytochrome b_{553}. The half-time for ubiquinone reduction is 4.5 seconds, and the extent of reduction is 90% or greater. About 30% of cytochrome b_{557} is reduced under these conditions with a half-time of 10 seconds; both cytochrome b_{562} and the fluorescent, high potential flavoprotein Fp_{hf} show little, if any, reduction. The two cytochromes c in these mitochondria, c_{547} and c_{549}, are reduced in synchrony with a half-time of 0.8 second. These two components are already 60% reduced in the presence of cyanide but absence of substrate, and they become completely reduced on addition of NADH. These results indicated that reducing equivalents enter the respiratory chain from exogenous NADH at flavoprotein Fp_{ha} and are rapidly transported through cytochrome b_{553} to the cytochromes c; once the latter are completely reduced, reduction of ubiquinone begins. Ubiquinone appears to act as a storage pool for reducing equivalents entering the respiratory chain on the substrate side of coupling site 2. It is suggested that flavoprotein Fp_{ha} and cytochrome b_{553} together may act as the branching point in the plant respiratory chain from which forward electron transport can take place to oxygen through the cytochrome chain via cytochrome oxidase, or to oxygen through the alternate, cyanide-insensitive oxidase via the fluorescent, high potential flavoprotein Fp_{hf}.

In the preceding paper describing the kinetic behavior of the high potential flavoproteins of mitochondria from skunk cabbage spadices (*Symplocarpus foetidus*), Erecinska and Storey (15) postulated that ubiquinone, cytochrome b_{553} (2, 3, 23), and the nonfluorescing high potential flavoprotein Fp_{ha} (24) are a trio of components in the plant respiratory chain which appear to act as a team. The oxidation rates of these three components are very similar, with half-time values clustering around 150 to 250 msec at 24 C and around 300 to 500 msec at 18 C (15, 22, 23, 27). They all become reduced fairly rapidly in oxygen pulse experiments upon exhaustion of oxygen, even when reducing equivalents can only trickle slowly into the respiratory chain from succinate in the presence of high concentrations of malonate. This behavior on anaerobiosis implies that they may have similar midpoint redox potentials at pH 7.2 at which all the kinetic studies were carried out, since the rate of input of reducing equivalents should be slow enough that the redox state of the respiratory chain components passes through a continuous series of near-equilibrium states during the process of reduction. The midpoint potentials of the electron transport carriers of the plant mitochondrial respiratory chain form a complex picture and will be dealt with in a future communication. The present study was designed to explore further the kinetic interactions of these carriers in the region of the respiratory chain encompassing the flavoproteins, the cytochromes *b* and *c*, and ubiquinone, with particular emphasis on the aforementioned trio of b_{553}, Fp_{ha}, and ubiquinone. The interaction among these components may provide the answer to an intriguing problem of plant mitochondria, which is simply stated: why *three* cytochromes *b*? The recent redox potential measurements of Wilson and Dutton (29) have uncovered two cytochromes *b* in mammalian and avian mitochondria with very similar spectra, in place of a single cytochrome *b*. Both of these are associated with coupling site 2 located between the *b* and *c* cytochromes (5, 14, 26). But plant mitochondria have three spectroscopically resolvable *b* cytochromes (2, 3, 4) and of the three, it is b_{553} which appears anomalous, both as regards oxidation kinetics and ease of reducibility (22).

The kinetic approach used here is complementary to the one used previously (15, 22, 24) in which a cycle of oxidation followed by reduction was initiated by a pulse of oxygen to mitochondria made anaerobic with succinate in the presence of malonate (6, 10, 11, 12, 13). In this study, reducing equivalents from NADH are fed into the respiratory chain in the presence of excess oxygen, taking advantage of the fact that mitochondria from plant tissues use exogenous NADH as substrate with respiratory control ADP/O ratios of about 1.5 indicating no interaction with coupling site 1, a K*m* of 70 μM, and no perceptible complications arising from the need to translocate the substrate inside the mitochondrion (18). It appears that exogenous NADH is dehydrogenated on the outside of the inner membrane and that the reducing equivalents go directly to that region of the respiratory chain encompassing ubiquinone, the cytochromes *b*, and the high potential flavoproteins. Because of the low K*m*, NADH pulses of 50 to 90 μM can be used to initiate a cycle of reduction followed by reoxidation

in a mitochondrial suspension kept aerobic by periodic treatment with air or oxygen. The results of NADH pulse experiments with depleted mung bean mitochondria are reported in this paper. Cyanide was used to inhibit cytochrome oxidase in order to increase the responses of the carriers to NADH pulses. The reoxidation of the carriers takes place through the alternate, cyanide-insensitive oxidase (1, 2, 21), and the time course of this reoxidation is readily followed. The nonfluorescent, high potential flavoprotein Fp_{ha} becomes nearly completely reduced by either NADH or succinate in the presence of cyanide, whereas its fluorescent companion, Fp_{hf}, remains highly oxidized (24). The reduction kinetics of Fp_{ha} alone can be studied under these conditions, free of complications from absorbance changes due to Fp_{hf}. This is not the case with antimycin A, nor with *m*CLAM,[1] a specific inhibitor of Fp_{hf} oxidation (15) and of electron transport through the alternate, cyanide-insensitive pathway (G. Schonbaum, W. D. Bonner, J. Bahr, and B. T. Storey, in preparation). For this reason, cyanide was the inhibitor of choice in these experiments which were designed primarily to examine Fp_{ha}, b_{553}, and ubiquinone. A comparison of reduction kinetics in the presence of antimycin A is also provided.

MATERIALS AND METHODS

Mitochondria were prepared from the hypocotyls of 6-day-old, etiolated mung bean (*Phaseolus aureus*) seedlings as described previously (22). The protein content of the mitochondrial suspension was determined by a modified Lowry method (19). NADH was obtained from Sigma; all other reagents were of the purest grade available. The uncoupler 1799 was the generous gift of Dr. Peter Heytler of E. I. duPont de Nemours Co. The redox state of the respiratory carriers was monitored as a function of time by means of the dual wave length spectrophotometer (7). The following wave length pairs were used for the various carriers: 468 to 493 nm for flavoprotein, 549 to 540 nm for c_{547}; 552 to 540 nm for c_{549}; 556 to 570 nm for b_{553}; 560 to 570 nm for b_{557}; 565 to 570 nm for b_{562}; and 282 to 295 nm for ubiquinone. (Subscripts give reduced minus oxidized difference absorbance maxima in spectra obtained at 77 K; Ref. 22 provides details concerning this nomenclature.) Rapid reactions were carried out in the manually operated, rapid mixing regenerative flow apparatus with 0.1-cm optical path (8, 9, 21) which has been used in previous studies. The time course of these reactions was recorded with a storage oscilloscope, and the traces were photographed to give permanent records.

The reaction medium was 0.3 M mannitol, 10 mM TES, 5 mM P_i, adjusted to pH 7.2 with KOH. The reduction of the carriers was initiated by addition of 90 μM NADH to the aerobic mitochondrial suspension depleted with 330 μM ADP and 7 μM 1799. The suspension was periodically treated with pure oxygen to make up for the oxygen consumed during NADH oxidation, during which time cyanide was also added to make up for cyanide consumed by reaction with NAD^+ accumulating in the mixture. The cyanide concentration was maintained between 0.2 and 0.4 mM.

RESULTS

The percentage reduction of the various carriers achieved by addition of 90 μM NADH to mung bean mitochondria in the presence of 0.27 mM cyanide can be estimated from the records of Figures 1 and 2. In these experiments, the pulse of 90 μM NADH is allowed to give a full cycle of reduction followed by complete

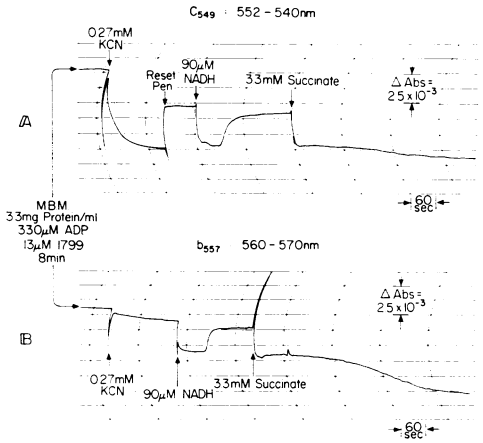

FIG. 1. Absorbance changes at 552 to 540 nm (A) and 560 to 570 nm (B) observed in depleted mung bean mitochondria (MBM) at 24 C upon addition of 90 μM NADH, followed by 3.3 mM succinate after consumption of NADH and reoxidation of cytochromes. A downward deflection of the trace indicates increased absorbance at 552 nm (A) and at 560 nm, corresponding to cytochrome reduction. The light path is 1.0 cm. The records were obtained with the Aminco-Chance dual wave length spectrophotometer, using an open cuvette containing a total reaction volume of 1.5 ml.

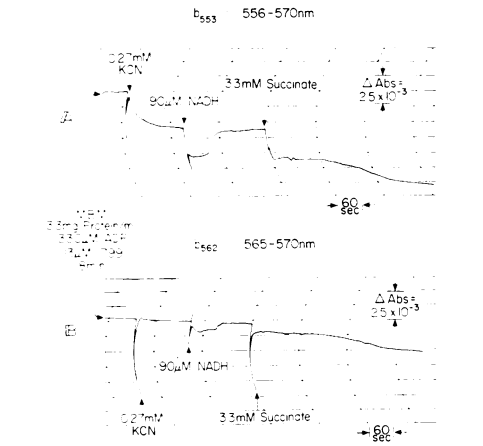

FIG. 2. Absorbance changes at 556 to 570 nm (A) and at 565 to 570 nm (B) observed in depleted mung bean mitochondria (MBM) under the same experimental conditions as in Figure 1. A downward deflection of the trace indicates increased absorbance at 556 nm (A) and at 565 nm (B), corresponding to cytochrome reduction.

reoxidation, and succinate is then added to give the aerobic steady state, followed by anaerobiosis. A similar set of experiments for the flavoprotein components of mung bean mitochondria has already been reported (24). Addition of cyanide to the aerobic mitochondrial suspension results in a 60% reduction of c_{549} (Fig. 1A), even in the absence of substrate (23); subsequent addition of 90 μM NADH gives essentially complete reduction of this component. Addition of succinate after exhaustion of the NADH and reoxidation of the cytochrome again produces complete reduction. The very small, slow absorbance change observed on anaerobiosis is attributed to cytochrome b_{557}, by comparison with Figure 1B. (The absorbance changes for c_{547} monitored at 549 to 540 nm in a parallel experiment are identical to those for c_{549}, but the slow absorbance change on anaerobiosis is so small as to be barely perceptible.)

The difference absorbance maxima for the three *b* cytochromes are sufficiently close together, and close to the *c* cytochromes, that no wave length pair is entirely specific for one component at room

[1] Abbreviations: *m*CLAM: *m*-chlorobenzhydroxamic acid; 1799: bis (hexafluoroacetonyl) acetone; TES: tris(hydroxymethyl)methylaminomethyl sulfonic acid; TTFA: 1-thienoyl-3,3,3-trifluoroacetone; MBM: mung bean mitochondria.

temperature, although the components can be separated kinetically (22). Thus, the absorbance changes recorded in Figure 1B, while largely due to b_{557}, also have contributions from b_{553} and b_{562}. The most conspicuous part of this trace is the large, slow change observed on anaerobiosis after addition of succinate, which is entirely attributable to b_{557}. The fast change observed on addition of NADH is attributable to b_{553}, the slower to b_{557}, by comparison with Figure 2A. This latter record was obtained with the wave length pair 556 to 570 nm, which is more suitable for monitoring b_{553}, although interference from c_{549} is evident from the absorbance change on prior addition of cyanide. The slow changes observed in Figure 1B are also seen but to only half the extent. On the other hand, the rapid change in Figure 2A, seen on adding NADH, is now larger than in Figure 1B. Of particular significance is the lack of a rapid change on anaerobiosis in either record, indicating that b_{553} is completely reduced in the presence of either 90 μM NADH or 3.3 mM succinate in the aerobic steady state with cyanide present. This result is expected from a previous study of the oxidation kinetics of this cytochrome (23). From Figures 1B and 2A, one can also estimate that about 30% of b_{557} is reduced in the aerobic steady state with cyanide, and that roughly half the absorbance change at 560 to 570 nm caused by NADH is attributable to b_{553}. The record shown in Figure 2B is very similar to that in 1B, except that the observed absorbance change is reduced by 60%. Most of this change is apparently attributable to b_{557}, rather than to b_{562}, which is evidently reduced only very slowly and to a very limited extent by 90 μM NADH pulses in depleted mung bean mitochondria.

In order to carry out experiments parallel to those of Figures 1 and 2 at wave lengths suitable for monitoring ubiquinone, it is necessary to use the shortest feasible optical path to minimize the interference provided by the absorbance of NADH itself in this region. This was accomplished with a rapid mixing regenerative flow apparatus of 0.1-cm path (8). The kinetics of ubiquinone reduction obtained with this apparatus are shown in Figure 3A for an intermediate time scale, corresponding to an oscilloscope sweep speed of 5 sec/cm. Upon addition of 90 μM NADH, there is a rapid downward deflection of the trace during the flow, corresponding to increased absorbance at 282 nm, caused by the

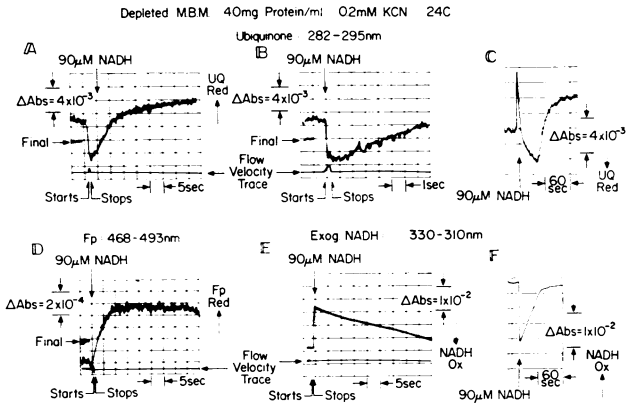

FIG. 3. Absorbance changes at 282 to 295 nm (A, B, C) observed on rapid mixing of 90 μM NADH with an aerobic suspension of mung bean mitochondria (MBM) in the regenerative flow apparatus with 0.1-cm optical path. An upward deflection of the trace in A and B, and a downward deflection in C, indicate decreased absorbance at 282 nm signifying ubiquinone reduction. Absorbance changes at 468 to 493 are shown in D for the same suspension under the same conditions as for A, B, and C. An upward deflection of the trace indicates decreased absorbance at 468 nm corresponding to flavoprotein reduction. Absorbance changes at 330 to 310 nm on addition of 90 μM NADH are shown in E and F on fast and slow time scales; a downward deflection of the trace in E indicates decreasing absorbance at 330 nm, corresponding to oxidation of the added NADH.

intrinsic absorbance of NADH itself. This is followed by an absorbance decrease, corresponding to ubiquinone reduction. About 90% of the ubiquinone in these mitochondria is reduced under these conditions; a like percentage is also reduced aerobically by succinate in the presence of either cyanide or antimycin A. There is a time lag of approximately 1.5 sec between addition of NADH and start of ubiquinone reduction, as shown in the record of Figure 3B, obtained with a sweep speed of 1 sec/cm. The reduction half-time, once reduction starts, is 4.5 sec. A complete cycle of ubiquinone reduction and reoxidation is shown in the record of Figure 3C which was obtained simultaneously with that of Figure 3A. What appears to be a slow phase of ubiquinone reduction lasting throughout the cycle in Figure 3C is actually decreased absorbance at 282 nm due to NADH consumption. This is evident by comparison of its time course with that observed directly for NADH consumption on the same time scale in Figure 3F. Once the exogenous NADH is used up, reoxidation of ubiquinone occurs quite rapidly through the alternate oxidase (Fig. 3C); the absorbance change observed for this process is due entirely to ubiquinone, and its extent is equal to that of the rapidly reduced component in the record of Figure 3A. The kinetics of ubiquinone reduction and reoxidation can be readily measured despite interference from NADH absorbance and from the small absorbance change at 282 to 295 nm as NADH is oxidized to NAD^+.

Also included in Figure 3 for comparison are the kinetics of flavoprotein reduction (Fig. 3D) and of NADH consumption (Fig. 3, E and F) obtained with same mitochondrial suspension. Measurement of flavoprotein reduction (Fig. 3D) is complicated in these experiments by an impurity in the NADH which absorbs at 493 nm relative to 468 nm but which fortunately undergoes no alteration in spectrum during these redox cycles. Addition of the NADH produces an absorbance decrease at 468 nm, which is in the direction of flavoprotein reduction, but this change actually occurs during the flow portion of the experiment corresponding to the addition process itself. This change is then followed by a further absorbance decrease at 468 nm with a half-time of 3 sec corresponding to flavoprotein reduction. Upon exhaustion of NADH, the trace returns to the point reached immediately after NADH addition (Fig. 3D). Reduction of flavoprotein, shown previously to be solely the complete reduction of Fp_{ha} under these conditions, has a shorter half-time than ubiquinone, and, as is shown more clearly in Figure 4A, shows no lag before reduction begins. The consumption of exogenous NADH (Fig. 3, E and F) is readily followed with the wave length pair 330 to 310 nm at which there should be minimal interference by the respiratory chain carriers. The first phase of NADH oxidation is the more rapid (Fig. 3E). The rate calculated from the steepest slope is 30 nmoles of NADH per min per mg of protein, which is about that expected in the presence of cyanide for mung bean mitochondria from the results of Ikuma and Bonner (18). It is considerably greater than that of 9 nmoles of ubiquinone per min per mg of protein calculated from the linear portion corresponding to ubiquinone reduction in Figure 3A, which is not unexpected since the alternate oxidase is fully active, and reducing equivalents are partitioned between this pathway to oxygen and the ubiquinone pool.

The reduction rates and redox cycles for Fp_{ha} and b_{553} observed upon adding 90 μM NADH to mitochondria in the presence of 0.4 mM KCN are shown in Figure 4. The reduction kinetics for Fp_{ha} were recorded with an oscilloscope sweep speed of 2 sec/cm in Figure 4A. It is evident that reduction begins immediately on addition of NADH without the time lag observed in ubiquinone reduction. The half-time of Fp_{ha} reduction in this experiment is 2.6 sec. The absorbance artifact due to the impurity in the added NADH is plain both in Figure 4A, where it manifests itself during the flow part of the experiment, and in Figure

4B, where it appears as an absorbance difference between the initial and final states of the redox cycle. Cytochrome b_{553} is reduced by NADH with a time course very similar to that of Fp_{ha} (Fig. 4C); the half-time is 2.8 sec. Examination of the record of Figure 4D, obtained simultaneously with that of Figure 4C, shows a more slowly reduced component, presumably b_{557}. Upon exhaustion of NADH, these components are reoxidized biphasically (Fig. 4D).

A similar set of experiments obtained with wave length pairs more suitable to b_{557} and b_{562} is shown in Figure 5. At 560 to 570 nm, both b_{553} and b_{557} are being monitored; the rough estimate from Figures 1B and 2A assigns to each component half the absorbance change caused by a 90 μM NADH pulse. The trace in Figure 5A shows biphasic kinetics. By splitting the absorbance change into two equal parts, one gets a half-time for the rapid phase of 2.5 sec, and a half-time for the slow phase of 10 sec. The first half-time is that expected for b_{553}, so the second is assigned to b_{557}. The redox cycle recorded at 560 to 570 nm (Fig. 5B) is very similar to that recorded at 556 to 570 nm (Fig. 4D). The absorbance change recorded at 565 to 570 nm on the more rapid time scale (Fig. 5C) is consistent with a half-time of about 10 sec. The change at these wave lengths recorded simultaneously on the slower time scale (Fig. 5D) also shows a reduction with similar half-time, followed by a barely perceptible further reduction attributable to b_{562}. Reoxidation on exhaustion of NADH is rapid. Since cytochrome b_{562} remains essentially completely oxidized under these conditions (Fig. 2B), the wave length pair 565 to 570 nm is primarily recording the redox behavior of b_{557} with little interference from b_{553}. The rapid reoxidation observed in Figure 5D is attributable to b_{557}, and this cytochrome then accounts for the rapid reoxidation phase observed in Figure 4A. It is cytochrome b_{553} which apparently shows the slower kinetics of reoxidation on exhaustion of NADH in these experiments.

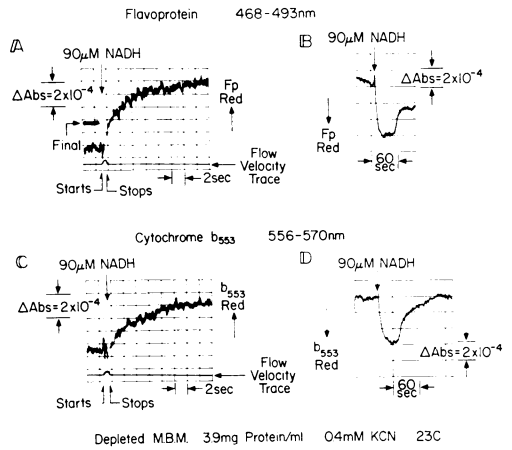

FIG. 4. Absorbance changes at 468 to 493 nm showing the kinetics of flavoprotein reduction (A) and a redox cycle (B) upon addition of 90 μM NADH to the aerobic mitochondrial suspension containing 0.4 mM KCN in the regenerative flow apparatus with 0.1-cm optical path. Reduction of flavoprotein gives decreased absorbance at 468 nm; the direction of the trace corresponding to reduction is shown in the figure. The records of A and B were obtained simultaneously. Absorbance changes at 556 to 570 nm observed with same mitochondrial suspension under the same conditions as in A and B are shown on fast (C) and slow (D) time scales in records obtained simultaneously. The direction of cytochrome reduction is shown in the figure; it is manifested by increased absorbance at 556 nm.

The kinetics of reduction by 90 μM NADH and the full redox cycles for c_{549} and c_{547} are shown in Figure 6. Both these components show a rapid reduction with a half-time of 0.8 sec (Fig. 6, A and C), the shortest half-time observed in these experiments with cyanide present. Both components show similar redox cycles characterized by a slow rate of reoxidation (Fig. 6, B and D). The reduction of the c components differs from that of the other components examined in that the two cytochromes c are already more than half reduced in the presence of cyanide but absence of added substrate. The reactions recorded in Figure 6 represent a transition from partial reduction to total reduction. In contrast, ubiquinone, Fp_{ha}, and the b cytochromes all remain oxidized in the presence of cyanide but absence of added substrate (23).

In the presence of antimycin A, reduction of flavoprotein and ubiquinone can also be accomplished by added NADH; the reduction kinetics upon addition of 50 μM NADH are shown in Figure 7, A and B. At the lower NADH concentration, the rates are slower than in the experiments with cyanide where 90 μM NADH was used. A slight lag in ubiquinone reduction is evident even on the slow scale of Figure 7B. The lag was determined to be 1.5 sec in a separate experiment on a faster time scale (not shown) which yielded a record virtually indistinguishable from Figure 3B. In this respect, ubiquinone shows consistent behavior, regardless of which respiratory inhibitor is used. Both Fp_{ha} and Fp_{hf} be-

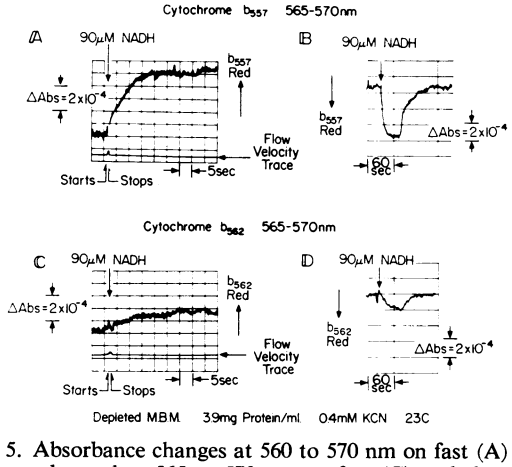

FIG. 5. Absorbance changes at 560 to 570 nm on fast (A) and slow (B) time scales and at 565 to 570 nm on fast (C) and slow (D) time scales are shown for 90 μM NADH additions carried out with the same suspension under the conditions described in Figure 4. Records A and B were obtained simultaneously, as were records C and D. Cytochrome reduction is manifested by increased absorbance at 560 nm(A, B) and at 565 nm (C, D).

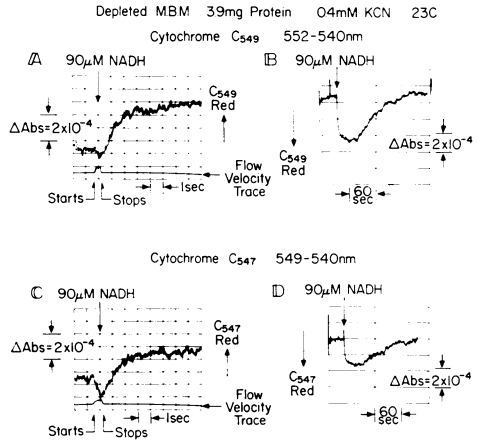

FIG. 6. Absorbance changes at 552 to 540 nm obtained simultaneously on fast (A) and slow (B) time scales and at 549 to 540 nm obtained simultaneously on fast (C) and slow (D) time scales are shown for 90 μM NADH additions carried out with the same suspension under the conditions described in Figure 4. Cytochrome reduction is manifested by increased absorbance at 552 nm (A, B) and at 549 nm (C, D).

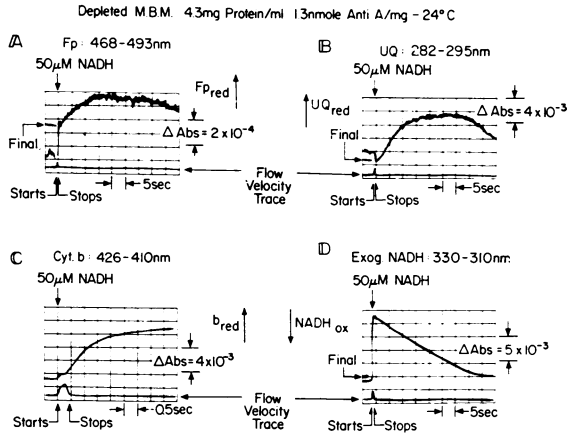

FIG. 7. Absorbance changes induced in depleted mung bean mitochondria treated with antimycin A upon addition of 50 μM NADH are shown at the wave length pairs: 468 to 493 nm (A) corresponding to flavoprotein, 282 to 295 nm (B) corresponding to ubiquinone, and 426 to 410 nm (C) corresponding to the cytochromes *b*. Flavoprotein and ubiquinone reduction result in an absorbance decrease at 468 and 282 nm, respectively; cytochromes *b* reduction results in an absorbance increase at 426 nm. The time course of NADH oxidation by the same mitochondrial suspension, which gives an absorbance decrease at 330 nm relative to 310 nm, is shown in D.

come partially reduced under these conditions (24), so that the absorbance changes in Figure 7A reflect the reduction of both components. No lag is observed, as verified in a separate experiment on a faster time scale, which is consistent with the result obtained with cyanide. In the absence of added substrate, antimycin A induces reduction of the *b* cytochromes. A series of experiments, identical to those of Figures 1 and 2 but with antimycin A replacing cyanide, showed that 70% of b_{553} and b_{557} are reduced while 40% of b_{562} is reduced in the absence of added substrate. Addition of 50 μM NADH gives complete reduction of the *b* cytochromes followed by reoxidation to the previous level of reduction as the NADH is consumed. (The *c* cytochromes remain fully oxidized under these conditions.) Reduction of the *b* cytochromes is rapid, as shown in Figure 7C, where these components are followed as a group in the Soret region at 426 to 410 nm. The reduction is biphasic, with the more rapid phase having a half-time of 0.6 sec. The more rapid reduction is attributed to b_{553} and b_{557} and the slower to b_{562}, by analogy with the reduction rates shown of these components observed in the presence of cyanide. The time course of NADH consumption in this experiment is shown in Figure 7D. Comparison of this record with Figure 7B shows that the reoxidation of ubiquinone starts rapidly upon exhaustion of added NADH. The kinetic behavior of ubiquinone in NADH pulse experiments with mitochondria treated with antimycin A is qualitatively similar to that observed with cyanide present, as would be expected from its kinetic behavior in oxygen pulse experiments (25).

DISCUSSION

The high extent and relative rapidity of the reduction of cytochrome b_{553}, flavoprotein Fp_{ha}, and ubiquinone by NADH in depleted mung bean mitochondria treated with cyanide is in agreement with the kinetic behavior of these carriers observed in oxidation experiments with both mung bean and skunk cabbage mitochondria. One surprising observation to emerge from this work is the lag of some 1.5 sec after NADH addition which occurs before reduction of ubiquinone begins. No such lag is observed with Fp_{ha} or with b_{553}. The lag time is the same whether electron transport through the cytochrome chain is inhibited by cyanide or by antimycin A, and it corresponds approximately to the time required for complete reduction of the cytochromes *c* in the former case and of the cytochromes *b* in the latter case. Only after these cytochromes are completely reduced and further electron transport to these components can no longer occur does ubiquinone start to be reduced. Thus, ubiquinone does not act in concert with either Fp_{ha} or b_{553} in electron transport; it appears to be acting as a storage pool for reducing equivalents entering the respiratory chain on the substrate side of coupling site 2, when these can no longer be accommodated by the cytochrome pathway to cytochrome oxidase. The simplest way to accommodate ubiquinone in the plant respiratory chain, given these results, is to have it interact directly with Fp_{ha} on a side path as previously suggested for skunk cabbage mitochondria (15, 25).

In NADH pulse experiments carried out in the presence of cyanide, electron transport between b_{553} and the *c* cytochromes is rapid on the time scale of reduction of the other carriers, in apparent conflict with the observation that the oxidation of b_{553} by the cytochrome *c* in both mung bean (23) and skunk cabbage mitochondria (15) is partially inhibited by cyanide. It is suggested that there is some inhibition of electron transport from b_{553} to the *c* cytochromes, and that the half-time for this reaction is given by the 0.8 sec for *c* reduction observed in these experiments. This value is four times that of 0.2 sec observed for the oxidation of b_{553} at 24 C in oxygen pulse experiments carried out in the absence of inhibitor. The half-time for Fp_{ha} oxidation by b_{553}, as measured in the presence of *m*CLAM to inhibit the alternate oxidase is also about 0.2 sec (15). A 4-fold decrease in the rate of electron transfer from b_{553} to the *c* cytochromes, with no concomitant decrease in the rate of electron transfer from Fp_{ha} (which in turn is fed by equivalents from reduced ubiquinone) will give a much reduced net rate of oxidation in oxygen pulse experiments. This problem of net rates is treated in detail elsewhere (22). It suffices to point out here that electron transport from Fp_{ha} to the cytochromes *c* in depleted plant mitochondria apparently takes place through b_{553} in both the absence and presence of cyanide, rather than through b_{557}. While the rate of electron transport from reduced b_{557} to the cytochromes *c* is very rapid as measured in oxidation experiments, the rate of electron transport to oxidized b_{557} is exceedingly slow in depleted mitochondria, particularly in the presence of cyanide, as is evident from Figure 1B.

The absence of a lag in Fp_{ha} reduction, and the further observation reported previously (24) that this component is reduced rapidly by NADH in the presence of both amytal and TTFA in anaerobicsis, while Fp_{hf} is reduced only slowly, imply that Fp_{ha} is closely linked to the exogenous NADH-dehydrogenase system. Electron transport apparently proceeds from NADH to Fp_{ha}, at which point three pathways become available. In depleted mitochondria, there is the pathway to the *c* cytochromes via b_{553} which is the dominant one, but which does not operate in coupled mitochondria (26). There is the pathway to b_{557} which is slow in depleted mitochondria, and there is the pathway to the alternate oxidase through Fp_{hf}. A detailed scheme of these interactions was presented in the preceding paper (15). These experiments with NADH pulses now make it possible to identify tentatively the combination of Fp_{ha} plus b_{553} with the branch point carrier *Y*, designated by Storey and Bahr (25) as the point at which the paths of electron transport in skunk cabbage mitochondria diverged from the main respiratory chain to the two terminal oxidases.

The rates of reoxidation of the carriers in the presence of cyanide, observed on exhaustion of NADH, reflect the interaction of the reduced carriers with only the alternate oxidase. In the presence of oxygen and 0.2 mM KCN, electron transport through cytochrome oxidase is effectively nil (18, 23), and all reducing equivalents in the respiratory chain have only the one outlet to oxygen. The reoxidations of Fp_{ha} and ubiquinone are relatively

rapid, the former being complete in 20 sec (Fig. 4A), the latter in 40 sec (Fig. 3C). Reoxidation of b_{557}, postulated to occur via Fp_{ha} (15), is also rapid (Fig. 5A), whereas that of b_{553} apparently requires nearly 80 sec for completion (Fig. 4B). This figure is probably high because of interference from the *c* cytochromes at the wave length pair used to monitor b_{553}. Reoxidation of the two cytochromes *c* requires some 100 to 120 sec for completion. Qualitatively, this order follows the expected midpoint potentials of the carriers, those with most positive midpoint potentials being the slowest to reoxidize. This may reflect the relatively slow rates of electron transport through the alternate oxidase in mung bean mitochondria, which makes it possible to attain the oxidized state on depletion of NADH through a continuous series of near-equilibrium states. On the other hand, these rates as measured by $t_{1/2\,off}$ (17) for the reduction-oxidation cycle also follow the kinetic ordering theorems of Higgins (17), assuming that Fp_{ha}, b_{553}, and the two *c* cytochromes form a linear sequence and that NADH interacts directly at Fp_{ha}. In any case, it is a novelty to observe a slow oxidation of the cytochromes *c*.

The experiments reported in this paper indicate that the duo Fp_{ha} and b_{553} operate as a team, rather than the trio including ubiquinone as previously suggested. Ubiquinone appears to function as a storage pool for reducing equivalents entering the respiratory chain at Fp_{ha}. As was pointed out in the previous paper (15), assignment of this function to ubiquinone would predict that its midpoint redox potential in these mitochondria at pH 7 lie between the 89 mv calculated by Slater (20) from solution measurements and the 66 mv determined in beef heart particles by Urban and Klingenberg (28). These experiments also indicate that the previously postulated branch point carrier *Y* is actually Fp_{ha} plus b_{553} in *depleted* mitochondria. In coupled mitochondria, it appears that electron transport proceeds from b_{553} to b_{557}, and that coupling site 2 is between b_{557} and the *c* cytochromes (10, 26). The need for three *b* cytochromes may arise from the fact that the system of reverse electron transport from ubiquinone to endogenous pyridine nucleotide in plant mitochondria has its own separate route, which operates in increments from b_{553} through b_{557} to b_{562}, from more positive to more negative redox potential. Such a system may be an "early design," which was streamlined in the course of evolution to that observed in mammalian and avian mitochondria. This route may utilize energy from any of the coupling sites, but not utilize the same carriers as energy coupling site 1. This hypothesis remains to be tested. It is evident that reliable midpoint redox potentials for the electron transport carriers of the plant respiratory chain are now required to supplement these kinetic data in order to develop an electron transport scheme with a firmer foundation. These measurements are in progress.

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