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The Respiratory Chain of Plant Mitochondria

IV. OXIDATION RATES OF THE RESPIRATORY CARRIERS OF MUNG BEAN MITOCHONDRIA IN THE PRESENCE OF CYANIDE

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ABSTRACT

The half-time for oxidation of cytochrome b_{557} in mitochondria from etiolated mung bean (*Phaseolus aureus*) hypocotyls is 5.8 milliseconds at 24 Celsius in the absence or presence of 0.3 mM KCN, when the oxidation is carried out by injecting a small amount of oxygenated medium into a suspension of mitochondria made anaerobic in the presence of succinate plus malonate. Since oxygen is consumed by the alternate, cyanide-insensitive respiratory pathway of these mitochondria, cycles of oxidation and reduction can be obtained with the oxygen pulses when cyanide is present. Reduced cytochromes ($a + a_3$) also become oxidized at nearly the uninhibited rate under these conditions, a_3 completely and a partially. The half-time for oxidation of c_{547} is also unaffected by 0.3 mM KCN, but c_{549} has a half-time equal to that of c_{547} in the presence of KCN, compared to the shorter one observed in the absence of inhibitor. The maximum extent of oxidation of the cytochromes c is about 70% in the presence of 0.3 mM KCN; this oxidation is rapidly followed by an extensive reduction which is synchronous with the reduction of cytochrome a observed under the same conditions. In the presence of cyanide, it appears likely that the cytochromes c and b_{557} are oxidized by cytochrome oxidase in oxygen pulse experiments, rather than by the alternate oxidase. The oxidation of cytochrome b_{553} is partially inhibited by KCN, but complete oxidation is attained in the aerobic steady state with excess oxygen. If the oxygen pulse experiment is carried out in the presence of sufficient malonate so that entry of reducing equivalents into the respiratory chain occurs at a rate negligible compared to intercarrier electron transport, the half-time for flavoprotein oxidation is unaffected by 0.3 mM KCN while that for ubiquinone oxidation is but 2-fold larger. The observed net oxidation rate of these two carriers in mung bean mitochondria is more sensitive to the entry rate of reducing equivalents, as set by succinate concentration and malonate to succinate ratio, then it is in skunk cabbage (*Symplocarpus foetidus*) mitochondria. These observations are interpreted in terms of a respiratory carrier Y, placed between flavoprotein plus ubiquinone and the cytochromes, which is the fork in the split respiratory pathway to the two terminal oxidases and which has lower electron transport capacity in mung bean mitochondria than in skunk cabbage mitochondria.

The case for three distinct cytochromes b in mitochondria from plant tissues has been convincingly argued by Bonner (5-7, 15), and Lance and Bonner (30) based on evidence from reduced-minus-oxidized difference spectra obtained at −196 C under a wide range of conditions with mitochondria from a variety of sources. The three b cytochromes in mitochondria from etiolated mung bean (*Phaseolus aureus*) hypocotyls are also differentiated by rates of oxidation and ease of reducibility (33). Of these, cytochrome b_{557}[1] is the most rapidly oxidized, with a half-time of around 8 msec at 18, and is completely reduced by the substrate ascorbate plus TMPD[2] in uncoupled, depleted mitochondria, conditions under which b_{553} is only partially reduced while b_{562} remains largely oxidized (33). Bonner (15) has suggested that b_{557} is identical to cytochrome b_7 which Bendall and Hill (4) found in mitochondria from the spadix of *Arum maculatum*, and to which they assigned a role in the cyanide-insensitive pathway so prominent in these mitochondria. The oxidation of cytochrome b_7 in mitochondria from *Arum* is not inhibited by cyanide or antimycin A (1, 4, 24), whereas b_{557} oxidation in mung bean mitochondria made anaerobic with succinate is severely inhibited in the presence of antimycin A (7, 33). With mitochondria isolated from the spadix of the skunk cabbage (*Symplocarpus foetidus*), which resemble mitochondria from *Arum* in having little or no sensitivity to respiratory inhibition by cyanide (3, 11, 25, 37), Storey and Bahr (35) found under the same conditions a high degree of inhibition of b_{557} oxidation in the presence of antimycin A, and some inhibition in the presence of cyanide. With the latter inhibitor present at concentrations saturating for respiration through the cytochrome oxidase pathway, they also observed a partial oxidation of cytochrome c at a rate comparable to the rate in the absence of inhibitor. These observations prompted a more detailed examination of the effect of cyanide on the redox reactions of the respiratory chain carriers of mung bean mitochondria. These mitochondria show a respiration rate with succinate amounting to about 20% of the maximum state 3 rate which is insensitive to both antimycin A and cyanide (29). The oxidation kinetics of different respiratory carriers in the presence of high cyanide concentrations can be measured in one experiment with the regenerative flow apparatus by means of redox cycles initiated by an oxygen pulse to anaerobic mitochondria (17), since oxygen consumption can still proceed by the cyanide-insensitive pathway. In this paper, the oxidation kinetics of the respiratory carriers of mung bean mitochondria are examined at cyanide concentrations known to be saturating

[1] The subscripts refer to the difference absorbance maxima observed for these cytochromes in reduced-minus-oxidized spectra of mitochondrial suspensions obtained at −196 C. The difference maxima observed at room temperature are shifted 3 mμ toward the red.

[2] Abbreviations: TMPD: N,N'-tetramethyl phenylenediamine; TES: tris(hydroxymethyl)methylaminoethyl sulfonic acid; 1799: bis-(hexafluoroacetonyl) acetone; E_{m7}: midpoint potential at pH = 7 referred to Normal Hydrogen Electrode, following the nomenclature of Clark (20).

for the cyanide-sensitive respiratory pathway in mung bean mitochondria (29) with the object of defining the role of the cytochromes *b* and *c* in the cyanide-resistant respiratory pathway of plant mitochondria. In the following paper (34), some further aspects of the oxidation of reduced cytochromes ($a + a_3$) in the presence of cyanide are reported.

MATERIALS AND METHODS

Mitochondria were prepared from the excised hypocotyls of 6-day-old mung bean (*Phaseolus aureus*) seedlings, following the method of Ikuma and Bonner (28) with the modifications described by Storey and Bahr (35). All chemicals used were of the purest grade available commercially. The uncoupler 1799 was kindly supplied by Dr. P. Heytler of E. I. DuPont de Nemours Co. This uncoupler is fully effective at 6 μM (35). Its absorption spectrum has but one maximum at 280 mμ with $\epsilon' = 0.03$ mM^{-1} cm^{-1}; as a result it does not interfere with ubiquinone determination in the ultraviolet region of the spectrum.

The respiratory activity and respiratory control quotient of each mitochondrial preparation were determined polarographically according to Estabrook (22) to ensure that the mitochondria were isolated intact (8). For this determination, mitochondria were suspended at 0.6 to 1.2 mg of protein per ml in a medium at pH 7.2 containing 0.3 M mannitol, 10 mM TES, and 4 mM phosphate. The same medium was used for all kinetic experiments. The protein content of each preparation was determined by a modified Lowry method (31).

The rates of oxidation of the respiratory carriers were measured by essentially the same method described in the previous paper (33). In all these experiments, the aerobic mitochondrial suspension was preincubated for 12 to 15 min with 130 μM ADP and 10 μM 1799 to deplete the mitochondria of energy conservation capacity. The mitochondrial suspension was then made anaerobic by addition of 3 to 5 mM succinate; from 6 to 30 mM malonate was added to inhibit partially succinate dehydrogenase. The flow experiment was initiated by mixing the anaerobic mitochondrial suspension with oxygen-saturated medium at a volume ratio of 64:1 or of 100:1 in a rapid mixing flow apparatus. Two of these instruments were used for this study. One was a manually operated regenerative flow apparatus with 0.1-cm light path designed by Chance (14). The other was a gas-driven regenerative flow apparatus similar to the one described recently by Chance and co-workers (16, 18) with a 0.6-cm light path and two observation points, one for monitoring reactions in the time domain 0.1 to 10 msec and the other for reactions in the time domain 5 to 500 msec or more. Absorbance changes corresponding to redox changes of the carriers were monitored with the dual wave length spectrophotometer (13). In order to obtain a kinetic spectrum, the reference wave length was set at 570 mμ, and the measuring wave length was varied over the range 540 mμ to 570 mμ as previously described. Cytochromes ($a + a_3$) were monitored with the wave length pair 445 to 455 mμ (10); for cytochromes c_{547}, c_{549}, b_{557}, and b_{553}, the wave length pairs 549 to 540 mμ, 552 to 540 mμ, 560 to 570 mμ, and 556 to 570 mμ, respectively, were used (33). Flavoprotein was monitored with the wave length pair 468 to 493 mμ; as in the previous experiments with skunk cabbage mitochondria (35), the kinetics of the absorbance change in depleted mung bean mitochondria were synchronous with the changes in fluorescence obtained with a fluorometer designed to measure flavoprotein fluorescence (19). Ubiquinone was monitored with the wave length pair 282 to 295 mμ (36). Fast changes were displayed on a storage oscilloscope and photographed to give permanent records; slow changes were displayed on a pen recorder chart.

Difference spectra of the mitochondrial suspensions at liquid nitrogen temperature were obtained with the split beam spectrophotometer described by Chance (13), using the technique described by Estabrook (21) as modified by Bonner (5).

RESULTS

An anaerobic-minus-aerobic difference spectrum at −196 C (77 K) of depleted mung bean mitochondria treated with succinate and 0.3 mM cyanide is shown in Figure 1A. Malonate is present in the mitochondrial suspension to ensure maximum oxidation of the carriers in the aerobic steady state which acts as reference in the difference spectrum. The maxima at 553 and 557 mμ, and the single maximum at 424 mμ, indicate the oxidation of b_{553} and b_{557} under these conditions in the presence of cyanide. The shoulder at 548 mμ shows partial oxidation of c_{547} and c_{549} in the aerobic reference sample. The small maximum at 588 mμ and shoulder at 445 mμ represent the reduced-minus-oxidized difference spectrum of cytochrome a_3 in the presence of cyanide (3). The cytochrome *a* is completely reduced in the aerobic steady state under these conditions. In the presence of cyanide, this component becomes reduced in aerobic depleted mitochondria in the absence of added substrate and presence of

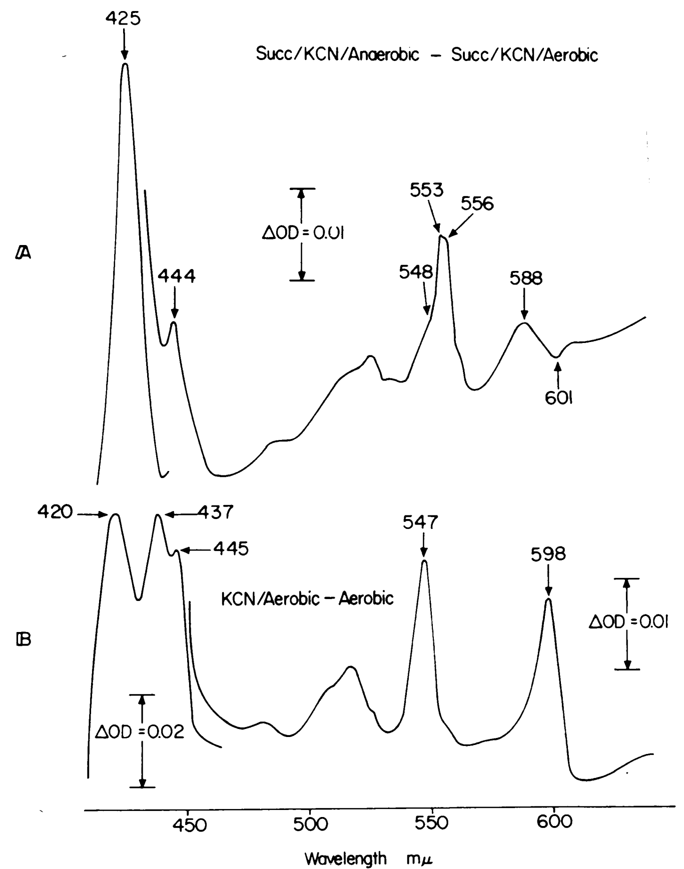

FIG. 1. Low temperature difference spectra obtained with depleted mung bean mitochondria. The optical path length is 0.2 cm. The mitochondrial suspension contained 11.2 mg of protein per ml, 0.2 mM ADP, and 10 μM 1799 and was preincubated aerobically for 12 min to achieve depletion. A: Succinate (5 mM) was added to the mitochondrial suspension, which was then allowed to go anaerobic; 50 mM malonate and 0.4 mM KCN were then added with minimal stirring. The reference sample was then treated vigorously with O_2, while the measured sample was treated with N_2. B: No substrate was added in this experiment. The aerobic suspension after depletion served as reference; the measured sample contained 0.4 mM KCN. The recording sensitivity is 2-fold less in the Soret then in the α-region of the spectrum.

malonate as do the cytochromes *c*, as shown in Figure 1B, but the cytochromes *b* remain oxidized. The extent of reduction with added cyanide under these conditions was checked with the dual wave length spectrophotometer, using the wave length pairs 549 mμ to 540 mμ and 552 mμ to 540 mμ for cytochromes c_{547} and c_{549}, respectively (33), and the wave length pair 603 mμ to 620 mμ for cytochrome *a* (34). For cytochrome c_{547}, the extent of reduction was found to be 57%; for c_{549}, it was 59%; and for cytochrome *a*, it was 89%. Both *c* cytochromes, therefore, became reduced to the same extent.

The time course of cytochrome *b* oxidation, initiated by a pulse of oxygenated medium to a suspension of mung bean mitochondria made anaerobic with succinate, is shown in Figure 2 as recorded with the regenerative flow apparatus at the wave length pair 560 to 570 mμ. In the absence of inhibitor (Fig. 2A), the oxidation is biphasic. There is a rapid reaction, partially complete during the flow, with a half-time of 5.8 msec, followed by a slower one with a half-time of 400 msec. The fast reaction has been shown to be that of b_{557}, while the slower one is b_{553} (33). In the presence of 0.3 mM cyanide (Fig. 2B) the rapid oxidation is still observed at 560 to 570 mμ with a half-time of 5.9 msec; the slower one does not appear on this time scale.

The time course of oxidation at 18 C of cytochrome c_{547} and c_{549} in the presence of 0.3 mM KCN, recorded at 549 to 540 mμ and 553 to 540 mμ, respectively, is shown in Figure 3 along with the time course for cytochrome $(a + a_3)$ oxidation recorded on the same sample at 445 to 455 mμ. The time scale for these records was chosen to show both the oxidation, which is well advanced during the flow, and the rapid partial rereduction which occurs after an aerobic steady state lasting about 0.3 sec. This partial reduction is not observed in the absence of cyanide. The oxidation half-times are 3.0 and 3.1 msec for c_{547} and c_{549}, respectively (Figs. 3A and 3B), compared to 3.1 and 2.5 msec in the absence of cyanide. The maximum extent of oxidation is about 70% of that observed with no cyanide. The partial reduction occurring after the brief aerobic steady state has a half-time of 0.7 sec for both cytochromes *c*. Its time course follows exactly that observed at 445 to 455 mμ for cytochromes $(a + a_3)$

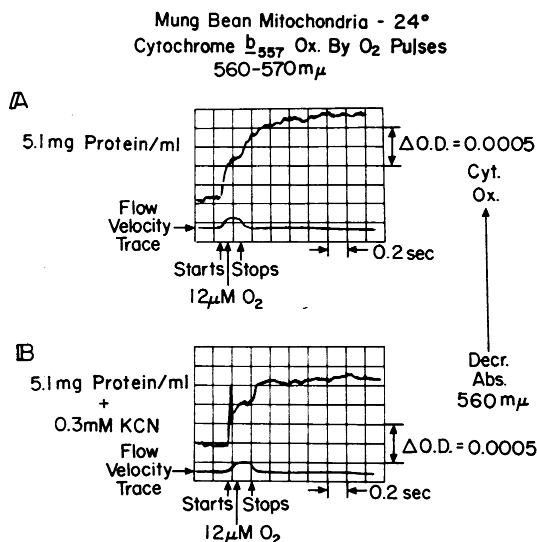

FIG. 2. Absorbance changes corresponding to oxidation of cytochrome b_{557} in mung bean mitochondria recorded with the dual wave length spectrophotometer at 24 C. The optical path length is 0.1 cm and the response time of the detection system is 60 msec. Reducing equivalents are provided by 2.5 mM succinate in the presence of 30 mM malonate. A: Oxidation with no inhibitor present; B: oxidation in the presence of 0.3 mM KCN. The "spike" in the record is caused by a flow disturbance as the flow starts.

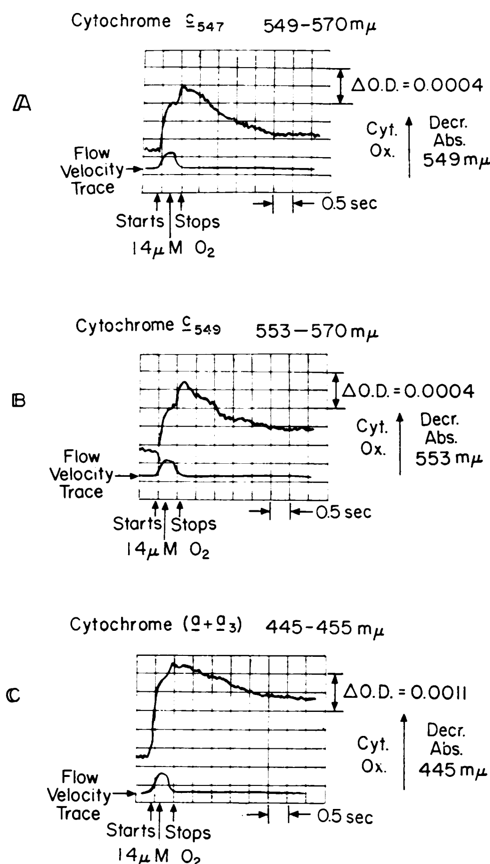

FIG. 3. Absorbance changes corresponding to oxidation of cytochrome c_{547}, cytochrome c_{549}, and cytochrome $(a + a_3)$ at 18 C in the presence of 0.2 mM KCN, with the wave length pairs 549 to 570 mμ (A), 553 to 570 mμ (B), and 445 to 455 (C), respectively. The optical path is 0.1 cm and the response time of the detection system is 50 msec. Reducing equivalents are provided by 5 mM succinate in the presence of 10 mM malonate.

(Fig. 3C), which is due to the *a* component (34). In the final aerobic steady state achieved after the rapid reduction, c_{547} is oxidized to the extent of 29% of that achieved initially and c_{549} to the extent of 36%, corresponding to 20 and 25% of the total contents, respectively. Cytochrome a_3 is fully oxidized.

A kinetic spectrum for the rapid oxidation reactions of the *b* and *c* cytochromes in the presence of 0.3 mM KCN at 24 C over the wave length range 540 to 570 mμ, with the use of the regenerative flow apparatus, is shown in Figure 4 for a single mitochondrial suspension. Cytochrome b_{557} appears clearly with an absorbance maximum at 560 mμ and a half-time of 5.8 msec. Cytochromes c_{547} and c_{549} also appear as a maximum at 552 mμ and a shoulder at 550 mμ. Their oxidation half-times are both about 2.5 msec, in contrast to the shorter half-time normally observed for c_{549} in the absence of respiratory inhibitor or presence of antimycin A (33). The difference absorbance values for the *c* cytochromes in Figure 4 are for the maximum extent of oxidation and thus appear greater in relation to the absorbance value for b_{557} than in the difference spectrum of Figure 1A.

Cytochrome b_{553}, with a room temperature absorbance maximum at 556 mμ, has too long a half-time to appear in the kinetic spectrum of Figure 4 which plots the absorbance changes for reactions rapid enough to proceed during the flow portion of the experiment. Cytochrome b_{553} has an oxidation time of 0.5 sec at 18 C in the absence of inhibitor (33) and accounts for the slower portion of the biphasic trace of Figure 2A. Its oxidation half-time is increased sufficiently by the presence of cya-

nide that it does not appear in the trace of Figure 2B. Another feature of the redox reactions of b_{553} becomes evident on comparison of the experimental records presented in Figure 5. These show complete oxidation-reduction cycles resulting from a pulse of O_2 to the anaerobic mitochondrial suspension. In Figure 5A, the cycle is monitored at 560 to 570 mμ to avoid interference from *c* cytochromes in the absence of cyanide. A third phase of oxidation with a half-time near 10 sec, followed by a biphasic reduction with a fast phase preceding a slower one, is the normal pattern observed with this wave length pair. In the presence of cyanide (Figs. 5B and 5C), the small "peak" observed in the record of Figure 5A is missing, and a steady state lasting some 20 sec is established. The record obtained with the wave length pair 556 to 570 mμ (Fig. 5C), which is more suitable for monitoring b_{553}, is very similar to that obtained at 560 to 570 mμ (Fig. 5B) but shows much less of the fast component. Interference from *c* cytochromes at this wave length pair is minimal in the presence of cyanide. The three records in Figures 5A, 5B, and 5C were obtained by an oxygen pulse of initial concentration 12 μM (24 natom O/ml) into an anaerobic mitochondrial suspension containing 3.8 mg of protein per ml, corresponding to a small excess of oxidizing equivalents over the reducing equivalents available in the reduced respiratory chain carriers. In Figure 5D, the initial oxygen concentration is 19 μM (38 natom O/ml) and the mitochondrial suspension contains 1.7 mg of protein per ml. Under these conditions of excess oxygen, in the presence of cyanide, there is a pronounced peak in the trace, the oxidation half-time being about 10 sec, followed by a biphasic reduction in which the first phase is more rapid than the second one. In the experiments with small excess of oxygen, the malonate to succinate ratio was 12; in the experiment with greater excess oxygen it was 10. Malonate to succinate ratios greater than this at 2 to 3 mM succinate have no further effect on the extent of carrier oxidation at these concentrations of mitochondrial protein (J. T. Bahr, unpublished results). In the presence of cyanide, cytochrome b_{553} is oxidized more slowly but appears to be reduced at nearly the same rate as in its absence. The balance between these two rates is such that, under the conditions of low

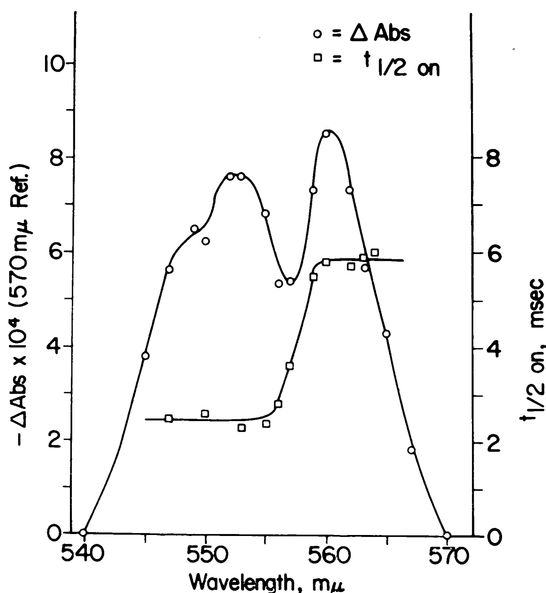

FIG. 4. Absorbance changes and half-times at 24 C for the cytochrome components of mung bean mitochondria, which are rapidly oxidized in the presence of 0.3 mM KCN, plotted as a function of wave length with 570 mμ as reference wave length. Experimental conditions are those described in Figure 2.

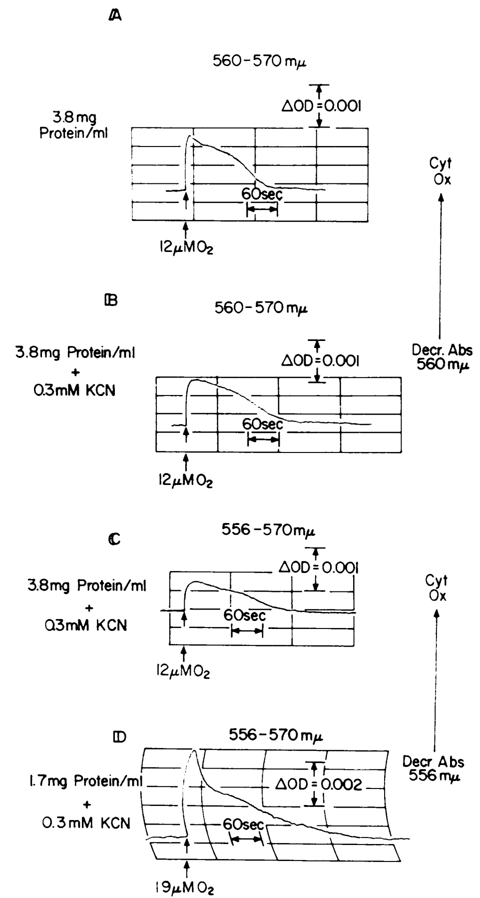

FIG. 5. Absorbance changes corresponding to oxidation-reduction cycles of cytochromes b_{557} and b_{553} in mung bean mitochondria monitored by absorbance changes at 560 to 570 mμ in the absence (A) and presence of 0.3 mM KCN (B). In both traces the rapidly oxidized major component is b_{557}. Absorbance changes at 556 to 570 mμ corresponding to redox cycles in the presence of 0.3 mM KCN of b_{553} and b_{557} at slight excess of oxygen over carriers (C) and substantial excess of oxygen over carriers (D). In both traces, the rapidly oxidized component is b_{557}, and the slowly oxidized component is b_{553}.

oxygen excess applicable to Figures 5A, 5B, 5C, little b_{553} becomes oxidized. Under the conditions of higher excess oxygen in Figure 5D, more b_{553} becomes oxidized. Under the conditions of oxygen surfeit and ample time allowed for oxidation of the aerobic sample used as reference in the difference spectrum of Figure 1A, b_{553} becomes completely oxidized.

The experiments described above are designed to maximize the extent of carrier oxidation in the aerobic steady state so that maximum absorbance changes can be used to measure maximum rates. Under the conditions normally used to measure the cyanide-insensitive respiration of mung bean mitochondria, which are those intended to maximize electron transport, succinate is present at 5 to 10 mM, and malonate is absent. Further, the mitochondria are not depleted, since depletion inhibits succinate oxidation. If mung bean mitochondria are first treated with ATP and then uncoupled, conditions which allow near maximal electron transport rates with succinate (28), the two cytochromes *c* in the aerobic steady state with 0.3 mM KCN and 5 mM succinate are completely reduced, as is cytochrome *a*. Cytochromes b_{557} and b_{553} both appear to be 58% oxidized, which implies that the reduction rates of these two carriers are still slow compared to their oxidation rates, even under these conditions. Certainly, the prominence of b_{557} in the kinetic spectrum of Figure 4 is a consequence of its rapid rate of oxidation and

very slow rate of reduction under the conditions of the experiment.

Cytochrome b_{562} is also missing from the kinetic spectrum of Figure 4. This component of the plant mitochondrial respiratory chain has been shown to form part of the energy-linked mechanism by which reducing equivalents are transferred from succinate to pyridine nucleotide (16, 33). In mitochondria depleted aerobically with uncoupler and ADP in the presence of phosphate, b_{562} becomes at best only partially reduced in anaerobiosis, and the presence of cyanide may further inhibit this reduction (29).

Storey and Bahr (35) showed with depleted skunk cabbage mitochondria that ubiquinone and the high potential flavoproteins which become reduced in anaerobiosis are oxidized at the same rate in the presence of either cyanide or antimycin A, in contrast to the differing effects of either inhibitor on the oxidation rates of the cytochromes *b* and *c*. They concluded that both components interacted with the alternate, cyanide-insensitive terminal oxidase present in these mitochondria in accord with the earlier proposal of Bendall, Bonner, and Plesnicar (3). The same result is obtained with mung bean mitochondria; both oxidation rates and extents, and redox cycle times, are affected in just the same way by either inhibitor. Ubiquinone and flavoprotein are thus convenient intrinsic probes for the redox reactions of the alternate oxidase. The time course of oxidation of ubiquinone in mung bean mitochondria initiated by an oxygen

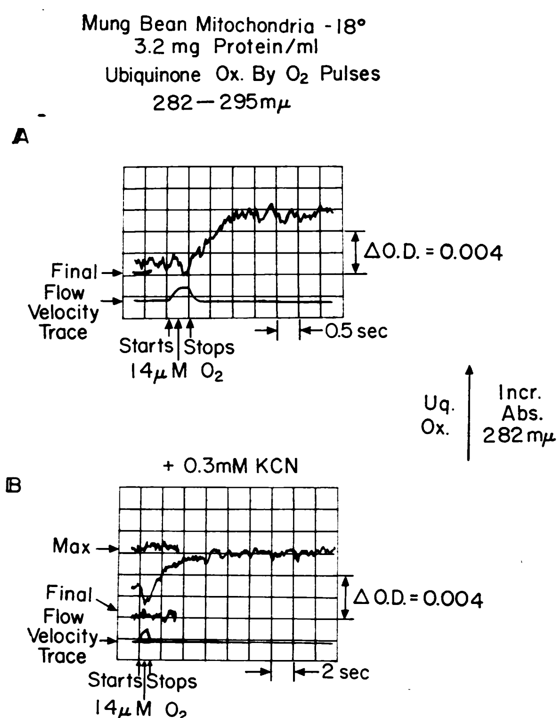

FIG. 6. Absorbance changes corresponding to ubiquinone oxidation in mung bean mitochondria recorded at 282 to 295 mμ. Optical path is 0.1 cm. In the absence of cyanide (A), oxidation is monophasic. Reducing equivalents for cycling are provided by 2.5 mM succinate in the presence of 30 mM malonate. Because of the dilution artifact, the trace returned to the point marked "final." The response time of the detection system is 100 msec. In the presence of 0.3 mM KCN (B), oxidation is biphasic. The maximum extent of oxidation is shown by the point marked "max." In this experiment, the malonate concentration had been increased to 50 mM. Because of the dilution artifact and because of slight drift due to the long cycling time at the higher malonate concentration, the trace returned to the point marked "final." The response time of the detection system is 200 msec.

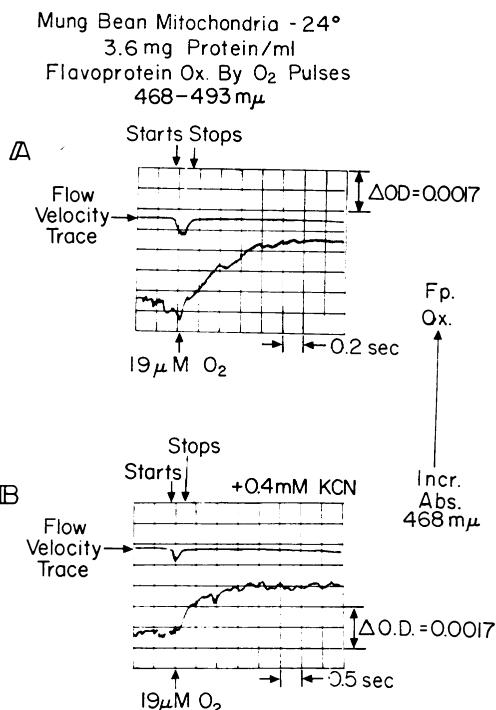

FIG. 7. Absorbance changes corresponding to oxidation of flavoprotein recorded at 468 to 493 mμ in the absence (A) and presence of 0.4 mM KCN (B). Optical path was 0.6 cm. Between the experiment recorded in A and that recorded in B, a sufficient number of injections of oxygenated medium were made to cause a 1.3-fold dilution of the suspension in B compared to A. Reducing equivalents were supplied by 2.7 mM succinate in the presence of 20 mM malonate.

pulse to an anaerobic suspension is shown in Figure 6A with no inhibitor present, and in Figure 6B with 0.3 mM KCN present (experiment carried out with J. T. Bahr). The half-time at 18 C is 500 msec in the absence of inhibitor. In the presence of 0.2 mM KCN, the oxidation of some 70% of the reduced quinone takes place with a half-time of 1 sec; the remaining 30% requires about 20 sec to reach the aerobic steady state, in which the quinone is fully oxidized. Oxidation of the flavoprotein component in the absence and presence of cyanide is shown in Figure 7. The half-time with cyanide is 0.3 sec (Fig. 7B), which is the same as that with no inhibitor (Fig. 7A). Oxidation is essentially complete in both cases.

The experiments of Figures 6 and 7 were carried out at malonate to succinate ratios of 12 and 7.5, respectively, at succinate concentrations around 2.5 mM. Doubling the malonate concentration in the latter experiment had no effect on the oxidation half-time. The maximum rates of flavoprotein and ubiquinone oxidation are being recorded, and it is evident that cyanide has little effect on the flavoprotein rate. This result is apparently in contrast to that obtained previously with skunk cabbage mitochondria: oxidation rates of flavoprotein and ubiquinone were about five times slower in the presence of cyanide or antimycin A (9). The experiments with skunk cabbage mitochondria were carried out with a malonate to succinate ratio of 2 at 5 mM succinate, however. Under these conditions, the input of reducing equivalents from succinate is not negligible when the alternate oxidase of these mitochondria alone provides the oxidizing equivalents to these two components. Further, the malonate to succinate ratio required to render this input negligible increases with increasing succinate concentration (J. T. Bahr, unpublished results). If a comparable experiment with mung bean mitochondria is carried out at 5 mM succinate with a malonate to succinate ratio as high as 5, the decrease in apparent oxidation rate of

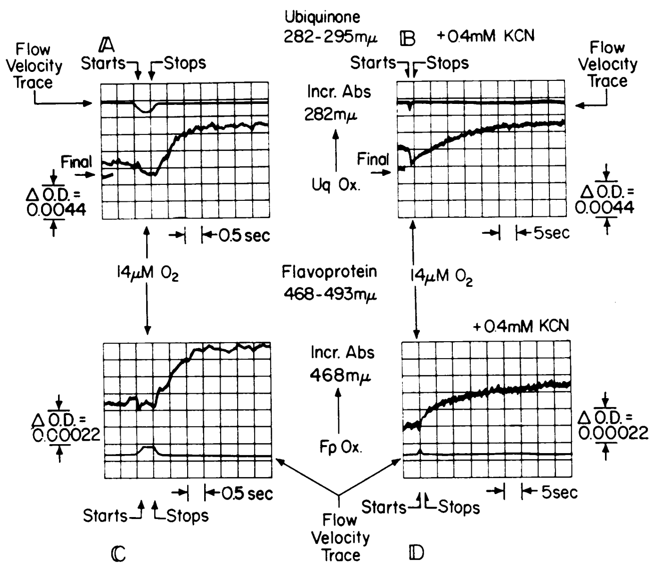

FIG. 8. Absorbance changes corresponding to ubiquinone and flavoprotein oxidation in mung bean mitochondria. Reducing equivalents are provided by 5 mM succinate in the presence of 25 mM malonate. Optical path is 0.1 cm. Ubiquinone is monitored at 282 to 295 mμ; in the absence of cyanide (A), the time scale is 0.5 sec/cm; in the presence of 0.4 mM KCN (B) it is 5 sec/cm. Flavoprotein is monitored at 468 to 493 mμ; the same time scales as for ubiquinone are used in the absence (C) and presence of 0.4 mM KCN (D).

flavoprotein and ubiquinone is quite pronounced, as shown in Figure 8 for a single preparation. The half-times are 0.5 sec for both components in the absence of cyanide; in its presence, the half-time for flavoprotein oxidation increases to 5 sec and that for ubiquinone to 8 sec, factors of 10 and 16, respectively. The oxidation of flavoprotein becomes biphasic, the second phase having a half-time of 20 sec; oxidation is substantially complete. The oxidation of ubiquinone is also complete in the aerobic steady state under these conditions. If an experiment similar to that of Figure 8 is carried out at 5 mM succinate with a malonate to succinate ratio of 2, as used for the skunk cabbage experiments (35), the half-time for ubiquinone oxidation rises to 12 sec and the extent of oxidation is only 44%. Flavoprotein oxidation is also incomplete and becomes difficult to record under these conditions. The more dramatic effect of modulating the input of reducing equivalents observed with mung bean mitochondria, as compared to skunk cabbage mitochondria, reflects the lower activity of their alternate oxidase pathway, resulting in a lower net oxidation rate for a given input of reducing equivalents from succinate. From the work of Lance and Bonner (30), this input is expected to be about the same for both types of mitochondria under the same experimental conditions.

DISCUSSION

Cytochromes c_{547} and c_{549}. Upon addition of cyanide to an aerobic suspension of mung bean mitochondria in the absence of substrate and presence of malonate, cytochrome *a* becomes 90% reduced and both cytochrome c_{547} and c_{549} become 60% reduced. This reduction occurs in uncoupled mitochondria whether depleted or not, but the rate of reduction decreases somewhat with increasing depletion. The same degree of reduction is obtained in all cases unless the mitochondria have been damaged by excessive depletion times. It is proposed that the reducing equivalents are available to the respiratory chain in a very slow trickle from endogenous substrate (the nature of which is at present unknown) and that, in the absence of substrate and presence of oxygen, this trickle is so slow that no measurable reduction of the carriers can take place. Upon addition of cyanide, the transport of electrons from reduced cytochrome *a* to oxygen slows to a virtual halt. As a result, the rate at which reducing equivalents are supplied to the cytochromes *c*, and the rate at which they depart from *a*, is close to zero when compared to the rate at which these equivalents are exchanged between the cytochromes *c* and *a*. A virtual redox equilibrium between these carriers is established in the aerobic steady state achieved by addition of cyanide. If a midpoint potential $E_{m7} = +0.25$ v is assigned to the cytochromes *c*, by analogy with mammalian cytochrome *c* and c_1 (12, 23, 32), the calculated potential of the equilibrium system is +0.24 v and $E_{m7} = +0.30$ v for cytochrome *a*. The cytochromes *b* remain oxidized under these conditions presumably because they have access to oxygen with $E_{m7} = 0.81$ v (20) through the alternate, cyanide-insensitive oxidase. The potential of +0.24 v maintained in the steady state appears to be sufficiently positive compared to the E_{m7} for the cytochromes *b* that cytochromes *c* and *a* are effectively insulated from reaction with oxygen via the alternate oxidase, in the presence of uncoupler which inhibits reverse electron transport through the coupling site between the *b* and *c* cytochromes (10, 36). This implies that the cytochromes *b* of plant mitochondria have values of E_{m7} comparable to those reported for mammalian cytochrome *b*, namely +0.03 to +0.08 v (12, 27). Cytochrome b_{562} may have an E_{m7} more negative than this. An E_{m7} value for b_{557} in this range is still perfectly compatible with its ready reduction by abscorbate plus TMPD, since ascorbate is the actual reductant in this system. The E_{m7} for the ascorbate to dehydroascorbate couple is +0.06 v as compared with +0.03 v for succinate to fumarate (20). In principle, any redox component reducible by succinate should be reducible by ascorbate. In practice, the reduction rates may be vastly different, and thus reduction of b_{557} by ascorbate plus TMPD is a consequence of its ease of reducibility (33)—a kinetic property—rather than its redox potential. If the coupling site between the *b* and *c* cytochromes is operative, reducing equivalents can be transferred from reduced *c* and *a* in the presence of cyanide to oxygen via the cytochromes *b* and the alternate oxidase in an energy-linked reaction sensitive to inhibition by uncouplers. Such a reaction requires an input of energy as primer, but, once in the steady state, may operate like a siphon, in which the equivalents move up a potential gradient of some 0.2 v, and then down a potential gradient of some 0.8 v to oxygen. Bonner and Bendall (9) have reported just such a reaction in mitochondria from *Arum*, but their results imply that a continuous input of energy may be necessary to maintain the siphon.

The results shown in Figure 3 and the kinetic spectrum of Figure 4 provide evidence that, even in the presence of 0.3 mM KCN, the two cytochromes *c* can be oxidized by an oxygen pulse from the reduced state in anaerobiosis through cytochromes $(a + a_3)$. The reaction of reduced $(a + a_3)$ with oxygen in the presence of cyanide is fast enough (34) to ensure that the rate of oxidation of c_{547} remains unchanged. The rate of c_{549} oxidation drops slightly to that of c_{547}; the reason for this is obscure. Both cytochromes follow in synchrony the rapid reduction of cytochrome *a* observed after the initial oxidation, in accord with the idea that forward electron transport from the two *c* components proceeds only to cytochrome *a* in uncoupled mung bean mitochondria.

Cytochrome b_{557}. The fact that the oxidation rate of reduced b_{557} is substantially unchanged in the presence of cyanide is evidence in favor of its identity with the cytochrome b_7 of Bendall and Hill (2). But the profound inhibition exerted by antimycin A on the oxidation of this and the other two *b* cytochromes would seem to rule out the participation of b_{557} as an integral part of the alternate, cyanide-insensitive terminal oxidase of

these mitochondria. In this regard, cytochrome b_{557} in mung bean mitochondria does behave differently from cytochrome b_7 in *Arum* mitochondria, since the latter remains oxidized in the presence of antimycin A (1). At present, the differences between b_{557} and b_7 appear to outweigh their similarities.

Since the oxidation half-time for b_{557} is in the time domain of the cytochromes *c*, and since the oxidation half-times of the latter respiratory carriers are relatively unaffected by cyanide, it seems plausible that reduced b_{557} is oxidized directly by the cytochromes *c* in the presence or absence of cyanide in oxygen pulse experiments. But none of the results from this and other studies (33, 35) completely rule out direct oxidation of b_{557} by cytochrome a_3 in uncoupled mitochondria; in fact, these are the only two cytochromes whose oxidation under these conditions proceeds as though quite unaware of the presence of cyanide. In coupled mitochondria, b_{557} appears to be located on the substrate side of the cytochromes *c* with a coupling site between, as mentioned above. The possibility does exist, however, that in uncoupled mitochondria a "short circuit" occurs between b_{557} and a_3; this point requires further investigation.

Once oxidized in an oxygen pulse experiment in the presence of cyanide, cytochrome b_{557} stays oxidized in the aerobic steady state, unlike the cytochromes *c*, and returns to the reduced state with a rate best described as tedious. Storey and Bahr (35) suggested that, in skunk caggage mitochondria, there exists a respiratory carrier Y which distributes reducing equivalents from ubiquinone and flavoprotein via three pathways to the alternate oxidase, to the cytochromes *b*, and to the cytochromes *c*. The last pathway does not operate in coupled mitochondria, while in uncoupled mitochondria it operates at the expense of the pathway to b_{557}. This suggestion appears applicable to mung bean mitochondria with the additional proviso that the branching point carrier Y have a lower capacity for electron transport in these as compared to skunk cabbage mitochondria. This proviso would explain the observation that, in the presence of cyanide, the $t_{1/2\text{off}}$ (26) for cytochrome c_{547} reduction is 150 msec in skunk cabbage mitochondria as opposed to 700 msec for mung bean mitochondria when both sets of mitochondria are tested under the conditions of Figure 3. It would also explain the observation, made under these same conditions, that the $t_{1/2\text{off}}$ (26) values for b_{557} reduction in mung bean and skunk cabbage mitochondria are 40 sec and 20 sec, respectively, with no inhibitor. These values increase to 80 and 50 sec in the presence of 0.3 mM cyanide. It would also explain the observation that b_{557} in skunk cabbage mitochondria showed an increase in oxidation half-time from 18 to 200 msec upon addition of 0.3 mM KCN under these same conditions, namely 5 mM succinate with a malonate to succinate ratio of 2. The oxidation rate appears slower in skunk cabbage because of significant feed-in of reducing equivalents to b_{557} through the carrier Y, while the rate in mung bean mitochondria is scarcely affected. On the substrate side of Y, the oxidation rates of ubiquinone and flavoprotein should be more rapid in skunk cabbage mitochondria than in mung bean mitochondria. Indeed, a malonate to succinate ratio of 5 is required at 5 mM succinate with mung bean mitochondria to achieve rates of ubiquinone and flavoprotein oxidation in the presence of cyanide (Fig. 8) which are comparable to those obtained with skunk cabbage at a malonate to succinate ratio of 2.

The experiments reported in this paper provide more support for the idea of a respiratory carrier unique to mitochondria from plant tissues which acts at a branched pathway leading to two terminal oxidases (3, 22, 35, 37). They also indicate that this carrier, tentatively designated Y, acts as a kinetic bottleneck between the cytochromes *b* and *c* and the ubiquinone-flavoprotein part of the respiratory chain and may well determine the capacity of the alternate, cyanide-insensitive pathway (3). The experiments also show that cytochrome oxidase of plant mitochondria in the reduced form can be oxidized by oxygen with cyanide present and, in oxygen pulse experiments, can oxidize those cytochromes lying between the oxidase and Y at least partially and, in the case of b_{557}, completely. This observation, the reverse of that of Bendall and Bonner (2), who found that oxidized cytochrome a_3 would become reduced in anaerobiosis in the presence of cyanide, is examined in more detail in the following paper (34).

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