The Respiratory Chain of Plant Mitochondria

V. REACTION OF REDUCED CYTOCHROMES $a$ AND $a_3$ IN MUNG BEAN MITOCHONDRIA WITH OXYGEN IN THE PRESENCE OF CYANIDE

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ABSTRACT
The half-times of oxidation by oxygen pulses of reduced cytochromes $a$ and $a_3$ in mung bean mitochondria made anaerobic with succinate have been measured by means of a rapid mixing flow apparatus coupled to a dual wave length spectrophotometer in the presence and absence of cyanide. The absorbance changes at 438 to 455 millimicrons and 603 to 620 millimicrons are suitable for recording the time course of cytochrome $a$ oxidation; the half-time is 2.0 milliseconds at 24 Celsius. This half-time does not change over the range 0 to 300 $\mu$M KCN, but the fraction of cytochrome $a$ oxidized falls to a limiting value of 0.3 at the higher cyanide concentrations. The absorbance changes at 445 to 455 millimicrons record the time course of both cytochrome $a$ and cytochrome $a_3$ oxidation; the former contributes 60% of the absorbance change and the latter 40%. The half-time for $a_3$ oxidation is calculated as 0.9 milliseconds at 24 Celsius. This half-time increases slightly to 1.3 milliseconds at 300 $\mu$M KCN. Reduced cytochrome $a_3$, whether uncomplexed or complexed with cyanide, becomes fully oxidized. The dissociation constant for the reduced cytochrome $a_3$-cyanide complex is estimated to be 30 $\mu$M, whereas that for the oxidized $a_3$-cyanide complex which inhibits electron transport is estimated to be 2 $\mu$M. This suggests two different binding sites for cyanide on the reduced and oxidized forms of cytochrome $a_3$. The fact that a limiting fraction of reduced cytochrome $a$ can be oxidized at high cyanide concentrations implies that there is no interference by cyanide with electron transport from $a$ to $a_3$, if cyanide remains bound to the site it occupies on reduced $a_3$ after this carrier becomes oxidized on reaction with molecular oxygen. Rearrangement of cyanide from this noninhibitory site to the inhibitory site occurs rapidly enough to compete with cytochrome $a$ oxidation. The half-time for the rearrangement is calculated to be 0.9 milliseconds.

Ray (21) have recently summarized the arguments for a cyanide binding site on reduced cytochrome oxidase (from heart muscle) which is different from that on the oxidized enzyme. The latter site is the locus of inhibition by cyanide of electron transport to oxidized cytochrome $a_3$ from reduced cytochrome $a$. These arguments derive in part from his work and from that of Chance (5) and of Kelin and Hartree (13), who first suggested that the cyanide complex of reduced $a_3$ in heart muscle oxidase was auto-oxidizable.

In the previous paper (16), the oxidation of reduced cytochromes ($a + a_3$) in mung bean (Phaseolus aureus) mitochondria was shown to occur rapidly when oxygen pulses were injected into the mitochondrial suspension made anaerobic with succinate, in the presence as well as the absence of cyanide, and the oxidizing equivalents could be transmitted to $a_{4+}$, $b_{4+}$, and $b_{5+}$. The reaction of reduced cytochromes ($a + a_3$) with oxygen in the presence of cyanide is more readily studied with plant mitochondria than with mammalian mitochondria, since the former have an alternate, cyanide-insensitive oxidase (2, 10, 12, 18). With these mitochondria, the oxygen pulse technique with the regenerative flow apparatus, developed by Chance (8), is readily used since, even at high cyanide concentrations, the oxygen is consumed by the alternate oxidase to give a full redox cycle. In this paper, the oxidation of reduced cytochromes ($a + a_3$) in mung bean mitochondria by molecular oxygen in the presence of cyanide is examined in more detail.

MATERIALS AND METHODS
Both mitochondrial preparations and kinetic studies were carried out in the same manner and with the same apparatuses as described in the previous paper (16). In all kinetic studies the mitochondria were depleted aerobically with 130 $\mu$M ADP and 10 $\mu$M 1799\(^2\); reducing equivalents were provided by succinate; the flux of reducing equivalents through the respiratory chain was controlled by added malonate. Steady state redox levels of cytochromes $a$ and $a_3$ were determined with the Amino-Change dual wave length spectrophotometer (American Instrument Co., Silver Spring, Maryland).

RESULTS
Separation of Oxidation Rates of Cytochromes $a$ and $a_3$
Bendall and Bonner (1) showed that both cytochrome $a$ and cytochrome $a_3$ of plant mitochondria have an absorption maxi-

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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 $\mu$$\mu$ toward the red.
2 Abbreviation: 1799: bis(hexafluoroacetylonyl)acetone.
Fig. 1. Absorbance changes recorded with the dual wave length spectrophotometer at 445 to 455 mμ (A) corresponding to oxidation of cytochromes (a + a2) at 438 to 455 mμ (B) and at 603 to 620 mμ (C) corresponding to oxidation of cytochrome a. The experiments were carried out in a rapid mixing flow apparatus driven by nitrogen (17) with an optical path of 0.6 cm. The flow velocities in the records of A and B are the same, and 1.6 times that in C. The mitochondrial suspension contains 1.3 mg of protein per ml; succinate is present at 2.5 mM and malonate at 25 mM.

The absence of this inhibitor, cytochrome b562 is at most only partially reduced in anaerobiosis. Its oxidation half-time is in the range 15 to 35 msec (15).

With the use of the improved rapid mixing regenerative flow apparatuses recently developed by Chance and co-workers (7, 9), the separation of cytochrome a oxidation kinetics from those of the cytochromes b can be accomplished. Three records of absorbance changes accompanying the oxidation of cytochromes (a + a2) upon injection of oxygen-saturated medium into an anaerobic suspension of mung bean mitochondria are presented in Figure 1 for the three wave length pairs, 445 to 455 mμ (Fig. 1A); 438 to 455 mμ (Fig. 1B); and 603 to 620 mμ (Fig. 1C). Partial oxidation is observed as a plateau of decreased absorbance at 445 mμ during the flow in Figure 1A, upon cessation of flow, the rapid oxidation proceeds to completion. The calculated half-time is 1.4 msec for this reaction. There follows a further slight and slower absorbance decrease. The record obtained at 438 to 445 mμ (Fig. 1B) shows a rapid absorbance decrease at the initiation of the reaction, the plateau corresponding to partial oxidation during the flow, followed by a rapid absorbance decrease as the flow stops. Immediately on cessation of flow, there is a further absorbance decrease, complete in 70 msec, which is followed by yet another slower absorbance decrease with a half-time of some 300 msec. The oxidation reaction monitored during the flow has a calculated half-time of 2.0 msec, and this absorbance change corresponds to cytochrome a; the succeeding ones are attributed to the cytochromes b. The third record obtained with a slower flow velocity at the wave length pair 603 to 620 mμ shows a single oxidation reaction with a half-time of 2.1 msec, again corresponding to oxidation of cytochrome a.

If the relative contributions of the absorbance changes at 445 to 455 mμ for cytochromes a and a2 can be assessed, then the half-time for cytochrome a oxidation can be calculated, assuming that the half-time for cytochrome a oxidation only is recorded at the other two wave length pairs in Figure 1. This
assessment is made in Figure 2 and departs from the observation that cytochrome $a$ undergoes nearly 90% reduction in the absence of added substrate when cyanide is added to an aerobic suspension of depleted mung bean mitochondria, while no reduction of the cytochromes $b$ occurs (16). Addition of cyanide to a depleted, aerobic mitochondrial suspension produces an increase in absorbance at 445 $\mu$m (Fig. 2A) which can be assigned entirely to cytochrome $a$. Addition of succinate produces another absorbance increase attributed in small part to further reduction of cytochrome $a$ and in large part to interference from the cytochromes $b$, since nearly the same increase is observed in the aerobic steady state with succinate in the presence of antimycin A at this wave length pair. Upon anaerobiosis, there is a further absorbance increase due to the formation of reduced cytochrome $a_3$ cyanide complex. Addition of succinate and cyanide to the depleted mitochondrial suspension are made in the reverse order in Figure 2B. The absorbance increase on adding succinate to attain the aerobic steady state is again equal to that expected for the cytochromes $b$. The absorbance change upon anaerobiosis is attributed to cytochromes $(a + a_3)$. Addi-
tion of KCN and aeration by stirring gives a steady state wherein $a$ is reduced and $a_3$ is oxidized; upon anaerobiosis there is again the absorbance change attributable to the reduced $a_3$-cyanide complex. From these results, one calculates that cyto-
ochrome $a$ accounts for 59% of the difference absorbance change at 445 to 455 $\mu$m while $a_3$ accounts for 41%; the difference absorbance change attributable to the $a_3$-cyanide complex at this wave length pair is 60% of the change attributable to $a_3$. From the low temperature difference spectra presented by Bendall and Bonner (1), one estimates that cytochrome $a$ contributes about 60% of the difference absorbance maximum at 445 $\mu$m, and cyto-
ochrome $a_3$ contributes about 40%, in good agreement with the values obtained from Figure 2. (A similar set of experiments at 603 to 620 $\mu$m shows that cytochrome $a$ contributes more than 90% to the reduced-minus-oxidized absorbance change observed with this wave length pair.)

Using the relative contributions to the absorbance change at 445 to 455 $\mu$m of 0.6 for cytochrome $a$ and 0.4 for cytochrome $a_3$ and an oxidation half-time of 2.0 msec for the oxidation of cyto-
ochrome $a$, one calculates from Figure 1A an oxidation half-time of 0.8 msec for cytochrome $a_3$. This is greater than, but still close to, the observed oxidation half-time of 0.6 msec for cytochrome $a_3$ in pigeon heart mitochondria reported by Chance and Pring (9).

**Oxidation Rates of Cytochromes $(a + a_3)$ in the Presence of Cyanide.** The absorbance changes accompanying the reaction of reduced cytochromes $(a + a_3)$ with molecular oxygen in the absence and presence of KCN are shown in Figure 3 as recorded with the wave length pair 445 to 455 $\mu$m. In the absence of in-
hibitor (Fig. 3A), the calculated half-time is 1.5 msec for the reaction occurring during the flow. Upon cessation of flow, there is the same slow absorbance decrease observed in Figure 1A. With 60 $\mu$m cyanide, which is some eight times the half-maximal concentration established by Ikuma and Bonner (12) for in-
hibition of the cyanide-sensitive respiratory pathway in mung bean mitochondria, the oxidation pattern is similar to that in Figure 3A. The calculated half-time is also 1.5 msec. The absorbance decrease during the reaction is reduced to 75% of the value in Figure 3A, and the slow absorbance decrease after cessation of flow is reduced but still evident. With a concentra-
tion of 300 $\mu$m, which is saturating for the cytochrome $a + a_3$ pathway (12), the record shown in Figure 3C is obtained. It shows clearly the reaction which occurs during the flow with a half-time of 1.6 msec. This is followed by a further small absorbance decrease in 60 msec leading to an aerobic steady state lasting about 0.2 sec, and then a slower absorbance increase. The maximum absorbance decrease in Figure 3C is about 45% that observed in Figure 3A. Using the relative contributions to the absorbance changes at 445 to 455 $\mu$m calculated from Figure 2, and assuming that cytochrome $a_3$ is completely oxidized in the presence of cyanide, one calculates that 30% of cyto-
ochrome $a$ is oxidized under the conditions of Figure 3C. The same value was obtained with the wave length pair 438 to 455 $\mu$m for a similar series of oxidation reactions with another mitochondrial suspension carried out under the same experimental conditions, making due allowance for interference from the cytochromes $b$.

The rate of oxidation of reduced cytochrome $a$ remains essentially unchanged over this concentration range of cyanide. The half-
time for oxidation of cytochrome $a$ was measured at 603 to 620 $\mu$m in the absence of inhibitor for the same mitochondrial suspension under the same conditions which yielded the records of Figure 3. The half-time was 2.0 msec, which yields a calculated value of 0.9 msec for the half-time of cytochrome $a_3$ oxidation with no cyanide present, and a half-time of 1.3 msec with 0.3 $\mu$m KCN. In this particular mitochondrial suspension, the half-
time for oxidation of $C_{457}$ was 2.5 msec with no cyanide present while that for $C_{450}$ was 2.0 msec, as determined in separate oxygen pulse experiments. It is a characteristic of mung bean mitochondria that the oxidation half-times of cytochromes $a$ and $C_{450}$ are very close in the absence of cyanide, while those of $C_{457}$ and $C_{450}$ are very close in its presence (16). These kinetic measurements show that, in mung bean mitochondria, reduced cyto-
ochrome $a_3$ complexed with cyanide is oxidized by reaction with oxygen completely and at a rate but slightly less than that in the absence of inhibitor. Even at cyanide concentrations known to be saturating for the cyanide-sensitive respiratory pathway in these mitochondria, as much as 30% of reduced cytochrome $a$ can become oxidized in oxygen pulse experiments.

**Effect of Cyanide Concentration on the Oxidation of Cyto-**
chromes \((a + a_3)\). The absorbance decrease corresponding to the rapid reaction observed at 445 to 455 nm upon oxidation of reduced cytochromes \((a + a_3)\) becomes smaller with increasing cyanide concentration (Fig. 3). A cyanide titration of the rapid absorbance change at 445 to 455 is shown in Figure 4A; the points were obtained by adding successive aliquots of KCN solution to the same mitochondrial suspension. The decrease in absorbance change is monotonic; it requires 30 \(\mu M\) KCN for half-maximal effect and becomes saturated above 200 \(\mu M\). Two factors contribute to the decrease in the absorbance change. First, the reduced-minus-oxidized extinction coefficient \(A_{455-400}\) for cytochrome \(a_3\) complexed with cyanide is 60% of that for cytochrome \(a_3\) itself. Second, the percentage of cytochrome \(a\) oxidized by an oxygen pulse drops from 100% with no cyanide to 30% or less at 200 \(\mu M\) or more cyanide. As a result, the titration curve is not strictly hyperbolic. The value of 30 \(\mu M\) KCN for half-maximal effect is 6-fold greater than the concentration of 5 \(\mu M\) required for half-maximal inhibition of cyanide-sensitive respiratory pathway of these mitochondria in state 3 with succinate as substrate (12).

An effect of increasing cyanide concentration on the absorbance change observed after the flow has stopped is also evident from Figure 3. At 0 and 60 \(\mu M\) KCN, there is a continued decrease in absorbance at 445 nm, while at 300 \(\mu M\) KCN, there is an absorbance increase after attainment of a short-lived steady state. A cyanide titration of this absorbance change, taking the point at cessation of flow as the baseline, is shown in Figure 4B. The curve is sigmoidal. At low cyanide concentrations, there is no effect. The direction of the change reverses from absorbance decrease to absorbance increase at 110 \(\mu M\), and the effect is saturated at 300 \(\mu M\) KCN. The half-time for the absorbance decrease at low or no KCN corresponds to that expected for \(b_{553}\) (15), while the half-time for the absorbance increase is about 700 msec at 18 C and about 400 msec at 24 C and is attributed to reoxidation of that fraction of cytochrome \(a\) oxidized at high cyanide concentration.

A series of oxidation-reduction cycles of cytochromes \((a + a_3)\), initiated by an oxygen pulse to mung bean mitochondria made anaerobic with succinate, is shown for a single mitochondrial suspension at six different concentrations of cyanide in Figure 5. In the absence of cyanide (Fig. 5A), the time from initiation of oxidation to half-reduction after the aerobic steady state—\(t_{1/2}\) off (11)—is 35 sec, and, except for a minor slow component, the reduction occurs rapidly in one stage. Even at cyanide concentrations as low as 5 \(\mu M\) (Fig. 5B), the reduction occurs in two stages. The first is like that observed with no cyanide, but the second stage appears as a plateau in the experimental record, followed by a slow return of the trace to the original base line. As the cyanide concentration is increased (Figs. 5C, 5D), the extent of the absorbance change on oxygenation not only decreases, but its reversal, corresponding to the first stage of reduction, begins directly. There is no apparent aerobic steady state on the time scale shown. At cyanide concentrations above 100 \(\mu M\) (Figs. 5E, 5F), the initial part of the absorption change no longer appears on the slow time scale, and the plateau and slow return of the trace to the base line for complete reduction is now dominant. (At the higher sensitivity used for Figures 5E and 5F, there are some minor, slow absorption changes attributed to cytochrome \(b_{553}\).

The values for \(t_{1/2}\) off (11) for the slow stage of the cycle involving reduction of cytochrome \(a_3\), taken from the experiments of Figure 5, are plotted in Figure 6 as a function of cyanide concentration. The concentration of cyanide required for half-maximal increase of \(t_{1/2}\) off is 2 \(\mu M\). This value is comparable to and, as expected under these conditions of minimal flux of reducing
remains bound fraction of that assuming high cyanide limiting dissociation 445 oxidase, oxygen, chrome the first recorded at incomplete with concentration of less equivalents, except concentration of shown 1. stored in the protein concentration 70 mg of the inhibition magnitude less than cyanide complex. It is shown in Figure 7, recorded at 445 to 455 mÅ (Fig. 7A) and 603 to 620 mÅ (Fig. 7B). The malonate to succinate ratios are 10 at 2.5 mm succinate in both experiments. Comparison of the two records shows that the first stage of reduction observed at 445 to 455 mÅ is due to reduction of cytochrome a1, since the characteristic plateau is missing from the trace in Figure 7B recorded at 603 to 620 mÅ and the reduction here is synchronous with the first stage in Figure 7A. Oxidations of both cytochrome c1 and c4 are complete at this intermediate cyanide concentration. Their redox cycles are similar to those observed with no cyanide, except for a slightly longer t1/2 att (11), as compared with incomplete oxidation and reduction cycles following cytochrome a in high cyanide concentrations. The experiment of Figure 7 was carried out with a mitochondrial protein concentration of 1.3 mg of protein per ml and an initial oxygen concentration of 19 μM, corresponding to a sizable excess of oxygen, as compared to the experiment of Figure 5 where oxygen is in moderate excess over reducing equivalents stored in the respiratory carriers.

**DISCUSSION**

The results of the oxygen pulse experiments reported here suggest that the cyanide binding site on the reduced cytochrome a1 of plant mitochondria is different from that on oxidized a1 as is the case for mitochondria from animal sources (5, 13, 20). The dissociation constant of the latter cytochrome complex, which is the one inhibiting electron transport through the cytochrome oxidase, is 2 μM from the titration curve of Figure 7. On the other hand, the dissociation constant for the reduced cytochrome a1-cytochrome a1 complex is about 30 μM, as estimated from Figure 4A, assuming that the fraction of cytochrome a oxidized reflects the fraction of a1 present as the cyanide complex. The fact that a limiting fraction of reduced cytochrome a can be oxidized at high cyanide concentrations implies that there is no interference by cyanide with electron transport between a and a1, if cyanide remains bound to the site it occupies on reduced cytochrome a1 after this carrier becomes oxidized on reaction with molecular oxygen. But rearrangement of cyanide from the noninhibitory to the inhibitory site on oxidized a1 occurs rapidly enough to compete with this carrier’s oxidation of reduced cytochrome a in a half-time of 2 msec. Since only 30% of reduced a becomes oxidized at saturating cyanide concentrations, the half-time for the rearrangement is calculated to be 0.9 msec, assuming simple partition between the oxidation and rearrangement reactions. This assumption is undoubtedly too simple for the complex sequence of enzymatic reactions between cytochromes (a + a1), oxygen, and the other respiratory carriers, but it is a reasonable approximation. Preliminary calculations with more complicated models did not yield a rearrangement half-time which is much different from 0.9 msec.

The effect of cyanide concentration on the redox cycles of cytochromes (a + a1) initiated by oxygen pulses delineates three regions of cyanide concentration in which the reaction shows differing behavior; this is reflected in the plot of Figure 4B. The first region is 0 to 30 μM KCN where reduced cytochrome a1 exists only partially as the cyanide complex. Oxidation of cytochrome a is still extensive in this region, and the oxidation of the other carriers of the respiratory chain is essentially complete. Both the uncomplexed and noninhibitory forms of the a1-cytochrome complex can still turn over sufficient cytochrome a that the oxidative flux is little affected by the inhibitor. Only upon reduction at the end of a redox cycle does the effect of cyanide become apparent when sufficient inhibitor a1-cytochrome complex has formed to markedly reduce the rate of a1 reduction by reduced cytochrome a1. (In all these experiments, the flux of reducing equivalents was deliberately limited at succinic dehydrogenase by the presence of malonate.) The effect of cyanide on oxidation of the cytochromes b is nil in this region, and thus there is no effect on the slow absorbance changes observed at 445 to 455 mÅ and plotted in Figure 4B.

As the cyanide concentration is increased beyond 30 μM toward 200 μM, there appears a transitional region where the fraction of reduced a1 in anaerobiosis present as the cyanide complex becomes predominant. The amount of uncomplexed a1 and noninhibitory a1-cytochrome complex turns over less cytochrome a1, and oxidation of cytochrome b is inhibited, resulting in a decrease of the slow absorbance change at 445 to 455 mÅ plotted in Figure 4B. Further, upon completion of the oxidation reaction, conversion of oxidized a1 to the inhibitor complex with a dissociation constant of 2 μM is essentially complete, and reduction of cytochrome a starts immediately with no intervening steady state, as seen in Figure 6. Oxidation of the cytochromes c and the respiratory carrier Y, the postulated fork in the respiratory pathway of plant mitochondria to the two terminal oxidases (16, 17), is sufficiently complete, however, that the slow reduction of oxidized Y becomes the step which limits reduction of cytochrome a and gives the rates seen in Figure 5D and Figures 6A and 6B.

At cyanide concentrations of 200 μM and above, there is a third region of cyanide concentration in which reduced cytochrome a1 in anaerobiosis is present almost entirely as the cyanide complex. The rate of rearrangement of oxidized a1-cytochrome complex from the noninhibitory to the inhibitory form is rapid and determines that a maximum of 30% of the total reduced cytochrome a becomes oxidized. The turnover of a is insufficient to effect more than 70% oxidation of the cytochromes c and, as described in the previous paper (16), there is a rapid, synchronous reduction of the two cytochromes c and cytochrome a with a half-time of 0.7 sec at 18°C and 0.5 sec at 24°C. This reaction is reflected in the slow absorbance increase observed in Figure 3C and plotted in Figure 4B. Under these conditions, oxidizing equivalents from the oxygen pulse do not proceed further than the cytochromes b and c; oxidation of ubiquinone.
and flavoprotein occurs entirely via the cyanide-insensitive alternate oxidase (2). The common reduction rate of the cytochromes $C_{453}, C_{494},$ and $a$ presumably mirror the oxidation rate of reduced carrier Y, and this rate is slow compared to that of the cytochromes, as required by the scheme of Storey and Bahr (17).

The cytochrome oxidase of plant mitochondria does resemble that of mitochondria from the animal kingdom in that the cytochrome complex of reduced cytochrome $a_3$ is oxidized by molecular oxygen. There is still no evidence, however, that the oxidase is a complex of cytochromes $(a + a_3)$ as is the oxidase isolated from heart muscle, this being in part due to the intransigence of the plant cytochromes $(a + a_3)$ toward isolation (9). The kinetic behavior reported here is fully compatible with $a$ and $a_3$ acting as independent carriers in plant mitochondria; taken together with results reported in the previous paper (16), it is possibly more compatible with $C_{494}$ plus $a$ acting in concert. The results reported by Bonner and Plesnicar (4), which imply that an energy conservation site exists between cytochromes $a$ and $a_3$, would indicate that these are separate carriers. Further clarification of this point must await isolation of these cytochromes, either together or separately.

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LITERATURE CITED


