The Respiratory Chain of Plant Mitochondria

XVII. FLAVOPROTEIN-CYTOCHROME \( b_{562} \) INTERACTION IN ANTIMYCIN-TREATED SKUNK_CABBAGE MITOCHONDRIA

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ABSTRACT

During the transition from the aerobic steady state with succinate as substrate to anaerobiosis, in suspensions of skunk cabbage (Symplrocus foetidus) mitochondria treated with antimycin A, cytochrome \( b_{562} \) becomes reoxidized to the extent of about 20%, synchronously with the reduction of cytochrome \( c_{562} \). This reoxidation occurs in both the absence and presence of m-chlorobenzhydroxamic acid, a specific inhibitor for the alternate terminal oxidase of plant mitochondria. A flavoprotein component, amounting to 13% to 15% of the total non-fluorescent mitochondrial flavoprotein, undergoes reduction synchronously with the oxidation of cytochrome \( b_{562} \) during the aerobic to anaerobic transition with succinate as substrate in the presence of both antimycin A and m-chlorobenzhydroxamic acid. This flavoprotein component remains reduced in the presence of cyanide. The half-time for reduction of the flavoprotein component and cytochrome \( c_{562} \) and for oxidation of cytochrome \( b_{562} \) during the aerobic to anaerobic transition with succinate as substrate in the presence of both antimycin A and m-chlorobenzhydroxamic acid is 2 seconds. The half-times for oxidation of cytochrome \( c_{562} \) and the flavoprotein component are 2.1 and 170 milliseconds, respectively, during the anaerobic to aerobic transition induced by addition of 14 \( \mu \)M O2 to the mitochondrial suspensions. The half-time for reduction of cytochrome \( b_{562} \) under these conditions is 150 milliseconds, synchronous with the flavoprotein component. The synchrony of the flavoprotein oxidation and of the cytochrome \( b_{562} \) reduction at a rate much slower than that of cytochrome \( c_{562} \) oxidation implies that, in antimycin-treated plant mitochondria, the state of the cytochrome \( b_{562}/ \)antimycin complex is regulated by the redox state of this flavoprotein component, rather than by cytochrome \( c_{562} \). It is tentatively suggested that these two components are not part of the main sequence of the respiratory chain, but may be part of a multienzyme complex active in the hydroxylation reactions required for ubiquinone biosynthesis in the inner mitochondrial membrane.

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Cytochrome \( b_{562} \), the cytochrome with the \( \alpha \)-band of longest wavelength in the reduced form of the three cytochromes \( b \) of plant mitochondria (1–3, 7, 12, 18), has the peculiar property that it is fully reduced in mung bean (Phaseolus aureus) mitochondria treated with antimycin A when the cytochromes \( c \) are fully oxidized, but is reoxidized to the extent of about 20% as the latter cytochromes become reduced (21). More recently, Lambowitz and Bonner (14) have confirmed this result and shown, in addition, that there is a further, very slow reoxidation of cytochrome \( b_{562} \) which can attain completion in 12 to 15 min. It is the rapid reoxidation reaction which is of interest in studying the interaction of the various redox components of the inner membrane of plant mitochondria. This reaction becomes of further interest in the light of the demonstration that cytochrome \( b_{562} \) is probably not a member of main sequence of carriers of the plant respiratory chain (22). In order to study this reaction in greater detail, the kinetics of the rapid partial reoxidation of cytochrome \( b_{562} \) on reduction of the cytochromes \( c \) and the reverse reaction, the reduction of cytochrome \( b_{562} \) on the oxidation of the cytochromes \( c \), in antimycin-treated mitochondria are examined in this paper. Mitochondria isolated from the spadices of skunk cabbage (Symplrocus foetidus) have been used, since the larger amounts of mitochondria needed for spectrophotometric kinetic studies involving small absorbance changes are readily available from this source.

MATERIALS AND METHODS

Mitochondria were prepared from the excised spadices of skunk cabbage (Symplrocus foetidus) flowers, using the method described by Bonner (4) and Ikuma and Bonner (13) with the modifications of Storey and Bahr (23), and assayed in medium TP as described in the previous paper by Estabrook (10). All experiments reported in this paper were carried out in medium T which is medium TP with phosphate omitted. Under these conditions, the anaerobic reduction of endogenous pyridine nucleotide and of the low potential fluorescent flavoprotein does not occur in uncoupled mitochondria utilizing succinate as substrate (20). Mitochondrial protein was determined by the method of Miller (15). Absorbance changes corresponding to the reduction or oxidation of the respiratory chain carriers were recorded by means of a dual wavelength spectrophotometer (5, 8) as described in previous papers (18, 22, 23).

RESULTS

The reoxidation of cytochrome \( b_{562} \) in skunk cabbage treated with antimycin A during the aerobic to anaerobic transition with succinate as substrate is shown in Figure 1A.
treated mung bean mitochondria, and that this oxidation occurs in the presence of mCLAM as well as in its absence. Further, part of the flavoprotein component undergoes rapid reduction on anaerobiosis, also in the presence of mCLAM. The time course of the rapid phase of cytochrome \( b_{\text{sa}} \) reoxidation was correlated with the concomitant reduction of cytochrome \( c_{\text{sa}} \) in mung bean mitochondria (14, 21); the same correlation can be made for skunk cabbage mitochondria, as shown in Figure 2. The transition from the aerobic steady state with succinate as substrate is shown for cytochrome \( b_{\text{sa}} \) (Fig. 2A), cytochrome \( c_{\text{sa}} \) (Fig. 2C), and also for the flavoprotein component (Fig. 2B). The rapid phases of cytochrome \( b_{\text{sa}} \) oxidation and flavoprotein reduction have the same time course as that of cytochrome \( c_{\text{sa}} \) reduction: the half-time is 2 sec for all three components.

In the presence of sulfide or cyanide, the cytochromes \( c \) in mung bean mitochondria treated with antimycin A are largely reduced, rather than fully oxidized as they are in the absence of these inhibitors of cytochrome oxidase (14, 21). Under these conditions, the degree of reduction of cytochrome \( b_{\text{sa}} \) in the aerobic steady state is about 80% and remains at that level in anaerobiosis; the rapid partial reoxidation of this cytochrome from full reduction to the 80% level which occurs with anti-

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### Abbreviations:
- mCLAM: m-chlorobenzydahxamic acid
- E\(_{\text{red}}\): midpoint potential at pH 7.2, referred to normal hydrogen electrode
- SCM: Succinate Concentration Mixture
- A.A: 2nmol/mg
- 0.6 mM mCLAM, antimycin A at 2 nmol/mg protein, oligomycin at 1 \( \mu \)g/mg protein, 0.3 mM ATP, and 13 mM 1799; substrate is 5 mM succinate.

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**Fig. 1.** Degree of reduction in the aerobic steady state with succinate as substrate, and on transition from this state to anaerobiosis, of cytochrome \( b_{\text{sa}} \) (A) and flavoprotein (B) in skunk cabbage mitochondria (S.C.M.) treated with antimycin A (A.A.). The sensitivity to absorbance changes and the time scale are the same in both experiments. Mitochondria are suspended in medium T (See "Material and Methods") at 5 mg protein/ml.

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**Fig. 2.** Time course of oxidation of cytochrome \( b_{\text{sa}} \) (A) and reduction of flavoprotein (B) and of cytochrome \( c_{\text{sa}} \) (C) in skunk cabbage mitochondria during the transition from the aerobic steady state to anaerobiosis. The mitochondria are suspended in medium T at 6.8 mg protein/ml and are treated with 0.6 mM mCLAM, antimycin A at 2 nmol/mg protein, oligomycin at 1 \( \mu \)g/mg protein, 0.3 mM ATP, and 13 mM 1799; substrate is 5 mM succinate.
mycin A alone in anaerobiosis is not observed. The effect of cyanide on the response of the flavoprotein component under the experimental conditions of Figures 1 and 2 is shown in Figure 3. The two traces represent parallel experiments on the same mitochondrial suspension. Advantage is taken of the residual respiration in these mitochondria, insensitive to both cyanide and mCLAM (16), to obtain an anaerobic transition. In the top trace, cyanide is absent; in the bottom trace, it is present. With cyanide, the degree of reduction of the flavoprotein components in the aerobic steady state with succinate is 66% and the component rapidly reduced on anaerobiosis is not observed. Without cyanide, the degree of reduction in the aerobic steady state with succinate is 52%, and the component rapidly reduced on anaerobiosis is observed, accounting for 13% of the total flavoprotein absorbance change. The two sum to 65%, which is the degree of reduction seen in the aerobic steady state with cyanide. This result implies that the latter component is reduced in the presence, but oxidized in the absence of cyanide in anticycin-treated mitochondria with succinate as substrate. The redox state of this component therefore correlates with the redox state of the cytochromes c, being reduced when they are reduced and oxidized when they are oxidized.

Addition of O₂ to the anaerobic suspension of skunk cabbage mitochondria during addition of mCLAM accompanied by stirring results in a biphasic oxidation of the flavoprotein components (Fig. 1B), with a rapid phase which is not time-resolved under the conditions of the experiment, followed by a slow phase. It is the rapid phase which is of interest in connection with the reduction of cytochrome b₅₆₃ under these conditions; the slow phase may be avoided by adding a limited amount of oxygen to the anaerobic suspension, using the rapid mixing regenerative flow apparatus (6). Cycles of oxidation followed by reduction are obtained for flavoprotein and cytochrome c₅₅₂ and one of reduction followed by oxidation for cytochrome b₅₆₃ (Fig. 4), on addition of 14 μM O₂ to skunk cabbage mitochondria treated with anticycin A and mCLAM and rendered anaerobic with succinate. Malonate, which was present in the O₂ pulse experiments reported in previous work (9), is omitted so that only those components, which are involved in the partial reduction of cytochrome b₅₆₃ on addition of O₂, change redox state. The response of the detector in the experiment of Figure 4 is too slow for resolution of the oxidation rate of these components but is adequate for resolution of the time of the induced redox cycles. All three components show synchronous cycles with time from half oxidation (reduction for cytochrome b₅₆₃) to half reduction (oxidation for cytochrome b₅₆₃), designated t₁/₂ off (11), equal to 7 sec. This result shows that the flavoprotein component which is reduced rapidly in the aerobic to anaerobic transition is also the one which is rapidly oxidized in the reverse transition.

The kinetics of the redox changes occurring during the anaerobic to aerobic transmission on addition of 14 μM O₂ to the anaerobic mitochondrial suspension, using the rapid mixing regenerative flow apparatus with fast detector response, are shown in Figure 5. The oxidation of flavoprotein has a half-time of 170 msec, and the reduction of cytochrome b₅₆₃ has a half-time of 150 msec; the two reactions are effectively synchronous. The oxidation of cytochrome c₅₅₂ is 85% complete.
is reversible in the sense that an aerobic to anaerobic transition gives partial oxidation of cytochrome \( b_{sa} \) while the reverse transition gives full reduction of this cytochrome from the partially oxidized state. The kinetic studies reported here show that, in the aerobic to anaerobic transition, the reduction of cytochrome \( c_{sa} \) in antimycin-treated mitochondria is sufficiently slow that the reaction which causes partial reoxidation of cytochrome \( b_{sa} \)-antimycin complex (21), is not rate-limiting, and the reoxidation proceeds synchronously with cytochrome \( c_{sa} \) reduction. In the anaerobic to aerobic transition, however, the oxidation of cytochrome \( c_{sa} \) in antimycin-treated mitochondria is so rapid that it far outpaces the reaction which results in cytochrome \( b_{sa} \) reduction. While the redox states of the two cytochromes can be correlated on an intermediate time scale, they can be separated on the very short time scale of Figure 5, as well as on the very long time scale of the experiments of Lambowitz and Bonner (14).

The great difference between the observed oxidation rates of cytochrome \( c_{sa} \) and cytochrome \( b_{sa} \) shows that there is essentially no interference from the former cytochrome in the spectrophotometric determination of the latter cytochrome. Lambowitz and Bonner (14) had calculated indirectly from potential measurements that about 50% of the absorbance change at 566 to 540 nm attributed to the rapid phase of cytochrome \( b_{sa} \) oxidation in the aerobic to anaerobic transition was due to the cytochromes \( c \). The direct kinetic determination shows that this cannot be the case.

The results also show that a flavoprotein component is involved in these peculiar redox transitions of cytochrome \( b_{sa} \). This component comprises 13% to 15% of the nonfluorescing flavoprotein components, is on the \( O_2 \) site of the antimycin site of inhibition, and its redox reactions are unaffected by the presence of mCLAM. The most probable candidate for this component is the flavoprotein with midpoint potential \( E_{m,v/2} = +170 \text{ mv} \) which, from potential measurements, comprises about 12% of the total substrate-reducible flavoproteins in skunk cabbage mitochondria (19). This component was designated \( F_{p,sa} \). A similar component with estimated \( E_{m,v/2} = +190 \text{ mv} \) has been found to comprise about 20% of the flavoproteins of mung bean mitochondria (B. T. Storey, unpublished observations). This component is apparently oxidized by one of the cytochromes \( c \), presumably the membrane-bound \( c_{sm} \) at a rate far too slow for it to function as main sequence member of the respiratory chain. Since oxidation of \( F_{p,sa} \) and reduction of cytochrome \( b_{sa} \) in the anaerobic to aerobic transition of antimycin-treated mitochondria are synchronous, it appears to be the redox state of \( F_{p,sa} \) which controls the state of the postulated cytochrome \( b_{sa} \)-antimycin complex (21). When \( F_{p,sa} \) is highly oxidized, the complex is tight and the midpoint potential is shifted to a more positive value; when \( F_{p,sa} \) is reduced, the complex is less tight, and the midpoint potential becomes more negative. This suggests that the two components are closely associated, possibly forming a complex which is functional in the absence of antimycin \( A \). Since this study also shows that \( F_{p,sa} \) is not part of the main sequence of the respiratory chain carriers, such a complex would be consistent with the finding that cytochrome \( b_{sa} \) is also not part of this main sequence (22). The suggestion was tentatively put forward that cytochrome \( b_{sa} \) might be part of an enzyme complex mediating the hydroxylation reactions required for the biosynthesis of ubiquinone in the inner mitochondrial membrane (22). We suggest here that the flavoprotein \( F_{p,sa} \) is another component of this enzyme complex.

**DISCUSSION**

The results reported here extend the results previously reported from this laboratory (21) and results recently reported by Lambowitz and Bonner (14) which show that full reduction of cytochrome \( b_{sa} \) in antimycin-treated plant mitochondria correlates with full oxidation of cytochrome \( c_{sa} \), while reduction of this latter cytochrome in anaerobiosis or by addition of cyanide or sulfide correlates with a partial oxidation of cytochrome \( b_{sa} \) on a time scale of the order of seconds. The effect

![Diagram](https://example.com/diagram.png)

**Figure 5.** Time course of the oxidation of flavoprotein (A) and cytochrome \( c_{sa} \) (C) and of the reduction of cytochrome \( b_{sa} \) (B) on addition of 14 \( \mu \)M \( O_2 \) to anaerobic skunk cabbage mitochondria, recorded at an oscilloscope sweep of 0.5 sec/cm. The experimental conditions are those of Fig. 4.

within the continuous flow portion of the rapid mixing in the flow apparatus. The half-time for oxidation is calculated (17) from this value to be 2.1 msec, in agreement with previous values obtained with both skunk cabbage (23) and mung bean mitochondria (18). The oxidation of cytochrome \( c_{sa} \) is thus complete before the oxidation of flavoprotein and reduction of cytochrome \( b_{sa} \) is well underway.

The flavoprotein component which is rapidly oxidizable has, in antimycin-treated mitochondria, the same oxidation half-time whether malonate is present to give a malonate/succinate ratio of 12, as in the previous study of these kinetics (9), or if malonate is absent as in this study. This result shows that this component must lie on the \( O_2 \) side of the antimycin site of inhibition where malonate would have little effect, rather than on the substrate side of this site where malonate would have a marked effect (24).

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LITERATURE CITED


