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THE RESPIRATORY CHAIN OF PLANT MITOCHONDRIA

XIII. REDOX STATE CHANGES OF CYTOCHROME b_{562} IN MUNG BEAN SEEDLING MITOCHONDRIA TREATED WITH ANTIMYCIN A

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SUMMARY

1. The response to antimycin A of the redox state changes of cytochrome b_{562}, the cytochrome b in mung bean seedling mitochondria with long wavelength absorbance maximum and low midpoint potential of -77 mV independent of mitochondrial energy state, has been examined with regard to possible spectral shifts, with regard to the energy state of the mitchondria, and with regard to the redox state of the cytochromes c.

2. Absorbance changes observed at 566 to 540 nm on addition of antimycin A to the mitochondrial suspension correspond primarily to changes in the redox state of cytochrome b_{562}. Spectral shifts contribute little or nothing to these changes.

3. When a suspension of antimycin A-treated mung bean mitochondria becomes anaerobic with succinate as substrate through the alternate oxidase pathway characteristic of plant mitochondria, cytochrome b_{562}, which is highly reduced in the aerobic steady state, becomes partially oxidized. The degree of reoxidation is maximal in the presence of ATP and decreased by the presence of uncoupler or oligomycin. Reoxidation of the anaerobic mitochondrial suspension causes re-reduction of cytochrome b_{562}.

4. The oxidation of cytochrome b_{562} in antimycin A-treated mitochondria in the presence of ATP on transition to anaerobiosis is synchronous with the reduction of cytochrome c_{549} (corresponding to c_1 in mammalian mitochondria) observed during this transition. Reduction of highly oxidized cytochrome c_{549} in antimycin A-treated mitochondria by addition of sulfide during the aerobic steady state results in a corresponding oxidation of highly reduced cytochrome b_{562}.

5. Oxidation of the b cytochromes by means of the alternate oxidase, which operates on the substrate side of these cytochromes with reference to cytochrome oxidase, is very slow in the presence of antimycin A when the c cytochromes are oxidized.

6. It is concluded that b_{562} and antimycin A form a stabilized complex analogous to the cytochrome a_3/CO complex, in which the reduced form is energetically favored when the c cytochromes are highly oxidized. The midpoint potential of the stabilized complex would be more positive than that of uncomplexed cytochrome b_{562} by an amount determined by the free energies of formation of the antimycin A

complex with oxidized and reduced b_{562}, as is the case with the CO complex of cytochrome a_3. When the *c* cytochromes become reduced, the stabilized cytochrome b_{562}/antimycin A complex relaxes, and in this form resumes a more negative midpoint potential. The cytochrome then is reoxidized by equilibration with oxidized carriers on its substrate side. It is proposed that this mechanism is also applicable to the changes in redox state of cytochrome b_T observed in solubilized mitochondria preparations from heart muscle treated with antimycin A.

INTRODUCTION

The antibiotic antimycin A, first isolated by Dunshee *et al.*[1], has proved to be a particularly useful tool for probing the mitochondrial respiratory chain, since it is a potent inhibitor of electron transport between the cytochromes *b* and the cytochromes *c*, *a*, a_3 (refs 2–5). It has been possible, as a result of this property, to isolate the reactions of the latter cytochromes from the former, and to obtain reduced *minus* oxidized spectra of the cytochromes *b* of mitochondria free of interference from the other cytochromes[4–7]. When such spectra are run at the temperature of liquid N_2, the absorbance maxima are sharper and better resolved[8,9]. Using this technique, Bonner[10–12] was able to show the existence of three cytochromes *b* in plant mitochondria with difference absorbance maxima at 553, 557 and 562 nm which are clearly resolved at 77 °K, but form an asymmetric broad peak centered around 560 nm at room temperature. The maxima observed at 77 °K correspond to maxima at room temperature of 556, 560, and 565 nm, respectively.

In addition to its action as an inhibitor of the oxidation of reduced cytochrome *b* in mitochondria and submitochondrial particles from mammalian tissues, antimycin A also induces changes in the spectra of this component under varying experimental conditions, as first noted by Chance[4,6]. In addition, the titration curve for antimycin A inhibition of respiration[3,13,14] and of the spectral changes[15–17] is sigmoidal rather than hyperbolic. The possible significance of these observations to energy coupling in mitochondria has recently been developed by Slater and associates[18,19]. The interactions of antimycin A with the *b* cytochromes in mitochondrial membrane fragments from heart muscle are complex. They have recently been elegantly reviewed and largely resolved by Brandon *et al.*[20] and need no further exposition here.

The effect of antimycin A on mitochondria isolated from higher plant tissues is similar to that on mammalian mitochondria. The site of inhibition is such as to maintain the cytochromes *b* reduced and cytochromes *c* and aa_3 oxidized in the presence of O_2. The titration curves for the inhibition of succinate and malate oxidation in mung bean (*Phaseolus aureus*) mitochondria by antimycin A are sigmoidal, as shown by Ikuma and Bonner[21]. Inhibition of respiration by antimycin A in mung bean mitochondria is not complete, however. The remaining respiration unaffected by antimycin A has the same rate as that unaffected by cyanide, and proceeds through the alternate, cyanide (and antimycin A)-insensitive oxidase peculiar to plant mitochondria[22–34]. (This oxidase will be referred to as the "alternate oxidase" and the corresponding respiratory pathway as the "alternate respiratory pathway" for convenience in this paper.) No spectral shifts of the cytochrome *b* absorbance maxima induced by antimycin A have been reported for mung bean mitochondria. Bonner and

Slater[35] have reported differential absorbance changes at the wavelength pair 560 to 565 nm when aerobic mitochondria from white potatoes (*Solanum tuberosum*) were treated with antimycin A. As pointed out by these authors, the changes could have been caused as well by changes in the redox state of cytochrome b_{562} as by spectral shifts, and indeed the former appears more probable.

One effect of antimycin A on cytochrome b_{562} in mung bean mitochondria has been observed consistently over the past few years in this laboratory. This cytochrome is highly reduced in State 4 *plus* antimycin A but is partially oxidized on anaerobiosis caused by consumption of O_2 *via* the alternate oxidase. A similar observation concerning cytochrome *b* in yeast cells treated with antimycin A was reported some years ago by Chance[36], and more recently by Kovac *et al.*[37] for both yeast cells and mitochondria isolated therefrom. The other cytochromes *b* in mung bean mitochondria are also highly reduced in State 4 *plus* antimycin A, but on anaerobiosis become slightly more reduced. This observation becomes increasingly interesting in the light of the recent report by Rieske[38] and by Wilson *et al.*[39] of similar behavior by cytochrome b_T[40,41,42], the cytochrome with the difference absorbance maximum at 566 nm, in solubilized mitochondrial preparations from heart muscle. They observed that, in the presence of antimycin A, oxidation of cytochrome c_1 by either ferricyanide or ferricytochrome *c* resulted in a concomitant reduction of cytochrome b_T in either chicken heart mitochondria solubilized with cholate or a purified, soluble preparation of succinate–cytochrome *c* reductase from beef heart muscle. Similar behavior of cytochrome b_T in intact pigeon heart mitochondria has been reported by Erecinska *et al.*[43]. Upon exhaustion of the oxidant, cytochrome b_T becomes reoxidized. The reaction is unaffected by added uncoupler. This interesting response was interpreted as an energization of cytochrome b_T by the residual electron transport which occurs through the antimycin A inhibition site, resulting in a shift of its midpoint potential to far more positive value, thus leading to its reduction[39].

That energy conservation coupled to electron transport might occur in solubilized mitochondria and in a purified soluble succinate–cytochrome *c* reductase is indeed surprising and evidently of great importance in formulating the mechanism of energy coupling. The fact that cytochrome b_{562} in plant mitochondria, whose midpoint potential of -77 mV is quite unaffected by the energy state of the mitochondria[44], behaves rather similarly to cytochrome b_T in the soluble preparations from heart muscle in the presence of antimycin A, suggests that a mechanism other than energy conservation might be operative in the succinate–cytochrome *c* reductase, and that this mechanism might be clarified by investigating the responses to antimycin A of the redox state changes of cytochrome b_{562} in plant mitochondria. The responses of the cytochromes *b* to antimycin A in mung bean and skunk cabbages (*Symplocarpus foetidus*) mitochondria are reported in this paper with particular emphasis on the changes in redox state of cytochrome b_{562} in mung bean mitochondria during transitions between the aerobic and anaerobic states.

METHODS

Mitochondria were prepared from the hypocotyls of 5- or 6-day-old etiolated seedlings of mung bean (*P. aureus*), using substantially the method described by Bonner[45] and Ikuma and Bonner[46], with the modifications of Storey and Bahr[31,47].

Skunk cabbage (*S. foetidus*) flowers were collected from selected marshy areas adjacent to the Wissahickon Creek in Whitemarsh Township, Pa., and stored at 4 °C. Mitochondria were prepared from the excised spadices by the method cited above. The mitochondria were assayed for respiratory control in a medium containing 0.3 M mannitol, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), 5 mM P_i, and brought to pH 7.2 with KOH. This medium is designated TP; the same medium without P_i is designated T. The experiments in this paper were carried out in Medium T, unless otherwise specifically noted. O_2 consumption by the mitochondria suspended in medium TP with succinate or malate as substrate and ADP as P_i acceptor was measured in a closed cuvette with a Clark elctrode (Yellow Springs Instrument Co.) as described by Estabrook[38]. Mitochondrial protein content was determined by a modified Lowry method[49].

Adenine nucleotides were obtained from Boehringer Mannheim Corp.; succinic acid from Aldrich Co.; sodium sulfide and mannitol from J. T. Baker Cemical Co.; oligomycin and antimycin A from Sigma Chemical Co.; these were used without further purification. The uncoupler 1799 was generously supplied by Dr. Peter Heytler of E.I. duPont de Nemours Co.

Absorbance changes on a slow time scale corresponding to the reduction or oxidation of the respiratory chain carriers were monitored using a dual wavelength spectrophotometer[50] with a compensation circuit to reduce noise from light source fluctuations[51], and read out on a strip chart recorder. The following wavelength pairs were used: 468 to 493 nm for flavoprotein; 560 to 540 nm or 560 to 570 nm for b_{557}; and 566 to 540 nm for b_{562}. The subscripts give the reduced *minus* oxidized difference absorbance maxima for the cytochromes in spectra obtained at 77 °K[52,53].

The kinetic measurements were carried out in the rapid mixing, regenerative flow apparatus [54]. Absorbance changes corresponding to the oxidation and reduction of flavoprotein and cytochrome b_{557} were monitored with a dual wavelength spectrophotometer as described above. The optical path length was 0.6 cm. The mitochondria were suspended in Medium TP and depleted with ADP and the uncoupler 1799, then made anaerobic with succinate. Malonate was subsequently added to inhibit succinate dehydrogenase, thereby severely reducing the flux of reducing equivalents into the respiratory chain[55]. The oxidation reaction was initiated by mixing the anaerobic mitochondrial suspension with O_2-saturated medium at volume ratio of 75, giving an initial O_2 concentration of 17 μM. Fast absorbance changes were displayed on a storage oscilloscope and photographed to give permanent records.

RESULTS

Spectral shift vs changes in redox state of cytochromes b_{557} and b_{562}

The first set of experiments reported in this paper deal with the response of the two longer wavelength cytochromes *b* on addition of antimycin A to mung bean mitochondria which have become anaerobic through oxidation of succinate. As the antimycin A is added, the mitochondrial suspension is vigorously stirred to induce a transient aerobic steady state, after which the suspension again becomes anaerobic as O_2 is consumed through the alternate respiratory pathway. The effect of antimycin A added in this manner on cytochrome b_{557} is shown in Fig. 1A. There is a slight apparent re-oxidation of the cytochrome during the transient aerobic steady state,

followed by slow apparent reduction which is not quite complete. Subsequent addition of uncoupler with stirring again causes a slow apparent oxidation of very limited extent; reduction of the cytochrome is slow and complete on anaerobiosis. The same experiment carried out at 566 to 540 nm, the wavelength pair suitable for monitoring the redox state of cytochrome b_{562}, is shown in Fig. 1B. In contrast to the effect on cytochrome b_{557}, antimycin A added in this manner causes a further apparent reduction of cytochrome b_{562} during the transient aerobic steady state; this is reversed rapidly on anaerobiosis. Subsequent addition of uncoupler again produces a rapid apparent reduction whose extent is slightly greater and whose reversal is less than in the absence of uncoupler. This result is in agreement with that observed by Bonner and Slater[35] with potato mitochondria, except that they did not report the reversal

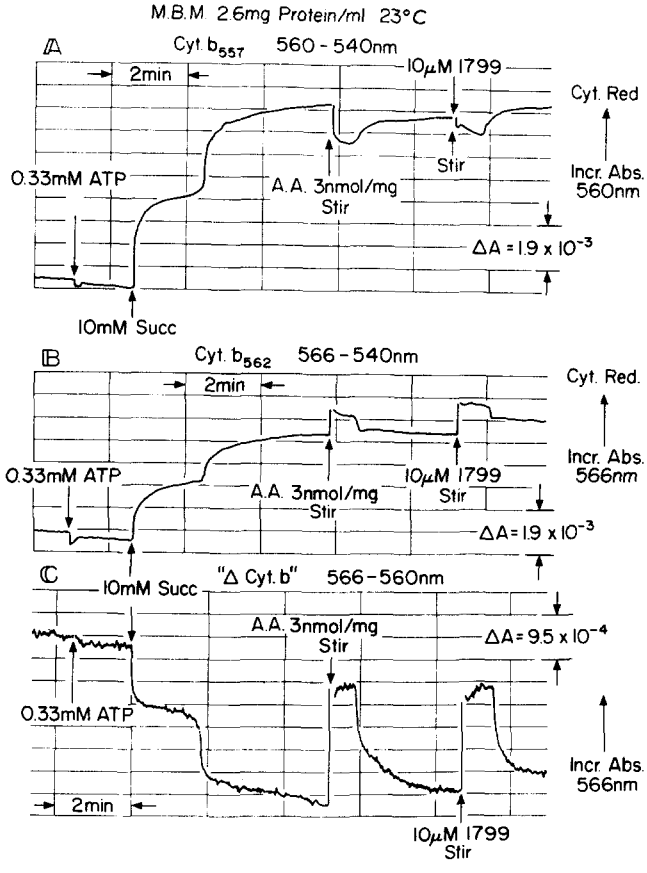

Fig. 1. Effect of adding antimycin A in anaerobiosis on cytochrome b_{557} and b_{562} of mung bean mitochondria (M.B.M.). (A) Absorbance change at 560 to 540 nm, corresponding to cytochrome b_{557}, on addition of antimycin A (A.A.) to mitochondria which have become anaerobic in the presence of succinate and ATP. The suspension is stirred vigorously on addition of antimycin A to give a transient aerobic steady state. After anaerobiosis, the uncoupler 1799 is added at the point indicated, also with vigorous stirring. (B) The same experiment as in (A), but carried out with the wavelength pair 566 to 540 nm corresponding to cytochrome b_{562}. (C) Same experiment as in (A) and (B), but carried out with the wavelength pair 566 to 560 nm, which gives the absorbance difference between cytochromes b_{562} and b_{557}. The sensitivity used for this recording is twice that used for (A) and (B).

of the apparent reduction on anaerobiosis after antimycin A addition. The absorbance changes observed under these same experimental conditions at 566 to 560 nm, the wavlength pair usually used to observe the antimycin A "red shift"[18,20], are shown in Fig. 1C. This trace was recorded at a sensitivity twofold greater than that used in Figs 1A and 1B. The decrease in absorbance at 566 nm on addition of antimycin A is in agreement with the results of Bonner and Slater[35] and with what one would expect from the records of Figs 1A and 1B: Fig. 1C is, in effect, Fig. 1B *minus* Fig. 1A. On addition of uncoupler, the response of the antimycin A-treated mitochondria to added O_2 is again what one would expect from the records of Figs 1A and 1B. The important difference between the responses of cytochrome b_{557} and b_{562} in these experiments is their time course. The initial oxidation of b_{557} and reduction of b_{562} on addition of antimycin are both rapid on the time scale shown here. The subsequent reduction on anaerobiosis of b_{557} is slow while the corresponding reduction of b_{562} is rapid. The second transient aerobic steady state induced by adding uncoupler with stirring gives rapid responses with cytochrome b_{562} and very slow responses with cytochrome b_{557}. If a spectral shift were involved, the responses should be in synchrony. Thus the absorbance changes observed with the wavelength pair 566 to 560 nm reflect primarily changes in the redox state of the two *b* cytochromes rather than a spectral shift.

Effect on cytochrome b_{562} response to antimycin A of mitochondrial energy state

The effect of the energy state of mitochondria on the redox state of cytochrome b_{562} induced by antimycin A addition in anaerobiosis with succinate as substrate is shown in Fig. 2. The four experimental records were obtained with the same mitochondrial suspension. The effect of adding antimycin A accompanied by oxygenation of the suspension, followed by a subsequent transition to anaerobiosis, is shown for coupled mitochondria treated with ATP in Fig. 2A. The result is similar to that observed in Fig. 1B; this experiment serves as a control for the series. If uncoupler is added to the mitochondrial suspension prior to succinate, cytochrome b_{562} remains oxidized in the aerobic steady state and is only partially reduced in anaerobiosis (Fig. 2B). Addition of antimycin A increases the reduction twofold in the transient aerobic steady state. The transition to anaerobiosis after this steady state is marked by a lower degree of oxidation of cytochrome b_{562} than is observed in the experiment of Fig. 2A. If the mitochondria become partially energized by oxidation of succinate in the absence of ATP (Fig. 2C), a condition in which the succinate dehydrogenase is not fully activated[56], the reduction of cytochrome b_{562} is slower in the aerobic steady state, and the transition to anaerobiosis is marked by a rapid reduction followed by reoxidation, since the energy generated by the respiratory chain is no longer provided. Addition of antimycin A to this mitochondrial suspension (Fig. 2C) gives a further reduction of the cytochrome to the maximum observable in the transient aerobic steady state: the reoxidation on anaerobiosis is comparable to that seen with uncoupler (Fig. 2B). Subsequent addition of ATP gives a greater degree of reduction in the transient aerobic steady state, while the transition to anaerobiosis gives a greater oxidation. The latter is, as expected, comparable to that observed in Fig. 2A. If the mitochondria are treated with oligomycin and then with ATP, as in the experiment of Fig. 2D, the degree of reduction in the aerobic steady state with succinate is higher because of activation of the succinate dehydrogenase by ATP. Since the ATP cannot interact

with the respiratory chain, free energy generation by the respiratory chain stops at anaerobiosis, and a cycle of reduction followed by oxidation occurs. Addition of antimycin A then gives complete reduction in the transient aerobic steady state, followed by a degree of reoxidation comparable to that observed in the experiments of Figs 2B and 2C. These experiments imply that the pronounced reoxidation of cytochrome b_{562} on anaerobiosis is energy linked, in the sense that it is observed only when ATP can interact with the respiratory chain. Lack of ATP, or the presence of

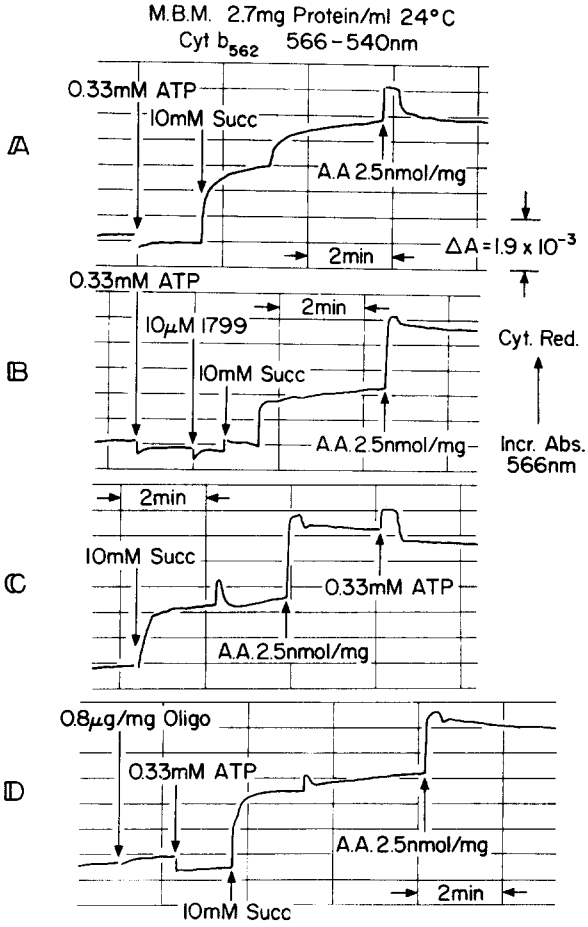

Fig. 2. Effect of adding antimycin A (A.A.) on cytochrome b_{562} in mung bean mitochondria (M.B.M.) which have become anaerobic with succinate in different energy states. The wavelength pair 566 to 540 nm is used in all the experiments; the absorbance changes are all recorded at the same sensitivity. The mitochondrial suspension is stirred vigorously on addition of antimycin A to induce a transient aerobic steady state. (A) Energized mitochondria, which have become anaerobic by oxidation of succinate in the presence of ATP. (B) Uncoupled mitochondria; the experiment was carried out as in (A), but the uncoupler 1799 was added prior to addition of succinate. (C) Partially energized mitochondria which have become anaerobic by oxidation of succinate alone; in this experiment, ATP is added after the addition of antimycin A. (D) Mitochondria energized by succinate oxidation in the presence of oligomycin (Oligo); this experiment was carried out as in (A), but oligomycin was added prior to the addition of ATP and succinate.

uncoupler or oligomycin partially inhibit this reaction but do not completely abolish it.

The question of an energy-linked oxidation of cytochrome b_{562} by ATP in mung bean mitochondria is examined more closely in the series of experiments presented in Fig. 3. Addition of antimycin A to the aerobic mitochondrial suspension causes a slow, partial reduction of cytochrome b_{562} due to endogenous substrate (Fig. 3A); subsquent addition of ATP causes reoxidation. Addition of succinate at this point gives a rapid reduction, followed by a much slower one proceeding to a very limited extent. The transition to anaerobiosis produces the expected oxidation. A transient aerobic

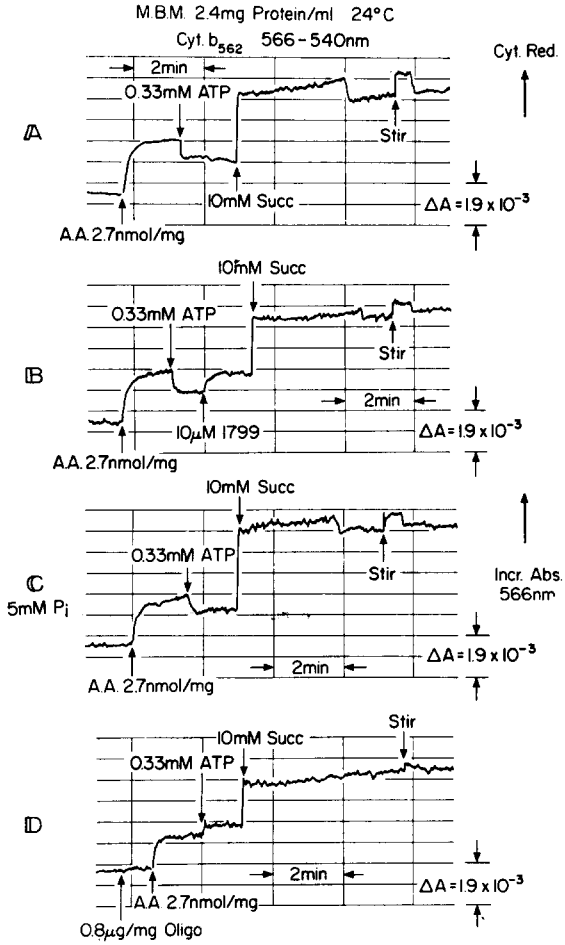

Fig. 3. Absorbance changes characteristic of cytochrome b_{562}, monitored at 566 to 540 nm, on addition of succinate to mung bean mitochondria (M.B.M.) treated with antimycin A (A.A.) and on reaching anaerobiosis by succinate oxidation through the alternate, cyanide (and antimycin A)-insensitive terminal oxidase. (A) Succinate added directly to mitochondria treated with antimycin A followed by ATP. At the point marked "stir", the suspension was stirred vigorously to induce a transient aerobic steady state. (B) The same experimental conditions as in (A), but the uncoupler 1799 was added just prior to the addition of succinate. (C) The same experimental conditions as in (A), but with 5 mM P_i added to the medium. (D) The same experimental conditions as in (A), but with oligomycin (Oligo) added prior to the addition of antimycin A.

steady state is induced by stirring which results in reduction of cytochrome b_{562}, followed by rapid oxidation on the second transition to anaerobiosis. In the experiment of Fig. 3B, uncoupler is added to the mitochondrial suspension directly after addition of ATP. The oxidation of cytochrome b_{562} induced by ATP is reversed, showing that this oxidation is indeed energy-linked. Addition of succinate results in essentially full reduction of the cytochrome; the extent of oxidation on anaerobiosis in the uncoupled mitochondria is one third that observed in the coupled mitochondria. The presence of 5 mM P_i in the reaction medium (Fig. 3C) reduces the extent of the energy-linked responses by about one half, as would be expected from the lower phosphate potential, but otherwise the responses of cytochrome b_{562} are much the same as in the absence of P_i. Addition of oligomycin to the mitochondrial suspension prior to the addition of antimycin (Fig. 3D) abolishes the oxidation of the cytochrome by subsequent addition of ATP. In fact, addition of ATP induces a slight further reduction, presumably by increasing the rate at which reducing equivalents are supplied from endogenous substrate. Subsequent addition of succinate produces the expected further reduction of the cytochrome but the extent is about 15 % less than that observed in the other experiments, and the transition to anaerobiosis is now hardly perceptible, as are the transitions induced by aeration through stirring. The changes in redox state in the presence of oligomycin under these conditions (Fig. 3D) are still smaller than those observed under the conditions of Fig. 2D. The experiments differ in that the conditions of the one in Fig. 2D allow generation of free energy by the cytochrome pathway during the aerobic steady state, but the conditions of the one in Fig. 3D do not: the oxidation occurs *via* the alternate pathway which does not conserve the free energy of succinate oxidation[56].

Effect of the redox state of cytochrome c_{549} on that of b_{562} in the presence of antimycin A

The record of Fig. 3A shows that the oxidation of cytochrome b_{562} in coupled mung bean mitochondria treated with antimycin A is rapid on transition to anaerobiosis with succinate as substrate. This rapid response of cytochrome b_{562} is also evident in the records shown in Figs 1B, 2A, and 2C. The reduction of the cytochromes *c* on the transition to anaerobiosis from the aerobic steady state in antimycin A-treated mitochondria oxidizing succinate is also rapid. The question then arises as to whether the two processes are synchronous. An experiment pertinent to this question is shown in Fig. 4. The pattern of addition of reagents to the mitochondrial suspension is that used in Fig. 3A: antimycin A, followed by ATP, followed by succinate. As the mitochondrial suspension approaches anaerobiosis (as determined in separate experiments), the speed of the strip chart recorder is increased in order to resolve the time course of the anaerobic transition. The record of Fig. 4A shows the response of cytochrome c_{549}. The redox state of this cytochrome is virtually unaffected by the addition of either antimycin A, ATP, or succinate; its degree of reduction in the aerobic steady state is effectively nil. On anaerobiosis, there is a rapid reduction of cytochrome c_{549} with an estimated half-time of 2 s. Induction of a transient, aerobic steady state by stirring air into the suspension produces a rapid, complete oxidation of the cytochrome, followed by a second, slightly slower reduction with an estimated half-time of 2.5 s on anaerobiosis. The record of Fig. 4B shows the comparable response of cytochrome b_{562}. On the transition to anaerobiosis from the aerobic steady state, in which the cytochrome is highly reduced, there is an oxidation which is essentially synchronous

with the reduction of cytochrome c_{549} with a half-time of 2 s. Induction of a transient steady state by stirring reduces the cytochrome, and reoxidation on anaerobiosis occurs synchronously with the reduction of cytochrome c_{549}.

From this experiment, it is evident that the anaerobic oxidation of cytochrome b_{562} follows directly the anaerobic reduction of cytochrome c_{549}. The question as to whether anaerobiosis *per se* or the actual redox state of the cytochrome c_{549} is the determining factor can be decided by treating the mitochondria with sulfide to induce cytochrome c_{549} reduction in the aerobic steady state. This experiment is shown in Fig. 5. Two parallel experiments were carried out at each wavelength pair. In one, the sulfide was added during the aerobic steady state with succinate and antimycin A. In the other, which acts as control, the mitochondrial suspension was allowed to go anaerobic to establish the duration of the aerobic steady state, and the sulfide was then added to the anaerobic suspension. The duration of the steady state is the same in the presence of antimycin A *plus* sulfide as it is in the presence of antimycin A alone, since the respiratory pathway through the alternate oxidase controls the rate of O_2 consumption[21]. The response of cytochrome c_{549} to sulfide added in the aerobic steady state is shown in the top record of Fig. 5A. There is a rapid partial reduction, followed by a slow reduction which lasts until anaerobiosis, at which point reduction is complete. The control experiment is shown in the lower trace. The duration of the aerobic steady state is the same in both experiments, and no change in the final state of reduction is observed on addition of sulfide after anaerobiosis. The transient aerobic

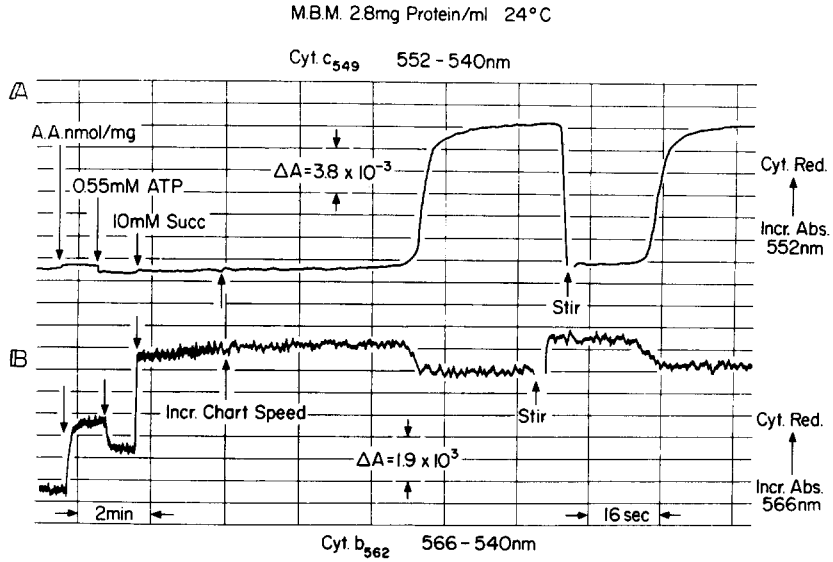

Fig. 4. Kinetics of the aerobic to anaerobic transition in the redox states of cytochrome c_{549} (A) and cytochrome b_{562} (B) in coupled mung bean mitochondria (M.B.M.) treated with antimycin A (A.A.) and ATP. The two experiments were carried out sequentially with the same mitochondrial suspension under conditions as nearly identical as possible. At the point indicated after addition of succinate, the recorder chart speed was increased 8-fold; the span corresponding to 16 s at the higher chart speed is shown in the figure. After the first transition to anaerobiosis, the mitochondrial suspension was stirred vigorously at the point indicated to induce a transient aerobic steady state, followed by a second transition to anaerobiosis. The response speed of the detection and recording system is shown by the speed of the deflection observed on stirring.

steady state induced by sulfide addition is characterized by highly reduced cytochrome c_{549}, rather than by the very highly oxidized cytochrome observed upon stirring in air with antimycin A alone as inhibitor (Fig. 4A). The response of cytochrome b_{562} to sulfide under these same conditions is shown in Fig. 5B. Addition of sulfide in the aerobic steady state induces a rapid oxidation of the cytochrome, as shown in the top trace. The transition to anaerobiosis is barely perceptible as a slight reduction. Addition of sulfide to the mitochondrial suspension after exhaustion of O_2, as shown in the lower trace of Fig. 5B induces a very small reduction immediately followed by

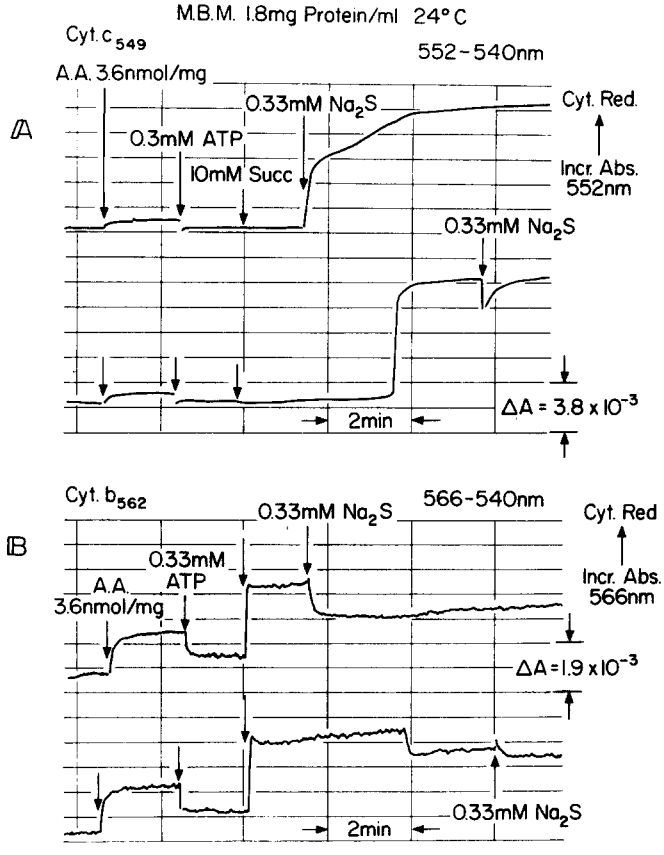

Fig. 5. Induction of cytochrome c_{549} reduction concomitant with cytochrome b_{562} oxidation on adding sulfide to coupled mung bean mitochondria (M.B.M.) oxidizing succinate in the aerobic steady state with antimycin A (A.A.) present. The four experiments were carried out with the same mitochondrial suspension under conditions as nearly identical as possible. (A) Cytochrome c_{549}. The effect of adding sulfide during the aerobic steady state with antimycin A is shown in the top trace; the bottom trace is the control in which sulfide is added after the attainment of anaerobiosis and consequent reduction of the cytochrome. The wavelength pair 552 tot 540 nm is used to monitor the redox state of c_{549}. (B) Cytochrome b_{562}. As in the experiment which yielded the records shown in (A), the top trace shows the effect of adding sulfide to the aerobic steady state with antimycin, while the lower trace is the control. The wavelength pair 566 to 540 nm is used to monitor the redox state of cytochrome b_{562}; all other experimental conditions as in (A).

reoxidation with the same time course observed for oxidation and re-reduction of cytochrome c_{549} (Fig. 5A).

These results imply that inhibition of the oxidation of reduced cytochrome b_{562} in the presence of antimycin A is relaxed when the cytochromes *c* become reduced. The oxidation is enhanced by ATP which also reverses the effect of the slow reduction by endogenous substrate. This suggests that the electron flow driven by ATP from the reduced cytochrome b_{562}/antimycin A complex in the presence of reduced cytochromes *c* is to the respiratory chain carriers on the dehydrogenase or substrate side of the cytochrome, and that electron flow in this direction is also inhibited by antimycin A when the cytochromes *c* are oxidized. All three cytochromes *b* are similarly affected in this respect by antimycin A. This suggestion can be tested in mitochondria treated with antimycin A by providing oxidizing equivalents to the cytochromes *b* from the substrate side with respect to cytochrome oxidase *via* the alternate oxidase

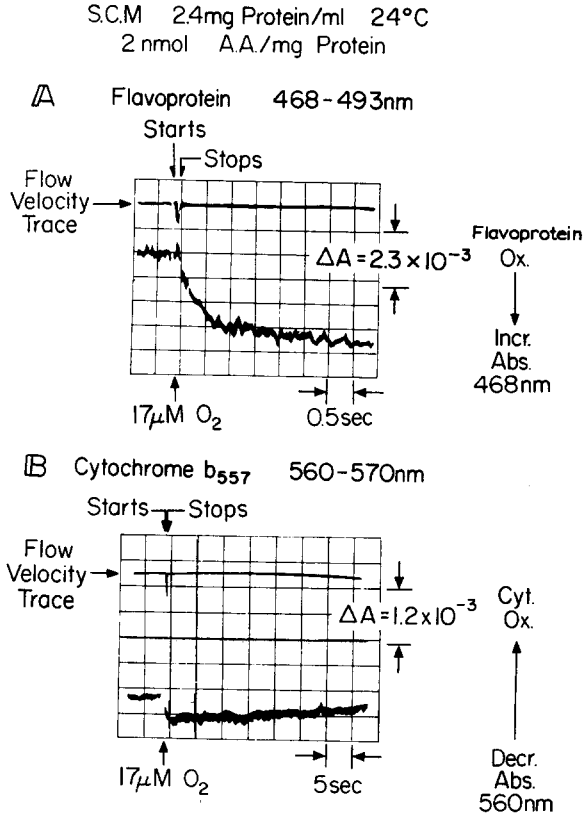

Fig. 6. Oscilloscope records showing the time course of the oxidation induced by a pulse of 17 μM O_2, of the flavoprotein component (A) and cytochrome *b* component (B) of skunk cabbage mitochondria (S.C.M.) in the presence of antimycin A (A.A.) at 2 nmoles/mg protein. The mitochondria are suspended in Medium TP (see Methods) containing 0,28 μM ADP, 6 μM 1799. The substrate is 2.8 mM succinate, and 33.5 mM malonate is added to give a malonate to succinate ratio of 12. The redox state of the flavoprotein component (A) is monitored with the wavelength pair 468 to 493 nm; that of the cytochrome *b* component (B) is monitored with the pair 560 to 570 nm. Note that record (A) was obtained at an oscilloscope sweep rate of 0.5 s/cm while record B was obtained at a sweep rate of 5 s/cm.

in O_2 pulse experiments[28,29,31,32]. The experiment was carried out using skunk cabbage mitochondria, since the activity of the alternate oxidase is much greater in these mitochondria than it is in mung bean mitochondria[34,57]. The pertinent experimental records (which were obtained in collaboration with Dr. M. Erecinska) are shown in Fig. 6. The time course of oxidation by an oxygen pulse of flavoprotein in skunk cabbage mitochondria treated with antimycin A is shown in Fig. 6A. The oxidation is biphasic on the time scale shown with half-times of 150 ms and 550 ms. The shorter half-time corresponds to the oxidation of the low-potential flavoproteins while the longer one corresponds to the oxidation of the principal high-potential flavoprotein component, FP_{ha} (refs 57, 58, 59). It is evident that the alternate oxidase can provide oxidizing equivalents to the flavoprotein components of the respiratory chain at quite a respectable rate, although this rate is still two orders of magnitude lower than that exhibited by the cytochromes *c* under these experimental conditions[31,53]. The same experiment was carried out with the wavelength pair 560 to 570 nm, which records the redox state primarily of cytochrome b_{557} and secondarily of cytochrome b_{562}[44], in order to compare the oxidation rates of these two cytochromes with that of flavoprotein. The record is shown in Fig. 6B. The initial absorbance change is essentially complete during the mixing time of the apparatus and corresponds to the time course of oxidation of the cytochromes *c*. This initial change amounts to only 11 % of the absorbance change observed in O_2 pulse experiments in the absence of antimycin A, and is in the direction of reduction rather than of oxidation. This initial response is attributed to cytochrome b_{562} on the evidence of the experiments presented in this paper. The subsequent oxidation of cytochrome b_{557} is very slow on the time scale of this experiment, which is 10-fold slower than that of Fig. 6A. It is clear that the oxidation of the reduced cytochromes *b* by the respiratory chain carriers, located on the substrate side with respect to cytochrome oxidase, is effectively inhibited by antimycin A in the presence of oxidized cytochromes *c*.

DISCUSSION

Two conclusions may be drawn from the experiments presented here concerning the effect of antimycin A on the redox responses of cytochrome b_{562} in plant mitochondria, as monitored with the spectrophotometer. First, there is little or no change in the difference absorbance spectrum of this cytochrome in the presence of antimycin A. Second, inhibition of electron transport from reduced cytochrome b_{562} to an oxidized carrier located on the substrate side is far more potent if the cytochromes *c* are oxidized than if the cytochromes *c* are reduced. The first conclusion implies that changes of redox state of cytochrome b_{562} are directly measured by absorbance changes at 566 to 540 nm with no complications from spectral shifts. The second conclusion implies that, in the presence of oxidized cytochromes *c*, reduced cytochrome b_{562} forms a complex with antimycin A which is analogous to the complex of reduced cytochrome a_3 with CO. In the latter complex, the CO ligand stabilizes the reduced cytochrome a_3/CO complex such that, in equilibration with cytochrome *a* located on the substrate side of cytochrome a_3, the equilibrium constant favors formation of reduced a_3/CO to a far greater extent than formation of reduced a_3 in the absence of CO. Reverse electron transport from reduced a_3/CO to oxidized *a* is, in effect, inhibited. One consequence of this stabilization is that the midpoint potential

of the a_3/CO complex is much more positive than that of the uncomplexed carrier. The shift in midpoint potential from that of uncomplexed cytochrome a_3 is given directly by the difference in free energies of formation, ΔG_f°, of the CO complexes of oxidized and reduced a_3, the reduced a_3/CO complex having by far the more negative ΔG_f° value. This property of cytochrome a_3 has been ingeniously utilized by Wilson and Dutton[60] to differentiate definitively between cytochromes a and a_3 in their measurement of the midpoint potentials under strictly anaerobic conditions. In this experimental situation, where O_2 cannot interfere, the cytochrome a_3/CO complex remains reduced even at the very positive potentials set by added redox mediators which give complete oxidation of cytochrome a, and the latter cytochrome can be titrated without interference from a_3.

The cytochrome b_{562}/antimycin A complex in the presence of oxidized cytochromes c appear to be very similar to the cytochrome a_3/CO complex. The rate of reduction of cytochrome b_{562} seems unimpaired when so complexed with antimycin A. But the rate of oxidation by carriers located on the substrate side is effectively inhibited since the complex has a much more positive midpoint potential as compared to the midpoint potential of the uncomplexed cytochrome. Again, this shift is given directly by the difference in ΔG_f° values of the antimycin A complexes of reduced and oxidized b_{562}.

The foregoing comments are valid only for the redox reactions which occur on the substrate side of cytochrome a_3 and cytochrome b_{562}. The ligand CO inhibits electron transport between reduced cytochrome a_3 to O_2 at the active site for that reaction, and antimycin A inhibits electron transport between cytochrome b_{562} (and the other b cytochromes) and the cytochromes c at the active site for that reaction, both sites being located on the O_2 side of the respective carriers. It is in the nature of an electron transport carrier of the mitochondrial respiratory chain that it have two active sites for reversible redox reactions with its partner carriers: one on the substrate side with respect to cytochrome oxidase and one on the O_2 side. Each site is presumably specific for the particular partner carrier. Determination of the sequence of the respiratory chain carriers has been facilitated in large part by the fact that certain inhibitors are specific for one or the other of the two sites. It is a peculiarity of both CO and antimycin A that, on binding to the active site located on the O_2 side of cytochrome a_3 and the cytochromes b, respectively, they produce a complex in which the reduced form of the carrier itself is stabilized relative to the uncomplexed carrier, leading to positive shift in midpoint potential. It is an additional peculiarity of antimycin A that this stabilization occurs only when the cytochromes c are fully oxidized.

It is evident from the records shown in Fig. 3 that ATP can reverse in part the slow reduction of cytochrome b_{562} induced by addition of antimycin A, in a reaction sensitive to both uncoupler and oligomycin. The rate of electron transport to the cytochrome from endogenous substrate is sufficiently slow that a slight acceleration of the reverse reaction linked to the free energy of ATP utilization can be observed, even though the reverse reaction is slow in the cytochrome b_{562}/antimycin A complex. (The same reaction is seen with the other two cytochromes b and is currently under investigation.) The addition of succinate so increases the rate of electron transport to the cytochrome b_{562}/antimycin A complex that the reverse reaction is overwhelmed and the cytochrome is completely reduced. When the cytochromes c become reduced,

either through anaerobiosis or by addition of sulfide, the stabilized reduced cytochrome b_{562}/antimycin A complex relaxes. Electron exchange with the appropriate carrier located on the substrate side of the cytochrome is no longer inhibited by the unfavorable midpoint potential of the stabilized complex, and the cytochrome b_{562} then becomes oxidized: to a very limited extent in uncoupled mitochondria, and to a substantial extent in coupled mitochondria with ATP available to displace the equilibrium by reverse electron transport. The carrier located on the substrate side appears at present to be flavoprotein, but this assignment is not yet definite.

In extending this model of a cytochrome *b*/antimycin A complex to account for the results reported for solubilized heart mitochondria[39], and purified succinate–cytochrome *c* reductase[38,39], one makes the following correspondences between the respiratory chain carriers of plant and heart muscle mitochondria. Cytochrome c_{549} is the cytochrome *c* strongly bound to the mitochondrial membrane[61]; it seems reasonable to assume that it corresponds to cytochrome c_1 in mammalian systems and is the one which influences the behavior of the cytochromes *b* in plant mitochondria. Since non-energized cytochrome b_T in mammalian mitochondria and cytochrome b_{562} are the cytochromes *b* of low midpoint potential[40,44], they are the ones incompletely reduced by succinate in the absence of some perturbation such as energy-linked reactions or complexation with antimycin A. The other cytochromes are essentially completely reduced, and it is the *b* cytochromes with long wavelength maxima which give anomalous effects under these experimental conditions in both systems. Despite the crucial difference that the midpoint potential of cytochrome b_T is changed by energization with ATP[40], while that of cytochrome b_{562} is not[44], the two carriers are considered to correspond in comparing the two systems. When cytochrome c_{549} (c_1) is oxidized, the stabilized cytochrome b_{562} (b_T)/antimycin A complex forms in which the cytochrome is now reduced. As the oxidant is used up, cytochrome c_{549} (c_1) becomes reduced, the stabilized cytochrome b_{562} (b_T)/antimycin A complex relaxes, and the cytochrome becomes reoxidized. In the case of mung bean mitochondria, the cytochrome b_{562} transfers electrons to an oxidized carrier on the substrate side, apparently flavoprotein. In the case of the solubilized mitochondria and purified succinate–cytochrome *c* reductase, the ultimate acceptor is added fumarate. But in both cases, the reaction sequence as interpreted by this model is essentially the same.

Energy coupling at Site II resulting in shift of midpoint potential cannot be invoked to explain the redox changes of cytochrome b_{562} since the midpoint potential of this cytochrome is a constant, independent of the energy state of the respiratory chain. One can invoke a more positive value of the midpoint potential for the cytochrome b_{562}/antimycin A complex than for cytochrome b_{562} itself, analogous to the more positive midpoint potential of the cytochrome a_3/CO complex compared to that of cytochrome a_3. Further, this particular cytochrome b_{562}/antimycin A complex of high midpoint potential — called the stabilized from of the complex — is formed in the presence of oxidized cytochrome c_{549} and reverts to a form with normal midpoint potential — called the relaxed form — in the presence of reduced cytochrome c_{549}. It is suggested that such a mechanism might also apply to the heart muscle systems with cytochrome b_T and cytochrome c_1 playing the role of b_{562} and c_{549}, respectively. This mechanism is perhaps simpler than one which invokes, at Site II of the respiratory chain, an energization reaction which is unaffected by uncoupler or solubilization of the membrane.

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