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REDUCIBILITY OF CYTOCHROMES *b* IN AEROBIC BEEF-HEART MITOCHONDRIA TREATED WITH ANTIMYCIN

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SUMMARY

Under anaerobic conditions cytochrome *b* in beef-heart mitochondria is partially reduced in the presence of NADH, whereas other cytochromes are completely reduced. Addition of antimycin together with oxygen under these conditions causes an immediate reduction of cytochromes *b*-558, *b*-562 and *b*-566 and oxidation of cytochrome *c*. During the subsequent transient aerobic steady state cytochromes *b*-558 and *b*-566 are rapidly re-oxidized without changes in redox state of cytochrome *c*, but cytochrome *b*-562 remains reduced. When oxygen is consumed by the leak through or around the antimycin-inhibition site, cytochrome *b*-562 becomes oxidized with concomitant reduction of cytochrome *c*.

The cytochromes *b* in lyophilized beef-heart mitochondria are more readily accessible to electrons from NADH, and in the presence of antimycin and NADH a complete and stable reduction is obtained under both aerobic and anaerobic conditions. Gradual addition of rotenone under these conditions causes re-oxidation of cytochromes *b* in which oxidation of cytochromes *b*-558 and *b*-566 precedes that of cytochrome *b*-562.

It is concluded that (1) the effect of antimycin in the presence of oxygen involves all three cytochromes *b*, (2) the reducibility of the cytochromes *b* in the aerobic steady state of antimycin-treated mitochondria is dependent upon the potential of the substrate redox couple registered on the cytochromes, and (3) the midpoint potential of cytochrome *b*-562 in the presence of antimycin is higher than that of cytochrome *b*-558 or *b*-566.

INTRODUCTION

The earlier observations of Chance[1] and Pumphrey[2] that cytochrome *b*, reduced by substrate in the presence of antimycin, is re-oxidized when the suspension becomes anaerobic have been explained by the demonstration that oxygen[3–7] or ferricyanide[5,8] is necessary as well as antimycin to induce or maintain the reduction of cytochrome *b*. Mechanisms have been proposed by Baum *et al.*[9], Erecińska *et al.*[6] and Wikström and Berden[7].

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Under the conditions in which these experiments are usually carried out, *b*-562 is already reduced by succinate before the addition of antimycin (with oxygen), and this addition induces the reduction of *b*-566[10,3-8] (and *b*-558)[4]. We have recently reported, however, that addition of antimycin together with oxygen to anaerobic, NADH-reduced pigeon-heart mitochondria causes a transitory reduction of cytochrome *b*-562, whereas in mitochondria reduced by succinate in the presence of glutamate it causes reduction of cytochromes *b*-558 and *b*-566[11]. It was concluded from these observations that the effect of oxygen in antimycin-treated pigeon-heart mitochondria is not exclusively on cytochrome *b*-566, but also on cytochrome *b*-562. The lack of reduction of *b*-558 and *b*-566 is presumably due to the poor accessibility of the electrons derived from NADH to the cytochromes *b* in these mitochondria, as shown by the fact that only 13 % of the cytochrome *b* is reduced after anaerobiosis[12].

The present report describes detailed studies on the redox response of cytochromes *b* in NADH-reduced beef-heart mitochondria on addition of antimycin and oxygen, which clearly demonstrate reduction of all three cytochromes *b*.

METHODS

Heavy beef-heart mitochondria were prepared according to the method of Löw and Vallin[13] and were kept at −20 °C for several months. These preparations, when thawed, show approximately two-thirds of the succinate oxidase activity of the freshly prepared mitochondria, but the NADH oxidase and phosphorylating capacity of submitochondrial particles derived from the frozen mitochondria are the same as those from the freshly prepared. Since damage to the mitochondrial membrane during the freezing–thawing makes the mitochondria permeable to NADH, no additional treatment (*e.g.* with proteinase) of mitochondria is necessary to render the mitochondria capable of oxidizing NADH. In order to obtain a clear-cut response of NADH-reduced mitochondria to addition of antimycin it was necessary to deplete the mitochondria of endogenous substrates, as much as possible, by prolonged incubation under a gentle stream of oxygen. Lyophilized mitochondria were prepared with frozen–thawed mitochondria suspended in 0.15 M KCl at a concentration of approximately 20 mg protein/ml. Lyophilization was carried out for 7–9 h. Changes in redox state of cytochromes *b* and *c* were followed with an Aminco-Chance dual-wavelength spectrophotometer.

RESULTS

Fig. 1 shows the kinetics of reduction and oxidation of cytochromes *b* and *c* after additions of NADH, antimycin, and succinate together with glutamate. As observed in pigeon-heart mitochondria[12], the reduction of cytochrome *b* upon anaerobiosis is partial, whereas that of cytochrome *c* (and aa_3, not shown) is complete. The degree of reduction of cytochrome *b* in frozen and thawed beef-heart mitochondria (40 %) is, however, greater than that in pigeon-heart mitochondria (13 %). Addition of antimycin (together with oxygen) after anaerobiosis causes an abrupt reduction of cytochrome *b* and oxidation of cytochrome *c*. The reduction of cytochrome *b* on addition of antimycin is only transitory, and slow re-oxidation occurs when the

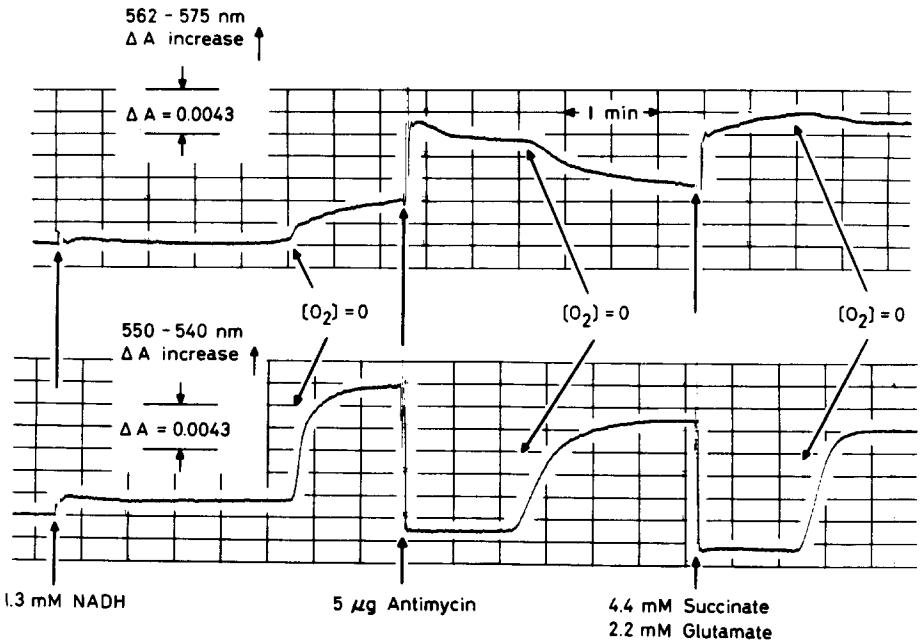

Fig. 1. Redox response of cytochromes *b* (upper) and *c* (lower) in anaerobic, NADH-reduced beef-heart mitochondria upon addition of antimycin together with oxygen and comparison with the response in the presence of succinate and glutamate. The mitochondria were suspended in 225 mM mannitol, 75 mM sucrose and 50 mM Tris–acetate buffer (pH 7.0) at a final concentration of 1.20 mg protein/ml. The preparation used in this experiment was kept at −20 °C for several months.

oxygen introduced during the addition of antimycin is consumed by the leak through or around the antimycin-inhibition site[14]. Upon anaerobiosis cytochrome *b* returns to the level that could be reached by slow reduction in the absence of antimycin after the same period of time. On the other hand, $\Delta A_{550\text{-}540}$ does not return to the original level. The difference spectrum of after addition of antimycin *minus* before addition shows a broad trough in the region of 548–552 nm. Depletion of ubiquinone from mitochondria results in a decrease of the trough to approximately one half (Lee, I. Y., unpublished). This suggests that the difference in $\Delta A_{550\text{-}540}$ before and after addition of antimycin is due to a component that is affected by depletion of ubiquinone, and by antimycin.

Close examination of the kinetics of re-oxidation of cytochrome *b* after addition of antimycin indicates that the oxidation occurs at least in two distinct steps. The initial oxidation proceeds without redox changes in cytochrome *c*, followed by a transient steady state, and later by a secondary oxidation with reduction of cytochrome *c*. Similar changes in the kinetics of oxidation of cytochrome *b* have been observed by Wikström and Berden[7] with succinate as substrate in the presence of a high concentration of fumarate. As shown in Fig. 2 the initial reaction can be clearly demonstrated by addition of rotenone which causes a shift of the transient steady state to a more oxidized state. The initial changes in cytochromes *b* and *c* after addition of succinate together with glutamate to anaerobic, antimycin-treated mitochondria (Fig. 1) are very similar to those obtained on addition of antimycin together

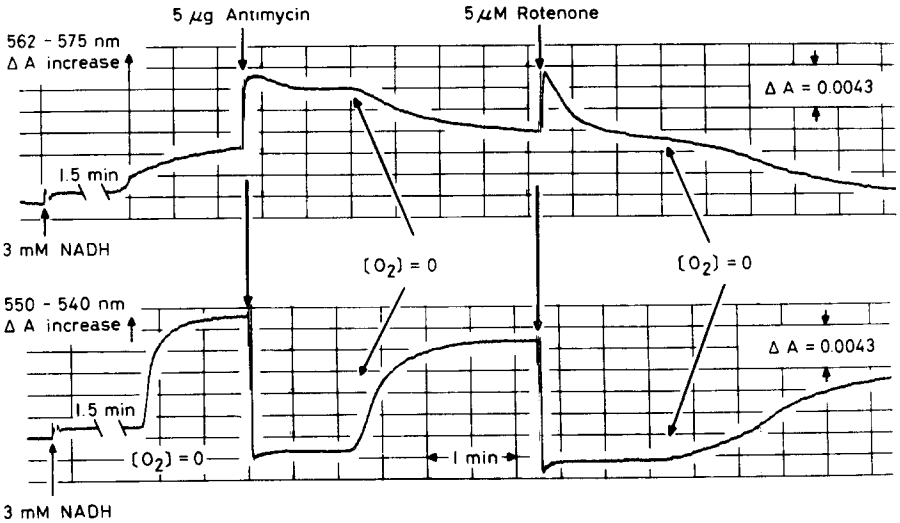

Fig. 2. Effect of rotenone on the redox response of cytochromes *b* (upper) and *c* (lower) in anaerobic, NADH-reduced beef-heart mitochondria upon addition of antimycin together with oxygen. Experimental conditions are identical to those described in Fig. 1. 1.20 mg protein/ml.

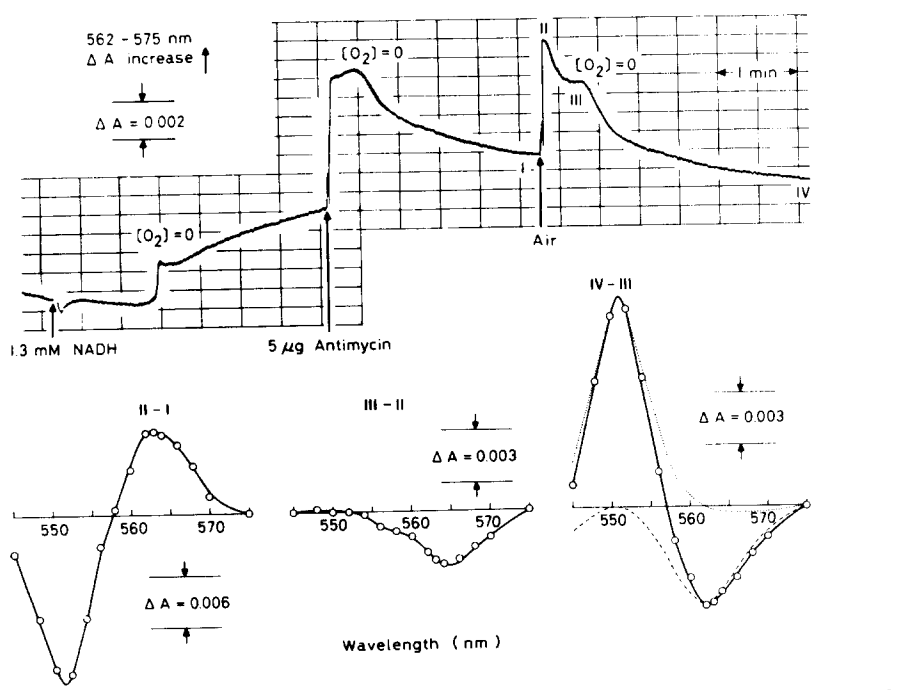

Fig. 3. Upper: Redox response of cytochromes *b* in NADH-reduced beef-heart mitochondria upon addition of antimycin together with oxygen and the effect of an additional stirring on the response. Experimental conditions are similar to those described in Fig. 1. 1.41 mg protein/ml. The preparation used in this experiment was kept at −20 °C for less than a week. Lower: Difference spectra of cytochromes *b* and *c* reduced and oxidized during the anaerobic–aerobic transitions in the presence of NADH and antimycin. The dotted line in the right-hand figure is a calculated spectrum for reduced *minus* oxidized cytochrome $c + c_1$ (molar ratio 2:1). The dashed line is the experimental spectrum *minus* the dotted line.

with oxygen to NADH-treated mitochondria. However, cytochrome *b* now remains reduced even after the anaerobiosis.

As shown in Fig. 3 it seems that a pre-incubation with antimycin is necessary to detect the two distinct kinetics of re-oxidation of cytochrome *b*. The difference spectra obtained at various states after addition of antimycin indicate that the component responsible for the initial rapid re-oxidation largely consists of cytochromes *b*-558 and *b*-566 (*cf.* Fig. 3, lower, middle). Spectral resolution of the components that are re-oxidized upon anaerobiosis (see Fig. 3, lower, right) is difficult since the changes of cytochromes *b* and *c* occur in the opposite direction. However, when the difference spectrum of a mixture of isolated cytochromes c_1 and *c* (molar ratio 1:2), calculated to give the same absorbance at the maximum (dotted line), is subtracted from the actual spectrum, the component responsible for the secondary re-oxidation gives a spectrum with a maximum at 562 nm (broken line).

Conclusive evidence that oxidation of cytochromes *b*-558 and *b*-566 precedes that of cytochrome *b*-562 is provided by the experiments shown in Figs 4 and 5. When mitochondria are lyophilized in the presence of 0.15 M KCl, a part of the cytochromes *b* is readily reduced upon anaerobiosis, and subsequent addition of antimycin further increases the reduction of cytochromes *b* (Fig. 4). The total amount of cytochromes *b* reduced after addition of antimycin is the same as that reduced by succinate in the presence of antimycin, or by dithionite. In contrast to the behaviour of frozen and thawed mitochondria, the cytochromes *b* in lyophilized mitochondria remain reduced in the presence of antimycin even after anaerobiosis, monitored by re-reduction of cytochrome *c* (see Fig. 4, lower trace).

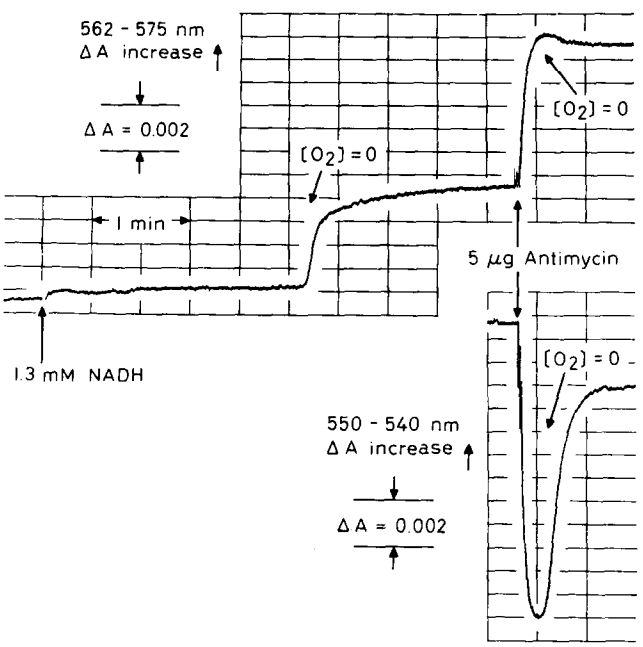

Fig. 4. Redox response of cytochromes *b* (upper) and *c* (lower) in lyophilized beef-heart mitochondria upon addition of NADH and antimycin. Experimental conditions are similar to those described in Fig. 1. 1.13 mg protein/ml.

As shown in Fig. 5 the reduced *minus* oxidized difference spectrum (lower left, Spectrum I) of cytochromes *b* in lyophilized mitochondria in the presence of NADH and antimycin shows a maximum at 562.5 nm and is the same under both anaerobic and aerobic conditions. Gradual addition of rotenone to aerobic, NADH-reduced mitochondria in the presence of antimycin causes gradual oxidation of cytochromes *b* (Fig. 5, upper trace) and results in a gradual shift of absorbance maximum of the remaining reduced cytochromes *b* toward shorter wavelength (*cf.* Fig. 5, lower, left). The difference spectra of cytochromes *b* oxidized after each addition of rotenone (Fig. 5, lower, right) clearly demonstrates that oxidation of cytochrome *b*-558 and *b*-566 is complete before that of *b*-562. The same order of oxidation of the cytochromes *b* is also obtained when the cytochromes reduced by succinate in the presence of antimycin are re-oxidized by gradual addition of malonate.

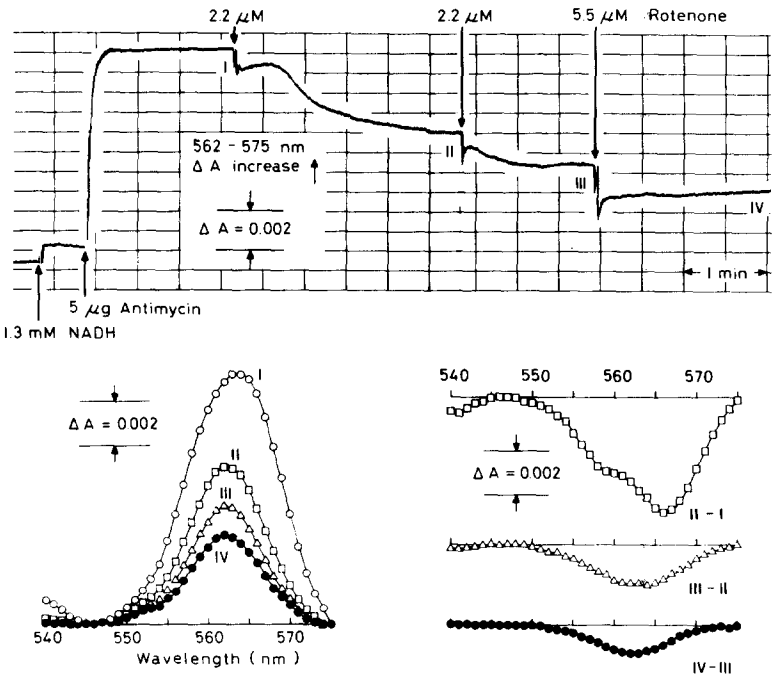

Fig. 5. Upper: Spectral titration of oxidation of cytochromes *b* with rotenone in lyophilized beef-heart mitochondria reduced with NADH and antimycin. Lower, left: Reduced *minus* oxidized difference spectrum of cytochromes *b* taken at various steady states (I–IV). Lower, right: Difference spectra of cytochromes *b* oxidized after each addition of rotenone. Experimental conditions were identical to those described in Fig. 4. 1.13 mg protein/ml.

DISCUSSION

The present communication provides clear-cut evidence that a transitory but complete reduction of all three cytochromes *b* occurs upon addition of antimycin together with oxygen. This observation is in agreement with the recent report by Rieske[8] that all of the cytochrome *b* in Complex III or submitochondrial particles undergoes an apparent increase in mid-point potential in the presence of antimycin

and an appropriate electron acceptor. Our observation is, however, at variance with the report by Erecińska *et al.*[6] that one half of cytochromes *b*, the so-called energy-transducing cytochrome b_T (*b*-566), is involved in the positive potential change. The discrepancy between our observation that all three cytochromes *b* are affected in the presence of antimycin and oxygen and that of Erecińska *et al.*[6] that cytochrome *b*-566 is affected can be explained on the basis of the difference in actual potential of the system employed in the two studies. In anaerobic, NADH-reduced mitochondria, used in our studies, the equilibrium between NADH/NAD^+ and cytochromes *b* cannot be reached owing to a barrier[12] that prevents the access of electrons from NADH to cytochromes *b*. Therefore, the effective potential of the NADH/NAD^+ redox couple registered on cytochromes *b* is much higher than the potential of the couple outside the mitochondria. The observations shown in Figs 1 and 2 that the cytochromes *b* in the transient aerobic steady state after the addition of antimycin are more reduced in the presence of succinate and glutamate, and more oxidized in the presence of rotenone, strongly support this conclusion and indicate further that the effective potential at the level of cytochromes *b* is lower in succinate-reduced than that in NADH-reduced mitochondria. The reduction of cytochrome *b*-566 upon addition of antimycin to succinate-reduced mitochondria is, therefore, due to the fact that cytochrome *b*-562 is already reduced before the addition of antimycin and, whatever the mechanism of antimycin-induced reduction may be, the addition of antimycin under these conditions can only result in the reduction of cytochrome *b*-566 (and *b*-558). Whether the transitory reduction of cytochromes *b* in the NADH-reduced mitochondria upon addition of antimycin and oxygen is as a result of the antimycin-induced removal of the accessibility barrier[12] cannot be clarified at present. However, preliminary EPR studies show that the transitory reduction of cytochromes *b* is accompanied by oxidation of all iron–sulphur centres of NADH dehydrogenase[15,16], except Centre 2. This suggests that NADH dehydrogenase and the cytochromes *b* are in equilibrium immediately after addition of antimycin and oxygen.

The increased reduction of cytochromes *b* in NADH-reduced lyophilized mitochondria shown in Figs 4 and 5 may be directly related to the observation by Jolly *et al.*[17] with the electron microscope that lyophilization of mitochondria in the presence of 0.15 M KCl causes aggregation of the tubules of the mitochondrial inner membrane and produces a vesicular structure. It is conceivable that such a structural change in the membrane enables the electrons from NADH to become more accessible to the cytochromes *b* so that the actual potential of the NADH/NAD^+ redox couple registered on cytochromes *b* is lower than that in frozen and thawed mitochondria.

The oxidation of cytochromes *b*-558 and *b*-566 prior to that of cytochrome *b*-562 shown in Figs 3 and 5 clearly indicates that the mid-point potential of the former species is higher than that of the latter. This observation leads to an interesting conclusion that although the spectrum of cytochrome *b*-562 is shifted by antimycin[18,11], its mid-point potential in the presence of antimycin is still higher than those of cytochromes *b*-558 and *b*-566.

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