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AN ANTIMYCIN-SENSITIVE CYTOCHROME *b* COMPONENT IN BEEF-HEART MITOCHONDRIA

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SUMMARY

1. Addition of antimycin to particles from beef heart, reduced by succinate or ascorbate in the presence of phenazine methosulphate and absence of oxygen, or by dithionite, results in a shift to the red of the cytochrome *b* absorption band without any concomitant further reduction.

2. Addition of antimycin to particles from beef heart, reduced by ascorbate in the presence of 4,4,4′,4′-tetramethyl-*p*-phenylenediamine and absence of oxygen, causes an oxidation of cytochrome *b*.

3. Potentiometric redox titrations of beef-heart mitochondria in the presence of uncoupler reveal high-potential and low-potential cytochrome *b* components. The midpoint potential of the component with highest potential is lowered by antimycin.

4. Absorption spectra measured during the redox titration show that the high-potential component (20% of total absorbance at 562–575 nm) is a b_{562}. Two low-potential components, with peaks at 562 nm and 565 nm, respectively, are present.

5. It is suggested that the antimycin-induced shift of the cytochrome *b* absorption band and oxidation of cytochrome *b* in these particles are due to the effect of antimycin on the high-potential cytochrome b_{562}.

INTRODUCTION

RIESKE *et al.*[1] showed that complete inhibition of the enzyme activity of the $b \cdot c_1$ complex is obtained with 1 molecule of antimycin per 1 molecule of cytochrome c_1 per 2 molecules of cytochrome *b*. The same stoicheiometry was found in direct binding studies[2]. Because of this stoicheiometry and the fact that antimycin, added to mitochondria or sub-mitochondrial particles after reduction by succinate, in the presence or absence of cyanide, causes reduction of a component absorbing at 565 nm, we distinguished between two components, *b* (562 nm) and b_i (565 nm in the presence of antimycin or dithionite), both sensitive to energization by ATP[3–5]. Chance *et al.*[6] also recognized two *b* species, b_K (562 nm) and b_T (565 nm), and

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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suggested that the former functions purely as an electron carrier, whereas b_T acts also as an energy-transducing enzyme. In this paper, the purely operational description of b_{562} and b_{565} will be used.

A third *b* species with a relatively high mid-point potential was found by Dutton *et al.*[7,8] to be present in beef-heart mitochondria or particles[7] and in pigeon-heart sub-mitochondrial particles[8]. This species will be the subject of this paper.

RESULTS

Fig. 1, Curve 1, shows, in agreement with earlier reports[4], that antimycin induces the reduction of a *b* component absorbing at 565–566 nm in succinate-reduced anaerobic phosphorylating sub-mitochondrial particles. In the presence of an electron-carrier mediator such as phenazine methosulphate (Curve 2), however, the antimycin-effect spectrum is typically a shift curve, indicating that, under these conditions, antimycin does not cause an increase in the amount of cytochrome *b* that is reduced (*cf.* ref. 9), but brings about a change in the absorption maximum (a 'red shift') of an existing component. Since it seems probable that this red shift occurs also in the absence of the mediator, Curve 1 is presumably composite, being due both to the antimycin-induced reduction of a component with the absorption spectrum shown by Curve 3, and to the effect of antimycin on the spectrum of this or of another component.

Spectra identical to Curve 2 of Fig. 1 were obtained with ascorbate, in the presence of phenazine methosulphate, or $Na_2S_2O_4$, despite the fact that b_{562} is only partially reduced by ascorbate, suggesting that b_{562} comprises more than one component, only one of which is affected by antimycin. If, however, tetramethyl-*p*-phenylenediamine is used instead of phenazine methosulphate, the addi-

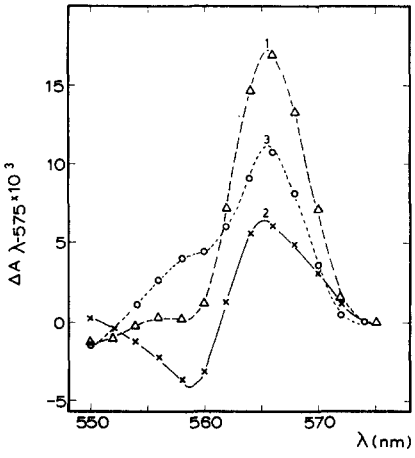

Fig. 1. Effect of antimycin on cytochrome *b*. To ATP–Mg particles (3.6 mg/ml), made anaerobic by oxidation of succinate (10 mM was added), 3 μM antimycin was added in the absence or presence of 10 μM phenazine methosulphate. The effect at different wavelengths was measured when $A_{565-575nm}$ was constant. ΔA_{575nm} was set to zero. Curve 1, effect of antimycin in the absence of phenazine methosulphate; Curve 2, effect of antimycin in the presence of phenazine methosulphate; Curve 3, Curve 1 *minus* Curve 2.

tion of antimycin to anaerobic mitochondria or sub-mitochondrial particles brings about the immediate and complete oxidation of cytochrome *b* (Fig. 2, Curve B). If cyanide (and oxygen) are present the oxidation is found only after a lag phase in which oxygen is consumed. The effect of oxygen on cytochrome *b* in the presence of antimycin will be described more extensively in a forthcoming paper.

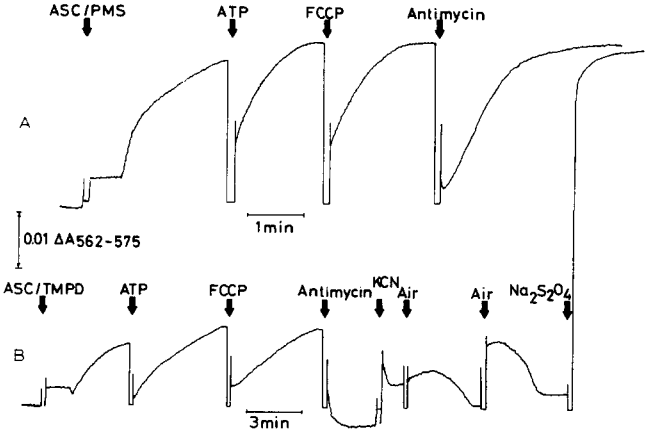

Fig. 2. Effect of antimycin on cytochrome *b*. A particles (3 mg/ml) were suspended in a medium containing 0.25 M sucrose, 10 mM Tris–HCl buffer (pH 7.4) and 1 mM EDTA. After successive additions of 4 mM ascorbate (ASC) – 10 μM phenazine methosulphate (PMS), 1 mM ATP and 0.5 μM FCCP (A), or 4 mM ascorbate – 60 μM tetramethyl-*p*-phenylenediamine (TMPD), 1 mM ATP and 0.5 μM FCCP (B), 4 μM antimycin was added. In B, 4 mM KCN was then added, followed by oxygen (by stirring) and finally $Na_2S_2O_4$.

In order further to study the effect of redox mediator on the response of cytochrome *b* to antimycin, in the absence of oxygen, potentiometric titrations of the type described by Dutton *et al.*[7] were carried out. Fig. 3A shows that, in the presence of phenazine ethosulphate, antimycin has an effect only at high potentials. The upper part of the two curves in Fig. 3A are identical to the titration curves for Complex III given by Rieske[10], who measured only above 80 mV. In Fig. 3B the potentiometric titration has been resolved, according to the procedure of Dutton *et al.*[7], into two components with E'_0 values (at pH 7.2) of 154 mV, comprising 21 % of the total $\Delta A_{562-575nm}$, and 28 mV, comprising the remainder. Antimycin causes a drop in the potential of the high-potential component to 107 mV. In the light of the potentiometric titrations of Dutton *et al.*[7], it is probable that the low-potential component, whose potential is not affected by antimycin, comprises two species that are not resolved in the experiment shown in Fig. 3.

Fig. 4 shows the absorption spectra measured during the potentiometric titration, in the absence and in the presence of antimycin. The high-potential component, specifically reduced between 210 and 110 mV in the absence of antimycin, absorbs at 562 nm, in disagreement with Dutton *et al.*[8], who assign 558 nm as the absorption maximum of this component. In the low-potential region a b_{562} (between 70 and 20 mV) and a b_{565} with shoulder at 558 nm (between 20 and —35 mV) can be distinguished.

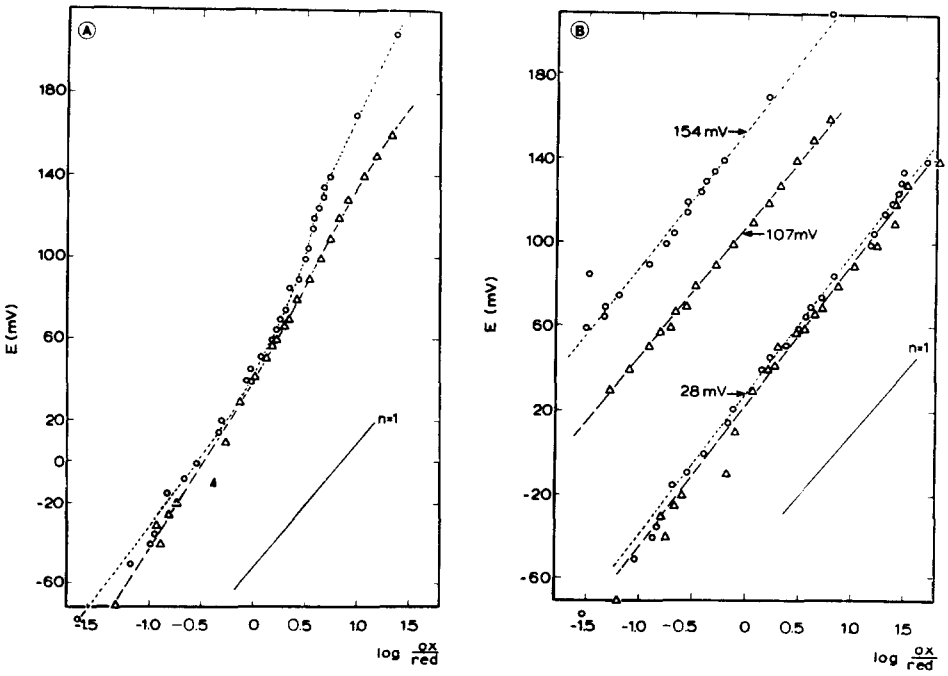

Fig. 3. Potentiometric redox titration of the *b*-cytochromes in beef-heart mitochondria. Beef-heart mitochondria (2.3 mg/ml) were suspended in a medium containing 0.25 M sucrose, 0.05 M Tris–HCl buffer (pH 7.2), 30 μM diaminodurene, 20 μM phenazine ethosulphate, 50 μM duroquinone, 4 μM pyocyanine, 25 μM 2-hydroxy-1,4-naphthoquinone, 25 μM anthraquinone-1,5-disulphonate and 2 μM FCCP. Anaerobiosis was reached by addition of a small amount of ascorbate. The potential of the system was made more positive by addition of ferricyanide and a reductive titration was carried out by adding small amounts of 200 mM NADH, and for the lower potentials freshly prepared aqueous $Na_2S_2O_4$. The subsequent addition of ferricyanide gave points lying on the same curve, showing that the titration is reversible. The ratio oxidized/reduced was calculated from the absorbance at 562–575 nm, assuming that 100% reduction was reached with dithionite. O, in the absence of antimycin; $\triangle$, in the presence of 10 μM antimycin. In B the curves of A are resolved into their component parts, assuming that the different components contribute 21% and 79%, respectively, to the total $\Delta A_{562-575nm}$.

In the presence of ascorbate and phenazine methosulphate antimycin causes a shift of the peak from 561.6 to 562.5 nm (Fig. 5, Curves 1 and 2). If allowance is made for the fact that only about one-half of the ascorbate-reducible *b* is sensitive to antimycin, according to the potentiometric titrations, it may be calculated that antimycin shifts the absorption peak from 561.6 nm to 563.1 nm, with a 10% increase in intensity (Fig. 5, Curves 3 and 4). The antimycin-effect spectrum (Curve 5) shows a trough at 558 nm and a peak at 565 nm.

Fig. 3 predicts that at 100 mV antimycin will cause an oxidation of the high-potential component. This is confirmed by the experiment shown in Fig. 6 in which antimycin was added to beef-heart mitochondria at a potential of 100 mV established by the addition of a small amount of NADH in the presence of mediators. A titration of this effect of antimycin is shown in Fig. 7, which includes for comparison the titration of the antimycin-induced shift in the presence of phenazine methosulphate. As shown by Pumphrey[12], the antimycin-effect curves are linear in the presence of artificial electron carriers, in contrast to the sigmoidal curves obtained

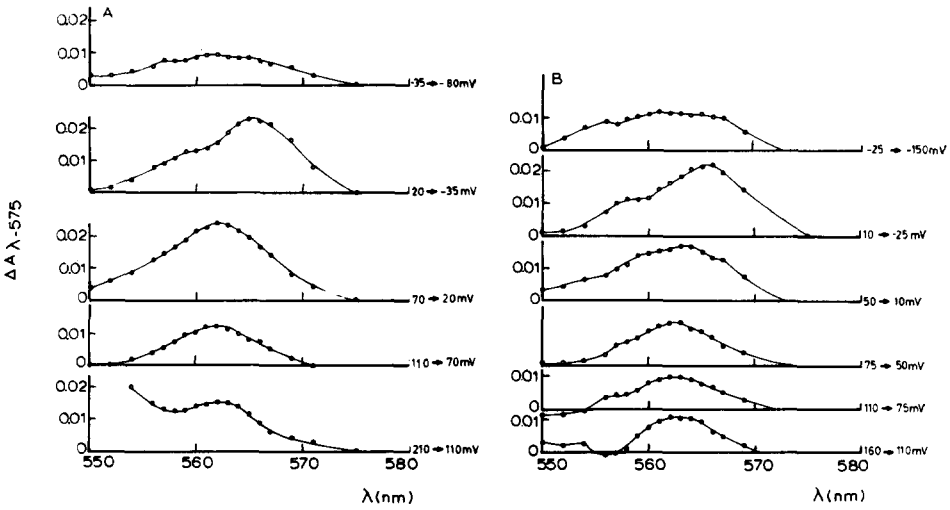

Fig. 4. Difference spectra of cytochrome *b* in beef-heart mitochondria, reduced at different redox potentials during the titration shown in Fig. 3. A, in the absence of antimycin; B, in the presence of antimycin.

with natural electron donors. The stoicheiometry in both titrations shown in Fig. 7 is the same, and the same as that found in previous titrations[2], namely 1 molecule antimycin per molecule of cytochrome c_1.

DISCUSSION

It is concluded that the 'red shift' induced by antimycin is due to an effect on the high-potential component absorbing at 562 nm. However, this is not the only effect of antimycin. In the absence of phenazine methosulphate, antimycin induces reduction of a component absorbing at 565 nm with a shoulder at 558 nm (see Fig. 1). The antimycin titration curve of the red shift is linear (Fig. 7), whereas that of the increased reduction is sigmoidal[12]. This is probably the explanation of the finding of Pumphrey[12], confirmed by Bryła *et al.*[13], that antimycin-effect curves are linear in the presence of artificial electron carriers and sigmoidal with natural electron donors. Indeed, the sigmoidal curves published by Bryła *et al.*[13], using the wavelength pair 566–560 nm, that records both the red shift and the increased reduction, have both linear and sigmoidal components (J. A. Berden and E. van Kuipers, unpublished). The former presumably represents the shift, the latter the increased reduction.

Two conditions have been described in which antimycin induces an oxidation of cytochrome *b*: (1) when a potential of 100 mV is established in the presence of phenazine methosulphate (Fig. 6); (2) when ascorbate is used as hydrogen donor in the presence of tetramethyl-*p*-phenylenediamine. Since the potential of the ascorbate-dehydroascorbate system, when anaerobiosis was established in Expt. 2B, is about 20 mV, this implies that tetramethyl-*p*-phenylenediamine is not able to establish an equilibrium between ascorbate and cytochrome *b*. Phenazine methosulphate seems to be a better mediator between ascorbate and cytochrome *b* (Fig. 2A).

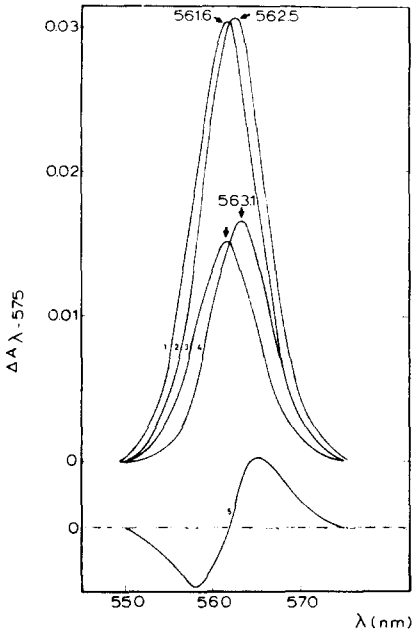

Fig. 5. Difference spectra (reduced *minus* oxidized) of A particles[11] (3 mg/ml) reduced with 5 mM ascorbate in the presence of 10 μM phenazine methosulphate and absence of oxygen. After a constant level was reached and the spectra recorded, 3 μM antimycin was added. The difference spectra were corrected (by subtraction) for the contribution of cytochromes *c* and c_1, whose combined absorption spectrum is known from the spectra measured during the potentiometric redox titrations between 310 and 200 mV where only the *c*-cytochromes are reduced. Curve 1, difference spectrum of cytochrome *b* after reduction with ascorbate. Curve 2, the same after addition of antimycin. Because ascorbate reduced somewhat more than 40% of dithionite-reducible *b*, the absorbance was divided by a factor of 2 to obtain the spectrum of the cytochrome *b* component sensitive to antimycin (21% of dithionite-reducible cytochrome *b*, with the same absorption spectrum as the rest of the ascorbate-reducible *b*). This is given in Curve 3. To obtain the spectrum of the antimycin-sensitive *b* component, in the presence of antimycin, one-half of the absorbance values shown in Curve 1 were subtracted from Curve 2. This is given in Curve 4. Curve 5 shows the effect of antimycin (difference between Curve 1 and Curve 2 or between Curve 3 and Curve 4).

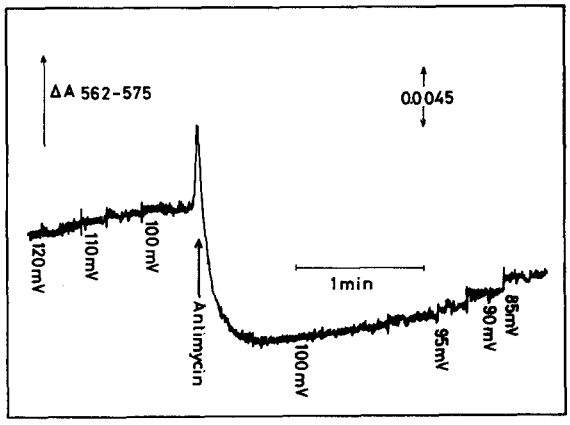

Fig. 6. Effect of antimycin on the high-potential cytochrome *b*. A redox potential of 100 mV was established under the conditions described in Fig. 3 and antimycin then added. The addition of antimycin had no effect on the redox potential measured.

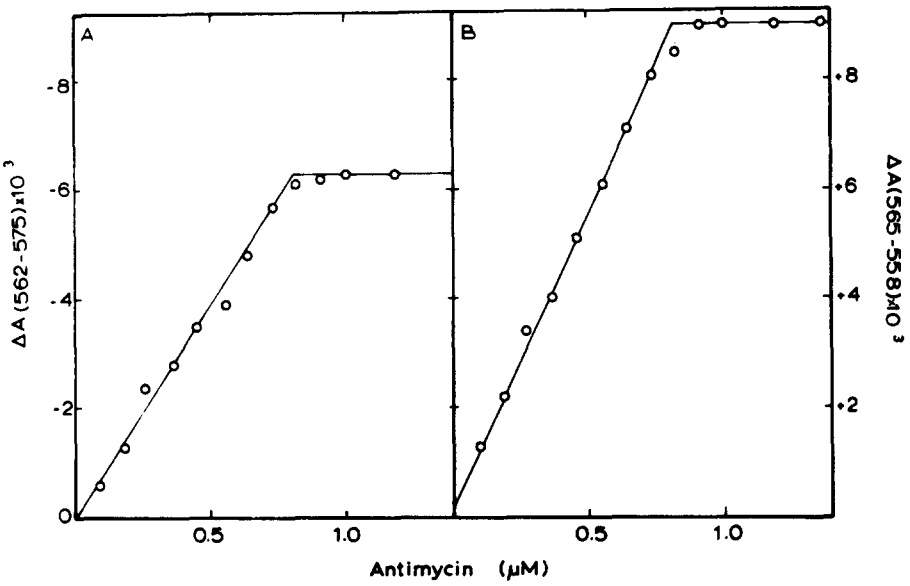

Fig. 7. Titration of A particles (1.6 mg/ml, light path 2 cm) with antimycin. A, titration at a constant redox potential (100 mV). Further conditions as in Fig. 3. B, titration after anaerobiosis by succinate *plus* phenazine methosulphate. $\Delta A_{565-558\ nm}$ measures the difference between the maximum and minimum of the shift spectrum.

The high-potential component absorbing at 562 nm should be distinguished from *b* (refs 3–5) or b_K (ref. 6), which absorbs at the same wavelength but which is only partly (about two-thirds) reduced by ascorbate in the presence of phenazine methosulphate.

METHODS

Beef-heart mitochondria and sub-mitochondrial particles were prepared according to the usual procedures[14,11]. Spectra were recorded on a Perkin-Elmer 356 double-beam, dual-wavelength spectrophotometer. Potentiometric measurements were carried out in a closed cuvette equipped with a calomel reference electrode and a platinum electrode and attached to an Aminco-Chance dual-wavelength spectrophotometer. Nitrogen was allowed to flow over the surface of the solution. Additions of reductant or oxidant were made with a syringe through a septum. A magnetic stirrer was attached to the side wall of the cuvette. The potentiometer was calibrated with a quinhydrone standard solution.

Diaminodurene was prepared from dinitrodurene[15] purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisc. The FCCP used was kindly supplied by Dr. P. G. Heytler.

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