Biochimica et Biophysica Acta, 376 (1975) 533–548

BBA 46888

ANTIMYCIN-INSENSITIVE MUTANTS OF *CANDIDA UTILIS*

II. THE EFFECTS OF ANTIMYCIN ON CYTOCHROME *b*

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(Received August 26th, 1974)

SUMMARY

1. Cytochrome *b*-562 is more reduced in submitochondrial particles of mutant 28 during the aerobic steady-state respiration with succinate than in particles of the wild type. When anaerobiosis is reached, the reduction of cytochrome *b* is preceded by a rapid reoxidation in the mutant. A similar reoxidation is observed in the wild type in the presence of low concentrations of antimycin.

2. In contrast to the wild type, inhibition of electron transport in the mutant has a much higher antimycin titre than effects on cytochromes *b* (viz., aerobic steady-state reduction; reduction in the presence of substrate, cyanide and oxygen; the 'red shift' and lowering of E'_0 of cytochrome *b*-562). Moreover, the titration curve of electron transport is hyperbolic whereas the curves for the reduction are sigmoidal. The conclusion is, that in both mutant and wild type, the actions of antimycin on electron transport and cytochromes *b* are separable.

3. The red shift in the mutant is more extensive than in the wild type.

4. Cytochrome *b*-558 and cytochrome *b*-566 (that absorbs in mutant and wild type at 564.5 nm) do not respond simultaneously to addition of antimycin, indicating that they are two separate cytochromes.

5. The difference between the effect of antimycin on electron transport and cytochromes *b* reduction is also found in intact cells of the mutant.

6. A model is suggested for the wild-type respiratory chain in which (i) the cytochromes *b* lie, in an uncoupled system, out of the main electron-transfer chain, (ii) antimycin induces a conformation change in QH_2-cytochrome *c* reductase resulting in effects on cytochrome *b* and inhibition of electron transport, (iii) a second antimycin-binding site with low affinity to the antibiotic is present, capable of inhibiting electron transport.

INTRODUCTION

Antimycin is a powerful inhibitor of the mitochondrial respiratory chain, acting between cytochromes *b* and c_1 (for a recent review, see ref. 1). It has also been shown to have effects on the spectral (visual and EPR) properties and redox potential

of cytochrome *b* [1], and it inhibits splitting into subunits, induced by guanidinium salts, of isolated QH_2-cytochrome *c* reductase (Complex III) [2].

Recently, Gupta and Rieske [3] have shown that a sub-unit of this enzyme, with molecular weight 11 500, is specifically labelled with an antimycin analogue, and have proposed that this sub-unit contains the antimycin-binding site.

In an attempt to obtain information on the mechanism of action of antimycin on the antimycin-binding site and, in particular, on the structure of QH_2-cytochrome *c* reductase, we are studying antimycin-resistant mutants of the yeast *Candida utilis* [4,5]. In a previous paper [5] we have shown that the lowered sensitivity to antimycin of respiration in one of these mutants (No. 28) is to only a small extent explained by a lower affinity of the mitochondria for the inhibitor. In this paper, the effects of antimycin on the cytochromes *b* are considered.

METHODS

The growth conditions of mutant and wild-type yeast cells as well as the preparation of submitochondrial particles have been given in our previous paper [5]. All experiments described in this paper, with the exception of those shown in Figs 3 and 4, were carried out with the same preparations of submitochondrial particles from mutant, or wild type. Small aliquots of these preparations were kept at liquid nitrogen temperature until use. Crucial experiments were confirmed with material from other preparations of mutant and wild type.

Respiratory activities were measured at 25 °C in a thermostated Oxygraph vessel supplied with a Clark electrode.

Spectroscopy was carried out in Aminco-Chance spectrophotometers. Some dual-wavelength traces and all the split-beam traces were recorded with an Aminco spectrophotometer model DW-2.

Protein was determined by the method of Cleland and Slater [6]. All chemicals were commercial products of the purest grade available. Antimycin was obtained from Nutritional Biochemicals Corporation and dissolved in methanol. Its concentration was determined spectrophotometrically, using an absorption coefficient at 320 nm of 4.8 $mM^{-1} \cdot cm^{-1}$ [7].

RESULTS

On addition of succinate to oxidized wild-type particles, cytochrome *b* is usually reduced to only a small extent during the steady-state respiration (see Fig. 1A). When the oxygen is consumed the reduction reaches a level that is about one half that obtained with dithionite. Addition of antimycin (together with a little oxygen) to the anaerobic succinate-reduced particles causes an increased reduction. Additional oxygen is necessary for maximal reduction .$Na_2S_2O_4$ causes further reduction. Since this additional reduction is CO sensitive (not shown), it is presumably caused by the presence of an auto-oxidizable cytochrome *b*, either present in the yeast or derived by denaturation of mitochondrial cytochrome *b* during preparation. The requirement for oxygen of the antimycin-induced reduction has been widely studied in other mitochondria (see, for example, refs 1 and 8).

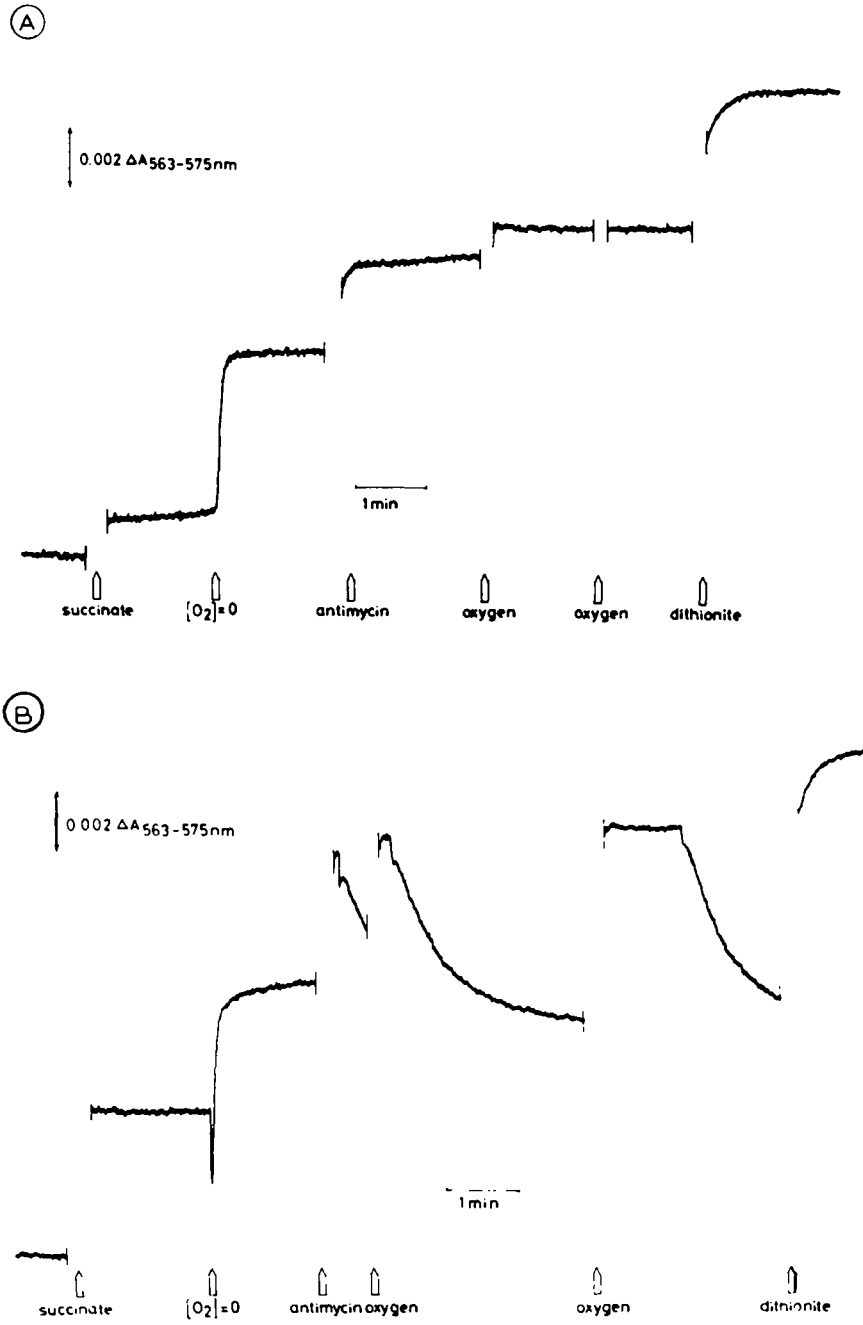

Fig. 1. Cytochrome *b* reduction after various additions to oxidized submitochondrial particles suspended in 250 mM sucrose, 20 mM Tris · HCl buffer and 1 mM EDTA, at pH 7.5. 15 mM succinate added as substrate. Light path, 1 cm. Band width, 1 nm. (A) Wild type 1.7 mg/ml. After anaerobiosis 0.27 μmol/g protein antimycin was added. (B) Mutant 1.3 mg/ml. After anaerobiosis 2.7 μmol/g protein antimycin was added.

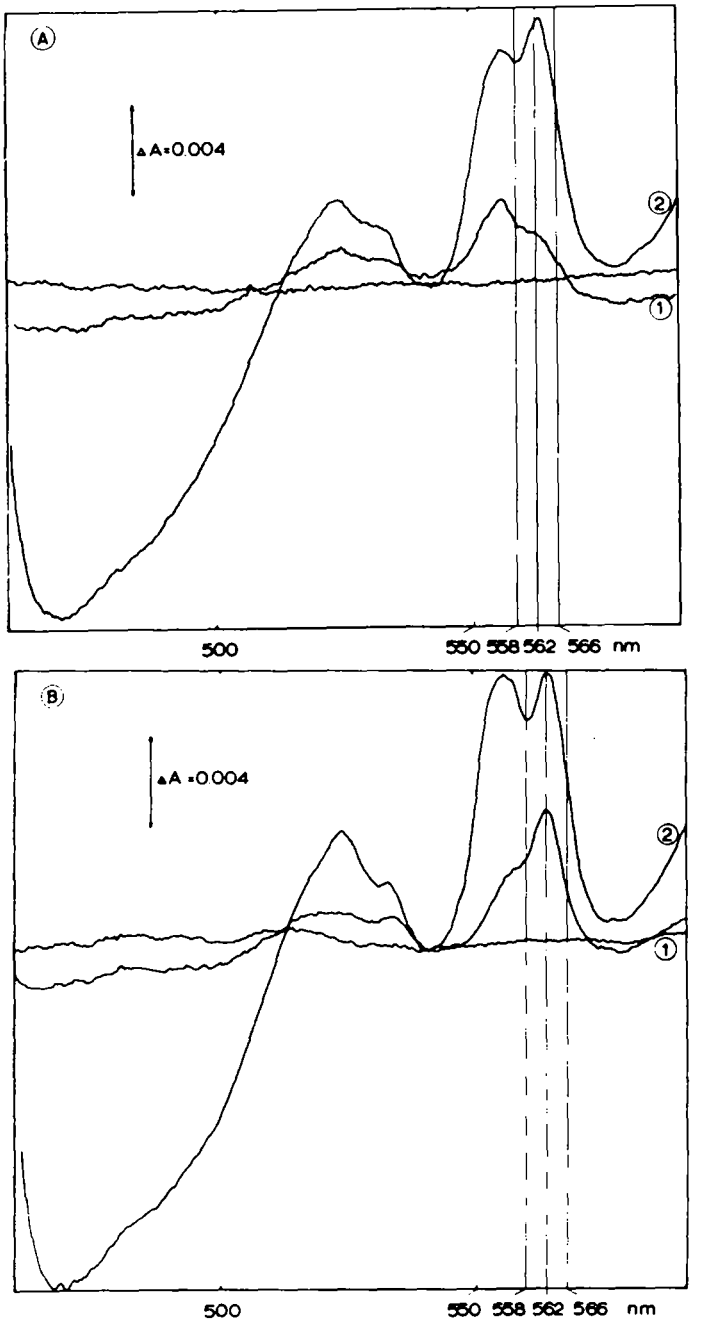

Fig. 2. Reduced minus oxidized spectra, measured in a split-beam spectrophotometer, of submitochondrial particles of wild-type and mutant. The curves 1 represent aerobically reduced by succinate minus oxidized and curves 2 represent anaerobically reduced by succinate minus oxidized. The nearly horizontal lines represent the baseline (oxidized minus oxidized). The particles were suspended in 250 mM sucrose, 20 mM Tris · HCl buffer and 1 mM EDTA, at pH 7.5. 15 mM succinate was used as substrate. The spectra were scanned at such a speed (2 nm/s) that the sample cuvettes of curves 1 remained aerobic. Band width, 1 nm. (A) Wild type, 1.7 mg/ml; (B) mutant, 1.3 mg/ml.

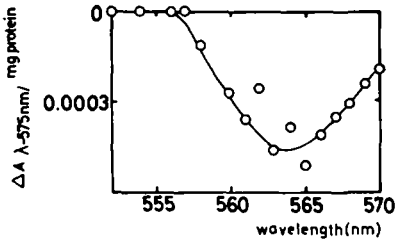

Fig. 3. Spectrum, measured in a dual-wavelength spectrophotometer, of the rapid reoxidation at anaerobiosis in submitochondrial particles of the mutant. The reoxidation shown in Fig. 1B was followed in separate runs at a different sample wavelength. The depth of the reoxidation is plotted then against the wavelength. Particles (2.3 mg/ml) were suspended in 100 mM potassium phosphate buffer (pH 7.5) and 0.1 mM EDTA. 10 mM succinate was used as substrate.

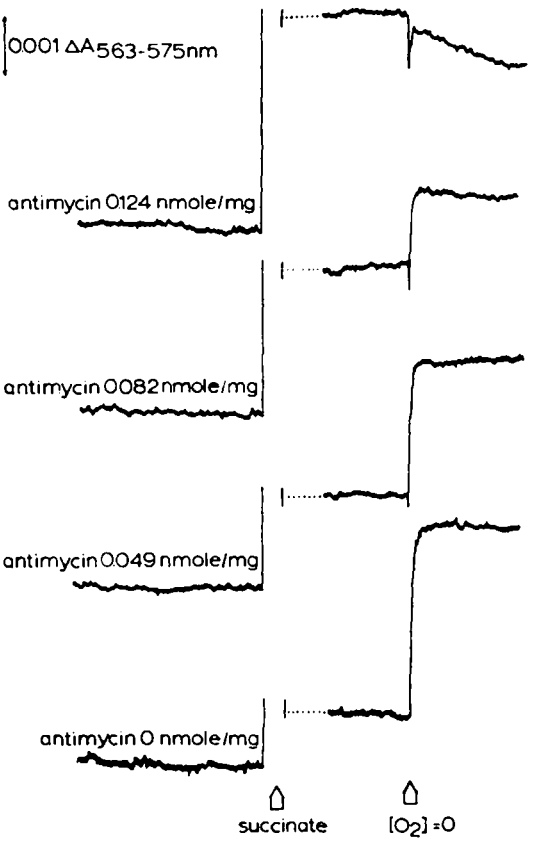

Fig. 4. Reoxidation of cytochrome *b* at anaerobiosis in particles of the wild type oxidizing 10 mM succinate in the presence of substoicheiometric amounts of antimycin. The particles were preincubated for 5 min with the inhibitor before the addition of succinate. Particles in a concentration of 1.8 mg/ml were suspended in 100 mM phosphate buffer (pH 7.5) containing 0.2 mM EDTA.

Fig. 1B shows the corresponding trace with mutant particles. The following differences with the wild type can be observed: (i) Cytochrome *b* is much more reduced in the aerobic steady state (30% compared with 8% in the wild type). The same reduction level was found when NADH was used as a substrate (not shown). (ii) When anaerobiosis is reached, the reduction of the cytochrome *b* is preceded by a short phase in which the cytochrome is more oxidized. (iii) Although the amount of antimycin

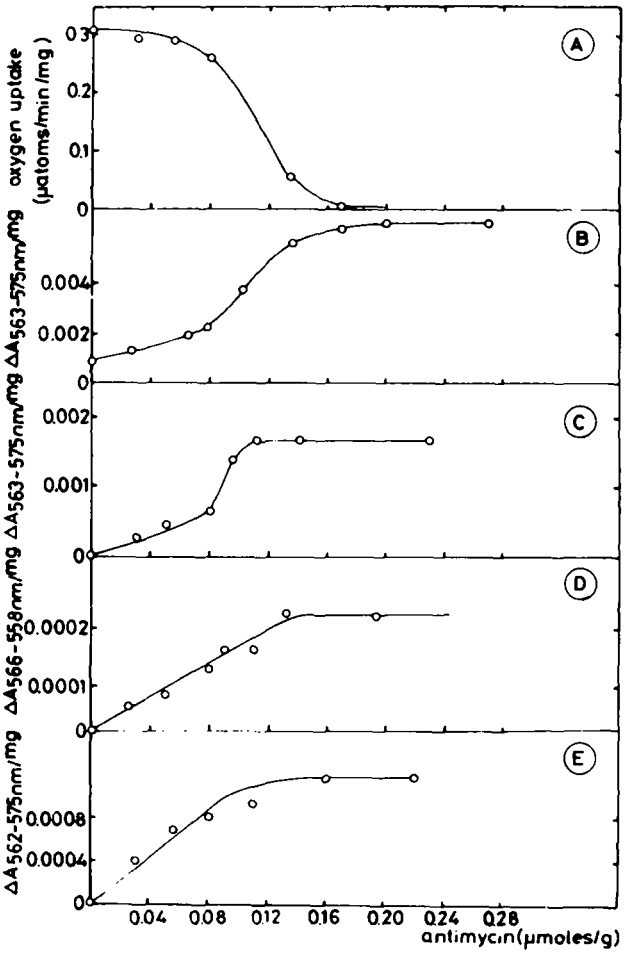

Fig. 5. An antimycin titration of electron transport and various effects on cytochrome *b* in a single submitochondrial preparation of the wild type suspended in the same reaction medium as in Fig. 2. The particles were pre-incubated with antimycin for 5 min before measuring the effect of the antibiotic. For the effects on cytochrome *b* appropriate wavelength pairs were chosen. (A) oxygen uptake with 15 mM succinate as substrate; (B) reduction of cytochrome *b* in the aerobic steady state with 15 mM succinate; (C) the reduction of cytochrome *b* in the presence of 1 mM cyanide, 15 mM succinate and oxygen; (D) the red shift of cytochrome *b* measured when the particles are maximally reduced with a few grains of dithionite; (E) lowering of E'_0 of cytochrome *b* measured as an oxidation after anaerobiosis is reached by respiration of 15 mM succinate. 10 μM phenazine metasulphate was added in order to abolish the reduction induced by antimycin [9]. The redox potential calculated from the succinate/fumarate couple was -45 mV.

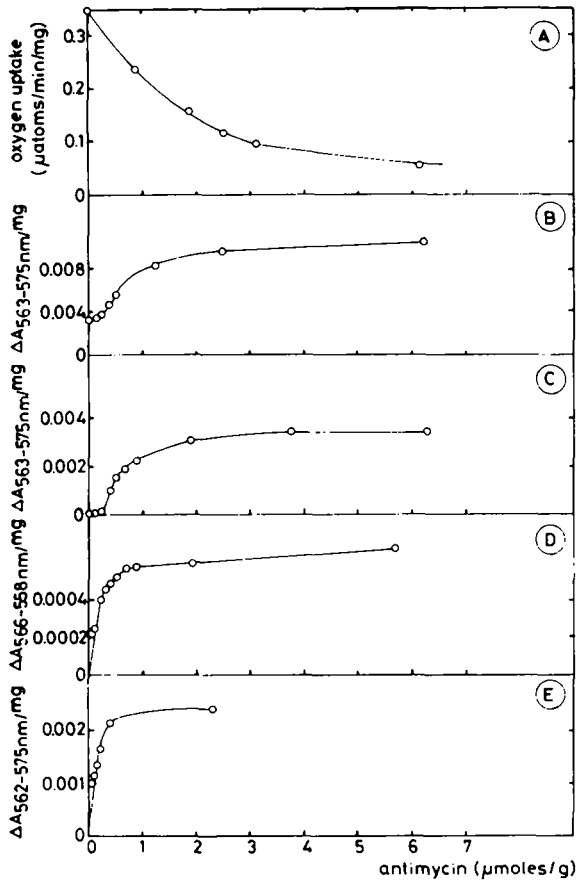

Fig. 6. An antimycin titration of electron transport and various effects on cytochrome *b* in a single submitochondrial preparation of mutant 28. The experiments were carried out as in Fig. 5.

added in the experiment shown in Fig. 1B (2.7 nmol/mg protein) is sufficient to inhibit the respiration only partially (65 %), the cytochrome *b* is highly reduced, but only temporarily, because the oxygen is rapidly consumed by the residual respiration. Mutant particles are, in fact, particularly suitable for the demonstration of the requirement of oxygen for the antimycin-induced reduction of cytochrome *b*.

In both wild-type (Fig. 2A) and mutant (Fig. 2B) particles, it is mainly the *b*-562 species among the cytochromes *b* that is reduced by succinate in the aerobic steady state or after anaerobiosis. As in other mitochondria [1], the complete reduction of *b*-566 requires the addition of antimycin. The spectrum of the species that is momentarily oxidized in mutant particles just before anaerobiosis sets in (Fig. 3) is that of a *b* cytochrome.

A similar reoxidation was also found in the wild type in the presence of antimycin in a concentration, insufficient for maximal inhibition (Fig. 4). Thus the mutant behaves in this respect like the wild type treated with low concentrations of antimycin.

Figs 5 and 6 record the antimycin-effect curves with wild-type and mutant particles, respectively, on the respiratory rate and on various parameters related to

cytochrome *b*. As is to be expected from results reported in the literature (reviewed in ref. 1), the curves for inhibition of electron transport (Fig. 5A), the reduction of cytochrome *b* in the aerobic steady state (Fig. 5B) and the reduction of cytochrome *b* in the presence of succinate, cyanide and oxygen (Fig. 5C) are sigmoidal, whereas those for the 'red shift' (Fig. 5D) and the lowering of the standard midpoint potential of cytochrome *b*-562 (Fig. 5E) are linear (cf. ref. 9). Maximal effects of antimycin are found in all cases with about the same concentration of added antimycin.

As reported earlier, the antimycin-effect curve for the respiration of the mutant (Fig. 6A) is not sigmoidal and much more antimycin is needed for inhibition than with the wild type (note different scales of abscissa in Figs 5 and 6). However, the

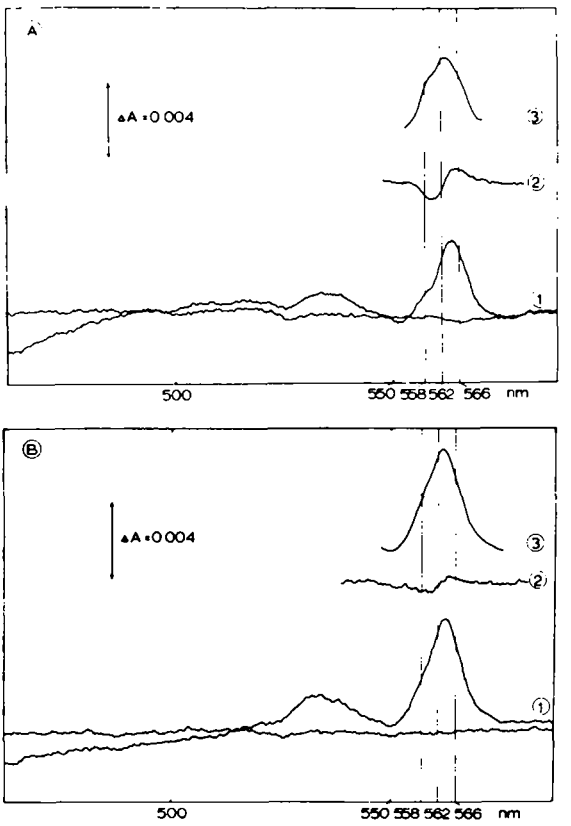

Fig. 7. Spectra, measured with a split-beam spectrophotometer, of respiratory-chain components in submitochondrial particles of mutant 28 reduced during the aerobic steady-state respiration of succinate in the presence of antimycin (cf. Fig. 6B). Protein concentration, 1.3 mg/ml. The particles were preincubated for 5 min with antimycin before the measurement was started by addition of succinate to sample and reference cuvettes. After the spectra were taken dithionite was added to both cuvettes in order to determine the red shift. (A) Curve 1, spectrum when 0.75 μmol antimycin/g protein was added to the sample cuvette (the nearly horizontal line shows the baseline measured before this addition); curve 2, the red shift under these conditions; curve 3, spectrum when curve 1 is corrected for the red shift. (B) Curve 1, spectrum when 5.5 μmol antimycin/g protein were added to the sample cuvette and 0.75 μmol/g protein to the reference cuvette (the nearly horizontal line is the baseline); curve 2, the red shift; curve 3, spectrum when curve 1 is corrected for the red shift.

curves for the reduction of cytochrome *b* (Figs 6B and 6C) are still sigmoidal in the mutant. The most striking difference between the wild type and the mutant is that, in the latter, the amounts of antimycin required for the various effects on cytochrome *b* are much less than those required to inhibit respiration. It is clear, then, that in the mutant the effects of antimycin on electron transport and on cytochrome *b* are separable. The degree of reduction of cytochrome *b* in the mutant induced by antimycin is about the same as that of the wild type, when the somewhat higher concentration of cytochromes in this preparation of the mutant (1.4-fold) is taken into account. The shift, however, is more pronounced in the mutant (Fig. 6D). The antimycin titres for shift and oxidation (Figs 6D and 6E), when corrected for the different cytochromes content, are similar to those of the wild type. The titres for reduction (Fig. 6B and 6C) are higher than in the wild type.

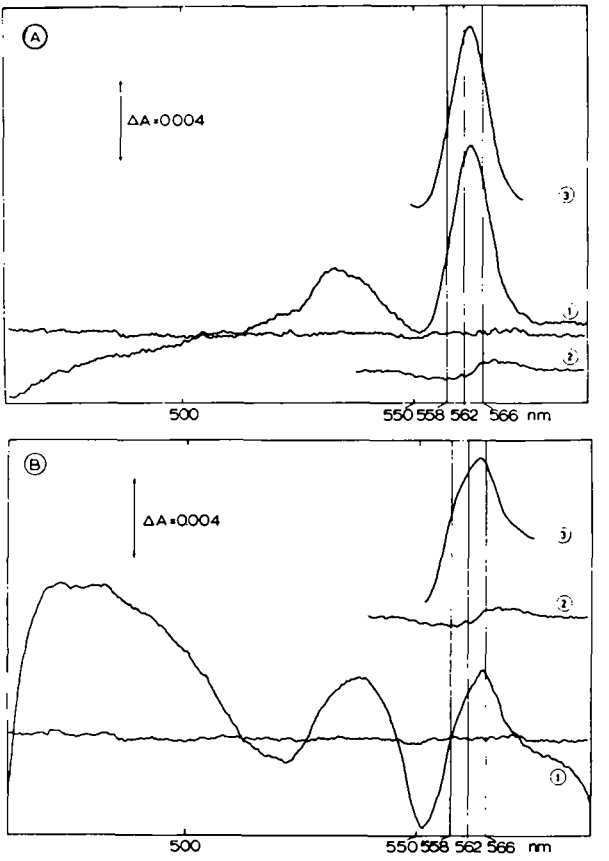

Fig. 8. Spectra, measured with the split-beam spectrophotometer, of submitochondrial particles of the wild type reduced by 15 mM succinate in the presence of 0.35 μmol antimycin/g protein and oxygen. No antimycin was added to the reference cuvette. Protein concentration, 1.7 mg/ml. Same conditions as in Fig. 7. (A) Curve 1, aerobically reduced by succinate in the presence of antimycin minus aerobically reduced by succinate (the nearly horizontal line is the baseline); curve 2, the red shift under these conditions; curve 3, represents curve 1 when corrected for the red shift. (B) Curve 1, aerobically reduced by antimycin and succinate minus anaerobically reduced by succinate; Curve 2, the red shift; curve 3, spectrum when curve 1 is corrected for the shift.

In Fig. 7A a difference spectrum is shown of the cytochromes *b* in the mutant that can be reduced by adding antimycin in a concentration that hardly inhibits electron transport, but substantially reduces *b*. When corrected for the antimycin-induced shift in *b*-562, a peak at 562.5 nm is observed showing that it is mainly cytochrome *b*-562 that is reduced. There is also a very pronounced shoulder at 558 nm belonging to cytochrome *b*-558, although no shoulder at 566 nm is visible. It appears, then, that the shoulder at 558 nm does not belong to cytochrome *b*-566. This is supported by the finding that at high concentrations of antimycin (Fig. 7B) the peak is found at 563.5 nm indicating that cytochrome *b*-566 as well as cytochrome *b*-562 contribute, but no clear shoulder at 558 nm is visible. For comparison a similar spectrum is recorded of the antimycin-induced reduction of the *b*-cytochromes in the wild type (Fig 8A). After correction for a shift, which is much smaller than in the mutant (see above), we find a peak at 563 nm, showing that both *b*-562 and *b*-566 participate.

The peak in Curve 3 of Fig. 8B, which represents the antimycin-induced reduction of cytochrome *b* by succinate, is at 564.5 nm rather than at 566 nm as found in mammalian mitochondria. Figs 9A and B, for wild type and mutant, respectively, show that this is also the case in the presence of cyanide. As is the case with mam-

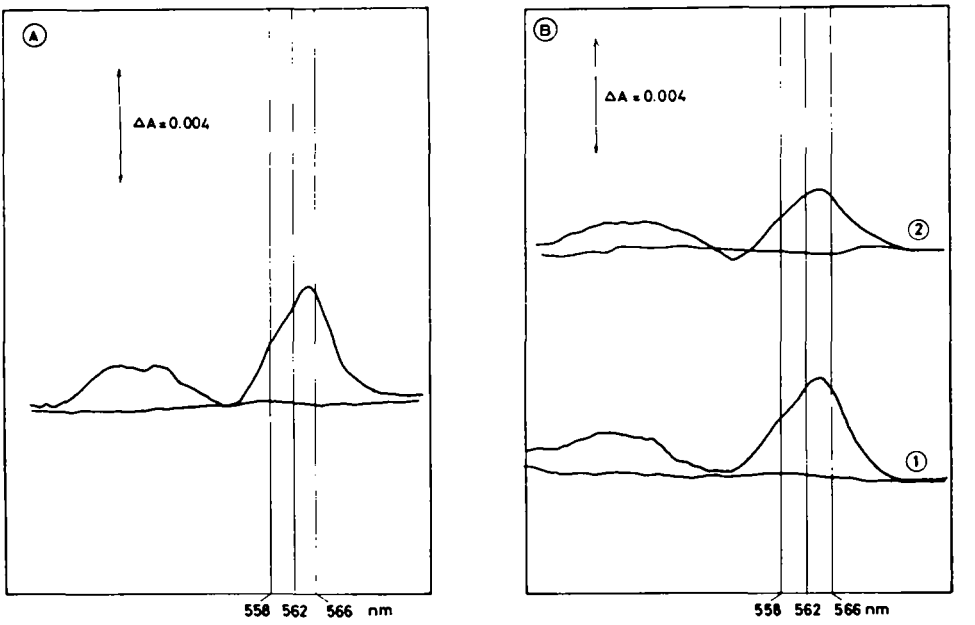

Fig. 9. Spectra, measured with the split-beam spectrophotometer, of the components that are reduced when antimycin and oxygen are added to submitochondrial particles reduced by 15 mM succinate and 1 mM cyanide. Same conditions as in Fig. 2. The particles were preincubated 5 min with antimycin. The spectra are corrected for the red shift that is measured by adding dithionite to both reference and sample cuvettes. (A) Particles of the wild type (1.7 mg/ml). 0.35 μmol antimycin/g protein was added to the sample cuvette (The nearly horizontal line is the baseline obtained before this addition). (B) Particles of the mutant (1.3 mg/ml). Curve 1, 0.75 μmol antimycin/g protein was added to the sample cuvette. (The corresponding baseline taken before adding the antimycin is also shown). In curve 2, 5.5 μmol antimycin/g protein were added to the sample cuvette and 0.75 μmol antimycin/g protein to the reference cuvette.

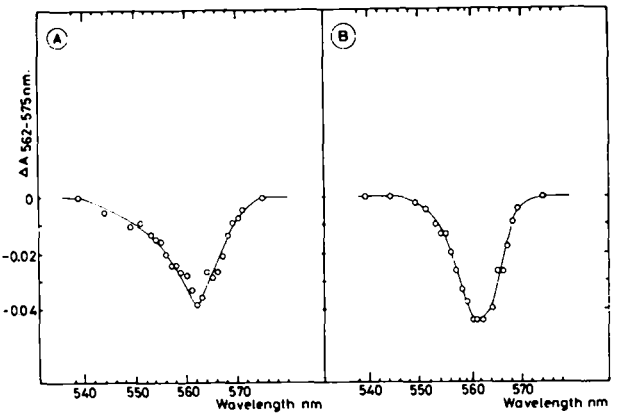

Fig. 10. Spectra measured in the dual-wavelength spectrophotometer, of cytochrome *b* oxidized by addition of antimycin. Submitochondrial particles were incubated in the same medium as in Fig. 1, to which was added 15 mM succinate and 10 μM phenazine metasulphate (see Fig. 5E). When the suspension became anaerobic, antimycin was added. At this moment the calculated redox potential of the succinate/fumarate couple was - 45 mV. The spectra drawn in the figure are the difference spectra anaerobic in presence of antimycin minus anaerobic, corrected for the red shift that was measured in a parallel experiment by adding antimycin to dithionite-reduced particles. (A) Wild type (3.7 mg/ml); antimycin, 0.2 μmol/g protein. (B) Mutant (2.7 mg/ml); antimycin, 7 μmol/g protein.

malian mitochondria, antimycin added to the particles in the presence of succinate, cyanide and oxygen induces the reduction of both *b*-566 and *b*-558, but not of *b*-562 since this was already reduced in the absence of antimycin.

In agreement with Berden and Opperdoes [9], the lowering by antimycin of the redox potential of *b*-562 is reflected by an oxidation of this cytochrome when antimycin is added to succinate-reduced particles poised at a suitable potential (Fig. 10). A lower potential than that found by Berden and Opperdoes in heart particles was necessary for maximal oxidation of *b*-562 in the yeast particles, suggesting that in this case antimycin affects the potential of all the *b*-562 and not just a high-potential component.

Fig. 11 shows that the difference between the sensitivity of the respiration to antimycin and its effects on cytochrome *b* can also be observed in intact whole yeast in the presence of uncoupler, demonstrating that it does not depend on the intactness of the mitochondrial preparation.

DISCUSSION

In the previous paper [5], it was shown that although the binding constant of antimycin to mutant particles is about one tenth that for the wild type, this is insufficient to explain the antimycin-resistant respiration, since little inhibition is found even when the binding site revealed in direct binding studies is almost completely saturated with inhibitor. In this paper, a similar separation is shown between the effects on cytochrome *b* and respiration. The separation of the two processes in the mutant implies that they are also separable in the wild type. The mutant provides the possibility of studying effects of antimycin on cytochrome *b* that are not caused per se by inhibition of the respiration.

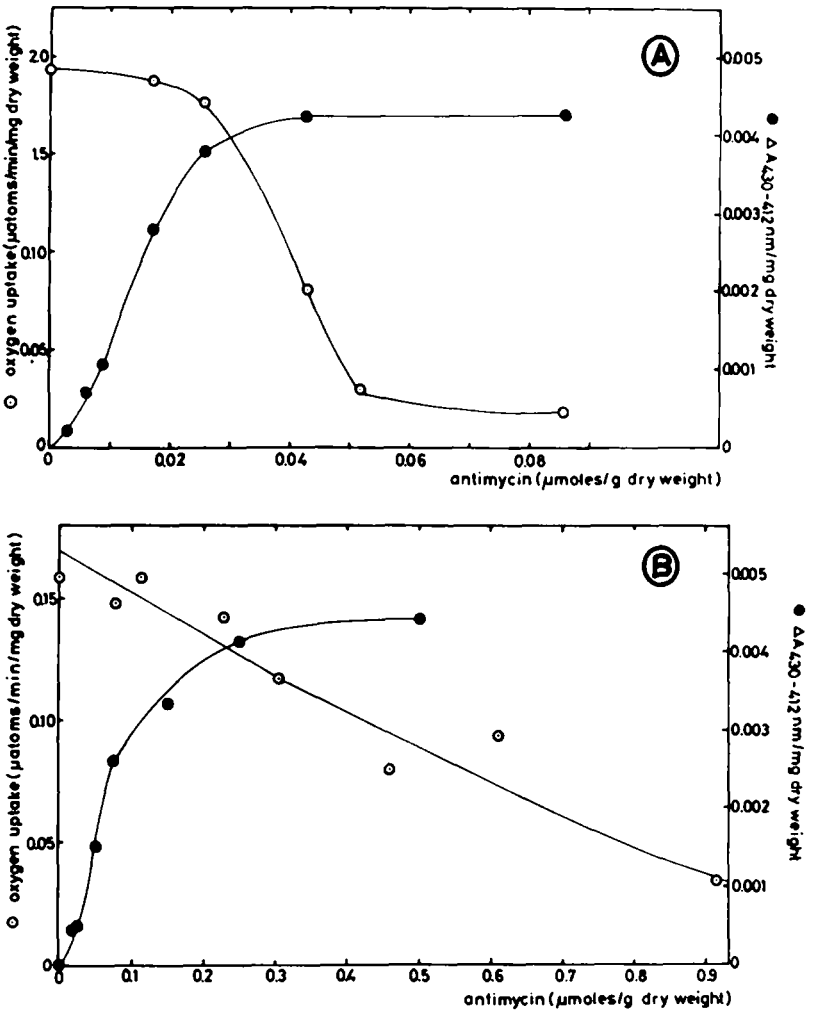

Fig. 11. Antimycin titration of the respiration and reduction of cytochrome *b* in whole cells of wild-type yeast and mutant 28. For measurement of oxygen uptake, freshly prepared cells were suspended in 50 mM phosphate buffer (pH 6.7). 4.5 mM ethanol was added as substrate and 62 μM 4,5,6,7-tetrachloro, 2-trifluoromethyl-benzimidazole in order to stimulate the respiration maximally. Preincubation with antimycin for 50 min was needed before respiration (○—○) and reduction in the presence of 1 mM cyanide (●—●) were constant, A, wild type cells. B, cells of mutant 28.

Two possible explanations for the different sensitivity to antimycin of respiration and cytochrome *b* may be considered. According to that represented in Fig. 12A, there are two antimycin-binding sites in the wild type with equal affinity, and the mutation causes changes in both binding sites. According to the second explanation, represented in Fig. 12B, the two binding sites have greatly different affinity in both the wild type and the mutant. In both cases, antimycin bound to the weak binding site inhibits respiration, but this is not seen in the wild type where binding to the strong site both inhibits respiration and affects the cytochrome b. The mutation invol-

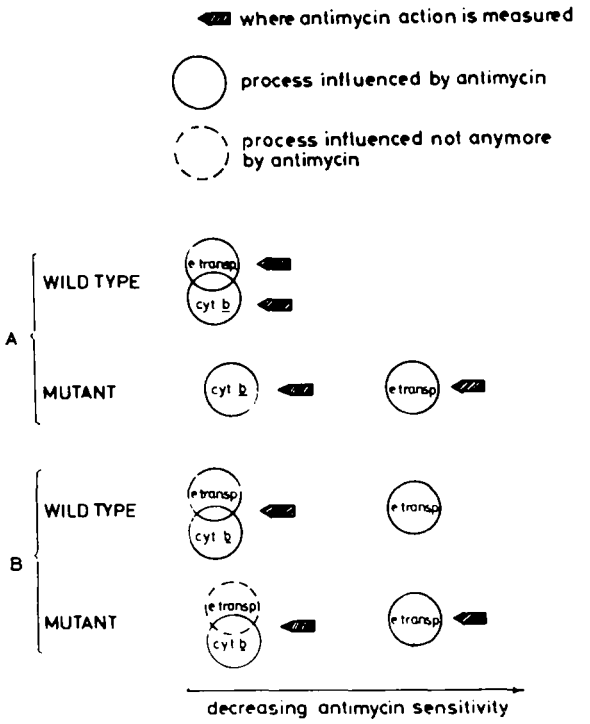

Fig. 12. Two alternative explanations of antimycin binding in wild type and the changes induced by a mutation. (A) Two binding sites in the wild type for the action on electron transport and cytochrome *b*, respectively, with equal high affinity to antimycin. Both binding sites are affected by the mutation. (B) One binding site in the wild type with high affinity where antimycin has an action on both cytochrome *b* and electron transport. There is also a second site present with low affinity to the antibiotic where the electron transport can be blocked. Only one binding site is affected by the mutation.

ves only the strong binding site, causing a lower binding constant. Moreover, the mutation in QH_2-cytochrome *c* reductase is such that, although binding of antimycin to the tight antimycin-binding site still has effects on the redox state, redox potential and absorption spectrum of the cytochrome *b*, it does not inhibit the respiration.

Although it is not possible to exclude the first explanation, the second seems the more likely, first because binding studies give no evidence of two tight binding sites in the wild type, and secondly because the first explanation would imply that the mutation involves two separate antimycin-binding sites.

The demonstration that, in the mutant, antimycin can cause complete reduction of the *b* cytochromes without having any effect on the respiration provides a further example, to be added to those reported by others [10–14], that it is possible to transfer electrons from substrate to oxygen without passing through the cytochromes *b*. This does not imply, however, that electrons do not pass through these cytochromes in a completely coupled system, in the absence of inhibitor. The experiments with intact mutant cells reported here show that the by-passing by the electrons of cytochromes *b* is not simply the result of mechanical damage to the mitochondria as has been suggested by Storey [13].

The transient oxidation of cytochrome *b* before anaerobiosis, observed in mutant particles in the absence, and in wild-type particles in the presence of low concentration of antimycin, suggests that the redox state of cytochrome *b* is influenced by the redox state of another component that becomes reduced before the cytochrome *b*, as has been proposed by Lee and Slater [15].

Bryła et al. [16] and Berden and Slater [17] have proposed, on the basis of sigmoidal binding curves of antimycin to substrate-reduced particles, that antimycin binds preferentially to an energized conformation (R state) of the QH_2-cytochrome *c* reductase in which cytochrome *b* is preferentially reduced and in which electrons cannot be transferred to cytochrome c_1. In terms of this proposal, the explanation of the mutation represented in Fig. 12B implies that electron flow to cytochrome c_1 is not inhibited in the R state of the mutant. This is further visualised in Fig. 13, which is only schematic and should not be taken to represent our views on the structure of QH_2-cytochrome *c* reductase.

According to this model, cytochrome *b* is not an obligatory electron carrier between QH_2 and cytochrome c_1. The tight antimycin-binding site is sufficiently close to the haem of cytochrome *b* to cause quenching of the antimycin fluorescence [17]. Binding of antimycin to this site induces a conformational change (or stabilizes one present in low concentration in equilibrium with a conformation with a lower affinity for antimycin) that is passed on to a component of the electron-transfer chain with the result that electron transfer is inhibited.

It is proposed that the mutation causes a change in one of the polypeptides of the QH_2-cytochrome *c* reductase, possibly the antimycin-binding peptide [3], but not necessarily. The change in this polypeptide results in a change in the antimycin-binding site so that the binding constant is decreased 10-fold and, more important, in the disappearance of interactions between the region of the antimycin-binding site and the electron-transfer chain in QH_2-cytochrome *c* reductase. Binding of antimycin, with induction of the R conformation, promotes the reduction of cytochrome *b*, but this is

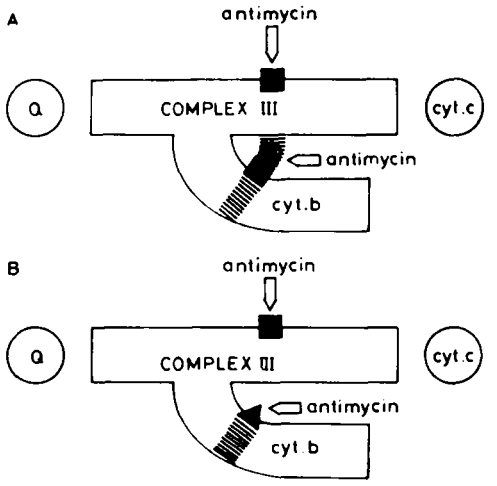

Fig. 13. A scheme illustrating some properties of the organization of Complex III in wild type (A) and mutant (B). For further details see text.

without inhibitory effect on respiration. This is caused by the second weak antimycin-binding site. Somewhat more antimycin must be added to particles of the mutant than to the wild type to reduce the cytochrome *b*. In terms of our hypothesis, this means that a greater degree of saturation of the tight antimycin-binding site is needed in the mutant to induce the R state. This difference between mutant and wild type is not seen when the 'shift' and the antimycin-induced oxidation of cytochrome *b* are titrated, because these effects are caused by mere binding of antimycin to its tight binding site [1,9].

The mutation of the region of the antimycin-binding site also has other minor effects. Possibly as a result of the disappearance of interactions with the electron-transfer chain in QH_2-cytochrome *c* reductase, cytochrome *b* is more reduced in the aerobic steady state. The antimycin-induced 'red-shift' of *b*-562 is also more pronounced in the mutant than in the wild type.

The separation, in the mutant, of the effects of antimycin on respiration and on the reducibility of cytochrome *b* in the presence of oxygen is difficult to reconcile with an alternative explanation of the latter effect that has been given [8], namely that the reduction of the cytochrome *b* is a direct consequence of the inhibition. Our results also give no support to the explanation of sigmoidicity of the antimycin-effect curve on respiration given by Kröger and Klingenberg [18,19], namely that it is due to the antimycin-sensitive site becoming rate-limiting only when it is highly saturated with antimycin. In our mutant, sigmoidal effect curves on the reducibility of cytochrome *b* are found with concentrations of antimycin that do not inhibit respiration and, moreover, the antimycin-effect curve on respiration is not sigmoidal.

The mutant has also proved useful in testing the proposal of Sato et al. [20,21] that the shoulder at 558 nm found in the cytochrome *b* spectrum belongs to cytochrome *b*-566. Wikström [22] and Slater and Lee [23] have provided some kinetic evidence that this is not the case, and Bandlow et al. [24] have isolated mutants of *Schizosaccharomyces pombe* containing a *b*-558, but lacking *b*-566. The view that *b*-558 and *b*-566 are different entities is supported by the observation that the addition of low concentrations of antimycin to the mutant particles in the presence of succinate and oxygen causes the appearance of a band at 562 nm and a clear shoulder at 558 nm, without any evidence of a shoulder at wavelengths above 562 nm. The addition of antimycin to succinate-reduced particles in the presence of cyanide that already show a band at 562 nm, causes a band at 564.5 nm with a shoulder at 558 nm. It seems likely that the three peaks, at 558, 562 and 564.5 nm. belong to different components.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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