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ANTIMYCIN-INSENSITIVE MUTANTS OF *CANDIDA UTILIS*

I. ISOLATION AND CHARACTERIZATION OF MUTANT 28

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SUMMARY

1. Mutants of *Candida utilis* have been isolated that are able to grow on non-fermentable substrate in the presence of 5 μg antimycin per ml.

2. The NADH oxidase activity of sub-mitochondrial particles isolated from one of these mutants (mutant 28) is 20 times less sensitive to antimycin than that of the wild type, judged by the amount required for 50% inhibition. The mutant has lost the characteristic sigmoidal titration curve, indicating that the mitochondrial Complex III has been significantly changed.

3. Particles isolated from the mutant are also much less sensitive to 4-heptyl-2-hydroxyquinoline-*N*-oxide, but are equally sensitive to treatment with 2,3-dimercaptopropanol and oxygen.

4. NADH and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine are equally rapidly oxidized by particles of the wild type and mutant. However, succinate and α-glycerol phosphate oxidase activities of mutant particles are about 50% and 20%, respectively, those of the wild type.

5. The oxidized *minus* reduced spectrum at room temperature of mutant particles is similar to those of the wild type. The concentration of total cytochrome *b* is the same in both strains.

6. There is no impairment of either Site-2 or -3 phosphorylation in mitochondria of the mutant.

7. The binding of antimycin to oxidized particles of wild type and mutant is non-co-operative with dissociation constants of $5.4 \cdot 10^{-11}$ M and $6.5 \cdot 10^{-10}$ M, respectively. The concentration of binding sites in the wild type is 0.13 μmole/g protein and in the mutant 0.22 μmole/g protein. The binding in succinate-reduced wild-type particles is positively co-operative with a dissociation constant of $1.7 \cdot 10^{-10}$ M at zero-bound antimycin and approaching the value of the oxidized particles at increasing antimycin concentration. The binding to succinate-reduced mutant particles is not positively co-operative. The dissociation constant at low antimycin concentrations is $1.3 \cdot 10^{-9}$ M and tends to increase with increasing antimycin.

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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8. The lower sensitivity of mutant particles to antimycin is, however, not explained by the lower binding constant *per se*, since inhibition in the mutant requires a higher degree of saturation with antimycin of the antimycin-binding sites revealed in the binding studies.

INTRODUCTION

Antimycin has been widely used as an inhibitor of the bc_1 region of the respiratory chain[1]. It is extremely firmly bound to sub-mitochondrial particles with a dissociation constant of $3.2 \cdot 10^{-11}$ M (ref. 2). From measurements of the quenching of its fluorescence when bound to Complex III, Berden and Slater[2] have concluded that it is bound to the complex at a distance from the *b* haem of 1.9 nm (19 Å) in the oxidized complex and 2.4 nm in the reduced.

In an attempt to obtain more information on the nature of the antimycin-binding site, an antimycin-resistant mutant of *Candida utilis* has been isolated. A similar mutant has been described by Butow and Zeydel[3], who have shown that, as in our mutant, the lesion resides in the mitochondrial respiratory chain and not in a permeability barrier to antimycin. Attempts to isolate similar mutants in *Saccharomyces cerevisiae* have failed. Although it is easy to isolate antimycin-resistant mutants of this organism, all of those isolated to date yielded sub-mitochondrial particles with a normal resistance to antimycin.

Some of the results of this investigation have been reported at a scientific meeting[4].

METHODS

Culture conditions

Candida utilis, strain CBS 621, obtained from the Centraal Bureau voor Schimmelcultures (Delft, The Netherlands), was cultured at 30 °C on a medium containing 0.5% Difco yeast extract, 0.6% $(NH_4)_2HPO_4$, 0.2% $MgSO_4 \cdot 7\,H_2O$ and 1% ethanol, brought to pH 5.5 with HCl. For plates and slants, ethanol was replaced by 3% glycerol and 2% agar was included. Antimycin was added, after sterilization of the culture medium in an autoclave, as a sterile solution in 70% aq. ethanol.

Isolation of mutant

Cells were harvested in the late logarithmic phase and washed twice with distilled water, under sterile conditions. The mutagenic procedure of Sherman[5] was applied to $2 \cdot 10^{10}$ cells, using 0.5 M $NaNO_2$ as mutagen. After washing, the cells were transferred to 100 ml growth medium and harvested after 16 h at 30 °C. All the cells were plated out on agar plates containing antimycin. Ten colonies growing on 5 μg antimycin/ml appeared, only 4 of which were stable after a single recloning. These 4 strains were twice recloned and transferred on slants containing antimycin. The 4 mutants differ in growth rate. The most rapidly growing (mutant 28) was used for the experiments described in this paper. After growth for 48 h in the absence of antimycin, harvesting in the logarithmic phase and plating in the absence of anti-

mycin, all 408 colonies replicated-plated on plates containing antimycin were still found to be resistant, showing that no appreciable reversion occurred.

Stable mutants were not obtained with ultraviolet light or addition of acriflavin as mutagenic treatment.

Preparation of mitochondria and sub-mitochondrial particles

Cells grown in 20-l flasks with thorough aeration for 24 h were harvested in their late-logarithmic phase. Mitochondria were isolated by the method of Kováč *et al.*[6]. Sub-mitochondrial particles were isolated by the procedure described by Mahler *et al.*[7]. Their fractions R_2L and R_2H were combined.

Respiratory activities

Respiratory activities were measured at 25 °C in an Oxygraph supplied with a Clark electrode. Submitochondrial particles were suspended in 0.1 M potassium phosphate buffer (pH 7.5), containing 0.2 mM EDTA. Mitochondria were suspended in 0.6 M sorbitol, 10 mM maleate, 10 mM KCl, 1 mM EDTA, 10 mM K_2HPO_4 and 2.5 mg bovine serum albumin/ml, free from fatty acids. The pH was brought to 6.5. The respiratory-control index and the P:O ratio was measured by the method of Chance and Williams[8].

Spectroscopy

Spectra were measured at 22 °C in the split-beam attachment of a Perkin–Elmer spectrophotometer, Model 356 with a 1-cm light path. The particles were suspended in 0.1 M phosphate buffer (pH 7.5), containing 0.2 mM EDTA.

Fluorimetry

Fluorescence was measured in an Eppendorf fluorimeter. The cell compartment was kept at 22 °C with a thermostat. A primary filter of 313+366 nm and a secondary filter of 420–3000 nm were used. Drift in the fluorimeter was allowed for by measuring the fluorescence of a standard antimycin solution in albumin-containing buffer.

Protein

Protein was determined by the biuret method after precipitation with trichloroacetic acid, as described by Cleland and Slater[9]. Essentially the same results were obtained by the Kjeldahl method.

RESULTS

Properties of mitochondria and sub-mitochondrial particles prepared from wild-type and mutant yeast

Particles prepared from the mutant oxidize NADH and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) at about the same rate as those from wild type (Table I). The succinate and α-glycerol phosphate oxidase activities are, however, considerably lower. As in wild type, the oxidation of succinate and α-glycerol phosphate is inhibited by theonyltrifluoroacetone (not shown).

The cytochrome spectra of the particles are similar in both wild type (Fig. 1A)

TABLE I

RESPIRATORY ACTIVITIES OF MUTANT AND WILD-TYPE PARTICLES WITH DIFFERENT SUBSTRATES IN THE ABSENCE AND PRESENCE OF ANTIMYCIN

The concentration of NADH was 1 mM and 5 μM bovine cytochrome *c* was added. The concentrations of succinate and α-glycerol phosphate were 20 mM, and 20 μM bovine cytochrome *c* was added. 20 mM ascorbate and 0.1 mM TMPD were used with 20 μM bovine cytochrome *c*. The particles were preincubated with 0.18 nmole antimycin per mg protein for 5 min before the activity was measured. The concentration of protein varied with the substrate.

Substrate	*Antimycin*	*Oxygen uptake (μatoms/min per mg)*	
		Wild type	*Mutant*
NADH	−	1.19	0.95
	+	0.03	0.89
Succinate	−	0.37	0.18
	+	0.02	0.18
α-Glycerol phosphate	−	0.24	0.05
	+	0.02	0.05
TMPD, ascorbate	−	1.39	1.30
	+	1.45	1.45

TABLE II

OXIDATIVE PHOSPHORYLATION IN WILD-TYPE AND MUTANT MITOCHONDRIA

The medium used in the oxygraph vessel is described in Methods. The concentration of NADH was 2 mM, α-glycerol phosphate 10 mM, and succinate 10 mM. The ADP concentration was 40 μM. The protein concentration with wild-type mitochondria was 0.44 mg/ml and with mitochondria of the mutant 0.69 mg/ml.

Substrate	*Wild type*		*Mutant*	
	P:O	*R.C.I.**	*P:O*	*R.C.I.**
NADH	1.5	1.5	1.4	1.6
α-Glycerol phosphate	1.2	1.6	1.0	1.8
Succinate	1.8	2.4	1.7	2.6

* Respiratory-control index

and mutant (Fig. 1B). Calculated from the wavelength pair 563–575 nm, using an absorbance coefficient of 25.6 mM^{-1} cm^{-1} (ref. 10), the amount of cytochrome *b* reduced by $Na_2S_2O_4$ was 0.33 μmole/g protein in wild type and 0.34 μmole/g protein in the mutant. As in mammalian mitochondria, only about one-half of the cytochrome *b* is reduced by NADH.

The P:O ratios in mitochondria prepared from the mutant are similar to those in mitochondria from wild type (Table II). The low P:O ratio with NADH is to be expected, since mitochondria from *Candida utilis* oxidize this substrate with a by-pass of the first phosphorylation site[11].

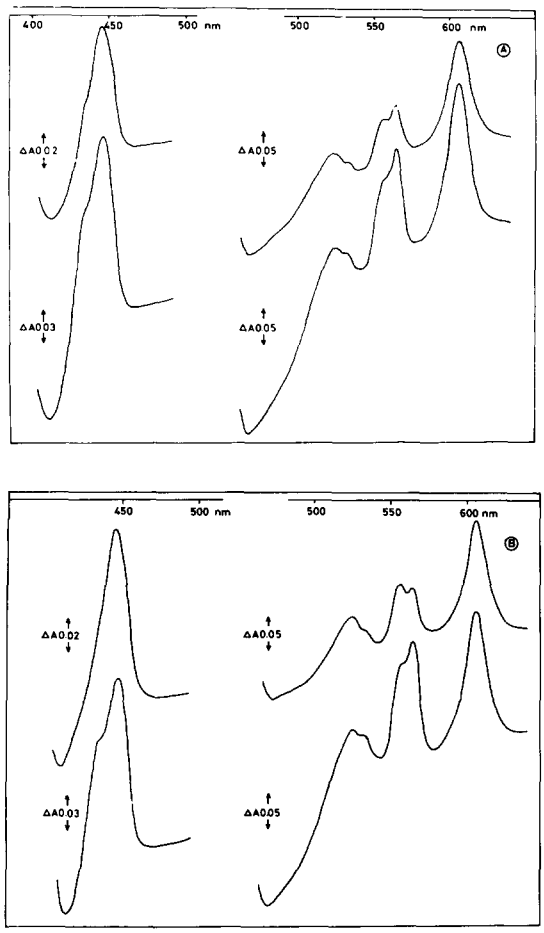

Fig. 1. Oxidized *minus* reduced spectra at room temperature. Sub-mitochondrial particles were suspended in the medium described in Methods. 2 mM NADH or a few grains of dithionite were used as reductants. (A) Wild type, 1.66 mg protein/ml. (B) Mutant, 1.76 mg protein/ml. The upper traces represent the NADH-reduced *minus* oxidized spectrum, the lower traces the dithionite *minus* oxidized.

Effect of antimycin and other inhibitors on the respiratory chain in wild-type and mutant mitochondria

The effect of antimycin on the NADH oxidase activities of sub-mitochondrial particles prepared from wild-type and mutant yeast cells are shown in Figs 2A and 2B, respectively. As is well established for mammalian mitochondria, the antimycin-titration curve for the wild type is strongly sigmoidal. The particles prepared from

the mutant are less sensitive to antimycin and the titration curve is hyperbolic. Half-maximal inhibition is obtained at concentrations of antimycin 20 times greater than that required for maximal inhibition of the wild type. Ethanol oxidation by whole cells of the mutant is 100 times less sensitive to antimycin than that by wild-type cells.

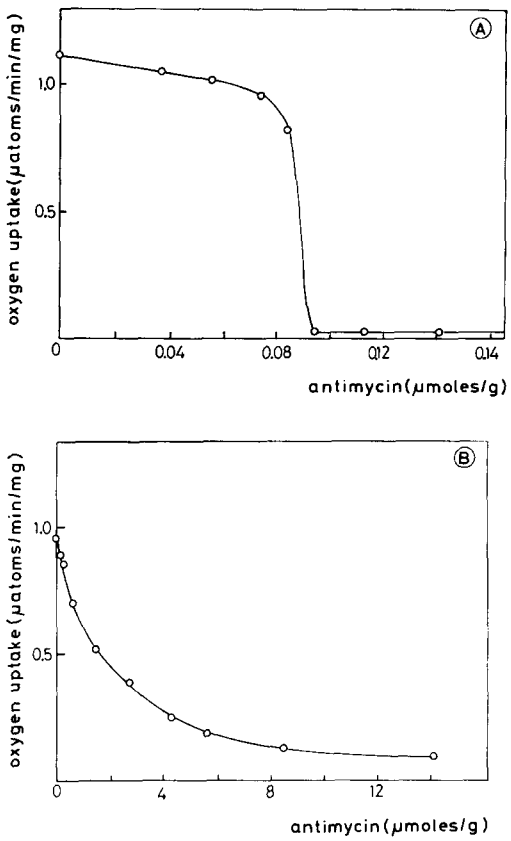

Fig. 2. Inhibition of NADH oxidase in sub-mitochondrial particles by antimycin. The medium is described in Methods with 2 mM NADH in presence of 5 μM bovine cytochrome *c*. Particles were preincubated with antimycin for 5 min. (A) Wild type, 0.124 mg protein/ml. (B) Mutant, 0.133 mg protein/ml.

Particles from the mutant are also less sensitive (about 10-fold) to 2-heptyl-4-hydroxyquinoline-*N*-oxide, an inhibitor resembling antimycin in many ways[12], than particles from the wild type (see Figs 3A and 3B). On the other hand, the mutant particles are equally sensitive to 2,3-dimercaptopropanol treatment (see Table III), indicating that, although 2,3-dimercaptopropanol-treated sub-mitochondrial particles behave in many respects like antimycin-treated particles[13], the site of action of 2,3-dimercaptopropanol is different. Table III shows too that the particles of the mutant are sensitive to rotenone and cyanide.

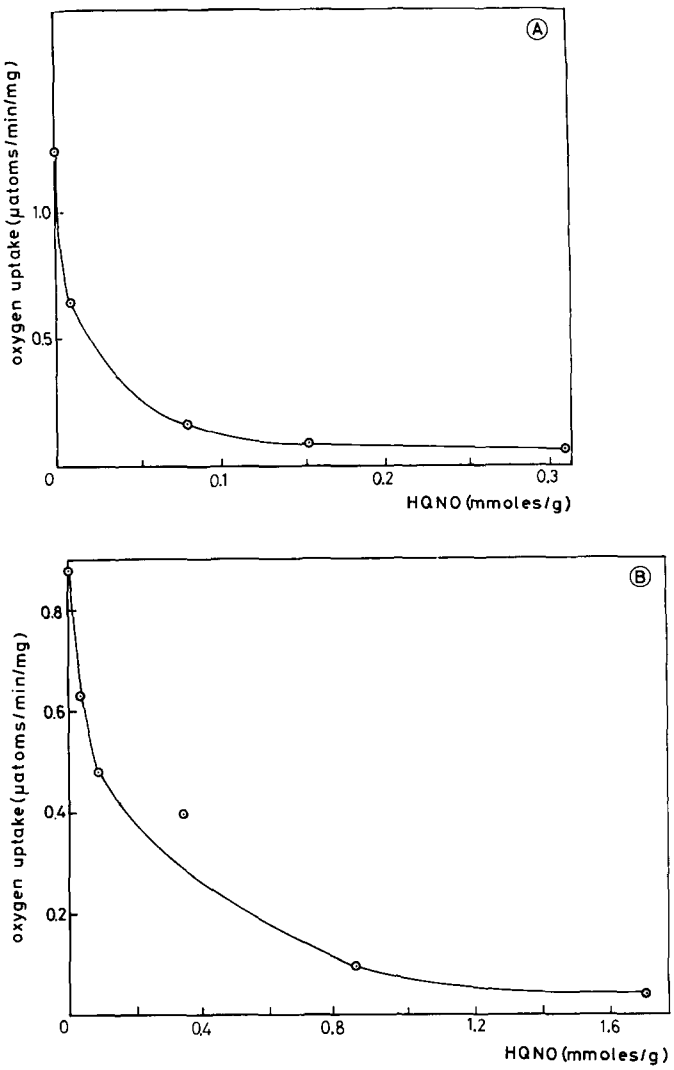

Fig. 3. Inhibition of NADH oxidase in sub-mitochondrial particles by 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO). Reaction mixture as in Fig. 2. The particles were preincubated with the inhibitor for 15 min. (A) Wild type, 0.124 mg protein/ml. (B) Mutant, 0.221 mg protein/ml.

Binding of antimycin to particles isolated from wild-type and mutant yeast

The binding of antimycin to particles was measured by the procedure of Butow and Zeydel[3] and Berden and Slater[2], in which different amounts of antimycin are added to a suspension of particles in a solution containing serum albumin. After 15 min, the particles were removed by centrifugation and the fluorescence of the supernatant measured. As a control, the same amounts of antimycin were added to a supernatant obtained after centrifuging a suspension of particles to which no antimycin was added. Figs 4A and 4B show, respectively, the results obtained with oxidized particles from wild-type yeast and those reduced with succinate in the

TABLE III

THE EFFECT OF SOME RESPIRATORY-CHAIN INHIBITORS ON NADH OXIDASE ACTIVITY OF SUB-MITOCHONDRIAL PARTICLES OF MUTANT AND WILD TYPE

Incubation with 2,3-dimercaptopropanol was carried out as described by Slater[13]; 1 mM NADH and 5 μM bovine cytochrome *c* were used in measurement of NADH oxidase. 0.118 mg protein/ml of the wild type and 0.147 mg protein/ml of the mutant.

Inhibitor	*% Inhibition of NADH oxidase*	
	Wild type	*Mutant*
2,3-Dimercaptopropanol (0.42 mM)	49	54
2,3-Dimercaptopropanol (6.7 mM)	97	95
Rotenone (10 μg/mg protein)	92	93
Cyanide (0.02 mM)	83	91

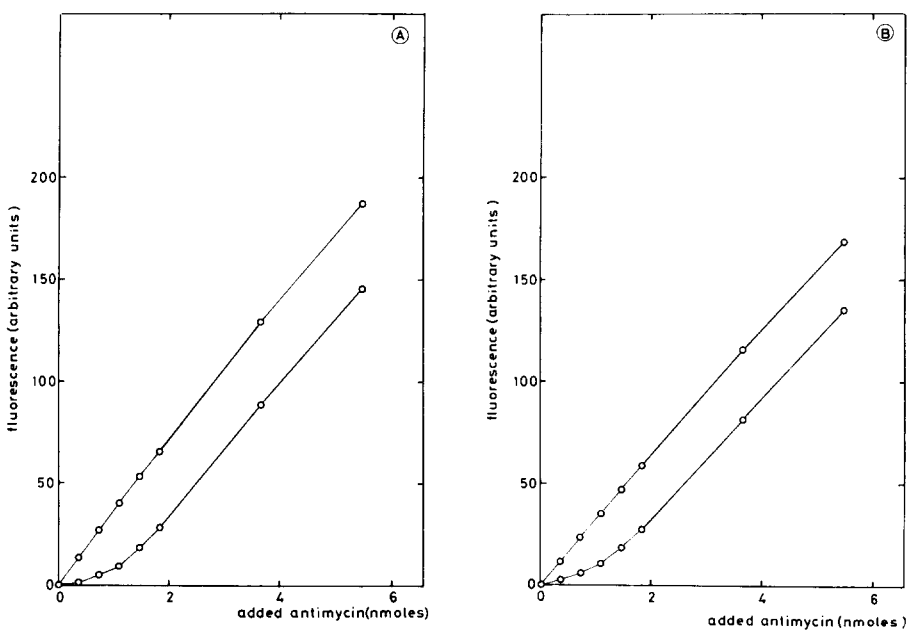

Fig. 4. Binding of antimycin to sub-mitochondrial particles of wild-type yeast. The binding was measured following the procedure of Berden and Slater[2]. The particles were incubated in a medium containing 250 mM sucrose, 20 mM Tris–HCl buffer, 1 mM EDTA and 6.0 mg/ml bovine serum albumin at pH 7.5. Antimycin was added as a methanol solution. The fluorescence of the supernatants after spinning down the particles is shown in the lower trace of each figure. The upper trace represents supernatants to which antimycin was added after removal of the particles. (A) Binding to oxidized particles (3.89 mg protein/ml). (B) Binding to particles reduced by 20 mM succinate in the presence of 5 mM cyanide (3.89 mg protein/ml).

presence of cyanide. The difference between the two lines in these figures represents the amount of bound antimycin.

The binding data are plotted in the form of a Scatchard plot in Fig. 5. The straight line obtained with oxidized wild-type particles (Fig. 5A) shows that antimycin

is bound non-co-operatively. The concentration of antimycin-binding sites, given by the intercept of the straight line on the abscissa, equals 0.13 μmole/g protein, or 0.4 mole/mole cytochrome *b*, compared with 0.5 mole/mole cytochrome *b* in particles from heart mitochondria[2]. The dissociation constant, taking into account the binding of antimycin to the albumin[2], equals $5.4 \cdot 10^{-11}$ M, compared with $3.2 \cdot 10^{-11}$ M in heart mitochondria[2].

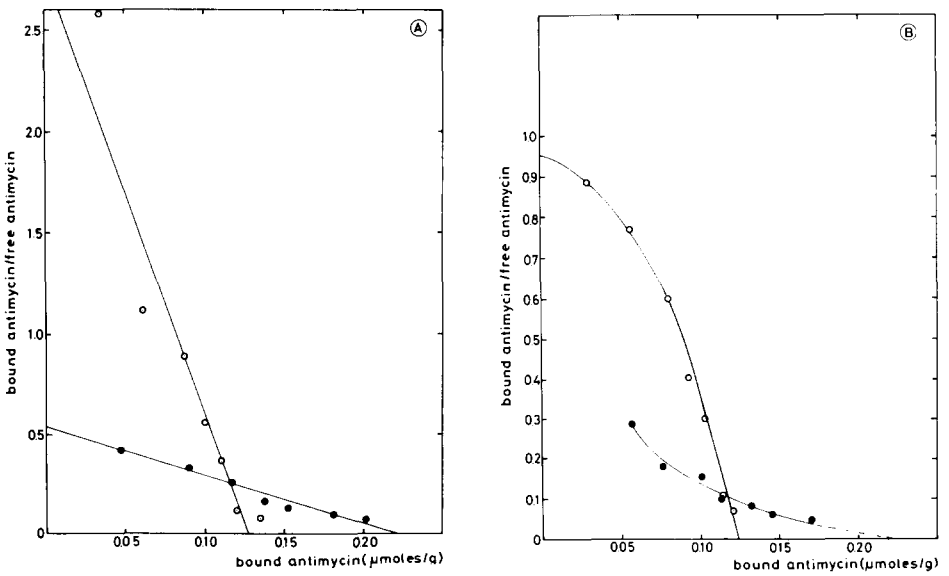

Fig. 5. A Scatchard plot for the binding of antimycin to particles of wild-type yeast and mutant. (A) The plot for oxidized particles of wild type (○—○), and mutant (●—●), calculated from Figs 4A and 6A, respectively. The dissociation constant for the wild type, after the correction for the competition between particles and albumin for antimycin[2], is $5.4 \cdot 10^{-11}$ M, and the concentration of binding sites is 0.13 μmole/g protein. The dissociation constant for the mutant is $6.5 \cdot 10^{-10}$ M, and the concentration of binding sites is 0.22 μmole/g protein. (B) The plot for succinate-reduced particles of wild type (○—○), and mutant (●—●), calculated from Figs 4B and 6B, respectively. Extrapolation of the curve for the wild type to zero bound antimycin gives a dissociation constant of $1.7 \cdot 10^{-10}$ M; at high amounts of antimycin the dissociation constant for the oxidized particles is reached. The curve for the mutant at low antimycin concentrations gives a dissociation constant of $1.3 \cdot 10^{-9}$ M.

A comparison of Figs 4A and 4B shows clearly that low concentrations of antimycin are less firmly bound to succinate-reduced particles than to oxidized particles. The Scatchard plot in Fig. 5B for succinate-reduced wild-type particles (open circles) shows that the binding is co-operative, the binding constant at high concentrations of antimycin approaching that found with oxidized particles. This is also in agreement with the findings with heart particles[2].

Fig. 6 shows the binding data for particles isolated from the mutant. A comparison with Figs 4A and 4B shows clearly that binding to the mutant is less firm than to the wild type. From the Scatchard plot (Fig. 5A, closed circles), it can be calculated that antimycin binds non-co-operatively to oxidized particles with a dissociation constant of $6.5 \cdot 10^{-10}$ M, *i.e.* 12 times higher than to wild type. The concentration of binding sites is 0.22 μmole/g protein, 1.7 times that in the wild type.

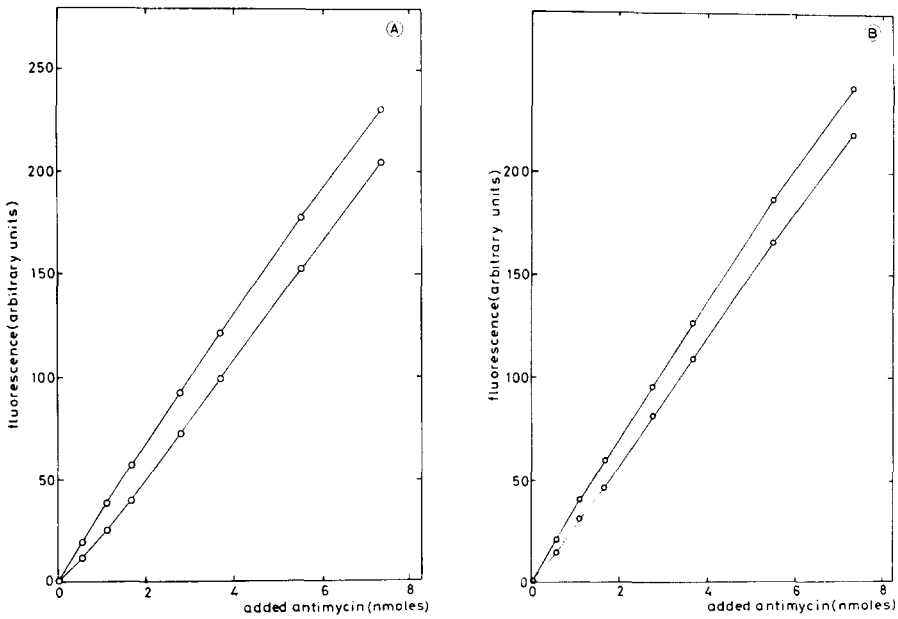

Fig. 6. Binding of antimycin to sub-mitochondrial particles of the mutant. The conditions were as in Fig. 4. The fluorescence of the supernatants after spinning down the particles is shown in the lower trace of each figure. The upper trace is a control. (A) Binding to oxidized particles (1.81 mg protein/ml). (B) Binding to particles reduced by 20 mM succinate in the presence of 5 mM cyanide (1.81 mg protein/ml).

The data for succinate-reduced particles (Fig. 5B, closed circles) show that the positive co-operativity, characteristic of the wild type, is absent in the mutant. It is replaced either by a negative co-operativity or by two binding sites, with different affinity. The dissociation constant, calculated from the data at low antimycin concentration, is about twice that for oxidized particles and 24 times higher than in succinate-reduced wild-type particles.

DISCUSSION

Except for somewhat lower activities of the succinate and α-glycerol phosphate oxidase systems, the only abnormality in the mutant yeast is the resistance of its respiration to antimycin and hydroxyquinoline-*N*-oxide. The resistance to antimycin is accompanied by a lower binding constant of the inhibitor to mutant particles.

The following differences in the binding of antimycin to mutant and wild-type particles have been observed: (i) the dissociation constant of antimycin bound to oxidized particles is 12 times, and in succinate-reduced particles 24 times greater with mutant particles than with wild-type; (ii) the concentration of binding sites is greater in mutant particles than in wild-type; (iii) the co-operative binding, characteristic of succinate-reduced particles from heart[2] and wild-type yeast, is absent in mutant particles.

However, the difference *per se* in binding constants of the inhibitor to the wild-type and mutant particles is insufficient to explain quantitatively the resistance

of the respiration to antimycin. This is shown in Fig. 7. Antimycin is bound to wild-type particles so strongly that the relationship between the amount of antimycin added to that bound is a straight line with a slope of 1:1 until more than 95% is bound. Inhibition becomes appreciable, however, only when more than about 70% of the binding sites are occupied. Berden and Slater[2] have proposed that the sigmoidal inhibition curve, which is also found when inhibition is plotted against the concentration of free antimycin, is due to the preferential binding of antimycin to an inhibited conformation of Complex III. Even though antimycin is bound to mutant particles less firmly than to those from wild-type, the relationship between added and bound antimycin is also linear, with a slope of 1:1, up to about 70% bound antimycin.

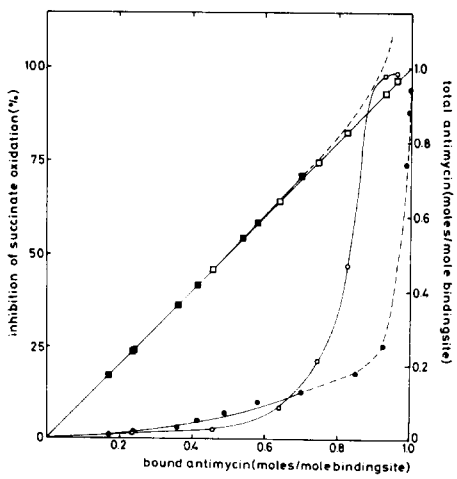

Fig. 7. The relation between the concentration of bound antimycin and added antimycin in wild-type (□—□) and mutant (■—■) sub-mitochondrial particles, calculated from the binding data in Figs 4B and 6B. The relationship between the inhibition of succinate oxidation and the degree of saturation of the antimycin-binding site for wild type (⊙—⊙) and mutant (●—●) is also shown. The inhibition experiments were carried out with the same mitochondrial preparations as in Figs 4B and 6B, suspended in a medium containing 250 mM sucrose, 20 mM Tris–HCl buffer, 1 mM EDTA, 20 μM bovine cytochrome *c*, 0.6 mg/ml bovine serum albumin and 10 mM succinate. The pH was 7.5. The particulate protein concentration was 0.389 mg/ml for the wild type and 0.181 mg/ml for the mutant. The respiratory activity was measured after preincubation for 10 min with succinate and antimycin. The dotted part of the line relating the inhibition with the degree of saturation in the mutant was calculated from a titration of inhibition in a medium from which the albumin was omitted. For this calculation, it was assumed that the concentration of binding sites was the same as found in the Scatchard plot given in Fig. 5 (0.22 μmole/g protein) and that the dissociation constant ($1.3 \cdot 10^{-9}$ M) applicable at 70% saturation was also applicable between 70% and 100% saturation (see Fig. 5).

The curve then deviates upwards, since some antimycin is not bound to the binding site revealed in the Scatchard plots. In this case, however, inhibition does not exceed 25% until 92% of the antimycin is bound to this site and is less than 75% when 99% of the antimycin is bound.

The concentrations of bound antimycin plotted in Fig. 7 were calculated from the binding data for succinate-reduced particles in the presence of cyanide. These may not be quite appropriate for particles in the aerobic steady state in which the

cytochrome *b* will be largely oxidized at concentrations of antimycin insufficient to inhibit respiration. If the binding data for oxidized particles are used, the two lower curves in Fig. 7 are both shifted to the right, but the difference between the two curves remains about the same.

It must be concluded from Fig. 7 that bound antimycin is less effective as an inhibitor in mutant particles than in the wild type. Two explanations may be considered. (1) The binding site measured in the Scatchard plot is relevant to the inhibition by antimycin. In terms of the explanation given by Berden and Slater[2], the parameters describing the equilibrium between the R and T states of the mutant particles in the presence of antimycin are such that a higher concentration of antimycin is required to stabilize the inhibited R state. (2) The binding site measured in the Scatchard plot with the mutant is irrelevant to the inhibition by antimycin, which is due to binding to a site with a much lower affinity for antimycin, that is not revealed in the Scatchard plot.

Data at present available are insufficient to choose between these two possible explanations.

The alteration in the binding properties of mutant mitochondria to antimycin presumably reflects a change in the structure of Complex III, which contains the antimycin-binding site[14]. This alteration in the structure does not impair the rate of oxidation of NADH or oxidative phosphorylation, but does impair the rate of oxidation of succinate and α-glycerol phosphate. In this connection, it is interesting to recall that Albracht *et al.*[15] have shown that antimycin affects the EPR spectrum of an iron–sulphur centre associated with succinate dehydrogenase, suggesting an intimate relationship between Complex II, containing succinate dehydrogenase, and Complex III.

The lowered sensitivity of the antimycin-resistant mutant to 2-heptyl-4-hydroxyquinoline-*N*-oxide shows that the binding sites of the two inhibitors must be close to one another. 2,3-Dimercaptopropanol treatment, on the other hand, although it has results similar to those of addition of antimycin, has the same effect in mutant particles as in those of wild type. This gives good support for the conclusion of Deul and Thorn[16] that the antimycin- and 2,3-dimercaptopropanol-sensitive sites are not identical.

The properties of the mutant described in this paper resemble in many respects, including its resistance to hydroxyquinoline-*N*-oxide and the lower binding constant for antimycin, those described by Butow and Zeydel[3] for a mutant of *Candida utilis* obtained by acriflavin treatment. However, our mutant differs from that of Butow and Zeydel in the somewhat lower activity of the NADH oxidase system and the much lower activity of the succinate oxidase system in the mutant compared with the wild type. The mutant of Butow and Zeydel has substantially higher activities of these oxidase systems.

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