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STUDIES ON THE MECHANISM OF ELECTRON TRANSPORT IN THE bc_1-SEGMENT OF THE RESPIRATORY CHAIN IN YEAST

II. THE BINDING OF ANTIMYCIN TO MITOCHONDRIAL PARTICLES AND THE FUNCTION OF TWO DIFFERENT BINDING SITES

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SUMMARY

1. In mitochondrial particles antimycin binds to two separate specific sites with dissociation constants $K_{D1} \leqq 4 \cdot 10^{-13}$ M and $K_{D2} = 3 \cdot 10^{-9}$ M, respectively.

2. The concentrations of the two antimycin binding sites are about equal. The absolute concentration for each binding site is about 100 – 150 pmol per mg of mitochondrial protein.

3. Antimycin bound to the stronger site mainly inhibits NADH- and succinate oxidase. Binding of antimycin to the weaker binding site inhibits the electron flux to exogenously added cytochrome *c* after blocking cytochrome oxidase by KCN.

4. Under certain conditions cytochrome *b* and c_1 are dispensible components for antimycin-sensitive electron transport.

5. A model of the respiratory chain in yeast is proposed which accounts for the results reported here and previously. (Lang, B., Burger, G. and Bandlow, W. (1974) Biochim. Biophys. Acta 368, 71–85).

INTRODUCTION

The existence of one antimycin binding site has been reported for yeast, *Neurospora* and beef heart [2–7]. However, recent studies have revealed that in preparations of mitochondrial particles from *Schizosaccharomyces pombe*, the concentration of antimycin binding sites [8, 9] is much less than the concentration of antimycin needed for inhibition [1]. On the one hand we showed [1] that the antimycin inhibition curve of QH_2-3 : cytochrome *c* oxidoreductase contains some activity which is only slightly affected by high concentrations of antimycin. This indicates that a second weaker site for antimycin exists. But we also found that QH_2-2 : cytochrome *c* oxidoreductase activity is linearly affected by increasing concentrations of antimycin, whereas the ef-

Abbreviations: QH_2, ubiquinol; $QH^{\cdot}$, ubiquinone radical; Q, ubiquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide-*p*-trifluormethoxyphenylhydrazone.

fect on QH_2-3 : cytochrome *c* oxidoreductase is sigmoidal. We postulated therefore that there may exist a dual electron transport chain in the bc_1-segment of yeast, having a separate antimycin inhibition site in each branch [1].

Of the models currently under consideration [4–7, 10–14], none takes into account the possibility of two types of antimycin binding sites. They also fail to explain satisfactorily the shapes of inhibition curves by antimycin of QH_2 : cytochrome *c* oxidoreductase. Thus, we were prompted to reinvestigate the correlation of antimycin binding and its inhibitory effect.

The experiments described here have been performed with mitochondria from *S. pombe*, as mutants exist in this genetically well known organism, which are resistant in vitro to antimycin [15–17]. Similar mutants have been described with *Candida utilis* [2, 18], a sexless yeast. These mutants are of considerable value in explaining the mechanism of inhibition of this antibiotic and in differentiating between its inhibitory function and its effect on the *b*-cytochromes [15, 16, 19].

MATERIALS AND METHODS

Chemicals

Ubiquinones were graciously donated by Dr Solms NADH and cytochrome *c* were obtained from Boehringer, antimycin from the Nutritional Biochemical Corporation, Lubrol WX from Imperial Chemical Industries, Urografin from Schering and bovine serum albumin from Behring Werke.

Cell growth and preparation of mitochondrial particles

Cell cultivation of *S. pombe* 972 h^- or 50 ade_7 h^-, cell disruption by shaking with glass beads and purification of mitochondrial particles by Urografin gradient centrifugation was performed as described previously [17].

Determination of enzymatic activities

Optical assays of enzymatic activities were performed at 25 °C as described previously [1], except that 10 % sorbitol was present in the buffer. With these conditions and with the type of mitochondrial particle preparation described above, QH_2 : cytochrome *c* oxidoreductase activites could be measured at a pH of 7.4, without interference by antimycin-insensitive reactions. A KCN concentration was chosen which inhibited cytochrome oxidase activity 98 %.

Oxygen consumption was measured with a Clark-type electrode in the same buffer (containing 0.1 mM cytochrome *c*). The volume of the thermostated vessel was 2.0 ml.

Concentrations of antimycin in ethanolic solution were determined spectroscopically (absorption coefficient $\varepsilon_{320} = 4.8\ mM^{-1}cm^{-1}$) [20].

Reduction and determination of the concentration of QH_2 has been described previously [1, 21].

Low temperature spectroscopy

Difference absorption spectra were recorded with a Cary model 14 spectrophotometer at 77 °K. As QH_2 is only sparingly soluble in the buffer, its effective concentration as an electron donor was increased by extraction of cytochrome *c* from the

particles by homogenisation (Potter-Elvehjem homogenizer, 2000 rev./min for 3 min) in the presence of 0.2 mg Lubrol WX per mg protein or in 0.2 M potassium phosphate buffer, pH 7.4, and subsequent centrifugation at 46 000 $\times$ g for 20 min.

Determination of cytochrome concentrations in mitochondrial particles

Cytochrome concentrations were determined at 25 °C in an Aminco DW 2 double-beam spectrophotometer from the difference in absorbance of dithionite-reduced and oxidized particles. Cytochrome *b* was determined by using the wavelength pair 563/575 nm and an absorption coefficient of $\varepsilon_{563/575} = 25.6\ mM^{-1}\ cm^{-1}$ [22]. The content in cytochrome aa_3 was measured using an absorption coefficient of $\varepsilon_{605/590} = 12\ mM^{-1}\ cm^{-1}$ [13].

Protein determination

Protein was determined according to the method of Lowry et al. [23]. Urografin was removed before protein estimation by chromatography on Bio-Gel A-5.

Binding of antimycin to mitochondrial particles

Binding studies were done with an Eppendorf fluorimeter as described by Berden and Slater [4], except that we used the same buffer as for determination of enzymatic activities. Only carefully purified mitochondrial particles, i.e. by the procedure given in Methods, were used.

The binding of antimycin to the particles was also measured in the absence of bovine serum albumin. It was carried out by the same method except that after centrifugation of the samples, 2 mg/ml serum albumin was added in order to increase the fluorescence.

The binding parameters were determined graphically starting from the Scatchard plot [24] as devised by Danchin [25]. By this method the concentration of sites varies by about $\pm$ 10 pmol and the dissociation constants by a factor of about $\pm$ 0.5; dissociation constants less than 10^{-13} M are not determinable. The apparent dissociation constant was corrected for the antimycin-binding capacity of bovine serum albumin [4].

RESULTS

Binding of antimycin to mitochondrial particles

In a number of binding experiments with mitochondrial particles, prepared as described in our previous paper [1], the amount of total antimycin bound per mg of protein varies. This indicates that further purification of the mitochondrial particles is necessary. Specially prepared particles [17] prove to contain a rather constant concentration of a strong binding site. In addition, this purification step removes components which are solubilized from normal preparations during the binding assay and which disturb the fluorimetric measurement. Fig. 1A shows a binding experiment in the absence of serum albumin and the Scatchard plot is drawn in Fig. 1B. Since this plot results in a hyperbolic curve, more than one site is involved in antimycin binding, presupposing non-interacting sites.

First, it was assumed that all the antimycin binding, except to a strong binding site, is non-specific. Therefore, the graphical parameter fitting was tried with a single

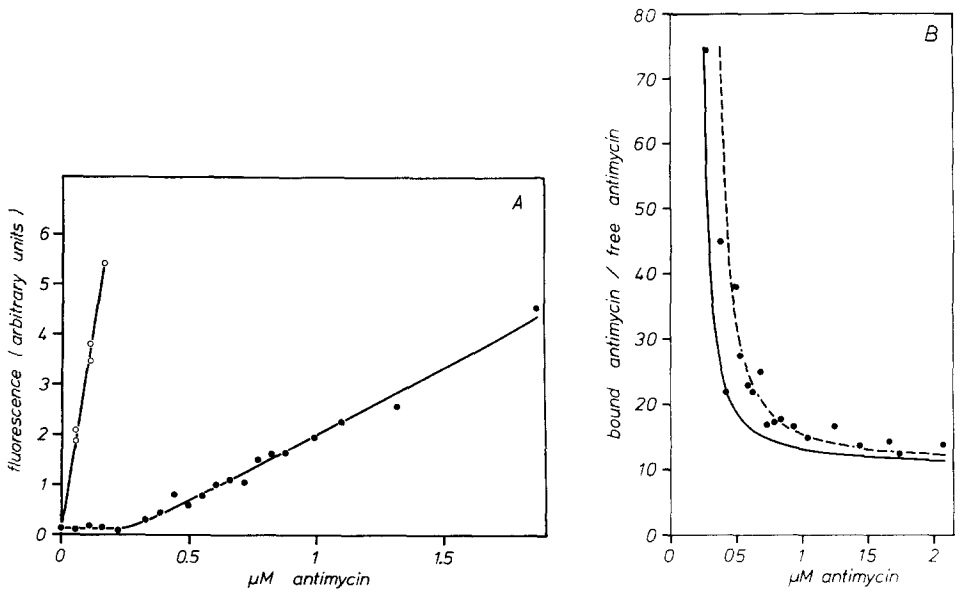

Fig. 1. Binding of antimycin to mitochondrial particles in the absence of serum albumin. The concentration of mitochondrial particles was 4.95 mg per 3.3 ml test volume. Antimycin was allowed to bind by gently shaking the suspension at 4 °C for 20 min. (A) Binding curve (●, test curve; ○ standard curve). (B) Scatchard plot. The dotted line is the theoretical hyperbola resulting from 3 different binding sites: A_1, $K_{D1} \leqq 1 \cdot 10^{-12}$ M, 150 pmol per mg protein; A_2, $K_{D2} = 3 \cdot 10^{-9}$ M, 100 pmol per mg protein; non-specific site, $K_{D3} \approx 10^{-4}$ M, approx. 70 mmol per mg protein. The non-interrupted line results from 2 binding sites, A_1 and the non-specific site. The points represent the measured data from (A).

specific site ($K_D \leqq 1 \cdot 10^{-12}$ M ; 150 pmol per mg protein; called A_1) and a non-specific site (Fig. 1B, —). But a fitting cannot be made unless the existence of at least a further specific binding site is accepted in addition to the non-specific binding, (Fig. 1B, ---).

The concentration of the second weaker binding site is (according to the parameter-fitting trial) 100 pmol per mg protein and the $K_{D2} = 3 \cdot 10^{-9}$ M (called A_2). The non-specific site then has a dissociation constant $K_{D3} \sim 10^{-4}$ M and is present at a concentration of approx. 70 mmol per mg protein.

To determine the dissociation constant of A_1 more precisely, a binding experiment was carried out in the presence of high concentrations of serum albumin (Fig. 2A). The Scatchard plot is shown in Fig. 2B. If the parameters for A_2 and the non-specific site are taken from Fig. 1B and are corrected for the binding capacity of serum albumin, no fitting with the data given in Fig. 2B is possible. Therefore, the steepest slope of the Scatchard curve is taken. The dissociation constant calculated in this manner must be higher than the real one. So, a $K_{D1} \leqq 4 \cdot 10^{-13}$ M can be determined.

Ratio of cytochromes to antimycin binding sites

In mitochondria of *S. pombe*, the content of cytochrome *a* is very much dependent on the growth conditions. When the cells are cultivated at approx. 10 % oxygen

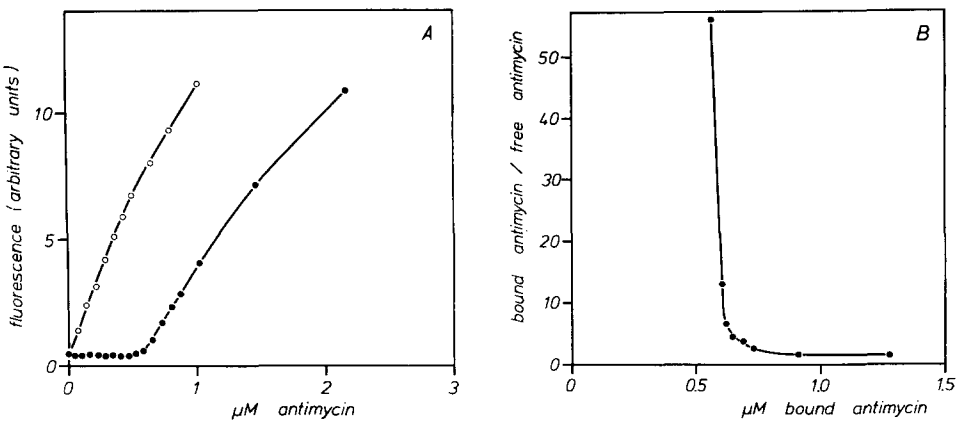

Fig. 2. Binding of antimycin to mitochondrial particles in the presence of serum albumin. The concentration of serum albumin was 10 mg per ml, that of mitochondrial particles 9.55 mg per 2.5 ml test volume. (A) Binding curve. (B) Scatchard plot. Using the steepest slope, an apparent $K_{D1} \leqq 6 \cdot 10^{-10}$ M is calculated. After correction for the competition of serum albumin, the real dissociation constant is $K_{D1} \leqq 4 \cdot 10^{-13}$ M.

saturation, much more cytochrome oxidase is present in the mitochondrial particles than with well-aerated, fermenter-grown cells (90% oxygen saturation). Therefore, no definite correlation between concentration of antimycin binding sites and cytochrome oxidase is to be expected. However, the concentration of cytochrome *b* and c_1 and its quantitative relation to A_1, is, on the contrary, constant ($\pm$ 10%) in different preparations. The determination of the exact concentration of cytochrome c_1 in yeast is problematic. Since the *b*-type cytochromes of yeast absorb at wavelengths different from beef heart, the overlap between cytochrome *b* and c_1 is different in cytochrome spectra of both these types of mitochondria. Therefore, the absorption coefficient of cytochrome c_1 found in beef heart mitochondria is not transferable to yeast. The exact concentration is not determinable until a specific absorption coefficient for yeast is calculated. With well-aerated cells, the content of cytochrome *b* is 475 pmol and of cytochrome aa_3 180 pmol per mg protein. The ratio A_1 to cytochrome *b* is1 : 3.

Inhibitory effect of antimycin

The mitochondrial particles used throughout these experiments are characterized by a general increase in enzymatic activities compared to those used in our previously published work [1]. In the presence of low concentrations of Lubrol WX, this increase is such that a significant difference in the activities of NADH : cytochrome *c* oxidoreductase, stimulated by exogenously added Q-3 and QH_2 : cytochrome *c* oxidoreductase could no longer be detected.

An increase was also observed in the activity of NADH oxidase which is now close to the value calculated from uncoupled whole cell respiration as shown in Table I. Furthermore, this table reveals that during the purification of the particles most of the NADH oxidase activity is preserved. The constant factor of stimulation by ubiquinones of NADH oxidase indicates that no natural ubiquinone and cytochrome *c*, essential for this activity is removed from their site of action in the membrane during

TABLE I

RESPIRATORY ACTIVITIES OF WHOLE CELLS AND MITOCHONDRIAL PARTICLES DURING THE PURIFICATION PROCEDURE

Cell respiration is given as μmol electrons per min per mg wet weight cells. Mitochondrial respiration is given in μmol electrons per min per mg protein. The values for mitochondrial respiration were calculated on the premise that 100 g wet weight cells yield 0.7 g protein of purified mitochondrial particles.

	Respiration	Purification step of mitochondrial particles	Succinate oxidase	NADH oxidase	NADH oxidase + Q-3	Stimulation factor by Q-3
Whole cells	0.03– 0.035	Differential centrifugation	0.6	1.9	2.5	1.32
Cells+FCCP + glucose	0.05– 0.08	Hypotonic treatment and recentrifugation	0.7	2.4	3.25	1.36
Expected highest value of mitochondrial respiration	7.00–11.00	Centrifugation through Urografin	0.9	3.7	5.2	1.41

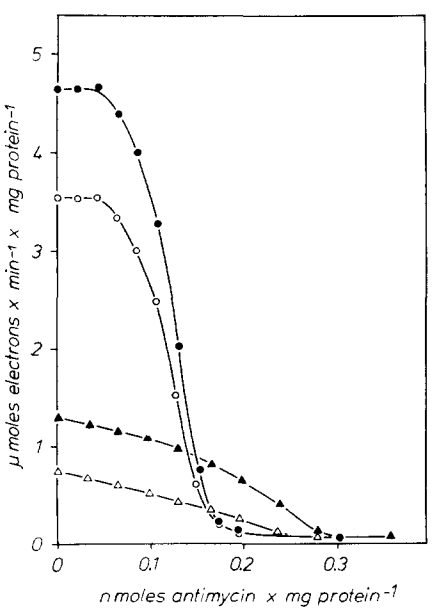

Fig. 3. Inhibition of NADH oxidase and NADH: cytochrome *c* oxidoreductase by antimycin. The concentration of mitochondrial protein was 75 μg and 5 μg, respectively, per test. Antimycin was added from a stock solution containing 1 μg and 0.1 μg, respectively, antimycin per ml. (○, unstimulated NADH oxidase; ●, stimulated by 26 μM Q-3; △, unstimulated NADH: cytochrome *c* oxidoreductase; ▲, stimulated by Q-3.)

purification. Parallel enrichment of NADH- and succinate : cytochrome *c* oxidoreductase is observed (not shown in Table I).

A principal change of the shapes of the inhibition curves by antimycin was not noticed with any preparation, so that our curves already published [1] remain valid.

Inhibition of the electron transport from NADH to oxygen by increasing concentrations of antimycin results in a sigmoidal inhibition curve (Fig. 3) as has also been shown earlier by others [3–8, 14]. In the presence of 150 pmol of antimycin per mg protein, the respiratory activity is inhibited by about 95 %. However, when NADH : cytochrome *c* oxidoreductase activity is titrated with antimycin, 95 % inhibition is not reached unless a concentration of 260 pmol antimycin per mg protein is added.

In the presence of detergent, the same degree of inhibition is reached by addition of 300 pmol antimycin per mg protein. The specific activity of NADH oxidase, stimulated by Q-3 is about 5.0 μmol electrons per min per mg protein and of NADH : cytochrome *c* oxidoreductase 1.0 μmol reduced cytochrome *c* per min per mg protein. The latter activity can be increased up to 2.5 μmol reduced cytochrome *c* per min per mg protein in these mitochondrial preparations by addition of 0.2 mg Lubrol WX per mg protein (Fig. 4). However, even in the presence of higher concentrations of detergent, the same rate of electron transport as obtained for NADH oxidase could not be achieved.

NADH : cytochrome *c* oxidoreductase activity can also be stimulated by the addition of Q-2 or Q-3 [1]. After the addition of detergent, the inhibition curve of

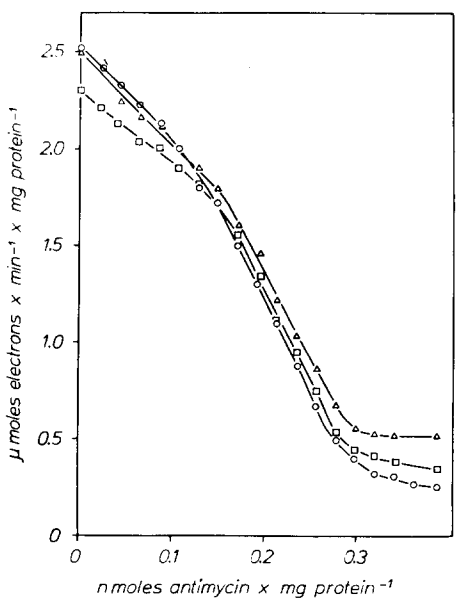

Fig. 4. Inhibition of NADH: cytochrome *c* oxidoreductase + Q-3 and QH_2-2 and QH_2-3: cytochrome *c* oxidoreductase by antimycin in the presence of Lubrol WX. 5 μg protein was used per test. The concentration of the stock solution of antimycin was 0.1 μg per ml. (○, NADH: cytochrome *c* oxidoreductase, stimulated by Q-3; □, QH_2-2: cytochrome *c* oxidoreductase, 75 μM QH_2-2; △, QH_2-3: cytochrome *c* oxidoreductase, 130 μM QH_2-3.)

NADH : cytochrome *c* oxidoreductase stimulated by Q-3 becomes superimposable with QH_2-2 and QH_2-3 : cytochrome *c* oxidoreductase.

The same experiments were carried out with an adenine-requiring mutant 50 h^-, derived from the wild type 972 h^-. The generation time in glycerol medium is 6 h (wild type 5 h). Respiration of whole cells and the activity of NADH oxidase of mitochondrial particles were the same. However, even in presence of detergents, the activity of QH_2 : cytochrome *c* oxidoreductase is less than 50% of that of the wild type. Nevertheless, the inhibition curves are similar to those obtained with the wild type.

Low temperature spectroscopy of cytochromes after reduction with QH_2-2 or QH_2-3

Since QH_2-2 and QH_2-3 are only sparingly soluble in aqueous buffer, it is impossible to measure the redox levels of cytochromes in the steady state or in the anaerobic state with ubiquinols like with NADH or succinate as substrate. Therefore, cytochrome *c* was extracted from the mitochondrial particles in order to abolish the rapid oxidation of ubiquinols by oxygen. It can be seen from Fig. 5 that there is no significant reduction of cytochrome *b* and c_1 with both ubiquinols as substrate. However, when KCN is added to the particles, a composite peak appears consisting of cytochrome c_1 absorbing at 549.5 – 550 nm and a *b*-type cytochrome absorbing at 553 nm, both at low temperature. In the presence of ascorbate, cytochrome c_1 is reduced to the same extent as with ubiquinols, but not this *b*-type cytochrome. In addition, after blocking of cytochrome oxidase with KCN, both *a*-cytochromes are al-

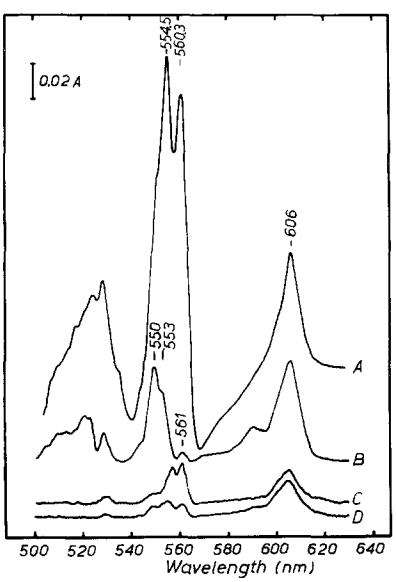

Fig. 5. Difference spectra at 77 °K (reduced minus oxidized) of cytochrome *c*-depleted particles, reduced by QH_2-2 or QH_2-3. Mitochondrial particles (12 mg per ml) were suspended in a buffer as described in Methods. The light path was 2 mm. In every case, the reference was oxidized by 0.2 mM $K_3(Fe(CN)_6)$. The samples were reduced (A) by a few crystals of $S_2O_4^{2-}$; (B) by 0.6 mM QH_2-3 or QH_2-2 and incubated for 5 min after addition of KCN; (C) as in (B) and incubated for 5 min after addition of 2 μM antimycin; (D) as in B, without further additions.

most totally reduced by the ubiquinols, though cytochrome *c* is removed. In order to look for an effect similar to the additional reduction of *b*-cytochromes with antimycin in the presence of succinate or NADH, spectra were repeated with the same type of particles in the presence of 3μM antimycin (Fig. 6). With both QH_2-2 and QH_2-3 only the same low amount of cytochrome *b* reduction (10–15 % of total) was obtained. However, the position of both peaks is shifted to the red compared with the spectra with succinate or NADH as substrate in the presence of antimycin.

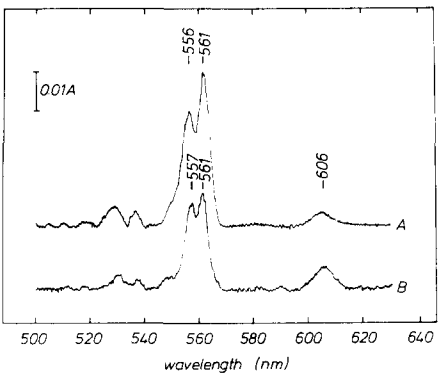

Fig. 6. Difference spectra at 77 °K of cytochrome *c*-depleted particles in the presence of antimycin. (A) mitochondrial particles (3 mg per ml) in the sample cell were reduced by 2 mM NADH after addition of 2 μM antimycin. The reference was oxidized by 0.2 mM $K_3(Fe(CN)_6)$. In (B), the same conditions were chosen as in Fig. 5C (12 mg protein per ml).

DISCUSSION

Previous models of electron flux through the bc_1-segment

From kinetic measurements, it has been inferred that the sigmoidal inhibition curve of NADH oxidase results from a rate-limiting step before the bc_1-segment in combination with the mobility of the intrinsic ubiquinone pool [6, 14, 26]. This model is inconsistent with our finding that (i) NADH oxidase is stimulable with added Q-2 or Q-3. This extension of the ubiquinone pool does not alter the shape of the antimycin inhibition curve at all; (ii) it does not explain the different shapes of QH_2-2 and QH_2-3 : cytochrome *c* oxidoreductase in the absence of Lubrol WX [1]; (iii) this kinetic model fails to explain the non-linear effect of antimycin on the reduction of cytochrome b_{566} in the anaerobic state, i.e. in the absence of electron flux.

Other authors [10] propose that the two electrons delivered to the respiratory chain by QH_2 pass separate pathways to cytochrome *c*; only the faster path contains cytochrome *b* and the binding site of antimycin. However, these conclusions were derived from the investigation of the redox behaviour of the *b*-cytochromes in beef heart mitochondria. Results obtained with yeast are not consistent with findings in beef heart: e.g. in an antimycin resistant mutant of *C. utilis*, the effects of antimycin on the redox behaviour of the *b*-cytochromes and on the electron flux are separable [19]. Therefore, a new model of the electron transport through the bc_1-segment of the respiratory chain in yeast is proposed.

The binding of antimycin to the respiratory chain

Under the presupposition of non-interacting sites, our results reveal the existence of two groups of specific binding sites.

In our inhibition experiments of NADH : cytochrome *c* oxidoreductase by antimycin in the absence of Lubrol WX a concentration of 260 pmol total inhibition sites per mg protein were found. Our binding studies reveal 250 pmol total binding sites for antimycin per mg protein. Because the detergent competes with the weaker binding site for the antibiotic, in the presence of Lubrol WX about 50 pmol more antimycin is needed for inhibition.

A model of electron transport through the bc_1-segment

In different preparations of mitochondrial particles showing different velocities of NADH oxidase and NADH : cytochrome *c* oxidoreductase the molar ratio of antimycin needed for inhibition of both activities is always approximately 1 : 2. Even in Lubrol WX-treated particles where the NADH oxidase activity is drastically lowered to the level of NADH : cytochrome *c* oxidoreductase activity, the same ratio is found.

The conclusion is that the binding site A_1, which is saturated first, is mainly responsible for inhibition of the electron flux to oxygen. The NADH : cytochrome *c* oxidoreductase in contrast is inhibited only after saturation of both binding sites.

Since the arrangement of A_1 and A_2 in a linear electron transport chain cannot explain the need for one or two times the amount of antibiotic for inhibition, one has to infer that the electrons pass on a branched way through A_1 and A_2. It would be likely that the two-electron transport from NADH dehydrogenase branches at ubiquinone, resulting in two one-electron transport chains, each of which contains one antimycin binding site (Fig. 7). Stabilized by lipoprotein complexes of the membrane, QH· should be able to act as an additional electron donor for a separate one-electron transport chain. QH· is formed during oxidation of QH_2 in acetonitrile [27] and is also present in mitochondrial particles in low concentrations [28].

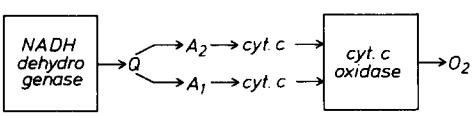

Fig. 7. Model of a split electron transport chain, containing one antimycin binding site per branch. The two-electron transport branches at ubiquinone into two one-electron transport chains. (Q, intrinsic ubiquinone; A_1, A_2, antimycin binding sites.)

Another model implies QH· to be rather stable [10]. But it is much more probable that QH· dissociates into QH_2 and Q, if QH· is not consumed. The reduction of Q by the NADH dehydrogenase should on the other hand be a two step reaction, too, so that QH· may be produced if the concentration of QH_2 is high and that of QH· low. Thus, it follows that one of the oxidation steps of QH_2 can be slower or faster than the other (Fig. 8).

The assumption that the electron transport chain in the bc_1-segment is branched affords an explanation for the various shapes of the composite inhibition curves

Fig. 8. Model of the two oxidation steps of QH_2, independent in their velocities. The number of arrows represents the velocity. (A), At high concentrations, QH· dissociates into QH_2 and Q. (B), At low concentrations of QH· and high concentrations of QH_2, QH· is produced by the NADH dehydrogenase.

presented in our previous paper [1]. The inhibition curve of NADH: cytochrome *c* oxidoreductase and QH_2 : cytochrome *c* oxidoreductase is almost linear. This reflects the coincidence that the two paths of electron transport have about the same velocity. This is confirmed by ubiquinone stimulation of the NADH : cytochrome *c* oxidoreductase activity. The increase in sigmoidicity of the inhibition curve could be due to the increase of substrate concentration in the A_2 branch by enlargement of the total ubiquinone pool. By addition of Lubrol WX to the particles, a stimulation of QH_2-2: cytochrome *c* oxidoreductase can be achieved. Under these conditions the inhibition curve becomes as sigmoidal as was observed with QH_2-3 as substrate. Addition of Lubrol WX accelerates the electron transport through the A_2 branch with all substrates tested and makes the sigmoidal shape of the inhibition curves more pronounced.

So, the inhibition curve of NADH : cytochrome *c* oxidoreductase is composed of (i) the curve representing the activity of the A_1-branch, which is abolished by the amount of antimycin necessary to saturate A_1 and (ii) the activity curve of the A_2 branch which declines to zero by binding of the antibiotic to A_2 after saturation of A_1. This is shown schematically in Fig. 9.

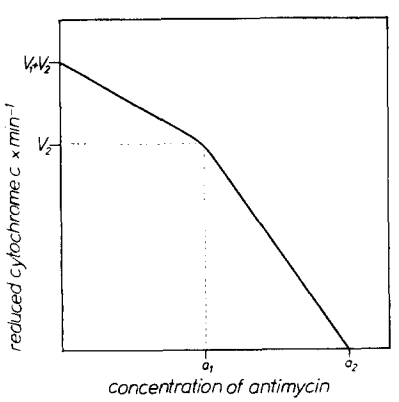

Fig. 9. Presumed composition of the antimycin inhibition curves with cytochrome *c* as electron acceptor. V_1, V_2, velocity of the electron transport over the A_1- and A_2-branch, respectively. a_1, a_2, concentration of A_1 and A_2.

Inhibition experiments in the presence of Lubrol WX can provide information why the A_1-path is so much faster in the NADH oxidase test than in the NADH : cytochrome *c* oxidoreductase test. As stated above, the activity of NADH oxidase can be drastically lowered by addition of even small concentrations of this detergent which removes most of the intrinsic cytochrome *c* from the membrane. Evidently, this property of the detergent affects the main activity of the NADH oxidase, which is mainly supplied by the electron transport over A_1 and apparently the A_1-branch runs through membrane-bound cytochrome *c*.

In the NADH : cytochrome *c* oxidoreductase test with externally added cytochrome *c* as electron acceptor, the cytochrome oxidase is inhibited by KCN. Thus, electrons transported over A_1 are forced to equilibrate with externally added cytochrome *c*. Therefore, the equilibration reaction with external horse heart cytochrome *c* should be the rate-limiting step of the A_1-branch in the NADH : cytochrome *c* oxidoreductase test. This assumption is supported by the fact that oxygen consumption of cytochrome *c* (horse heart) oxidase is much slower than that of NADH oxidase.

The velocity of the A_2-branch is higher in the NADH : cytochrome *c* oxidoreductase test than in the NADH oxidase test. Therefore the electrons coming from A_2 must equilibrate better with externally added cytochrome *c* than with cytochrome *c* oxidase. In Fig. 10, it is illustrated that the electrons passing A_2 run over externally added cytochrome *c*.

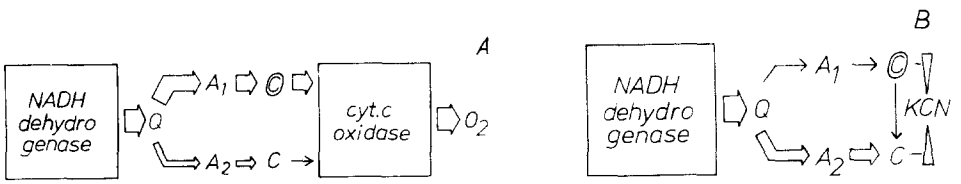

Fig. 10. Model of the distribution of electron transport velocities over the two branches in NADH oxidase and NADH: cytochrome *c* oxidoreductase. (A), Oxygen as electron acceptor. (B), cytochrome *c* as electron acceptor (c, externally added cytochrome *c*; ©, intrinsic cytochrome *c*). The size of the arrows represents the velocity.

The main electron flux in the NADH oxidase runs over A_1 only. If the interpretations of the sigmoidal shape by a rate limiting step before cytochrome *b* [10, 13–15] are correct, one should expect that under conditions where the rate limitation is eliminated, (i.e. in the presence of detergents, additional ubiquinones enlarging the intrinsic pool, or isolated Complex III) the respiratory chain should run much faster than in the NADH oxidase test. For beef heart mitochondria this explanation seems to be correct, because in the presence of deoxycholate the activity of QH_2-2 : cytochrome *c* oxidoreductase is about six times higher than of NADH oxidase [3]. However, this is in contrast to findings in yeast [e.g. 29] where NADH oxidase is always much faster than QH_2 : cytochrome *c* oxidoreductase.

Our explanation of the sigmoidal shape of the NADH oxidase inhibition curve in yeast is that this curve is also composite, though in a different manner than that of NADH : cytochrome *c* oxidoreductase. In the NADH oxidase test, the substrate of the A_1-branch is quickly oxidized and thereby more substrate for the A_2-branch is produced. Since the equilibration between internal and external cytochrome *c* is rate

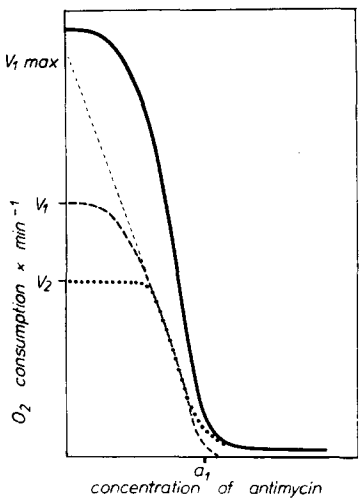

Fig. 11. Presumed composition of the antimycin inhibition curve of succinate and NADH oxidase. V_1, V_2, see Fig. 9. $V_{1\,max}$, maximum velocity of the A_1 branch by substrate saturation.

limiting, most of the electrons will reduce externally added cytochrome *c*. The electron flux over the A_2-branch to oxygen should therefore depend on the concentration of reduced external cytochrome *c*. This concentration depends on the availability of substrate for this path. Binding of antimycin to A_1 consequently lowers the concentration of the substrate available for the A_2-branch. This is in fact observed. In the steady state oxidation of NADH the amount of reduced external cytochrome *c* decreases proportionally with the decrease in activity of NADH oxidase from 12 % to ≦ 2 % of the total. The residual concentration seems to be too low to induce an electron flux over A_2 to oxygen that could be measured by the polarographic method.

The inhibition curve of NADH oxidase by antimycin is then composed of (i) the strongly sigmoidal curve of the activity of the A_2 branch and (ii) the much steeper curve of the activity of the A_1 branch, both inhibited by saturation of A_1 by the antibiotic. The shape of the inhibition curve of the A_1 branch could be linear or slightly sigmoidal. (If it is sigmoidal, then there is not sufficient substrate for the A_1-branch.) (Fig. 11.)

It can be seen in this figure that the maximum velocity of NADH oxidase which is the sum of the velocities V_1 and V_2 cannot be derived by extrapolation of the steepest slope to the ordinate which would lead to an unreasonably high V of the NADH oxidase. This extrapolation would be correct if the sigmoidicity of the inhibition curve of NADH oxidase by antimycin would merely be brought about by a rate-limiting step before the antimycin binding site.

It cannot be decided whether QH_2 or QH· is fed into the A_1 branch. We can only postulate that possibly QH· is the substrate of the A_2 branch, because the stimulation of its activity by Lubrol WX could be due to a stabilization and in that way to an increase in the concentration of QH·.

Arrangement of cytochromes b and c_1 in the respiratory chain

Since in cytochrome *c*-depleted particles no significant reduction of cytochrome

b and c_1 is observed in the presence of synthetic ubiquinols, we conclude that cytochromes are not essential for electron transport through A_2 but are presumably arranged in or close to the path through A_1. After addition of KCN, cytochrome c_1, a *b*-type cytochrome absorbing at 553 nm [30] and cytochrome aa_3 are reduced by ubiquinols.

Cytochromes may be dispensible for electron transport in QH_2 : cytochrome *c* oxidoreductase. Isolated enzyme in which the activity is 20-fold enriched is essentially free of cytochromes and yet fully sensitive to antimycin (Lang, B., unpublished).

It seems to be reasonable to assume that *b*-cytochromes are not accessible for exogenously added ubiquinols. The fact that they are only sparingly reduced could explain the observation that in enzyme kinetic measurements electrons coming from synthetic ubiquinols are not able to run the path over A_1 to cytochrome *c* as quickly as electrons passing the natural ubiquinone pool and the *b*-cytochromes [1]. In addition, *b*-cytochromes reduced by NADH or succinate in the presence of antimycin (561 nm and 555.5 nm) are not the same as that reduced by QH_2-2 or QH_2-3 (561 nm and 557 nm). Therefore, in the presence of antimycin at least two different cytochrome *b* species are reduced which are different from the *b*-cytochrome absorbing at about 553 nm in the presence of ubiquinol and KCN. The existence of 3 types of *b*-cytochromes would be in agreement with the ratio of 3 cytochrome *b* to $1A_1$, which we find in yeast.

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