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THE ALLOSTERIC BINDING OF ANTIMYCIN TO CYTOCHROME *b* IN THE MITOCHONDRIAL MEMBRANE

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SUMMARY

1. Based on the assumptions that the quenching of the fluorescence of antimycin on binding to the bc_1 segment of the respiratory chain (Complex III) is caused by energy transfer from antimycin to the cytochrome *b* haem, and the intrinsic fluorescence of antimycin bound to the complex is the same as that in ethanolic solution, the distance between the fluorescent group of antimycin and the *b* haem was found to be 1.9 nm in oxidized Complex III and 2.4 nm in the reduced Complex. If the intrinsic enhancement of the fluorescence on binding to the Complex is the same as that on binding to serum albumin (about 9-fold), these distances become 1.35 nm and 1.7 nm, respectively.

2. The binding of antimycin to oxidized (sub-)mitochondrial particles from beef heart is non-co-operative, with a binding constant of about $3 \cdot 10^{10}$ M^{-1}.

3. In succinate-reduced mitochondria and sub-mitochondrial-particles from beef heart the binding of antimycin increases with increasing amounts of antimycin. At zero antimycin concentration the binding constant is about $3 \cdot 10^9$ M^{-1}. With high concentrations of antimycin the binding constant equals that for the oxidized particles. It is assumed that in succinate-reduced particles the antimycin-binding site is predominantly in a conformation (T state) with a lower binding constant than the conformation present in oxidized particles (R state) and that antimycin promotes the formation of the R state in succinate-reduced particles.

4. Particles reduced with dithionite and beef-heart mitochondria reduced with succinate in the presence of ATP bind antimycin non-co-operatively with the same binding constant as for oxidized particles. Rat-liver mitochondria behave qualitatively the same as beef-heart mitochondria, but with a smaller affinity for antimycin.

5. In cytochrome *c*-depleted particles reduced with succinate, the antimycin-effect curves are linear, and the antimycin-binding curves are non-co-operative with a binding constant equal to that of the T state.

6. In pentane-extracted particles reduced with succinate, the binding is non-co-operative, with a binding constant a little less than in oxidized particles.

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

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7. Isolated Complex III in the oxidized state binds antimycin much more strongly than oxidized particles. The binding to Complex III reduced by succinate or dithionite is about the same as to oxidized particles. It is suggested that the site of splitting with detergent and salt is the same as the antimycin-binding site.

8. The results with particles and mitochondria are explained on the basis of the allosteric model of Monod. Both ubiquinone and cytochrome *c* are allosteric effectors of the bc_1 complex, while antimycin is an allosteric inhibitor. In mitochondria ATP also causes a shift of the T $\rightleftharpoons$ R equilibrium towards the R state.

9. Using the antimycin-induced inhibition of succinate oxidation or increased reduction of cytochrome *b* by succinate as a measure for the amount of R state present at a given antimycin concentration, the values of n and $\log L$ in the Monod allosteric model were calculated to be 7.5 and 5.85, respectively. The latter corresponds to an energy difference between T and R states of about 8 kcal/mole.

INTRODUCTION

In previous papers from this laboratory, in collaboration with the Laboratory of Biochemistry of the University of Warsaw, it has been proposed that the sigmoidal curves describing the relation between the inhibitory and spectral effects of antimycin on mitochondria or sub-mitochondrial particles and the antimycin concentration reflect a co-operative binding of antimycin to the particles[1–3]. KLINGENBERG AND KRÖGER[4] have favoured an alternative explanation, namely that the sigmoidal curves reflect the pool function of ubiquinone in the respiratory chain. Although this explanation does not, in our view, adequately account for the sigmoidal curves describing the effects of antimycin on the cytochrome *b* spectrum, under conditions in which there is no turnover of ubiquinone or other components of the respiratory chain[5], it remained desirable to test our conclusion by direct binding studies.

The quenching of the fluorescence of antimycin that occurs when it is bound to respiratory-chain preparations[6,7] provides a suitable method for measuring the binding constant. Using this method we have shown previously[7] that antimycin is bound stoicheiometrically in the proportion of 1 molecule of antimycin to two molecules of native cytochrome *b* and that the binding is stoicheiometric with the concentration of native cytochrome *b* and not with the cytochrome c_1 concentration. In agreement with YAMASHITA AND RACKER[8] we found no binding at all of antimycin to a reconstitutively active cytochrome c_1 preparation. On the basis of this stoicheiometry (see also RIESKE *et al.*[9]) it has been proposed that two species of cytochrome *b* are present in the mitochondrial membrane in equal concentrations, only one of which (termed b_1) is affected by antimycin[10,11]. From the parameters of the fluorescence quenching, the distance between the antimycin-binding site and the cytochrome b_1 haem are calculated in this paper.

In our previous papers[1–3], we have interpreted the co-operativity of the effects of antimycin in terms of the allosteric model of MONOD *et al.*[12], that is, we have assumed that the antimycin-binding site exists in two conformational states, the R and the T state, in equilibrium with one another, and that antimycin is an allosteric inhibitor that combines preferentially with the conformation in low concentration (the R state) that, at equilibrium in substrate-reduced particles in the absence of antimycin,

is present in low concentration. Furthermore, it is necessary to assume more than one antimycin-binding site in each reacting entity. Indeed, Complex III, which has only one antimycin-binding site per molecule, has a linear antimycin inhibition curve[9], and dispersal of particulate preparations with cholate[3], which would be expected to decrease the number of binding sites per particle causes a decrease of the sigmoidicity.

Since linear or hyperbolic* antimycin-effect curves are found with mitochondria in the presence of ATP[13,14], our interpretation implies that energization of the mitochondrial membrane shifts the $R \rightleftharpoons T$ equilibrium towards R, and that binding of low concentrations of antimycin is greater in the presence of ATP than in the presence of uncoupler. Linear or hyperbolic effect curves are also found with non-energized particles when $Na_2S_2O_4$ is used as electron donor instead of a substrate of the respiratory chain. $Na_2S_2O_4$ differs from substrate in that it is able to reduce b_i^{3+} in the absence of antimycin or energy. Our interpretation implies then that in the hybrid $b^{2+} \cdot b_i^{2+}$, the conformation of the antimycin-binding site is much more in the R state than in the substrate-reduced hybrid $b^{2+} \cdot b_i^{3+}$ and that $Na_2S_2O_4$-reduced particles will bind low concentrations of antimycin more firmly than substrate-reduced particles.

These predictions have been tested by the direct binding studies in this paper. Some of these results have been reported to a symposium[15].

RESULTS

The distance between the antimycin-binding site and the cytochrome b_i haem

Since the wavelength of the emission peak of excited antimycin at 420 nm is in the same region of the spectrum as the absorption maxima of cytochromes *b*, b_i and c_1, it is likely that the fluorescence quenching is due to energy transfer from the excited antimycin to the cytochromes. In a previous paper[7], reasons were given for favouring a cytochrome *b* over cytochrome c_1 as the quencher. Since, by definition, b_i is influenced by antimycin, it is most likely that this is the component responsible. From the titration curve it is clear that simple absorption by b_i of the emitted light cannot be the reason for the quenching. Transition metal ions in general are good fluorescence quenchers, so that a different as yet not understood quenching mechanism[16] may also be present, but since these effects operate over a short distance, it will be assumed in what follows that the quenching is due to energy transfer.

To obtain R_0, *i.e.* the distance at which quenching is 50 %, we need the values of the overlap integral of the cytochrome bb_i absorption and the antimycin emission, and the fluorescence lifetime[17]. The overlap integral is given by the expression

$$J\bar{\nu} = \int_0^\infty \varepsilon^D(2\bar{\nu}_0 - \bar{\nu}_a)\varepsilon^A(\bar{\nu})d\bar{\nu}$$

where $\varepsilon^D(2\bar{\nu}_0 - \bar{\nu}_a)$ is the absorbance coefficient of donor at a wavenumber equal to $(2\bar{\nu}_0 - \bar{\nu}_a)$, $\bar{\nu}_0$ is the arithmetic mean of the wavenumbers of absorption and emission maxima of the donor, $\bar{\nu}_a$ is a given wavenumber, and ε^A is the absorbance coefficient of acceptor at this wavenumber. It was determined by multiplying at each wavenumber the absorbance coefficient (per haem) of cytochrome bb_i by the coefficient of the correc-

* Because of the very high binding constant of antimycin, hyperbolic curves relating the effect of antimycin to *free* antimycin concentration become virtually linear when the effect is plotted against *total* antimycin concentration.

ted antimycin emission. This corrected antimycin emission spectrum was constructed by drawing the mirror image of the absorption spectrum around $\bar{\nu}_0$. The product of absorbance and corrected emission (the latter is also in units of absorbance) can be plotted against the wavenumber and the overlap integral is given by the area under the curve.

The absolute absorption spectrum of cytochrome bb_i was calculated from that of Complex III, correcting for the contribution of cytochrome c_1. This was preferred to the spectrum of isolated cytochrome b (refs. 7 and 8), since this is modified in the sense that it is catalytically inactive, combines with CO and does not quench the fluorescence of antimycin[7]. The correction for the contribution of cytochrome c_1 was based on the spectrum of isolated, reconstitutively active cytochrome c_1 and an absorbance coefficient of 17.1 $mM^{-1} \cdot cm^{-1}$ for reduced *minus* oxidized at 553 nm[7].

The absorption spectrum of antimycin was measured in ethanolic solution and the concentration was calculated using a millimolar absorbance coefficient at 320 nm of 4.8 $mM^{-1} \cdot cm^{-1}$ (ref. 18).

The values of the overlap integrals calculated in this way were $1.5 \cdot 10^{12}$ and $1.35 \cdot 10^{12}$ $cm^3 \cdot nmole\ haem^{-1} \cdot nmole\ antimycin^{-1}$ for oxidized and reduced Complex III, respectively.

The lifetime of fluorescence τ_s in ethanolic solution, measured as described in EXPERIMENTAL, was found to be $1.3 \pm 0.5 \cdot 10^{-9}$ sec.

R_0 was calculated from the formula

$$R_0 = \left[\frac{9 \cdot 10^6 (\ln 10)^2 k^2 c \tau_s J \bar{\nu}}{16\pi^4 n^2 N^2 \bar{\nu}_0^2}\right]^{1/6}$$

where c ($msec^{-1}$) is the velocity of light, n is the refractive index of the solvent and N is Avogadro's number, with the additional assumption that the orientation is random, so that $k^2 = 2/3$ (ref. 17). The calculated values are $35 \cdot 10^{-8}$ and $34 \cdot 10^{-8}$ cm for the oxidized and reduced complex, respectively. If only b_1 and not b is responsible for the quenching these values become $31 \cdot 10^{-8}$ and $30 \cdot 10^{-8}$ cm, respectively.

The uncertainty about the orientation factor introduces a small uncertainty in the calculation of R_0. If the orientation is optimal for energy transfer, k reaches its maximal value of 1. In this case, the value of R_0 would be increased by a factor of $(3/2)^{1/6}$, *i.e.* 1.07. If the orientation is highly unfavourable, k^2 will be less than 2/3 and R_0 will be correspondingly smaller. The value of R_0 calculated on the assumption of random orientation is, then, close to the upper limit.

The actual distance R equals $R_0[(1-T)/T]^{1/6}$, where T is the degree of quenching, which was found to be 95 % for oxidized Complex III and 80 % for reduced (see Fig. 1). This yields 1.9 nm for the distance between antimycin and the haem chromophore in oxidized Complex III and 2.4 nm in reduced. In this calculation it has been assumed that the intrinsic fluorescence of antimycin bound to Complex III is the same as that in ethanolic solution. If, in fact, the intrinsic fluorescence is greater, as in the case of antimycin bound to serum albumin[19], the degree of quenching is greater and the distance smaller than those calculated. If the intrinsic enhancement of the fluorescence of antimycin on binding to Complex III is the same as that on binding to serum albumin (about 9-fold), the distances become 1.35 nm and 1.7 nm for oxidized and reduced complex, respectively.

No effect of antimycin binding on the circular-dichroism spectrum of Complex III in the region of 220 nm was detected, with either oxidized or reduced complex. A difference was, however, detected between oxidized and reduced complex, independent of the presence of antimycin, the negative band at 220 nm being more intense in the reduced complex.

Binding of antimycin to non-phosphorylating sub-mitochondrial particles

The binding of antimycin to particles was followed by adding different amounts to a suspension of the particles in 0.5–0.75 % serum albumin. The serum albumin served three purposes: (1) the fluorescence of antimycin not bound to the particles is increased by binding to albumin[19,20]; (2) it removes antimycin from any low-affinity binding sites in the particles, thereby simplifying the analysis (*cf.* ref. 21); (3) by competing with the particles for antimycin, albumin increases the apparent dissociation constant of antimycin from the particles thereby increasing the sensitivity of the method. The binding of antimycin to serum albumin was studied in a control experiment, making use of the fluorescence enhancement on binding. The results, reported in a Scatchard plot in Fig. 2, give a dissociation constant of $1.2 \cdot 10^{-7}$ M with 1 binding site per 67000 daltons, *i.e.* 1 binding site per molecule of molecular weight 67500 (ref. 22).

The results of the experiments on the binding of antimycin to particles suspended in sucrose and Tris buffer (pH 7.4) containing serum albumin are shown in Fig. 3. On the ordinate is plotted the fluorescence of the supernatant after adding the various amounts of antimycin shown on the abscissa and spinning down the particles after 15 min at 22°. A control without particles is also included. The amount of bound antimycin is given by the difference between the two lines. By inspection it is clear that oxidized particles have a higher affinity for low concentrations of antimycin than

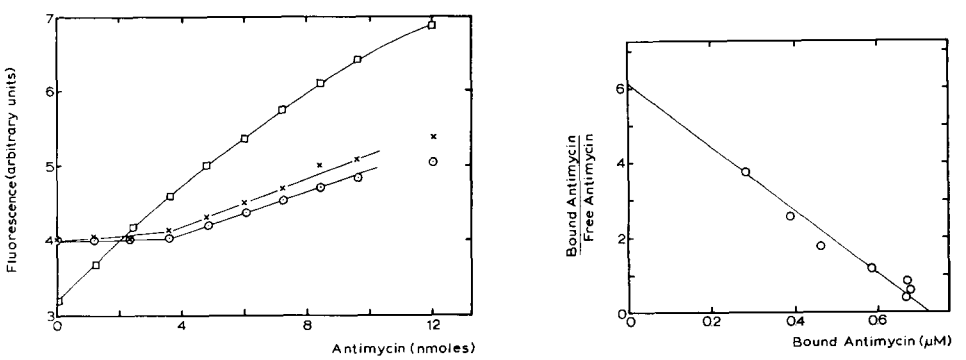

Fig. 1. Quenching of antimycin fluorescence by Complex III. Complex III (1.2 mg containing 4 nmoles cytochrome c_1 in 2 ml 0.25 M sucrose, 50 mM Tris–HCl buffer, pH 7.4) was titrated with antimycin. The fluorescence was measured with a fluorimeter described in ref. 40. Excitation wavelength was 350 nm, measuring wavelength 470 nm. The fluorescence of the unbound antimycin is somewhat quenched because of absorption of both the exciting and emitted light by Complex III. □—□, no Complex III; ⊙—⊙, Complex III; ×—×, Complex III + $Na_2S_2O_4$.

Fig. 2. Binding of antimycin to bovine serum albumin. The amount of antimycin bound to bovine serum albumin (0.05 mg/ml) in 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 7.3) was calculated from a fluorimetric titration curve with antimycin. The fluorescence of the bound antimycin was about 9 times higher than that of the free antimycin. The Scatchard plot gives a dissociation constant of $1.2 \cdot 10^{-7}$ M. The number of binding sites is 1 per 67000 daltons serum albumin.

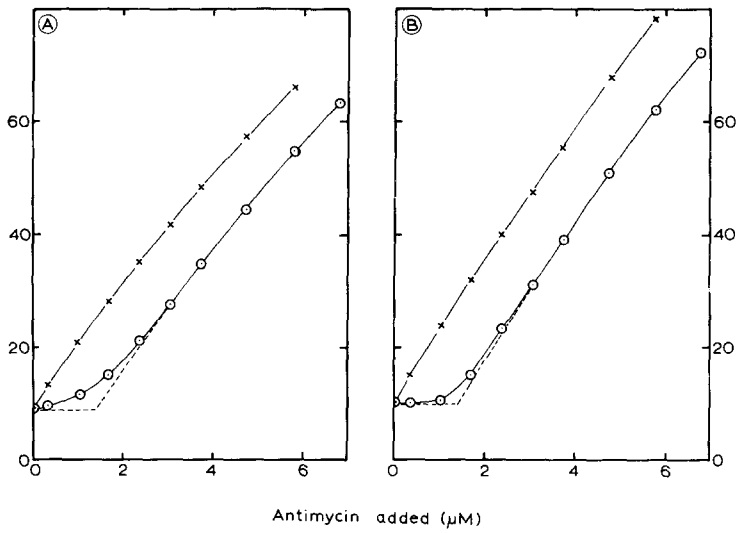

Fig. 3. Binding of antimycin to heart-muscle particles. The particles (5 mg/ml) were incubated in 0.2 M sucrose, 25 mM Tris–HCl buffer (pH 7.3) in the presence of 6 mg/ml bovine serum albumin for about 15 min at room temperature with varying amounts of antimycin. After spinning down the particles (20 min, 100000 × *g*), the fluorescence of the supernatant was measured with an Eppendorf fluorimeter. (A) Particles + 20 mM succinate + 10 mM cyanide. (B) Particles without further additions. ×—×, no particles.

succinate-reduced particles. The data are plotted in the form of a Scatchard plot in Fig. 4. The straight line obtained for the oxidized preparation shows that the binding is non-co-operative. The apparent dissociation constant in the presence of albumin is $2.4 \cdot 10^{-8}$ M. When allowance is made for binding to albumin, the dissociation constant of antimycin bound to particles is $3.2 \cdot 10^{-11}$ M. The curved line, concave to the abscissa, obtained with the reduced preparation reveals binding with a positive co-operativity. The dissociation constant, given by the negative of the inverse of the slope of the line joining a point with the intersection of the abscissa, is $3.6 \cdot 10^{-10}$ M (corrected) for zero bound antimycin. With higher concentrations of antimycin the points join the straight line obtained with oxidized preparations. In terms, then, of the allosteric model of MONOD *et al.*[12], we may equate $3.2 \cdot 10^{-11}$ M with K_R, the intrinsic dissociation constant of the complex with the R state, and $3.6 \cdot 10^{-10}$ M with K_T, the intrinsic dissociation constant of the complex with the T state. This yields 0.09 as value for $c = K_R/K_T$.

Fig. 5 shows that the binding of antimycin to $Na_2S_2O_4$-reduced particles is about the same as to oxidized particles, in contrast to the marked difference with succinate-reduced particles (*cf.* Fig. 5 with Fig. 3). Thus the antimycin-binding site in $Na_2S_2O_4$-reduced particles must be to a considerable degree in the R state which agrees with the linear antimycin-effect curve reported by BRYŁA *et al.*[3] in the presence of $Na_2S_2O_4$. Since BRYŁA *et al.*[3] explain the hyperbolic shift curve with antimycin in dithionite-reduced particles by the fact that dithionite is not a natural substrate, it was interesting to see what effect extraction of ubiquinone has on the binding of antimycin. The curve relating the antimycin-induced reduction of long-wavelength *b* by succinate to antimycin concentration is still sigmoidal (Fig. 6). Although the

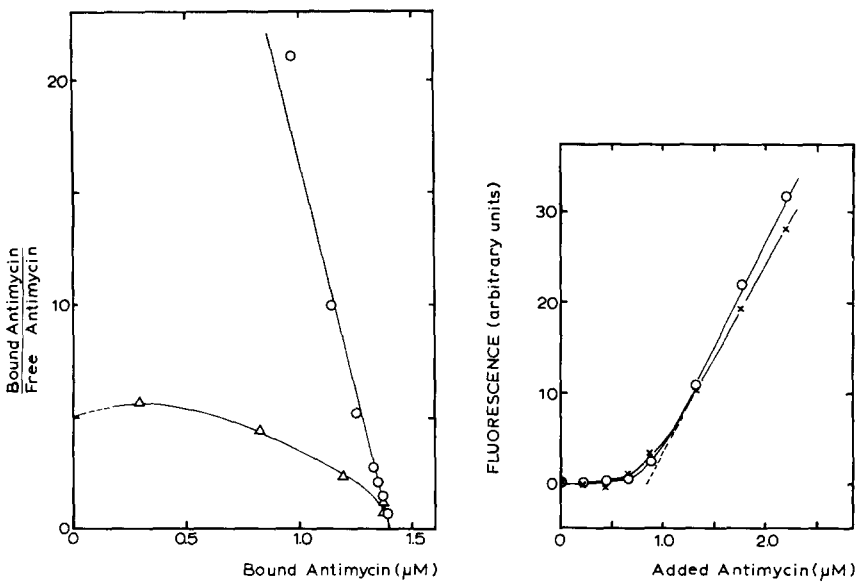

Fig. 4. Scatchard plot for the binding of antimycin to heart-muscle particles. From Fig. 3 the bound and the 'free' (in reality bound to albumin) antimycin concentrations were calculated on the assumption that the fluorescence of the particle-bound antimycin is not detectable. ○—○, points taken from the curve in Fig. 3B (oxidized particles); △—△, experimental points in Fig. 3A (reduced particles). The apparent dissociation constant for the oxidized particles is $2.4 \cdot 10^{-8}$ M. When corrected for the competition between particles and albumin for antimycin, the real dissociation constant for the particles is $3.2 \cdot 10^{-11}$ M. For succinate-reduced particles the dissociation constant is dependent on the antimycin concentration. Extrapolation to zero bound antimycin gives as corrected dissociation constant $3.6 \cdot 10^{-10}$ M. With high amounts of antimycin the dissociation constant for oxidized particles is reached. The number of binding sites is 0.28 nmole/mg protein.

Fig. 5. Effect of reduction by $Na_2S_2O_4$ on binding of antimycin to heart-muscle particles. Experimental conditions as in Fig. 3, with 3 mg/ml particles and 7.5 mg/ml albumin. ○—○, without further additions (oxidized particles); ×—×, $Na_2S_2O_4$ added.

preparations used had no residual NADH oxidase activity, they oxidized succinate slowly, and succinate reduced cytochrome *b* in cyanide-inhibited particles slowly but completely, indicating that they contain small concentrations of 'P', the lipid-soluble component involved in the succinate oxidase system[23], but no Q. P-reconstituted preparations[23] also show sigmoidal antimycin-effect curves. In the binding studies, however, succinate-reduced pentane-extracted preparations were found to bind antimycin more firmly than controls, indicating that more of the R state is present in these preparations, as shown clearly by the Scatchard plot (see Fig. 7)).

KLINGENBERG and co-workers[24,25] have brought forward in support of their explanation[4] of the sigmoidal antimycin-effect curves their finding that linear inhibition curves are obtained with both cyanide-inhibited and cytochrome *c*-deficient particles. Their results with cyanide-inhibited particles are difficult to reconcile with the binding of antimycin, which is clearly co-operative in the presence of higher concentrations of cyanide than those used by LEE *et al.*[24], and, in fact, we have been unable to confirm the effect of cyanide reported by these authors. In our hands, the inhibition curve remains sigmoidal in the presence of cyanide. Fig. 8 shows the results

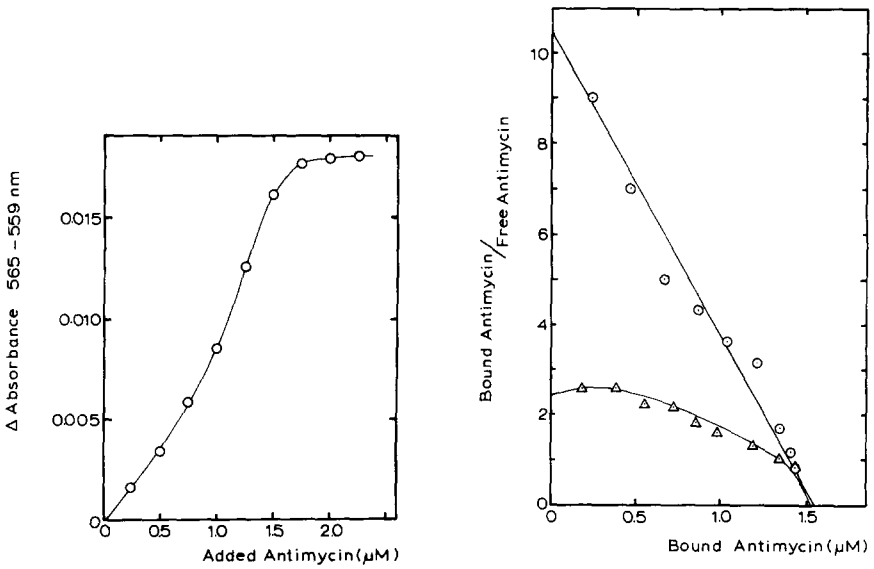

Fig. 6. Antimycin-induced shift of the cytochrome *b* spectrum of pentane-extracted heart-muscle particles. Heart-muscle particles extracted 8 times with pentane were suspended at 7.5 mg protein per ml in 0.25 M sucrose, 25 mM Tris–HCl buffer (pH 7.4) in the presence of 20 mM succinate and 10 mM cyanide. When the reduction of cytochrome *b* was constant successive amounts of antimycin were added and the increase in $\Delta A_{565-559\ nm}$ measured (*cf.* ref. 3).

Fig. 7. Binding of antimycin to pentane-extracted heart-muscle particles. The same particles as used for the experiment of Fig. 6 were used in this experiment. The experimental conditions were the same as in Fig. 3, except that the concentration of serum albumin was 7.5 mg/ml. The concentration of the particles was 5 mg/ml. 20 mM succinate and 10 mM cyanide were present. ⊙—⊙, pentane-extracted particles; △---△, control with non-extracted particles. The real dissociation constant (see legend to Fig. 4) for extracted particles equals $1.6 \cdot 10^{-10}$ M. The dissociation constant for the non-extracted particles at zero antimycin concentration equals $7 \cdot 10^{-10}$ M.

with 'A' (phosphorylating sub-mitochondrial) particles, and similar results were obtained with non-phosphorylating particles and mitochondria and with azide instead of cyanide. Because of the slow reaction between cytochrome oxidase and cyanide[26], we found it necessary to preincubate the particles with cyanide first without substrate and then with a low concentration of NADH before starting the measurements with a second addition of NADH or with succinate. Otherwise, linear oxygen uptake curves were not obtained. Each point in Fig. 8 represents a separate incubation.

We do agree with LEE *et al.*[24] and KRÖGER AND KLINGENBERG[25] that extraction of cytochrome *c* results in linear (see Fig. 9) antimycin-effect curves. However, this cannot be used in support of the explanation for the sigmoidal inhibition curves favoured by these authors, since extraction of cytochrome *c* also results in non-cooperative binding (Fig. 10) with a dissociation constant ($3.4 \cdot 10^{-10}$ M) characteristic of the T state of the antimycin-binding site. It appears, then, that the transformation T → R is not possible in the absence of cytochrome *c*. After re-incorporation of cytochrome *c* by sonication in a solution of cytochrome *c*, and removal of unbound cytochrome *c* by washing the particles three times, a sigmoidal effect curve was again obtained (Fig. 9).

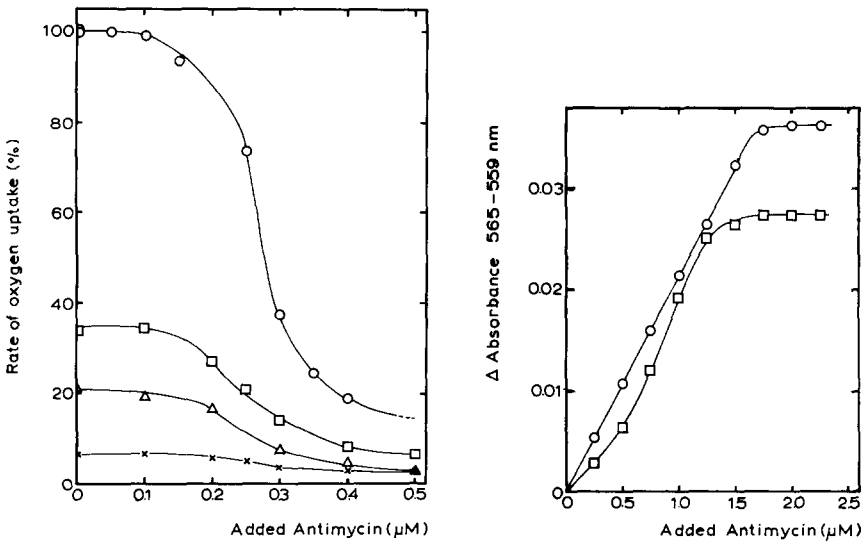

Fig. 8. Inhibition of succinate oxidation in 'A' particles by antimycin in the presence of cyanide. 'A' particles (23 mg/ml) were incubated for 1 h at 0° with varying concentrations of cyanide, and were diluted 15 times in the Oxygraph vessel in a medium containing 0.25 M sucrose, 25 mM Tris–HCl buffer (pH 7.4), 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and varying concentrations of antimycin. 25 μM NADH was then added. When this was all oxidized, as revealed by the cessation of the oxygen consumption, 10 mM succinate was added. The rate of the subsequent oxygen uptake remained constant. ○—○, no cyanide present during the incubation; □—□, 40 μM cyanide present during the incubation; △—△, 60 μM cyanide; ×—×, 300 μM cyanide.

Fig. 9. Antimycin-induced reduction of long-wavelength cytochrome *b* in cytochrome *c*-depleted and re-incorporated heart-muscle particles with antimycin. Conditions as in Fig. 6. ○—○, cytochrome *c*-depleted particles (7.5 mg/ml); □—□, cytochrome *c* re-incorporated particles (6 mg/ml). The cytochrome *c*-depleted particles were sonicated in 0.25 M sucrose, 25 mM Tris–HCl buffer (pH 7.4) containing 100 μM cytochrome *c*, and the excess cytochrome *c* removed by centrifugation and washing 3 times.

Binding of antimycin to phosphorylating sub-mitochondrial particles and to mitochondria

Oxidized and succinate-reduced 'A' particles and beef-heart mitochondria bind antimycin in the same way as non-phosphorylating particles. Both the 'A' particles and the non-phosphorylating particles were prepared from beef heart. Rat-liver mitochondria have a much lower affinity for antimycin than the beef-heart preparations, but show the same difference in behaviour between oxidized and succinate-reduced preparations. Moreover, in the presence of dithionite the binding is the same as with oxidized mitochondria.

In agreement with the findings of BONNER AND SLATER[13] and WEGDAM *et al.*[14] that linear antimycin-effect curves are obtained with succinate-reduced mitochondria in the presence of ATP, the addition of ATP to succinate reduced mitochondria resulted in an antimycin-binding curve characteristic of the oxidized state, *i.e.* of the R state of the antimycin-binding site (see Fig. 11). Qualitatively similar results were obtained with rat-liver mitochondria.

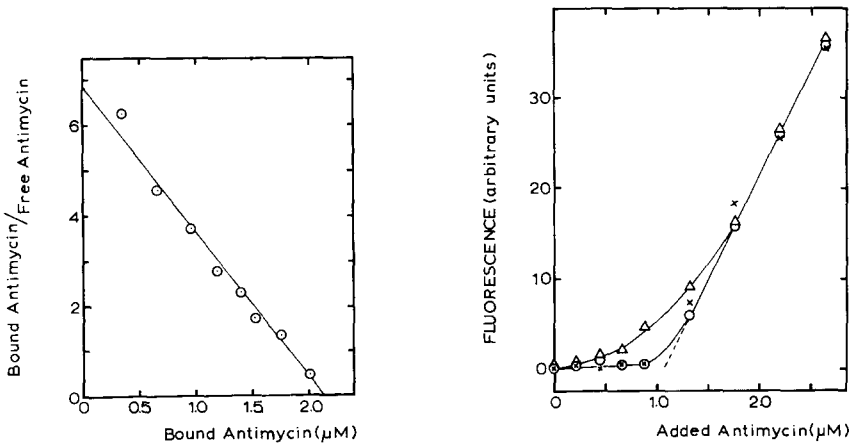

Fig. 10. Binding of antimycin to cytochrome *c*-depleted particles. The experiment was carried out as described in the legend for Fig. 3. 20 mM succinate and 10 mM cyanide were added. The concentration of the particles was 7.5 mg/ml, the concentration of serum albumin 7.5 mg/ml. The real dissociation constant (see Fig. 4) equals $3.4 \cdot 10^{-10}$ M.

Fig. 11. Effect of ATP on the binding of antimycin to beef-heart mitochondria. Beef-heart mitochondria (4.0 mg/ml) were incubated in a medium containing 6 mg/ml bovine serum albumin. For further details, see legend for Fig. 3. ○—○, oxidized mitochondria; △—△, 20 mM succinate and 10 mM cyanide added; ×—×, 20 mM succinate, 10 mM cyanide and 4 mM ATP added.

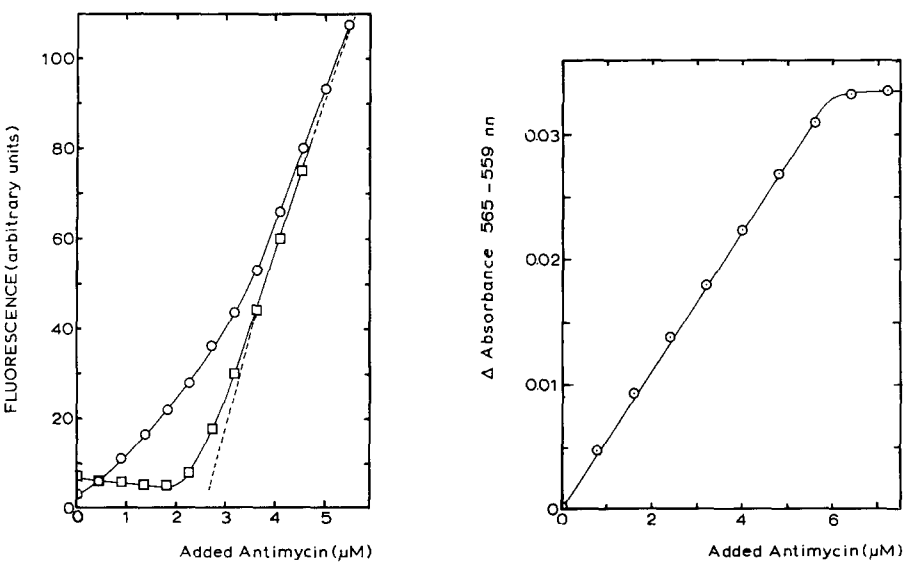

Fig. 12. Binding of antimycin to Complex III. Complex III (0.9 mg/ml, containing 3.0 μM cytochrome c_1), in 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 7.4) and 67 mg/ml albumin was titrated with antimycin and the fluorescence was measured. □—□, oxidized Complex III; ○—○, Complex III with 20 mM succinate and 5 mM cyanide. The binding constant for oxidized Complex III exceeds 10^{13} M^{-1}. For reduced Complex III the dissociation constant is about $6 \cdot 10^{-11}$ M.

Fig. 13. Antimycin-induced reduction of long-wavelength cytochrome *b* in Complex III. Complex III (2 mg/ml containing 6.5 μM cytochrome c_1), dissolved in 0.25 M sucrose, 25 mM Tris–HCl buffer (pH 7.4), 20 mM succinate and 5 mM cyanide was titrated with antimycin as in Fig. 6.

Binding of antimycin to Complex III

BRYŁA *et al.*[27] showed that Complex III can remove antimycin from non-phosphorylating particles indicating that it binds antimycin more firmly. This was confirmed in the present studies. Even in the presence of 6.7 % serum albumin, no fluorescence appeared in the supernatant until an amount of antimycin equal to the amount of binding sites was added (Fig. 12). The binding is much less in the presence of succinate but, in contrast to the results with particles, the binding is non-co-operative (K_D is about $6 \cdot 10^{-11}$ M). A linear inhibition curve[9] and antimycin induced reduction by succinate of high-wavelength *b* (Fig. 13) is also found.

DISCUSSION

In earlier publications the sigmoidal curves, found when the inhibition by antimycin of the respiratory chain or the extent of the antimycin-induced reduction of a long-wavelength *b* species is plotted against the antimycin concentration, were explained by the allosteric model. The binding studies reported in this paper are completely consistent with this model. The sigmoidal curves are obtained with substrate-reduced non-phosphorylating particles or phosphorylating particles in the absence of ATP. Under these conditions, the binding of antimycin is co-operative. Linear or hyperbolic effect curves are found with $Na_2S_2O_4$ as electron donor, with substrate-reduced cytochrome *c*-deficient sub-mitochondrial particles, and with substrate-reduced mitochondria in the presence of ATP. In all three cases, non-co-operative binding of antimycin was found.

By making use of the experimentally determined value of K_D, at each antimycin concentration, which can be read off from Fig. 4 for succinate-reduced particles, it is possible to transform the usual type of inhibition curve, in which the degree of inhibition is plotted against amount of antimycin added, into a curve relating degree of inhibition to $\alpha = [\text{free antimycin}]/K_R$. This is shown in Fig. 14. If we now assume that inhibition of the respiratory chain by antimycin is due to formation of the R state, Fig. 14 is at the same time a plot of $\bar{R}$ (the fraction in the R state) against α. The equation[12]

$$\bar{R} = \frac{(1+\alpha)^n}{(1+\alpha)^n + L(1+c\alpha)^n}$$

where $L = [T]/[R]$, in the absence of inhibitor, and n is the number of interacting binding sites, may be transformed into

$$\log \frac{1-\bar{R}}{\bar{R}} = \log L - n[\log(1+\alpha) - \log(1+c\alpha)]$$

so that a plot of $\log (1-R)/\bar{R}$ against $\log (1+\alpha) - \log (1+c\alpha)$ should yield a straight line with intercept on the ordinate equal to $\log L$, and a negative slope equal to n. The data of Fig. 14, plotted in this way in Fig. 15, yield $\log L = 5.85$ and $n = 7.5$. This value of L corresponds to a $\Delta G_0'$ of -8 kcal/mole for the reaction $R \rightleftharpoons T$. The same values for n and L are obtained if we use the increase of cytochrome *b* reduction by antimycin in the presence of succinate and cyanide ($\Delta A_{562-575\,nm}$)[3] as indicator for the amount of R state present (Fig. 15). The conditions of this measure-

ment are the same as used to measure the binding constant with antimycin. The antimycin induced 'red shift' cannot be used as a measure for the R state because this curve is a composite curve representing two different phenomena (J. A. BERDEN AND E. VAN KUIPERS, unpublished observations).

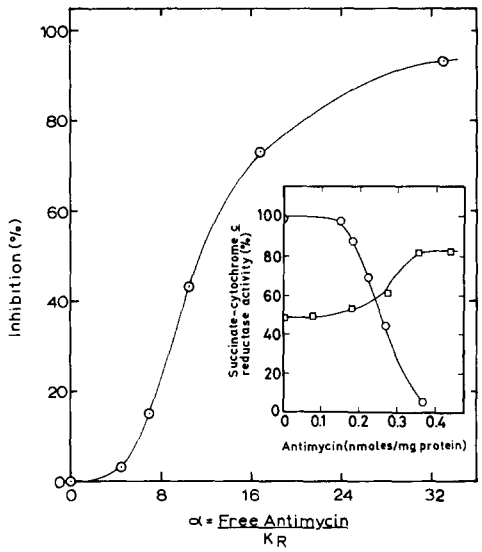

Fig. 14. Inhibition of succinate-cytochrome *c* reductase activity of heart-muscle particles by antimycin. The inhibition curve, given in Fig. 3A of ref. 3 (reproduced by the curve ○—○ in the inset), is converted to a curve relating inhibition with α, *i.e.* [free antimycin]/K_R. The free antimycin concentration was calculated by making use of the dissociation constants at varying concentrations of antimycin that can be calculated from curve △—△ in Fig. 4. $K_R = 3.2 \cdot 10^{-11}$ M (see Fig. 4). The curve □—□ of the inset, also reproduced from Fig. 3A of ref. 3, shows the increase of cytochrome *b* reduction, measured as $\Delta A_{563-575\,nm}$ and expressed as percentage of $Na_2S_2O_4$-reducible cytochrome *b*, with increasing amounts of antimycin added, in the presence of 20 mM succinate and 10 mM cyanide.

KLINGENBERG and co-workers[4,24,25] have suggested that the sigmoidal inhibition curves are a reflection of the pool function of ubiquinone. As already stressed elsewhere[5], this cannot be the explanation of the sigmoidal curve relating the antimycin-induced reduction of long-wavelength *b* with antimycin concentration, since these measurements are carried out under conditions in which there is no turnover of respiratory-chain components. These antimycin-effect curves are adequately explained by the allosteric model. The sigmoidal inhibition curves must also reflect at least partly the co-operative binding. The question will now be discussed whether they also reflect partly the type of phenomenon referred to by KLINGENBERG and co-workers[4,24,25].

There is no question that, as pointed out by this group, sigmoidal inhibition curves could be obtained even with non-co-operative binding when the activity of the antimycin-sensitive region of the chain is much greater than that of the chain as a whole. An example is provided by Complex III which is usually contaminated by succinate dehydrogenase. The binding is non-co-operative as is the effect curve describing the reduction of a long-wavelength *b* species. The inhibition of the highly

active QH_2–cytochrome *c* reductase is also described by a linear effect curve[9]. In contrast, since the succinate–cytochrome *c* reductase activity is limited by the succinate dehydrogenase, the antimycin-inhibition curve is clearly sigmoidal (Fig. 16). This is, however, an extreme case since succinate dehydrogenase is present as a contaminant

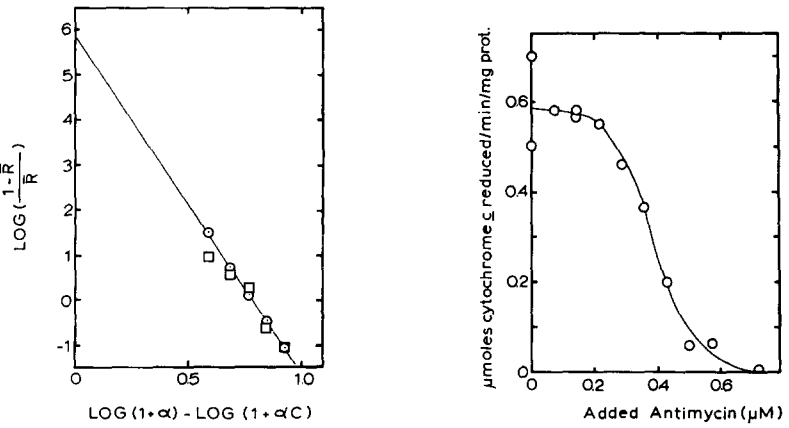

Fig. 15. Determination of the allosteric parameters n and L, using the formula $\log (1-\bar{R})/\bar{R} = \log L - n[\log(1+\alpha) - \log(1+\alpha c)]$. The points ⊙ are calculated from the inhibition curve given in Fig. 14 with the assumption that the degree of inhibition is a measure for the amount of R state. The points □ are calculated from a curve relating the increase of cytochrome *b* reduction with antimycin concentration in heart-muscle particles (see curve □—□ of the inset of Fig. 14), on the assumption that the relative increase of reduction is a measure for the R state. The negative slope of the curve (n) equals 7.5 and the intersection point with the ordinate ($\log L$) equals 5.85.

Fig. 16. Inhibition of succinate–cytochrome *c* reductase activity of impure Complex III by antimycin. Complex III (0.2 mg/ml containing 0.66 μM cytochrome c_1) was incubated in 0.25 M sucrose, 50 mM Tris–HCl buffer (pH 7.4), 20 mM succinate, 2 μM cytochrome *c* and varying amounts of antimycin for 4 min to activate the succinate dehydrogenase and to be sure that the antimycin inhibition was maximal. The reaction was started with 25 μM cytochrome *c* and the absorbance at 550 nm was followed.

in preparations of Complex III. In preparations containing the intact respiratory chain, the activity of the antimycin-sensitive region is not greatly in excess of that of the succinate dehydrogenase. Nevertheless, the sigmoidicity is much greater with preparations of the respiratory chain (see Fig. 14, from which a Hill coefficient of 4 can be calculated) than in the case illustrated in Fig. 16 (Hill coefficient is 1.9). It seems reasonable to conclude, then, that the sigmoidal inhibition curves reflect the allosteric binding of antimycin and that it is justified to use also these curves to calculate the allosteric parameters.

A second example of a sigmoidal inhibition curve with non-co-operative binding of antimycin is provided by cytochrome *c*-deficient particles in the presence of cyanide. Fig. 10 shows that antimycin binding to such particles in the presence of cyanide is non-co-operative. In the absence of cyanide, the antimycin-inhibition curve of cytochrome *c*-deficient preparations is linear[25], but in the presence of sufficient cyanide to inhibit the residual oxidase activity the inhibition curve becomes sigmoidal (J. A. BERDEN AND E. VAN KUIPERS, unpublished observations). This can be explained by a pool function for cytochrome *c*.

In oxidized particles containing the hybrid $b^{3+} \cdot h_i^{3+}$, the antimycin-binding site

is largely in the R state. Reduction *via* the respiratory chain in non-phosphorylating particles results in the formation of $b^{2+} \cdot b_i^{3+}$ in which the antimycin-binding site is in the T state. Antimycin is bound to the latter conformation with a binding constant of about $3 \cdot 10^9$ M^{-1}, without, however, causing inhibition of the respiratory chain. Thus, $b^{2+} \cdot b_i^{3+}$ in the T state is an active electron carrier, even when bound to antimycin. Higher concentrations of antimycin induce the transformation of the antimycin-binding site into the R state with concomitant reduction of b_i^{3+} by succinate, and the hybrid $b^{2+} \cdot b_i^{2+}$, when bound to antimycin, can no longer donate electrons to cytochrome c_1, so that inhibition of the respiratory chain results. Since there is a clear correlation between the conformation state of the antimycin-binding state and the behaviour of cytochrome *b*, we may extend the concept of the R and T states to the whole Complex III.

The sharp increase in $A_{566-560\,nm}$, in the presence of cyanide, found with amounts of antimycin that begin to inhibit respiration is due to the reduction of b_i^{3+} in the R state to b_i^{2+} which absorbs at a longer wavelength than b^{2+}. Thus, antimycin, by promoting the formation of the inhibitory R state, acts as an allosteric inhibitor. Electrons from substrate, by promoting the formation of the active $b^{2+} \cdot b_i^{3+}$ in the T state, act as an allosteric effector. They may do this directly by reducing b^{3+} in the hybrid, or indirectly by reducing other components, such as ubiquinone, which has been proposed as an allosteric activator of the b–c_1 complex[28]. Pentane extraction of the particles results in increased binding of antimycin (Fig. 7), consistent with an increased proportion of the R state.

Reduction of particles by $Na_2S_2O_4$ yields the hybrid $b^{2+} \cdot b_i^{2+}$. Since the binding is non-co-operative, with a binding constant similar to that for oxidized particles, the *b* complex is presumably largely in the R state. The increase in $A_{566-560\,nm}$ brought about by antimycin added to $Na_2S_2O_4$-reduced particles is due to a shift of the absorption maximum, and not to increased reduction[29].

Two independent lines of evidence indicate that reduction of b_i^{3+} is associated with a conformation change. First, antimycin is 0.5 nm closer to the iron atom in b_i^{3+} than in b_i^{2+}. Secondly, the circular dichroism spectrum around 220 nm is changed on reduction. WILLIAMS[30] has pointed out that a conformational change is to be expected on reduction of a haemoprotein. No changes corresponding to a conformational change could, however, be detected by circular dichroism on binding of antimycin to Complex III. The fact that in particles reduction by substrate favours the T state while binding of antimycin favours the R state confirms that antimycin does not induce the reduced conformation. The similar inhibitory effect of reduction by substrate and addition of antimycin on the splitting of Complex III by detergent plus salt can be understood, if splitting takes place at the antimycin-binding site (*cf.* ref. 31), and if this site is less accessible to detergents (as well as to antimycin) in the substrate-reduced complex, or when antimycin is bound to this site. It may be significant in this respect that the iron atom of cytochrome *b* split from the complex by this procedure is, in contrast to the molecule present in the complex, readily accessible to CO (ref. 7).

Cytochrome *c*, the electron acceptor of the bc_1 segment of the respiratory chain, appears to be a second allosteric effector of this segment since removal of the cytochrome *c* results in binding behaviour characteristic of the T state, and the inability of antimycin to convert the T state into the R state. The fact that antimycin

still inhibits the residual activity of cytochrome *c*-deficient particles can be explained by postulating that this activity is a property of the remaining bc_1 complex still bound to cytochrome *c*. This small fraction of the complex can be brought into the R state and is inhibited sigmoidally. The bulk of the complex, however, will still bind antimycin, albeit with lower affinity and non-co-operatively. The net result is an inhibition curve that is much less sigmoidal than with particles containing cytochrome *c*.

Although succinate has the same effect on Complex III as on particles in decreasing the binding of antimycin, the binding to succinate-reduced Complex III is still non-co-operative. This does not, however, necessarily mean that only one state is present in succinate-reduced Complex III since co-operative binding requires more than one interacting binding site as well as more than one conformation of the binding site. The value of $n = 7.5$, calculated from Fig. 6, indicates that 7–8 hybrid molecules interact in the binding of antimycin to succinate-reduced particles.

EXPERIMENTAL

Except where otherwise stated the heart-muscle particles were prepared by the method of KEILIN AND HARTREE[32].

'A' particles were prepared from heavy beef-heart mitochondria according to the method of FESSENDEN AND RACKER[33].

Rat-liver mitochondria were prepared as described by MYERS AND SLATER[34]. Complex III was prepared from Keilin and Hartree heart-muscle particles according to HATEFI *et al.*[35] and RIESKE *et al.*[36] as described previously[7]. Cytochrome *c*-depleted heart-muscle particles were prepared by washing the mince with 0.15 M phosphate buffer (pH 7.4) before grinding[37].

Protein was determined by the biuret method as described by CLELAND AND SLATER[38]. Ubiquinone was extracted with pentane[39]. Antimycin was added in ethanolic solution, the concentration of which was determined spectrophotometrically, using an absorbance coefficient at 320 nm of 4.8 $mM^{-1} \cdot cm^{-1}$ (ref. 18).

The determination of the quenching of the antimycin fluorescence upon binding to Complex III was carried out with the fluorimeter described by BROCKLEHURST *et al.*[40]. The fluorescence lifetime was measured with a TRW model 75 A decay-time fluorimeter, coupled to a Tektronix 556 dual-beam oscilloscope with a Type 151 sampling unit. The lamp was a N_2 flash lamp, operating at 75 V and with a repetition rate of 5 kHz. Excitation was cut off above 390 nm, and the emission was cut off below 440 nm with a Corning 3387 filter. Other fluorimetric measurements were carried out in an Eppendorf fluorimeter, primary filter 313 + 366 nm and secondary filter 420–3000 nm. Absorption measurements were carried out in an Aminco–Chance dual-wavelength spectrophotometer.

Succinate–cytochrome *c* reductase activities were measured by following the absorption at 550 nm, using 21.1 $mM^{-1} \cdot cm^{-1}$ as the absorbance coefficient for cytochrome *c* (reduced *minus* oxidized)[41]. Oxygen uptake was measured with an Oxygraph, equipped with a Clark electrode.

The binding of antimycin to particles was studied by incubating the particles in a medium containing 0.20 M sucrose, 25 mM Tris–HCl buffer (pH 7.3) and 5–7.5 mg/ml bovine serum albumin. Further additions are given in the text and the

legends to the figures. Varying amounts of antimycin were added in a constant volume of ethanol and the mixture was incubated for 15 min at room temperature before centrifugation at 100000 × *g* for 20 min. The fluorescence of the supernatant was measured.

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