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# THE REACTION OF ANTIMYCIN WITH A CYTOCHROME *b* PREPARATION ACTIVE IN RECONSTITUTION OF THE RESPIRATORY CHAIN

J. A. BERDEN AND E. C. SLATER

*Laboratory of Biochemistry, B. C. P. Jansen Institute*, University of Amsterdam, Amsterdam (The Netherlands)*

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SUMMARY

1. Succinate–cytochrome *c* reductase activity was reconstituted by incubating a mixture of succinate dehydrogenase, cytochrome $c_1$, ubiquinone-10, phospholipid and a preparation of cytochrome *b*, made by the method of YAMASHITA AND RACKER.

2. Preparations of cytochrome *b* active in reconstitution contained 5–28% native cytochrome *b*, as adjudged by reducibility with succinate in the reconstituted preparation and by lack of reaction with CO. Preparations of cytochrome *b* containing no native cytochrome *b* according to this criterion were inactive in reconstitution.

3. With a fixed amount of cytochrome *b*, the activity of the reconstituted preparation increased with increasing amounts of cytochrome $c_1$ until a ratio of about $2b$ (total):$1c_1$ (allowing for the cytochrome $c_1$ present in the cytochrome *b* preparation) was reached.

4. The amount of antimycin necessary for maximal inhibition of the reconstituted enzyme is a function of the amount of the cytochrome *b* and is independent of the amount of cytochrome $c_1$. It is equal to about one half the amount of native cytochrome *b*.

5. Preparations of intact or reconstituted succinate–cytochrome *c* reductase or of cytochrome *b* completely quench the fluorescence of added antimycin, until an amount of antimycin equal to onehalf the amount of native cytochrome *b* present was added. Antimycin added in excess of this amount fluoresces with normal intensity. The quenching is only partial in the presence of $Na_2S_2O_4$. Denatured cytochrome *b* does not quench the fluorescence.

6. Since preparations of cytochrome *b* active in reconstitution contained cytochrome $c_1$ in an amount exceeding one half the amount of native cytochrome *b* present in the preparation, there is no evidence that native cytochrome *b* has been resolved from cytochrome $c_1$. The stimulatory action of cytochrome $c_1$ may be due to the restoration of a damaged membrane conformation.

7. Based on the assumption that the $bc_1$ segment of the respiratory chain contains $2b$:$1c_1$:1 antimycin-binding sites, the specific quenching of antimycin fluorescence by binding to cytochrome *b* enables an accurate determination of the absorbance coefficients of cytochromes *b* and $c_1$. These are 25.6 and 20.1 $mM^{-1} \cdot cm^{-1}$

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

for the wavelength pairs 563–577 nm and 553–539 nm, respectively, in the difference spectrum reduced *minus* oxidized.

## INTRODUCTION

Complete resolution and reconstitution of the mitochondrial respiratory chain has not been achieved. Cytochrome *c* can be readily extracted by salt from swollen mitochondria[1,2] and re-incorporated at low salt concentrations[2]. Succinate dehydrogenase is extracted by treatment of sub-mitochondrial particles with alkali and the purified enzyme may be re-incorporated into the particles[3], provided that, during its isolation, succinate dehydrogenase is not treated with cyanide and it is kept fully activated by the presence of succinate[4]. Ubiquinone may also be extracted from dried preparations with organic solvents and re-incorporated with restoration of activity[5]. GREEN and his co-workers[6] have split sub-mitochondrial particles into four fragments—Complexes I, II, III and IV—containing, respectively, the segments of the chain between NADH and Q, succinate and Q, $QH_2$ and ferricytochrome *c*, ferrocytochrome *c* and oxygen. When the four complexes, together with Q and cytochrome *c*, are brought together, the original respiratory chain is reconstituted. However, the individual complexes have not been resolved and reconstituted.

YAMASHITA AND RACKER[7] have recently reported the reconstitution of the succinate–cytochrome *c* reductase segment of the chain by pre-incubating at 37° a mixture of succinate dehydrogenase, cytochrome $c_1$, Q-10, phospholipid and cytochrome *b*. The latter was isolated by splitting a preparation of succinate–cytochrome *c* reductase (Complex II + III) by guanidine in the presence of glycerol and succinate. An almost complete dependence on all five components of the mixture was reported.

The present investigation arose out of our interest in cytochrome *b* (refs. 8–11). The reconstitution experiments of YAMASHITA AND RACKER[7] provide a new system in which the function of cytochrome *b* in the respiratory chain and the action of antimycin can be studied.

## METHODS

### *Enzyme preparations*

The succinate–cytochrome *c* reductase preparation normally used was the supernatant obtained after isolating NADH–cytochrome *c* reductase from beef-heart mitochondria, according to the procedure of HATEFI *et al.*[12]. In some experiments, the succinate–cytochrome *c* reductase was prepared by the method of YAMASHITA AND RACKER[7]. No difference was observed between the two preparations. Complex III ($bc_1$ particle) was prepared from succinate–cytochrome *c* reductase essentially according to RIESKE *et al.*[13], the only difference being that 0.5 mg cholate was used per mg protein instead of 0.4 mg. Cytochrome *b* was prepared as described by YAMASHITA AND RACKER[7].

Cytochrome $c_1$ active in reconstitution was made either from succinate–cytochrome *c* reductase as described by YAMASHITA AND RACKER[7] (in some cases the purification on the calcium phosphate gel was omitted), or from Complex III by splitting the oxidized complex with taurocholate and ammonium sulphate as described by SILMAN *et al.*[14]. The taurocholate–cytochrome $c_1$ complex was precipitated with

ammonium sulphate to 38 % and dissolved in 0.1 M phosphate buffer (pH 7.4) containing 1 mM EDTA. The taurocholate was removed by dialysis against phosphate buffer followed by passage through a column of Sephadex G-100. The cytochrome $c_1$ was precipitated from the eluate by bringing to 60 % saturation with ammonium sulphate and dissolved in 0.05 M Tris–HCl buffer (pH 8) – 0.66 M sucrose. The concentrated preparation was treated with ferricyanide to oxidize it or with $Na_2S_2O_4$ to reduce it, and then again passed through the Sephadex column.

Cytochrome $c_1$ free of core protein and containing 18–19 $\mu$moles haem per g protein was isolated from oxidized Complex III by splitting with taurocholate and ammonium sulphate, after which mersalyl was added, as described by SILMAN *et al.*[14]. After removal of the precipitated core protein, the taurocholate–cytochrome $c_1$ complex was precipitated with ammonium sulphate to 38 % saturation, and dissolved in 0.1 M phosphate buffer, containing 1 mM EDTA and an amount of cysteine equal to twice the amount of mersalyl used. The subsequent procedure was the same as for cytochrome $c_1$ containing core protein. The yield of cytochrome $c_1$ is 60 % based on the amount in the Complex III.

Succinate dehydrogenase was isolated from Keilin and Hartree heart-muscle preparation as described by ZEYLEMAKER[15], modified so that 20 mM succinate was present during the final fractionation with ammonium sulphate. The preparation was stored at −192°. Reconstitution activity was rapidly lost in air (6 h at 0°).

*Reconstitution of succinate–cytochrome c reductase*

Succinate–cytochrome *c* reductase was reconstituted from succinate dehydrogenase, cytochrome *b*, cytochrome $c_1$, Q-10 and phospholipid by Method II of YAMASHITA AND RACKER[7]. The only modification was to replace phosphate buffer by 30 mM glycylglycine–HCl buffer (pH 8.4). The phospholipid used was purified soya phosphatides (asolectin) obtained from Associated Concentrates, Inc., New York. A suspension (20 mg/ml in 20 mM phosphate buffer, pH 7.4) was prepared by sonication for 15 min in a M.S.E. sonifer at an amplitude of 4 $\mu$m. The suspension was clarified by centrifugation at 20000 × *g*, and stored at 5° under nitrogen. When Q-10 (obtained from Sigma) was used as well as phospholipid, an ethanolic solution of Q was added to the phospholipid suspension. When phospholipid was not used, Q was added as an ethanolic solution. The concentration of the ethanolic solution was determined from $\Delta A_{275\ nm}$ (oxidized *minus* reduced with $KBH_4$) using an absorbance coefficient of 12.2 $mM^{-1} \cdot cm^{-1}$ (ref. 16). The succinate–cytochrome *c* reductase activity was measured at 550 nm in a medium containing 30 mM glycylglycine–HCl buffer, 20 mM succinate, 1 mM KCN and 1 mM EDTA. The temperature was 24°. The pH was 8.4. The activity is expressed as nmoles cytochrome *c* reduced per min per mg protein based on an absorbance coefficient of cytochrome *c* (reduced *minus* oxidized) of 21.1 $mM^{-1} \cdot cm^{-1}$ (ref. 17).

*Measurement of cytochrome b and cytochrome $c_1$ concentrations in enzyme preparations*

Protein was determined as described by CLELAND AND SLATER[18].

The cytochrome *b* and cytochrome $c_1$ concentrations were determined from the absorption difference spectra (reduced *minus* oxidized). An absorbance coefficient of 28.0 for the maximum (at 563 nm) – minimum (at 577 nm) difference was used for cytochrome *b*. This coefficient is the mean of three determinations (range

27.3–28.9; *cf.* 28.5 reported by ZAUGG AND RIESKE[19]) made by the method of ZAUGG AND RIESKE[19] using pyridine haemochrome prepared from haemoglobin as standard. This gave an absorbance coefficient of 34.7 $mM^{-1} \cdot cm^{-1}$ at 556 nm. Using the wavelength pair 563–577 nm for cytochrome *b* no correction was necessary for absorption of cytochrome $c_1$.

For calculating the concentration of cytochrome $c_1$ the absorbance coefficient of 19.1 $mM^{-1} \cdot cm^{-1}$ for the wavelength pair 553–539 nm in the difference spectrum, reduced *minus* oxidized, was used. This value is calculated for the wavelength pair 554–540 nm from the coefficient at 554 nm and the spectrum measured by GREEN *et al.*[20] (*cf.* 18.8 calculated by WILLIAMS[21]). Interference for absorption by cytochrome *b* was allowed for by subtracting 0.11 times $\Delta A_{563-577\ nm}$ from the $\Delta A_{553-539\ nm}$.

In the DISCUSSION more accurate absorbance coefficients based on binding studies with antimycin reported in this paper will be proposed.

*Antimycin*

Antimycin was obtained from Sigma. Its concentration was determined from its absorption in ethanol at 320 nm, using 4.8 $mM^{-1} \cdot cm^{-1}$ as the absorption coefficient[22].

*Optical measurements*

Absorption spectra were recorded on a Cary 15 spectrophotometer. The light path was 1 cm. Fluorescence was measured with an Eppendorf fluorimeter, with a mercury high-pressure lamp, using 313 + 366 nm as the primary filter, and 380–3000 nm as secondary.

## RESULTS

*Requirements for reconstitution*

Succinate–cytochrome *c* reductase activity was restored to about 30–70 % of its original value (about 1 μmole cytochrome *c* per min per mg protein), by preincubating at 37° for 3 h a mixture of succinate dehydrogenase, cytochrome $c_1$ and cytochrome *b*, prepared by the method of YAMASHITA AND RACKER[7]. In our hands, neither Q-10 nor phospholipid increase the activity markedly when the cytochrome *b* is isolated from the succinate–cytochrome *c* reductase preparations of HATEFI *et al.*[12] and RIESKE *et al.*[13] (see Table I, Expt. 1). The lack of requirement for Q is due to its presence in the preparations of cytochromes *b* and $c_1$. When cytochrome *b* was isolated from a pentane-extracted[5] succinate–cytochrome *c* reductase preparation, stimulation by both Q-10 and phospholipid was observed (Table I, Expts. 2 and 3). The residual activity in the absence of added Q or phospholipid is probably due to the presence of these components in the preparations of cytochrome $c_1$ or succinate dehydrogenase.

There is an absolute requirement for succinate dehydrogenase (Table II). The enzyme prepared by the method of ZEYLEMAKER[15] was inactive in reconstitution. It was essential to have succinate present during the preparation of the dehydrogenase, even during the last fractionation with ammonium sulphate (*cf.* ref. 4).

There was some activity in the absence of cytochrome $c_1$. Cytochrome $c_1$

TABLE I

REQUIREMENT FOR Q AND PHOSPHOLIPID FOR RECONSTITUTION OF SUCCINATE–CYTOCHROME *c* REDUCTASE

*Complete system. Expt. 1.* 5 nmoles cytochrome *b* (15% native and containing 1 nmole cytochrome $c_1$), 3 nmoles cytochrome $c_1$ (prepared by method of YAMASHITA AND RACKER[7]), 0.9 mg succinate dehydrogenase, 300 μg phospholipid and 30 nmoles Q-10. *Expt. 2.* 7.2 nmoles cytochrome *b* (containing 1.6 nmoles cytochrome $c_1$) prepared from pentane-extracted succinate–cytochrome *c* reductase, 5 nmoles cytochrome $c_1$ (prepared by method of YAMASHITA AND RACKER[7]), 1 mg succinate dehydrogenase, 300 μg phospholipid and 40 nmoles Q-10. *Expt. 3.* 9 nmoles cytochrome *b* (containing 2 nmoles cytochrome $c_1$) prepared from pentane-extracted succinate–cytochrome *c* reductase, 5 nmoles cytochrome $c_1$ (prepared from Complex III without mersalyl treatment), 1.2 mg succinate dehydrogenase, 400 μg phospholipid and 50 nmoles Q-10. In all cases the mixture was preincubated for 3 h at 37° in 0.5 ml 20 mM succinate –30 mM glycylglycine–HCl buffer (pH 8.4) – 1 mM EDTA.

| *Expt.* | *System* | *Succinate–cytochrome c reductase activity (μmole cytochrome c per min per mg protein)* |
|---|---|---|
| 1 | Complete | 0.40 |
| | *minus* Q | 0.30 |
| | *minus* phospholipid | 0.43 |
| | *minus* phospholipid and Q | 0.34 |
| 2 | Complete | 0.7 |
| | *minus* Q | 0.37 |
| | *minus* phospholipid | 0.35 |
| 3 | Complete | 0.37 |
| | *minus* phospholipid | 0.25 |
| | *minus* phospholipid and Q | 0.10 |

freed from 'core protein'[14] was inactive in the reconstitution, even though it reacted with cytochrome *c* and was reduced by succinate in the presence of succinate–cytochrome *c* reductase. Attempts to restore the reconstitution activity by pre-incubating the purified cytochrome $c_1$ with 'core protein' either at pH 10 (Table II) or at pH 8 (*cf.* ref. 23) were unsuccessful. The addition of 'core protein' to the complete system had no effect.

There is an almost absolute requirement for cytochrome *b* (Table II). The difference spectrum, reduced with $Na_2S_2O_4$ *minus* oxidized, of a typical cytochrome *b* preparation shows peaks at 560 and 530 nm (Fig. 1, Curve 1). The spectrum, isolated *minus* oxidized with ferricyanide (Curve 2), shows a residual peak of ferrocytochrome $c_1$ at 553 nm. Apparently, cytochrome $c_1$, but not cytochrome *b*, is present in the reduced form in the preparation as isolated. Much of the cytochrome *b* is denatured, judging by the decline of the peak on treating the reduced preparation with CO (*cf.* ref. 24). By this criterion, a second preparation, whose spectra are shown in Fig. 2 and which was completely inactive in reconstitution, is completely denatured. The proportion of native cytochrome *b* in the preparation was taken to be equal to $\Delta A_{560-575\ nm}$ (reduced + CO *minus* preparation as isolated): $\Delta A_{560-575\ nm}$ (reduced *minus* preparation as isolated). According to this criterion, the cytochrome *b* in the preparation shown in Fig. 1 is 25 % active and 75 % denatured.

TABLE II

REQUIREMENT FOR SUCCINATE DEHYDROGENASE, CYTOCHROME $c_1$ AND CYTOCHROME *b* FOR RECONSTITUTION OF SUCCINATE–CYTOCHROME *c* REDUCTASE

The complete system contained 3 nmoles cytochrome *b* (18% native and containing 0.8 nmole cytochrome $c_1$), 1.8 nmoles cytochrome $c_1$ (ref. 7), 0.45 mg succinate dehydrogenase, 380 μg phospholipid and 40 nmoles Q-10, preincubated as in Table I.

| *Omissions* | *Additions* | *Activity (μmole cytochrome c per min per mg protein)* |
|---|---|---|
| None | None | 0.54 |
| Succinate dehydrogenase | None | 0 |
| Cytochrome $c_1$ | None | 0.06 |
| | Core protein * (300 μg) | 0.02 |
| | Purified cytochrome $c_1$ ** (1.5 nmoles) | 0.06 |
| | Core protein (300 μg) *plus* purified cytochrome $c_1$ ** (1.5 nmoles) *** | 0.02 |
| Cytochrome *b* | None | 0 |
| None | CO | 0.53 |

* Prepared by method of SILMAN *et al.*[14].

** Prepared as described in METHODS.

*** The cytochrome $c_1$ was added to a solution of core protein at pH 10.5. After 30 min at room temperature, the pH was brought to 8 and the suspension used in the reconstitution mixture.

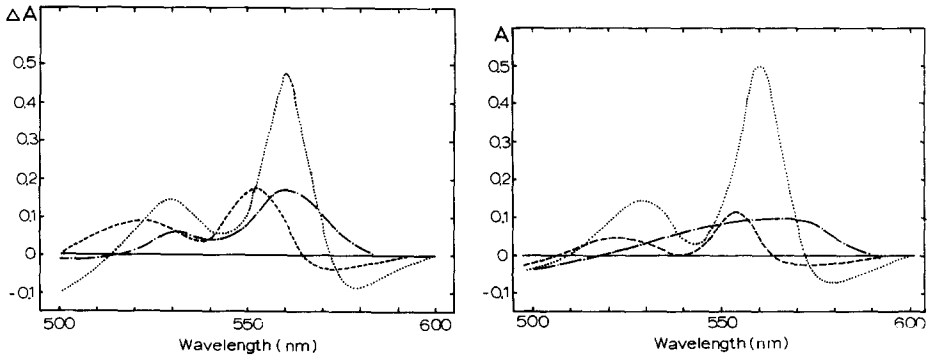



Fig. 1. Difference absorption spectra of a cytochrome *b* preparation active in reconstitution. ———, preparation as isolated *minus* preparation as isolated; - - - -, preparation as isolated *minus* oxidized with $K_3Fe(CN)_6$; . . . ., reduced with $Na_2S_2O_4$ *minus* preparation as isolated; —.—, reduced with $Na_2S_2O_4$ and treated with CO *minus* preparation as isolated. The spectra are adjusted to zero at 600 nm.

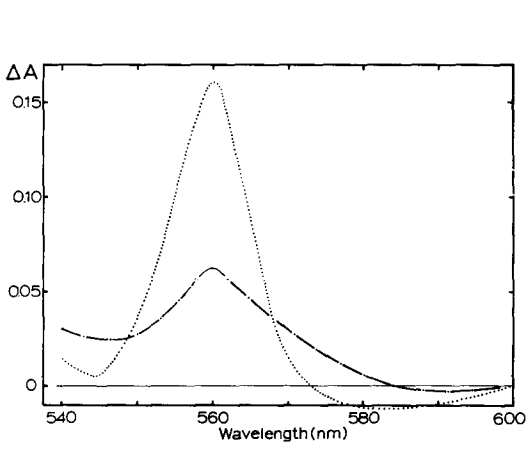
Fig. 2. Difference absorption spectra of a cytochrome *b* preparation inactive in reconstitution. See legend to Fig. 1.

Fig. 3 shows the effect of CO on the *b* spectrum in a reconstituted preparation, using the same preparation of cytochrome *b* as in Fig. 1. Cytochrome $c_1$ was first reduced with ascorbate and then the cytochrome *b* was reduced with $Na_2S_2O_4$. The $\Delta A_{560-575\ nm}$ ($Na_2S_2O_4$ *minus* ascorbate) in the presence of CO was 27% that in its absence. Thus the proportion of denatured to native cytochrome *b* remains the same

after reconstitution of the succcinate–cytochrome *c* reductase. CO has no effect on the enzymic activity of the reconstituted preparation (Table II).

*Stoicheiometry of cytochromes b and $c_1$ in reconstituted preparations*

In preparations of the intact respiratory chain[25] and Complex III[19], isolated from beef heart a stoicheiometry of $2b:1c_1$ is found. The stoicheiometry of the reconstitution was investigated by varying the amount of cytochrome $c_1$ with fixed amounts of cytochrome *b*. Two types of results were obtained, depending upon both the preparation of cytochrome *b* used and that of cytochrome $c_1$.



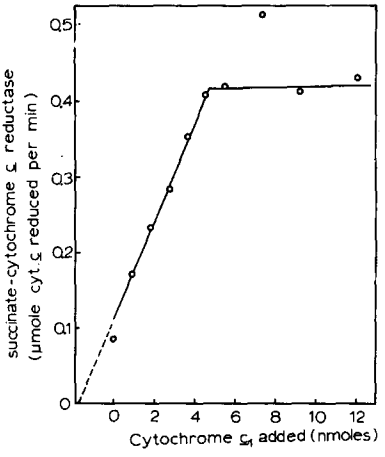




Fig. 3. Effect of CO on cytochrome *b* in reconstituted succinate–cytochrome *c* reductase, obtained by incubating for 4 h at 37° 7.5 nmoles cytochrome *b* (25% native (see Fig. 1), and containing 1.5 nmoles cytochrome $c_1$), 1.2 mg succinate dehydrogenase, 250 μg phospholipid, 30 nmoles Q-10, and 5 nmoles cytochrome $c_1$ (prepared by the method of YAMASHITA AND RACKER[7]) in 0.5 ml 30 mM glycylglycine–HCl buffer (pH 8.4), containing 20 mM succinate and EDTA. . . . . , reduced with $Na_2S_2O_4$ *minus* reduced with 5.6 mM ascorbate (pH 7.4); —.—, reduced with $Na_2S_2O_4$ and treated with CO *minus* reduced with ascorbate. The spectra are adjusted to zero at 600 nm.

Fig. 4. Reconstitution of succinate–cytochrome *c* reductase by incubating for 3.5 h at 37° 12 nmoles cytochrome *b* (5% native, and containing 3.2 nmoles cytochrome $c_1$), 1.2 mg succinate dehydrogenase, 300 μg phospholipid, 30 nmoles Q-10 and varying amounts of cytochrome $c_1$ (prepared from Complex III without mersalyl treatment) in 0.5 ml 30 mM glycylglycine–HCl buffer (pH 8.4), containing 20 mM succinate and 1 mM EDTA. The activity was measured on a sample of the incubation mixture and calculated for the total mixture.

One type of result is shown in Fig. 4. In this experiment, varying amounts of cytochrome $c_1$ were incubated for 4 h at 37° with 12 nmoles cytochrome *b* (5% native) and excess succinate dehydrogenase. A straight-line titration was found, with a sharp cut-off at 4.75 nmoles of added cytochrome $c_1$. Extrapolation of the straight-line to zero activity shows that the preparation of cytochrome *b* had an activity corresponding to 1.8 nmoles of added cytochrome $c_1$ (*cf.* 3.2 nmoles according to spectrophotometric analysis), so that the stoicheiometry is $12b:6.55c_1$, or $2b:1.1c_1$. Thus, the stoicheiometry characteristic of the intact system is obtained, even though 95% of the cytochrome *b* is denatured. The cytochrome $c_1$ present in the cytochrome *b* preparation was less active (by a factor of 1.8/3.2) than that added, and the straight

line shows that the endogenous cytochrome $c_1$ is not activated by added cytochrome $c_1$.

The second type of result is illustrated in Fig. 5, which shows an experiment carried out with the same cytochrome $b$ preparation as in Fig. 4 but with a different preparation of cytochrome $c_1$. The results differ from those given in Fig. 4 in three respects. First, there is an immediate restoration of activity, without pre-incubation, and pre-incubation for longer than 45 min causes inactivation. Secondly, higher concentrations of cytochrome $c_1$ inhibit. Thirdly, a straight-line titration is not obtained. Maximal activity is found a ratio of $2b$:1.07$c_1$, assuming that all the endogenous cytochrome $c_1$ is active. From the shape of the curves, it is clear that small amounts of added cytochrome $c_1$ have an effect much greater than to be expected from a stoicheiometric binding, suggesting that the endogenous cytochrome $c_1$ is activated by that added.

Fig. 6 shows the effect of varying the amount of cytochrome $b$ with a fixed amount of added cytochrome $c_1$. The dotted line shows the titration curve to be expected from a stoicheiometric binding of two molecules of cytochrome $b$ with one molecule of cytochrome $c_1$, ignoring the cytochrome $c_1$ present in the preparation of cytochrome $b$.

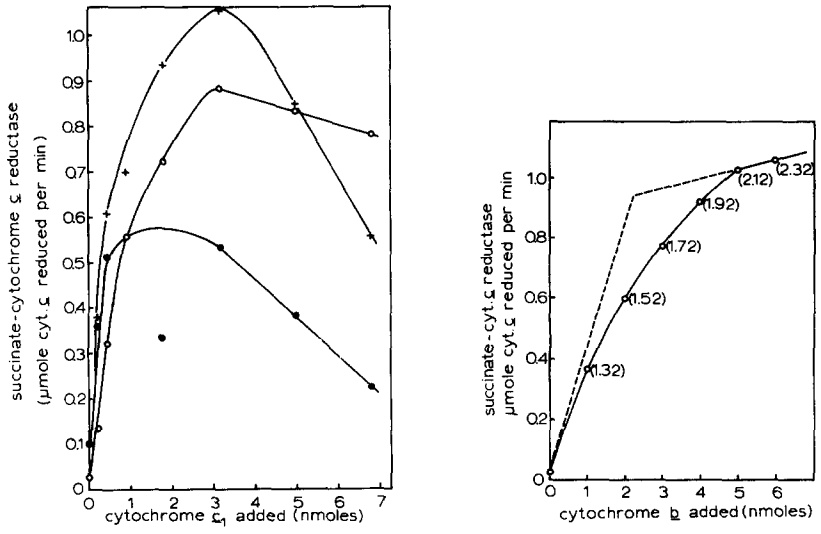



Fig. 5. Reconstitution of succinate–cytochrome $c$ reductase by incubating at 37° for different periods of time 12 nmoles cytochrome $b$ (the same preparation as in Fig. 4), 1.2 mg succinate dehydrogenase, 300 $\mu$g phospholipid, 30 nmoles Q-10 and varying amounts of cytochrome $c_1$ (prepared by the method of YAMASHITA AND RACKER[7]) in 0.5 ml 30 mM glycylglycine–HCl buffer (pH 8.4) containing 20 mM succinate and 1 mM EDTA. The activity was measured on a sample of the incubation mixture and calculated for the total mixture. ○—○, sample taken immediately after mixing; + — +, pre-incubation for 45 min; ●—●, preincubation for 5 h.

Fig. 6. Reconstitution of succinate–cytochrome $c$ reductase by incubating for 4 h at 37° 0.8 mg succinate dehydrogenase, 300 $\mu$g phospholipid, 40 nmoles Q-10, 1.12 nmoles cytochrome $c_1$ (ref. 7) and varying amounts of cytochrome $b$ (15% native) in 0.5 ml 30 mM glycylglycine–HCl buffer (pH 8.4), containing 20 mM succinate and 1 mM EDTA. The activity was measured on a sample of the incubation mixture and calculated for the total mixture. The values in brackets are the total amounts of cytochrome $c_1$ present, taking into account the amount present in the cytochrome $b$ preparation. The dotted line shows the titration curve to be expected from a stoicheiometric binding of two molecules of cytochrome $b$ with one molecule of cytochrome $c_1$, ignoring the cytochrome $c_1$ present in the cytochrome $b$ preparation.

*Effect of antimycin*

Inhibition by antimycin of mitochondria or sub-mitochondrial particles is described by a sigmoidal antimycin effect curve[26,10]. Complex III[27] or cholate-treated sub-mitochondrial particles[10], on the other hand, show a linear titration curve. In this respect, the reconstituted system behaves like a particulate preparation (Fig. 7).

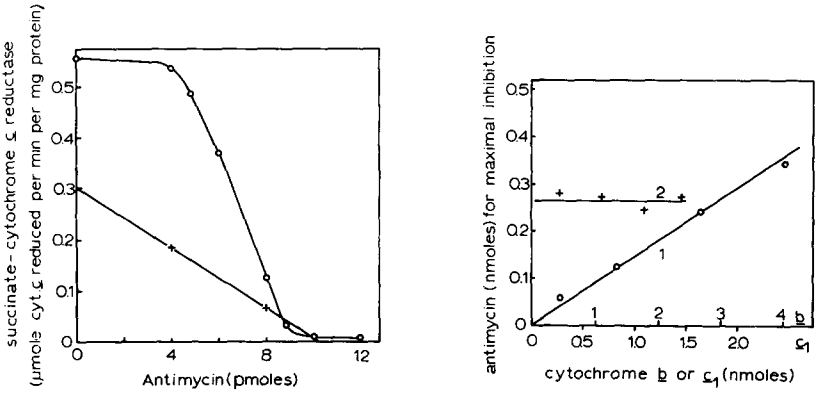



Fig. 7. Inhibition by antimycin of reconstituted succinate–cytochrome *c* reductase, in presence and absence of cholate. Succinate–cytochrome *c* reductase reconstituted as in Fig. 5, with 2.7 nmoles cytochrome *b* (15% native, and containing 0.5 nmole cytochrome $c_1$), 1.12 nmoles cytochrome $c_1$, 0.8 mg succinate dehydrogenase, 300 μg phospholipid and 40 nmoles Q-10 (all preparations are the same as used in Fig. 5). The succinate–cytochrome *c* reductase activity was measured on a sample of the incubation mixture containing 108 pmoles cytochrome *b*. The assay mixture was allowed to stand for 5 min after adding antimycin (or ethanol in control) before the reaction was started by adding the cytochrome *c*. O—O, without cholate; + — +, cholate to 5% (18.5 mg/mg protein) added to incubation mixture before sample was taken for measurement of activity.

Fig. 8. Amount of antimycin required for inhibition of reconstituted succinate–cytochrome *c* reductase as a function of amount of cytochrome *b* or $c_1$. Reconstitution carried out by incubating for 4 h at 37° 0.8 mg succinate dehydrogenase, 300 μg phospholipid, 40 nmoles Q-10 and either (Curve 1) 1.12 nmoles cytochrome $c_1$ (ref. 7) and varying amounts of cytochrome *b*, or (Curve 2) 3.0 nmoles cytochrome *b* (15% native and containing 0.6 nmole cytochrome $c_1$) and varying amounts of cytochrome $c_1$. The minimum amount of antimycin required for maximal inhibition was determined on a sample of the incubation mixture and calculated for the total mixture.

Fig. 8 shows that the amount of antimycin necessary for maximal inhibition is a function of the cytochrome *b* concentration and is independent of the cytochrome $c_1$ concentration. Thus, in agreement with YAMASHITA AND RACKER[7], the antimycin-binding site is in the cytochrome *b* preparation and not in the cytochrome $c_1$. The stoicheiometry is 0.086 mole antimycin per mole total cytochrome *b*. According to the effect of CO, this preparation contained 85% denatured and 15% native cytochrome *b*. In agreement with this calculation, 14% of the cytochrome *b* was reducible by succinate. Thus, the amount of antimycin necessary for maximal inhibition is 0.086/0.15 = 0.57 mole per mole native cytochrome *b*. This strongly suggests that antimycin binds only to native cytochrome *b*.

This conclusion is supported by measurements of the binding of antimycin by a fluorimetric procedure. SLATER AND LEE[28] found that the fluorescence of antimycin bound to sub-mitochondrial particles is quenched, and that antimycin added in

excess of that necessary for maximal inhibition fluoresces with normal intensity*. Fig. 9 shows this method applied to intact succinate–cytochrome *c* reductase. On adding increasing amounts of antimycin there was no increase in fluorescence until 0.56 mole antimycin per mole cytochrome *b* was added. The quenching was less in the presence of $Na_2S_2O_4$, but the same intersection point was obtained. This is understandable if the quenching is due to energy transfer from anticycin to the haem of cytochrome *b*, since the overlap of the emission band of antimycin (at 420 nm)

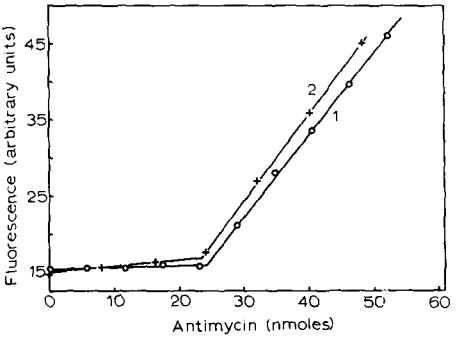



Fig. 9. Quenching of fluorescence of antimycin by binding to succinate–cytochrome *c* reductase. The fluorescence of a solution of 10 mg Complex II + III (containing 26 nmoles cytochrome $c_1$ and 43 nmoles cytochrome *b*) in 2 ml 0.05 M Tris–HCl, 0.66 M sucrose, 1 mM histidine–HCl (pH 8.0) was measured, in the presence of the indicated amounts of antimycin, in absence (Curve 1) or presence (Curve 2 )of $Na_2S_2O_4$.

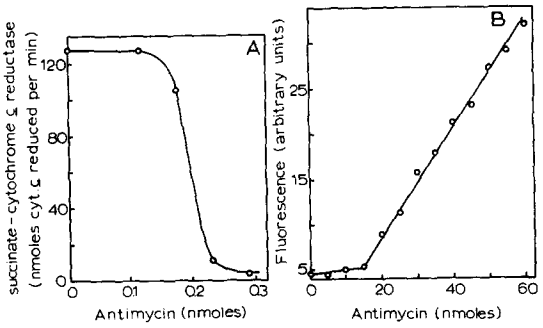



Fig. 10. Inhibition by antimycin of enzyme activity in reconstituted succinate–cytochrome *c* reductase (A) and quenching of antimycin fluorescence by cytochrome *b* preparation used in reconstitution (B). A. Reconstitution carried out by incubation for 4 h at 37° of 1.0 mg succinate dehydrogenase, 150 μg phospholipid, 15 nmoles Q-10, 7.1 nmoles cytochrome *b* (28% native, and containing 1.7 nmoles cytochrome $c_1$) and 5 nmoles cytochrome $c_1$ (ref. 7) in 0.5 ml 30 mM glycylglycine–HCl buffer (pH 8.4), containing 20 mM succinate and 1 mM EDTA. Samples containing 1.42 nmoles cytochrome *b* were removed for testing succinate–cytochrome *c* reductase activity in the presence of the indicated amounts of antimycin. B. The fluorescence of a suspension in 2 ml of 0.05 M Tris–HCl, 0.66 M sucrose, 1 mM histidine–HCl (pH 8.0) of 83 nmoles of the cytochrome *b* preparation used for reconstitution was measured in the presence of the indicated amounts of antimycin.

* This *quenching* of the fluororescence of antimycin by binding to submitochondrial particles is a different phenomenon from the fluorescence *enhancement* that occurs on binding to serum albumin[29,30]. The quenching of the fluorescence of the albumin–antimycin complex on the addition of a protein fraction isolated from chick-liver mitochondria, observed by REPORTER[29], can be ascribed to preferable binding of antimycin to this protein fraction, and the fact that the fluorescence enhancement of antimycin is less when bound to this protein than when bound to albumin.

with the Soret band of ferricytochrome *b* (also at 420 nm) is greater than the overlap of ferrocytochrome *b* (at 429 nm). Since the peak of ferrocytochrome $c_1$ is the same as that of the emission spectrum of antimycin and that of ferricytochrome $c_1$ (at 410 nm) is removed from this peak, it is clear that energy transfer from antimycin to the haem of cytochrome $c_1$ is not involved in the quenching.

Fig. 10 shows antimycin titrations of a reconstituted preparation made both by measuring the enzyme activity and the fluorescence. The cytochrome *b* used was 72 % denatured according to the CO test. The amount of antimycin necessary for maximal inhibition was 0.62 mole per mole native cytochrome *b* (Fig. 10A), and the break in the fluorescence curve was obtained at 0.61 mole antimycin per mole native cytochrome *b* (Fig. 10B). In an experiment not shown, the amount of antimycin required for a maximal 'red shift'[31] in the absorption band of ferrocytochrome *b* in the reconstituted preparation was 0.57 mole antimycin per mole of native cytochrome *b*. However, in contrast to what is found with particulate preparations[10], the antimycin-effect curve of the red shift was not detectably sigmoidal.

According to the fluorescence method a preparation of cytochrome *b* that by the CO test was nearly completely denatured and was inactive in reconstitution bound less than one antimycin molecule per 60 molecules of cytochrome *b* (Fig. 11).

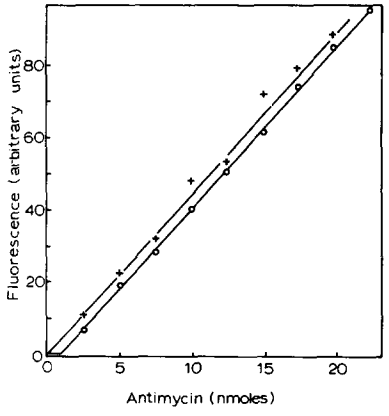



Fig. 11. Absence of quenching of fluorescence of antimycin by denatured cytochrome *b*. The cytochrome *b* preparation was almost completely denatured and was inactive in reconstitution. 60 nmoles cytochrome *b* suspended in 2 ml of 0.05 M Tris–HCl, 0.66 M sucrose, 1 mM histidine–HCl (pH 8.0) and fluorescence measured in the presence of the indicated amount of antimycin.

## DISCUSSION

It is clear from the experiments described that native cytochrome *b* is necessary for reconstitution of the succinate–cytochrome *c* reductase activity. Our preparations of cytochrome *b* made by YAMASHITA AND RACKER's[7] method are 72–95 % denatured, and it is the residual 28–5 % of native cytochrome *b* that is active in the reconstitution. The native cytochrome *b* is reducible by succinate, reacts with antimycin in the stoicheiometry 2*b*:1 antimycin, and does not react with CO. The denatured cytochrome *b* in the reconstituted preparation, on the other hand, is not reducible by succinate, does not bind antimycin and reacts with CO when reduced by $Na_2S_2O_4$. The stoicheiometry of $1c_1$:2 total *b* found in the reconstitution suggests that denatured

cytochrome *b* has the same affinity for cytochrome $c_1$ as the native cytochrome, under the conditions of these experiments.

It is also of considerably interest that the site of action of antimycin is definitely associated with the cytochrome *b* (*cf.* ref. 7), despite the fact the stoicheiometry is close to 1 antimycin : $1c_1$ : $2b$. However, the close association of the two cytochromes, which is clear from previous work, is again illustrated by our difficulty in isolating cytochrome *b* free from cytochrome $c_1$.

Indeed, there is no evidence that native cytochrome *b* has been resolved from cytochrome $c_1$, since the amount of cytochrome $c_1$ present in the cytochrome *b* preparations always exceeded one half the amount of *native* cytochrome *b*. YAMASHITA AND RACKER[7] reported that their preparation of cytochrome *b* required a ratio of antimycin to haem of less than 1:100 to inhibit the enzyme activity by about 30%. According to our finding that antimycin combines with native cytochrome *b* in the ratio of about 1:2, it follows that the preparation of cytochrome *b* used by YAMASHITA AND RACKER[7] contained at most about 6% native cytochrome *b*. According to our criteria (see METHODS) applied to the spectrum of cytochrome *b* published by YAMASHITA AND RACKER[7], their preparation contained an amount of cytochrome $c_1$ equal to 16% of the total cytochrome *b*.

Since only those preparations of cytochrome $c_1$ containing 'core protein' are active in reconstitution, it is possible that the stimulatory action of added cytochrome $c_1$ on the reconstitution of the enzyme activity, which increases with increasing cytochrome $c_1$ until a ratio of 2 *total* $b$:$1c_1$ is reached, represents the restoration of a damaged membrane conformation rather than a true reconstitution. In support of this possibility is the observation that the cytochrome $c_1$ reconstituted into the complex is reduced by succinate no faster than added cytochrome $c_1$.

It may be that we shall have to consider cytochromes *b* and $c_1$ as a single protein, cytochrome $bc_1$ (*cf.* cytochrome $aa_3$), containing 2 *b* sub-units, 1 $c_1$ sub-unit and one antimycin-binding site.

*Stoicheiometry of cytochromes b and $c_1$ in $bc_1$ particle and absorbance coefficients of cytochromes b and $c_1$*

On the basis of the absorbance coefficients for cytochromes *b* and $c_1$ and antimycin specified in METHODS, the stoicheiometry of the $bc_1$ particle is $b$:$c_1$:antimycin-binding sites = 1.83:1.05:1.0. However, this and earlier work (see, *e.g.* ref. 27) have made it clear (i) that antimycin combines stoicheiometrically with the cytochrome *b* moiety, and (ii) that the stoicheiometry is close to one molecule of antimycin to two molecules of cytochrome *b*. It seems highly likely, then, that the stoicheiometry is integral, *viz.* precisely 2:1:1.

If this is the case, the specific quenching of antimycin fluorescence by binding to cytochrome *b* enables a separate determination of the absorption coefficients of cytochromes *b* and $c_1$, dependent only on the extinction coefficient of antimycin. Seven determinations on preparations of succinate–cytochrome *c* reductase and Complex III gave mean values of 25.6 $\pm$ 0.1 (S.E.) $mM^{-1} \cdot cm^{-1}$ for cytochrome *b* for the wavelength pair 563–577 nm, and 20.1 $\pm$ 0.2 $mM^{-1} \cdot cm^{-1}$ for cytochrome $c_1$ for the wavelength pair 553–539 nm. The slightly lower value for cytochrome *b* than that obtained by the method of ZAUGG AND RIESKE[19] could be explained if

some of the precipitated cytochrome *b* was not extracted into the alkaline pyridine in the latter procedure.

These values for the absorption coefficients of cytochromes *b* and $c_1$ are probably more accurate than others reported in the literature. They correspond to 21.0 and about 17.1 $mM^{-1} \cdot cm^{-1}$, respectively, for the peak (at 563 and 553 nm, respectively) in the difference spectra. The latter value is identical with that reported by GREEN *et al.*[20] and used by RIESKE *et al.*[27], who also reported a precise stoicheiometry of $c_1$:antimycin-binding site of 1:1. However, in our experience, the ratio peak-trough difference to peak varied with the preparation of cytochrome $c_1$, which probably accounts for the difference between our value of 20.1 $mM^{-1} \cdot cm^{-1}$ for the absorbance coefficient of the peak-trough difference, and the value of 19.1 $mM^{-1} \cdot cm^{-1}$, which was calculated from GREEN *et al.*'s[20] value for the peak.

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