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ELECTRON TRANSFER THROUGH THE ISOLATED MITOCHONDRIAL CYTOCHROME b-c_1 COMPLEX

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(1) A kinetic analysis of electron donation into and through the cytochrome b-c_1 complex isolated from bovine heart mitochondria has been undertaken, using trimethylquinol as the donor. (2) Rate constants of two routes of redox equilibration with quinols have been defined by kinetic measurements and with the use of the inhibitors antimycin A and myxothiazol. (3) A model of electron transfer based upon the original Q-cycle formulation is presented to explain these and related results.

Introduction

The mitochondrial cytochrome b-c_1 complex and its electron transfer reactions have been investigated extensively. These studies have become of more general interest with the realisation of similarities with the bacterial photosynthetic cytochrome system [1,2] and with the chloroplast cytochrome b-f complex [3–5]. The use of specific inhibitors to define electron-transfer pathways within the complex has been particularly informative. The most notable of these inhibitors is antimycin A [6] and several recent reviews have discussed its effects on the cytochrome b-c_1 complex [7–9]. However, in recent reports there is some discrepancy in the observations obtained by different groups. In particular, there are various reports that antimycin A inhibits [10,11], inhibits a fraction of [12,13], or does not affect [14] the profile of reduction of cytochrome c_1 when a reductant is added. Further differences in the redox behaviour of the b haems on addition of a reductant are also found in the published data.

At least part of these discrepancies may arise from the different types of preparations used, ranging from the pure complex to whole mitochondria, and from the different substrates employed, usually an artificial quinol or succinate. In order to simplify the system, the data of the present report were obtained using the direct donor trimethylquinol to the purified bovine heart mitochondrial cytochrome b-c_1 complex. This quinol was used since its mechanism of reduction was direct, its donation rate could be made slow enough so that kinetics could be easily followed, and its reactions retained normal inhibitor sensitivities. The model which results from these studies is similar to the Q-cycle formulation [15] and, with the postulation of minor E_m differences between different cytochrome b-c_1 complex preparations, may accommodate a large part of the data already available in the literature.

Abbreviations: DBMIB, 2.5 dibromo-3-methyl-6-isopropyl-p-benzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; HOQNO, 2-alkyl-4-hydroxyquinoline N-oxide; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; UHDBQ, 3,6-dimethyl-2-hydroxy-5-undecyl-1,4-benzoquinone. All potentials are with reference to the standard hydrogen electrode with E_m the midpoint potential at pH 7.0 and E_h the ambient potential at the pH of the experiment.

Methods

The cytochrome b-c_1 complex was isolated from bovine heart mitochondria by the methods of Hatefi and Rieske [16,17].

Trimethylquinol had been recrystallised from the commercially available compound. Trimethylquinone was prepared from the quinol by oxidation with aqueous ferric chloride solution of a sample of quinol in diethyl ether. The resulting ethereal quinone solution was washed with saturated NaCl, dried with anhydrous sodium sulphate and evaporated to dryness with a vacuum rotary evaporator. Extinction coefficients used were: quinol at 290 nm, $\epsilon = 2.75\ mM^{-1} \cdot cm^{-1}$; quinone at 256 nm, $\epsilon = 17.3\ mM^{-1} \cdot cm^{-1}$.

Myxothiazol was the kind gift of Dr. W. Trowitzch and DBMIB and DNP-INT of Professor A. Trebst. UHDBQ was purchased from Aldrich Chemical Co.

All assays were performed with a buffer of 50 mM potassium phosphate, 2 mM potassium EDTA and 1 mM KCN at pH 7.0 and 25°C. The reaction was started by addition of trimethylquinol. For the multiple-turnover reactions, a non-enzymatic rate of quinol reduction of cytochrome c was subtracted from the observed rate to give the measured enzymatic rate of reaction. For slow reactions, spectra were taken by repetitive scanning of 580 to 540 nm with a Cary 219 instrument. Faster kinetic traces and their analyses were performed with a single-beam Applied Photophysics instrument linked via a Datalab transient recorder to an Apple microcomputer.

Results

The kinetic equation of the enzyme

Experiments were performed with the assay of trimethylquinol reduction of cytochrome c. Within the concentrations of reagents used (1 mM trimethylquinol and 20 μM ferricytochrome c), titrations of the cytochrome b-c_1 complex-catalysed enzymatic rate with [quinol] or with [cytochrome c] produced plots consistent with two second-order reactions. The highest concentrations of reagents tested were 4 mM trimethylquinol and 55 μM ferricytochrome c. Although the reactions were not fully second order at these rather non-ideal solution concentrations, the cytochrome b-c_1 complex was still not saturated and had a turnover number of 50 s^{-1}.The reactions are:

$$QH_2 + [\text{cytochrome } b\text{-}c_{1ox}] \xrightarrow{k_{in}} Q + [\text{cytochrome } b\text{-}c_{1red}]$$

$$[\text{cytochrome } b\text{-}c_{1red}] + \text{cytochrome } c^{3+} \xrightarrow{k_{out}}$$

$$[\text{cytochrome } b\text{-}c_{1ox}] + \text{cytochrome } c^{2+}$$

Then, in the steady state the rate of reduction of the complex is equal to its rate of reoxidation so that:

$$k_{in}[QH_2][\text{cytochrome } b\text{-}c_{1ox}] = k_{out}[\text{cytochrome } c^{3+}][\text{cytochrome } b\text{-}c_{1red}]$$

Hence, if we have [cytochrome b-c_{1ox}] + [cytochrome b-c_{1red}] = [cytochrome b-c_{1total}], then by substituting and rearranging we have:

$$\text{Rate} = [\text{cytochrome } b\text{-}c_{1total}] \times \frac{k_{in}[QH_2]k_{out}[\text{cytochrome } c^{3+}]}{k_{in}[QH_2] + k_{out}[\text{cytochrome } c^{3+}]}$$

Hence, one may measure apparent k_m values even although the reactions are both second order. For example, at low [cytochrome c^{3+}] the reaction will rapidly become rate limited by oxidation of the complex when titrating rate with quinol and will appear to become zero order with quinol, since the equation will approximate to:

$$\text{Rate} = k_{out}[\text{cytochrome } b\text{-}c_{1total}][\text{cytochrome } c^{3+}]$$

Similarly, at low [QH_2] the reaction may easily become zero order with [cytochrome c^{3+}], since the equation approximates to:

$$\text{Rate} = k_{in}[\text{cytochrome } b\text{-}c_{1total}][QH_2]$$

By measuring rates at these two extreme situations we may obtain the two multiple-turnover second-order rate constants, k_{in} and k_{out}, of $2.5 \pm 0.2 \cdot 10^4$ and $1.8 \pm 0.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$ at 25°C and pH 7.0.

It may be noted that k_{in} refers to the reaction of QH_2 with cytochrome b-c_1 complex. It appears that the reaction involves the formation of a $QH^- \ldots {}^+$cytochrome b-c_1 complex, by analogy with plastoquinol reduction of the chloroplast cytochrome b-f complex. This notion has been based upon an analysis of the pH profile of the reaction and on the relative rates of donation by a range of benzoquinol donors of known physical properties [18]. My previous rate constants have referred to the rate constants for the anionic quinol reaction. To convert the k_{in} value into the k_1 values described previously the pK_a of trimethylquinol of 10.8 [19] and the pK_x of the complex at the binding site of the quinol of 6.5 [20] must be taken into account. A k_{in} value of $2.5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ at 25°C corresponds to a k_1 value of $6.6 \cdot 10^8\ M^{-1} \cdot s^{-1}$ at 25°C where we have:

$$QH_2 \overset{pK_a}{\rightleftharpoons} QH^- + H^+$$

$$^+\text{cytochrome } b\text{-}c_{1ox} \overset{pK_x}{\rightleftharpoons} \text{cytochrome } b\text{-}c_{1ox} + H^+$$

$$QH^- + {}^+\text{cytochrome } b\text{-}c_{1ox} \overset{k_1}{\rightarrow}$$

$$QH^- \ldots {}^+\text{cytochrome } b\text{-}c_{1ox} \overset{\text{fast subsequent reactions}}{\dashrightarrow}$$

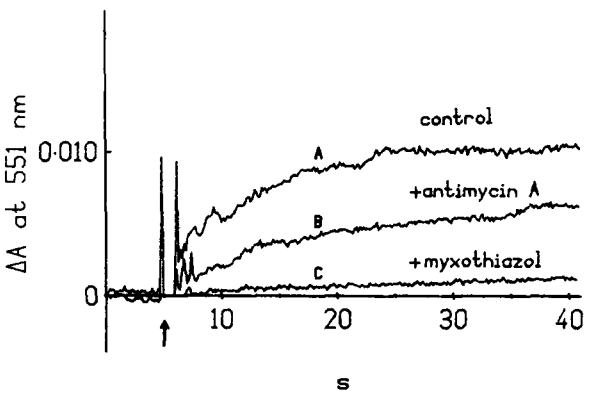

Fig. 1. The pulsed reduction of cytochrome c_1 by added trimethylquinol. The cytochrome b-c_1 complex was dissolved to approx. 0.5 μM in 50 mM potassium phosphate, 2 mM EDTA and 1 mM KCN at pH 7.0 and 25°C. Reaction was monitored at 551 nm with no inhibitors, with 2 μg/ml antimycin A or with 4 μM myxothiazol. The reaction was started by addition of 12.5 μM trimethylquinol at the point indicated by the arrow.

Reduction of components by added trimethylquinol

Fig. 1 illustrates the effects of a pulse of quinol on the reduction of cytochromes c_1. It may be seen that cytochrome c_1 reduction is approximately monophasic, as judged by a virtually linear semilogarithmic plot of the decay curve (Fig. 2A). In most experiments there was a very slight indication in the semilogarithmic plot of a small lag in this decay – such an indication may be noted from close examination of Fig. 2. A rate constant for the reaction of QH_2 + cytochrome c_1 could be extracted from this plot and gave a value of $1.3 \pm 0.2 \cdot 10^4\ M^{-1} \cdot s^{-1}$ at 25°C and pH 7.0.

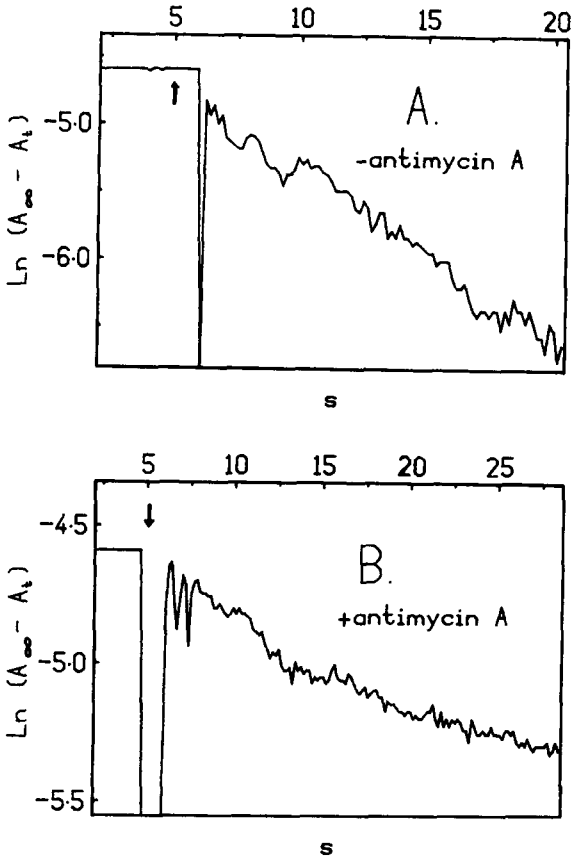

Fig. 2. Semilogarithmic plots of the pulsed reduction of cytochrome c_1 in the absence or presence of antimycin A. The data of Fig. 1A and B were converted by computer in semilogarithmic plots of ln (signal remaining) vs. time. It may be seen that the decay is reasonably monophasic in the absence of antimycin A (panel A) but is biphasic in the presence of this inhibitor. A k_{in} of $1.3 \pm 0.2 \cdot 10^4\ M^{-1} \cdot s^{-1}$ may be derived from panel A.

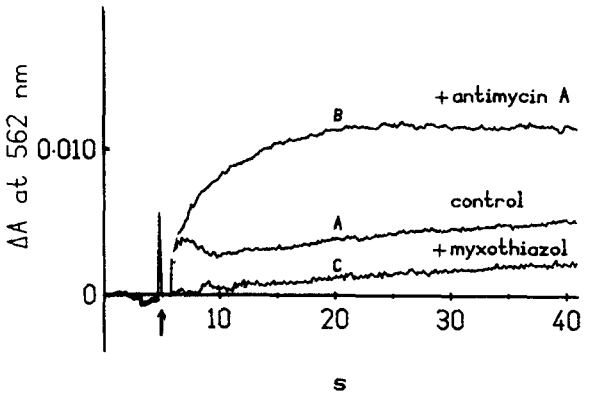

Fig. 3. The pulsed reduction of cytochrome *b*-561 by added trimethylquinol. The cytochrome *b*-c_1 complex was dissolved to around 0.5 μM in 50 mM potassium phosphate, 2 mM EDTA and 1 mM KCN at pH 7.0 and 25°C. Reaction was monitored at 562 nm with no inhibitors, with 2 μg/ml antimycin A or with 4 μM myxothiazol. The reaction was started by addition of 25 μM trimethylquinol at the point indicated by the arrow.

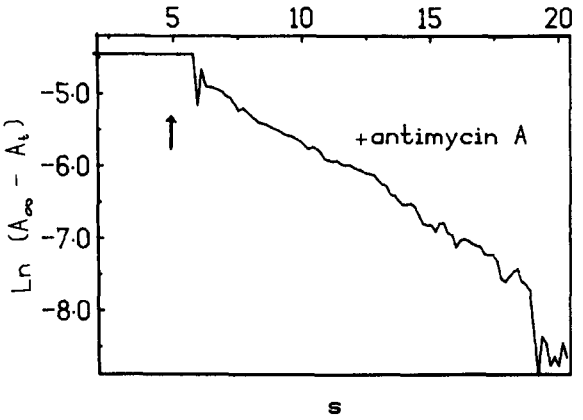

Fig. 4. Semilogarithmic plot of the pulsed reduction of cytochrome *b*-561 in the presence of antimycin A. The data of Fig. 3B were converted into a semilogarithmic plot by computer. The profile can be seen to be reasonably monophasic and a second-order rate constant of about $1.25 \pm 0.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ may be extracted from the gradient of the plot.

The reduction kinetics of cytochrome *b* were much more complex. Under the particular conditions of Fig. 3, the profile was clearly triphasic. An initial rapid reduction was followed by partial reoxiation and again followed by a slower rereduction to a redox level commensurate with the final E_h of the quinol/quinone couple of the surrounding solution. Such results are consistent with those reported by Tsou and co-workers [21,22]. Higher quinol concentrations produced a more rapid transient reduction and reoxidation of the cytochrome *b*-561 and the final redox state was accordingly more reduced because of the lower ambient E_h of the final quinol/quinone ratio. In these experiments, only cytochrome *b*-561 was observed to undergo redox changes.

The effects of antimycin A

When identical experiments were performed in the presence of 2 μg/ml antimycin A, rather different results were obtained (Fig. 1B and 3B). Cytochrome *b* reduction was now monophasic and rapid (Fig. 4). The rate constant extracted from the plot of Fig. 4 of $1.25 \pm 0.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 25°C was approximately the same as that obtained for the pulsed reduction of cytochrome c_1 in the absence of antimycin A.

Cytochrome c_1 reduction in the presence of antimycin A was more complex. A semilogarithmic plot of the decay curve produced a profile with a rapid initial phase followed by a much slower reaction to full cytochrome c_1 reduction (Fig. 2B). Extrapolation of the slow phase (Fig. 2B) indicated that 35 ± 10% of the cytochrome c_1 was rapidly reduced (with a rate constant of approx. $0.6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$, i.e., about one-half of the rate constant obtained in the absence of antimycin A). The remainder was reduced slowly with a rate constant of less than one-tenth of this value.

Comparison of the single-turnover and steady-state flux rate constants through the complex

It was of interest to compare rate constants for pulsed reduction of the complex with the multiple-turnover rate constant for quinol donation into the complex when the complex was working catalytically. This latter value was obtained by measuring the enzymatic rate of cytochrome *c* reduction in the presence of excess cytochrome *c* and at low quinol concentrations such that quinol donation into the complex was rate-limiting, as discussed in the first section of these results. The experimental value obtained for k_{in} of $2.5 \pm 0.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 25°C in 50 mM potassium phosphate, 2 mM EDTA and 1 mM KCN at pH 7.0 was approximately twice the value of $1.3 \pm 0.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$

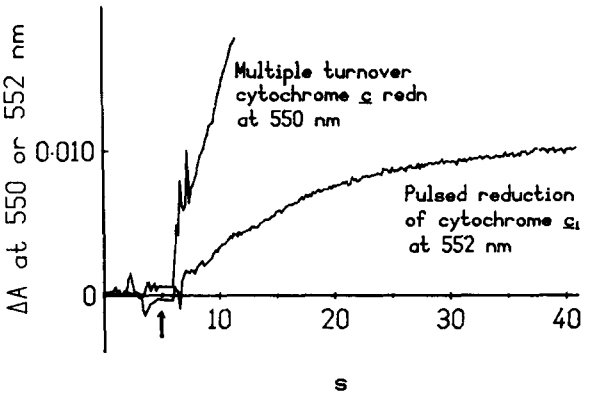

Fig. 5. Comparison of single-turnover and multiple-turnover cytochrome c_1 (c) reduction. Reaction buffer was 50 mM potassium phosphate, 2 mM EDTA and 1 mM KCN at pH 7.0 and 25°C. In both cases cytochrome b-c_1 complex was present at 0.5 μM and the reaction was started by addition of a pulse of 6.25 μM trimethylquinol at the point indicated by the arrow. The reaction was followed at 552 nm (single-turnover cytochrome c_1 reduction, no added cytochrome c) or at 550 nm (multiple-turnover flux condition, 20 μM added cytochrome c).

determined by pulsed reduction of the complex. This factor of two difference in multiple flux and single-turnover values of k_{in} is more directly illustrated in Fig. 5. Here, the initial rate of reduction of cytochrome c_1 on addition of quinol was compared with the initial rate of cytochrome c reduction catalysed by identical quinol and cytochrome b-c_1 complex concentrations. The soluble cytochrome c concentration was large enough so that quinol donation into the complex was limiting. The approximately 2-fold greater rate of multiple-turnover cytochrome c reduction when compared with the initial cytochrome c_1 reduction rate can clearly be seen.

Effects of other inhibitors

Several other compounds were tested for inhibitory effects on the complex. The most potent of these was myxothiazol which clearly inhibited electon donation by quinol into the complex (Figs. 1C and 3C). UHDBT, UHDMQ and HOQNO also slowed down the rate of donation into the complex by added quinols and also inhibited multiple-turnover flux. DNP-INT was without effect on either pulsed reduction of cytochrome c_1 or on multiple-turnover flux. DBMIB was without effect or, depending upon experimental conditions, could actually stimulate pulsed reduction of the complex, even although it actually inhibited multiple-turnover flux. A possible explanation for this paradoxical result with DBMIB has already been offered [20].

Further effects of myxothiazol and the oxidant for cytochrome b-561

The profile of reduction of cytochrome b-561 in the absence of antimycin A (Fig. 3A) may be examined in the presence of varying initial concentrations of quinone. It was found that if sufficiently high concentrations of quinone were used, then transient cytochrome b reduction/reoxidation steps could be eliminated and the approach to equilibrium was rapid. It is concluded that the liquid state quinone is therefore able to act as the reoxidant for cytochrome b if sufficient amounts are present.

It is apparent that antimycin A inhibits the site of cytochrome b-561 reoxidation, whereas myxothiazol inhibits donation into the complex. Use was made of myxothiazol to assess directly the rate constant of trimethylquinone oxidation of cytochrome b-561. In such an experiment, a large pulse of quinol was given to the myxothiazol-inhibited cytochrome b-c_1 complex and the kinetics of cytochrome b-561 reduction via the antimycin A-sensitive site were monitored. That this reaction was indeed occurring via the antimycin A-sensitive site is demonstrated in Fig. 6c where it may be seen that antimycin A slowed down this myxothiazol-insensitive reduction process enormously. Analysis of the decay curve of Fig. 6B gave a rate constant, assuming the reaction to be:

$$QH_2 + 2 \text{ cytochrome } b\text{-}561 \rightarrow$$

$$Q + 2 \text{ cytochrome } b\text{-}561^- + 2H^+$$

of $1.1 \pm 0.2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. A pulse of trimethylquinone could then be added to this reduced complex and the rate constant for trimethylquinone oxidation of cytochrome b-561 could be ascertained as approx. $5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. These are in reasonable agreement with the overall equilibrium constant for the reaction at pH 7.0 of +78 mV. This was calculated using E_m (QH_2/Q) = +109

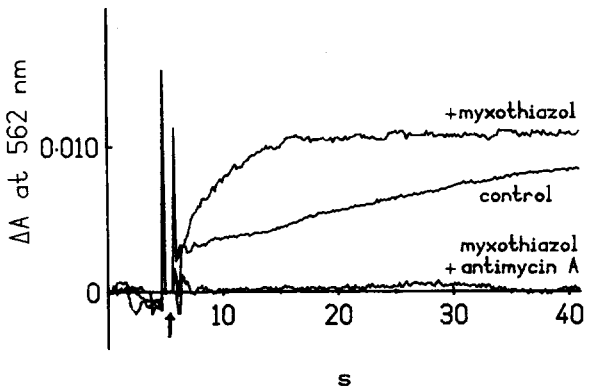

Fig. 6. The pulsed reduction of cytochrome *b*-561 with a high concentration of quinol. The cytochrome b-c_1 complex was dissolved to approx. 0.5 μM in 50 mM potassium phosphate, 2 mM EDTA and 1 mM KCN at pH 7.0 and 25°C. Reaction was monitored at 562 nm with no inhibitors, with 4 μM myxothiazol or with 4 μM myxothiazol plus 2 μg/ml antimycin A. The reaction was started by addition of 125 μM trimethylquinol at the point indicated by the arrow. On the same time scale a similar experiment performed in the presence of antimycin A alone would have caused full reduction of cytochrome *b*-561 within the mixing time of quinol addition.

mV [19] and E_m(cytochrome b-561_{red}/cytochrome b-561_{ox}) = +70 mV (measured by titration with a variety of trimethylquinol/ trimethylquinone ratios, Rich, P.R., unpublished data). The equation QH_2 + 2 cytochrome b-561_{ox} ⇋ Q + 2 cytochrome b-561_{red} + $2H^+$ was assumed. An appraisal of whether such rate constants are adequate for the observed redox behaviour of cytochrome *b*-561 is given in the Discussion.

A further experiment was performed with the myxothiazol-inhibited cytochrome b-c_1 complex to determine whether the cytochrome *b*-561 reduction was concerted with the concurrent reduction of the Rieske centre or cytochrome c_1, in a manner analogous to the reduction process at the myxothiazol-sensitive site. This was achieved by measuring the rate of cytochrome *b*-561 reduction under conditions where cytochrome c_1 and Rieske centre were either oxidised or reduced (with ascorbate plus TMPD) before the pulse of quinol was added. The rate of cytochrome *b*-561 reduction was identical in both cases and it is therefore deduced that reduction (and hence reoxidation) of cytochrome *b*-561 is not concerted with Rieske centre/cytochrome c_1 reduction (reoxidation) at this myxothiazol-insensitive site.

Discussion

The data of the present report, analysed in the light of recent results from several other groups [7–14, 21–30], have led to a working model consisting of the following points:

(1) The isolated cytochrome b-c_1 complex has two sites for rapid interaction with added quinol/quinone couples. The first of these is myxothiazol sensitive and antimycin A insensitive and may be identified with the 'centre o' described by Mitchell [15]. The rate constant for donation into the site by trimethylquinol is $1.3 \pm 0.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 25°C as measured by pulsed reduction. The second site is myxothiazol insensitive and antimycin A sensitive. Donation to this site is governed by a rate constant for trimethylquinol of $1.1 \pm 0.2 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ at 25°C. It may be equated with 'centre i' of Mitchell [15] and the equilibrium constant is such that the site would primarily be used for oxidation of the complex by quinone in the catalytically cycling complex;

(2) The Rieske centre and cytochrome c_1 are in rapid equilibrium with each other. Hence, when one electron is donated to this region it will distribute between them according to the difference in midpoint potentials of the two components. The Rieske centre E_m is about +280 mV at pH 7 [31]. It is suggested that the relative E_m values of the Rieske centre and cytochrome c_1 are somewhat variable in different cytochrome b-c_1 complex preparations so that a single electron shared between these two components will distribute differently. In the present authors preparation, cytochrome c_1 has a midpoint potential of 242 ± 5 mV (data not shown). It is because of sharing of electrons between the Rieske centre and cytochrome c_1 that the steady-state flux k_{in} is greater than the pulsed reduction k_{in} value – in the pulsed reduction experiment only one-half of the electrons which enter Rieske centre/cytochrome c_1 are observed whereas in steady-state flux to excess cytochrome *c* all electrons which pass through Rieske centre/cytochrome c_1 wiil be observed to reduce cytochrome *c*;

(3) When a quinol donates to the cytochrome

b-c_1 complex a concerted reaction occurs at centre o such that one electron is delivered to the Rieske centre/cytochrome c_1 region and one is delivered to the cytochromes b. This latter electron rapidly ends up on cytochrome b-561 because of the higher E_m of cytochrome b-561 compared to cytochrome b-566 [32] although it may pass through cytochrome b-566 transiently [33];

(4) When an electron is present in the Rieske centre cytochrome c_1 region and when an electron is already present on cytochrome b-561, a concerted reaction at centre o with another quinol may not occur. This has been suggested by Crofts [33] to be because of an unfavourable equilibrium constant which is then developed. In the intact respiratory chain, the constant removal of electrons from the Rieske centre/cytochrome c_1 region by cytochrome oxidase does allow another concerted electron transfer so that cytochrome b-566 in addition to cytochrome b-561 may be reduced.

When a quinol pulse is added to the cytochrome b-c_1 complex in the presence of antimycin A, one electron is rapidly delivered to the Rieske centre/cytochrome c_1 region and one to cytochrome b-561 and the cytochrome b-561 is observed to be reduced with a fast rate constant, since its reoxidation has been inhibited by the antimycin A. The observed behaviour of the cytochrome c_1 will depend upon its E_m relative to the Rieske centre. If the E_m values are equal, one-half of the cytochrome c_1 will be observed to be rapidly reduced. If cytochrome c_1 has a lower E_m than Rieske centre, then progressively less will be reduced in the rapid phase. Alternatively, if cytochrome c_1 has a higher potential than the Rieske centre, then more of the cytochrome c_1 will be observed to be reduced rapidly in the presence of antimycin A. This difference in relative E_m values of Rieske centre and cytochrome c_1 in different cytochrome b-c_1 complex preparations is suggested to be the cause of the variable results of different groups for cytochrome c_1 reduction in the presence of antimycin A [10–14]. Fig. 7 illustrates the relation between the midpoint potential difference between the Rieske centre and cytochrome c_1 and the amount of cytochrome c_1 which will be observed to be rapidly reduced in the presence of antimycin A. The equation is derived assuming equal concentrations of Rieske centre and cytochrome c_1, rapid equilibration between these components, and only one electron delivered rapidly into the Rieske centre/cytochrome c_1 part of each cytochrome b-c_1 complex. That is:

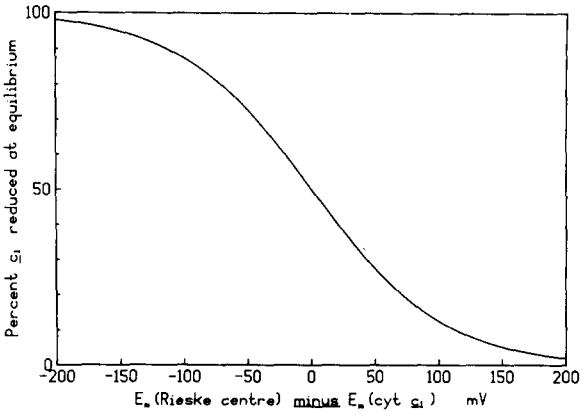

Fig. 7. Calculated relation between the E_m difference between Rieske centre and cytochrome c_1 and the percentage of cytochrome c_1 which is reduced rapidly in the presence of antimycin A. The derivation of the equation for this effect is given in the text.

$$FeS^- + FeS = \text{cytochrome } c_1^- + \text{cytochrome } c_1$$

$$FeS^- + \text{cytochrome } c_1^- = FeS + \text{cytochrome } c_1$$

$$FeS^- + \text{cytochrome } c_1 \overset{K}{\rightleftharpoons} FeS + \text{cytochrome } c_1^-$$

At equilibrium:

$$E_h = E_m(\text{Rieske}) + \frac{2.303RT}{nF}\log_{10}\frac{[FeS]}{[FeS^-]}$$

$$= E_m(\text{cytochrome } c_1) + \frac{2.303RT}{nF}\log_{10}\frac{[\text{cytochrome } c_1]}{[\text{cytochrome } c_1^-]}$$

$$K = \frac{[FeS][\text{cytochrome } c_1^-]}{[FeS^-][\text{cytochrome } c_1]} = \left(\frac{[\text{cytochrome } c_1^-]}{[\text{cytochrome } c_1]}\right)^2$$

Then:

$$\Delta E_m = E_m(\text{Rieske}) - E_m(\text{cytochrome } c_1)$$

$$= \frac{2.303RT}{nF}\log_{10}(1/K) = 59.18\log_{10}(1/K) \text{ at } 25°C.$$

Normalising so that cytochrome $c_1 + c_1^- =$

100%, we have the percentage of cytochrome c_1 rapidly reduced in the presence of antimycin A,

$$\text{cytochrome } c_1^- = \frac{100}{\sqrt{\text{antilog } \Delta E_m/59.18} + 1}$$

A fairly constant value for E_m(Rieske) of 280 mV has been obtained by previous workers [31] and E_m(cytochrome c_1) in this system is 242 ± 5 mV by equilibrium redox titration so that ΔE_m is about 38 mV. Fig. 7 gives an expected rapid phase of cytochrome c_1 reduction of approx. 32%, well within the experimentally observed value of 35 ± 10%.

The oxidation of cytochrome b-561

Most workers are agreed that the site of action of antimycin A is on the oxidation of cytochrome *b*-561. The above explanation of the observed variable effects of antimycin A on substrate-pulsed cytochrome c_1 reduction makes this suggestion generally applicable. A point of particular interest lies in the identification of the antimycin A-sensitive oxidant for cytochrome *b*-561 in the absence of antimycin A. In the present author's model experimental system, solution quinone can fulfil this role at sufficiently high concentrations. However, the observed triphasic kinetics of cytochrome *b*-561 reduction rule out the possibility that this solution quinone is the rapid oxidant in the second phase of oxidation of cytochrome *b*-561 (Fig. 3A). This is for the following reason: the cytochrome *b*-561 is transiently reoxidised to an extent beyond that expected from the ambient final E_h of the soluble quinol/quinone couple, since the final redox level of cytochrome *b*-561 becomes more reduced as it slowly equilibrates with the solution E_h in the third phase of its redox reaction profile. It is therefore not feasible for the cytochrome *b*-561 to be so rapidly reoxidised by soluble quinone in this second phase because we would set up a possible perpetual motion machine for cytochrome *b*-561 interaction with the soluble quinol/quinone couple.

A number of possible explanations for the phenomenon of rapid antimycin A-sensitive cytochrome *b*-561 reoxidation after reduction with a pulse of quinol may be mentioned:

(A) Transient conformational changes which alter midpoint potentials of components are occurring;

(B) The direct oxidant for cytochrome *b*-561 is the Rieske centre/cytochrome c_1 pair. This seems unlikely on the basis of the evidence that a quinone species of some form is involved in the oxidation of cytochrome *b*-561 [25,26];

(C) The cytochrome *b*-561 is reoxidised by a bound quinone [34]. This explanation remains a possibility since the cytochrome *b*-c_1 complex preparation generally retains about one ubiquinone per complex during purification. Whether this retained quinone is active at the site of cytochrome *b* reoxidation remains to be established;

(D) The cytochrome *b*-561 is reoxidised by the quinone species produced in the initial concerted reaction of quinol donation into the complex at centre o. The quinone produced would be postulated to be in the vicinity of the cytochrome *b*-561 for long enough to act as a high quinone concentration temporarily out of equilibrium with the soluble quinol/quinone couple. This interaction with the cytochrome *b*-561 would be antimycin A sensitive and equivalent to centre i of Mitchell [15].

A direct indication that the oxidant for cytochrome *b*-561 is actually produced at the myxothiazol-sensitive centre o may be deduced by consideration of Fig. 6. It may be seen that in the presence of myxothiazol, the cytochrome *b*-561 is actually fully reduced by quinol faster than in the absence of this inhibitor. Such would be the case if in the uninhibited reaction the cytochrome *b*-561 were undergoing an extremely rapid reduction followed by rapid reoxidation by the quinone product of the concerted reaction. This localised quinone (in its semiquinone form after reduction by cytochrome *b*-561) would then hinder reduction by added quinol at the myxothiazol-insensitive centre i.

A semiquinone associated with cytochrome *b*-561 would hence be formed when the electron transfer from cytochrome *b*-561 to quinone occurred. The semiquinone could then either re-equilibrate with the quinone pool (a Q-cycle formulation [15]), reduced the Rieske centre/cytochrome c_1 pair (a linear branched scheme [35,36]) or remain associated with the cytochrome *b*-561 until a second electron entered the cytochromes *b* via another concerted reaction with a second quinol at centre o (a Q-cycle formulation with a kinetically

stable semiquinone). These possibilities are illustrated in Fig. 8.

The schemes illustrated have incorporated into them the locations of the protons which would be translocated in the intact system. It may be noted that the two Q-cycle schemes produce the required reaction of:

$$QH_2 + 2H^+_{in} + 2(\text{oxidised acceptor}) \rightarrow$$

$$Q + 4H^+_{out} + 2(\text{reduced acceptor})$$

whereas the linear branched scheme would have to incorporate an additional proton-pumping mechanism of some sort. The desired proton stoicheiometry in this scheme could be achieved in the operating system by the quinone species which links cytochrome *b* reoxidation and Rieske centre/cytochrome c_1 reduction actually cycling only between the semiquinone, $Q^{\cdot-}$, and quinol, QH_2, forms.

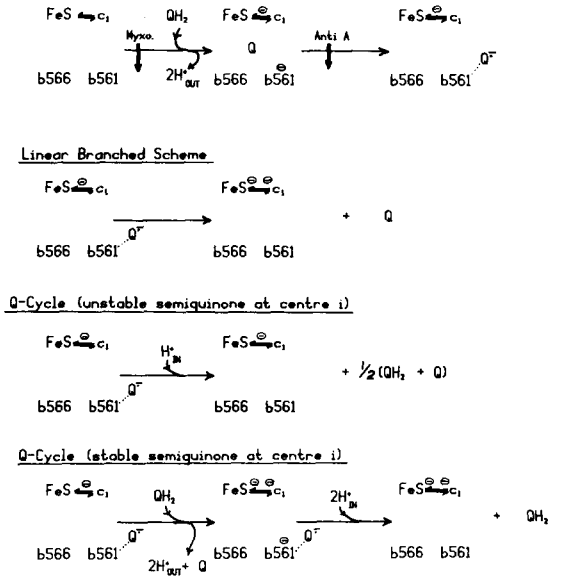

Fig. 8. Electron flux pathways through the isolated cytochrome *b*-c_1 complex. The top reactions indicate the initial reactions suggested to occur in the isolated complex. These involve a myxothiazol-sensitive concerted reduction of the Rieske centre/cytochrome c_1 region and of the cytochrome *b*, followed by an antimycin A-sensitive reoxidation of the cytochrome *b* by the quinone formed in the initial reaction or by the quinone co-purified with the complex. The subsequent three schemes illustrate the possible fate of the complex produced by these initial reaction.

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