Cytochrome *c*-mediated electron transfer between ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase

Kinetic evidence for a mobile cytochrome *c* pool

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Ubiquinol oxidase has been reconstituted from ubiquinol-cytochrome *c* reductase (Complex III), cytochrome *c* and cytochrome *c* oxidase (Complex IV). The steady-state level of reduction of cytochrome *c* by ubiquinol-2 varies with the molar ratios of the complexes and with the presence of antimycin in a way that can be quantitatively accounted for by a model in which cytochrome *c* acts as a freely diffusible pool on the membrane. This model was based on that of Kröger & Klingenberg [(1973) *Eur. J. Biochem.* **34**, 358–368] for ubiquinone-pool behaviour. Further confirmation of the pool model was provided by analysis of ubiquinol oxidase activity as a function of the molar ratio of the complexes and prediction of the degree of inhibition by antimycin.

The mechanism by which cytochrome *c* transfers electrons from ubiquinol-cytochrome *c* reductase (ubiquinol: ferricytochrome *c* oxidoreductase, EC 1.10.2.2) to cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) has received considerable attention in recent years. In principle, cytochrome *c* could mediate electron transfer as part of a rigid ternary complex of the three proteins, whereas, at the other extreme, cytochrome *c* could act as a laterally mobile agent, alternatively oxidized and reduced via random interactions with its oxidase and reductase. Between these two extremes could lie other models, and, more recently, opinion has tended to favour those that involve independently mobile cytochrome *c*. The most influential experiments in this respect are those that have shown that the domain on cytochrome *c* which interacts with the reductase is virtually identical with that which interacts with the oxidase (Rieder & Bosshard, 1980; Koppenol & Margoliash, 1982). Therefore, cytochrome *c* must, at the very least, rotate while bound to one redox partner to allow interaction with another, although this is considered unlikely by some (Koppenol & Margoliash, 1982). Whether cytochrome *c* must dissociate from one partner before interaction with the other has been the subject of some dispute. Ferguson-Miller *et al.* (1979) maintain that the off-rate for cytochrome *c* from its high-affinity site on the oxidase is insufficient to account for known rates of electron transfer.

Likewise, Dixit *et al.* (1982), using fluorescence and phosphorescence decay, found that cytochrome *c* in mitochondrial membranes had rotational diffusion rates comparable with those measured for cytochrome *c* oxidase, and these were considerably lower than those for cytochrome *c* bound to lipid vesicles. They concluded that cytochrome *c* in the membrane was always associated with one or other of its redox partners. On the other hand, evidence that binding of cytochrome *c* into low-affinity sites might increase the off-rate from the high-affinity sites (Wilms *et al.*, 1981) has led Capaldi *et al.* (1982) to propose that the dissociation of cytochrome *c* is indeed rapid enough, and therefore that cytochrome *c* mediates electron transfer by free diffusion. Similar conclusions have been reached by Hackenbrock's group (e.g. Höchli & Hackenbrock, 1979; Hackenbrock, 1981; Höchli *et al.*, 1982) from studies of lipid-enriched mitoplasts. They found no evidence for any association of the cytochrome *c* reductase and oxidase, implying that these enzymes were functionally linked by freely diffusible cytochrome *c*. Measurements of rotational diffusion of cytochrome oxidase by Cherry and co-workers also failed to reveal any association between the two enzymes (Kawato *et al.*, 1981), and estimates of cytochrome *c* diffusion rates were considered more than adequate to account for electron-transfer rates.

A freely diffusing cytochrome *c* pool is reminis-

cent of the freely diffusing ubiquinone pool widely held to be the functional link between dehydrogenases and the cytochrome *c* reductase (e.g. Rich, 1981). The kinetic applications of the ubiquinone-pool model have been described by Kröger & Klingenberg (1973*a,b*) and applied successfully to a number of different systems (e.g. De Troostem-burgh & Nyns, 1978; Heron *et al.*, 1978).

In an attempt to find similar kinetic evidence for a cytochrome *c* pool, we described the preparation and properties of a ubiquinol oxidase system, reconstituted from the purified cytochrome *c* reductase, cytochrome *c* and cytochrome oxidase (Diggins & Ragan, 1982). We showed that electron transfer was mediated preferentially by membrane-bound cytochrome *c*, that rates were higher when both low- and high-affinity cytochrome *c*-binding sites were occupied (Ferguson-Miller *et al.*, 1976; Errede & Kamen, 1978; Smith *et al.*, 1979) and we described the effects of changes in lipid-to-protein ratio and the relative concentrations of the constituent proteins. However, experiments designed to test the cytochrome *c*-pool model produced ambiguous results; in particular, those involving inhibitor titrations.

In the present paper we have extended our previous observations and find excellent agreement between experiments and the predictions of a very simple cytochrome *c*-pool model. We are also able to explain previous ambiguities on the basis of this model and conclude that, in the reconstituted system at least, free diffusion of cytochrome *c* is an obligatory step in electron transfer from ubiquinol to oxygen. In the following paper (Froud & Ragan, 1984), we provide other evidence for this from the effects of lipid-phase transition on ubiquinol oxidase activity and from e.s.r. studies of spin-labelled cytochrome *c*, cytochrome *c* oxidase and cytochrome *c* reductase.

Materials and methods

Preparations

Ubiquinol-cytochrome *c* reductase (Complex III) and cytochrome *c* oxidase (Complex IV) were purified from bovine heart mitochondria by the methods of Rieske (1967) and Capaldi & Hayashi (1972) respectively. The molarity of Complex III solutions was based on the cytochrome *c*₁ content (3.6–4.2 nmol/mg of protein) determined from ascorbate-reduced minus $K_3Fe(CN)_6$ -oxidized difference spectra. An absorption coefficient of $17\,500\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 552.5 nm minus 540 nm was used (King, 1978) and one cytochrome *c*₁ molecule per Complex-III molecule was assumed. The molarity of Complex-IV solutions was based on the haem *a* content (7–12 nmol/mg of protein)

determined from dithionite-reduced minus $K_3Fe(CN)_6$ -oxidized difference spectra. An absorption coefficient of $12\,000\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 605 nm minus 630 nm was used (Ferguson-Miller *et al.*, 1976) and two haem *a* molecules per molecule of Complex IV were assumed. Cytochrome *c* (type III, horse heart) was from Sigma, Poole, Dorset, U.K., and was used without further purification. Solutions were standardized spectrophotometrically by using a dithionite-reduced minus $K_3Fe(CN)_6$ -oxidized absorption coefficient at 550 nm of $20\,000\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (Ferguson-Miller *et al.*, 1976).

Chemicals

Ubiquinone-2 was reduced to ubiquinol-2 by the method of Rieske (1967) and stored in ethanol acidified to pH 2 with HCl at -20°C . Soya-bean phospholipids were purified from 'L- α -lecithin' (Sigma) as described by Ragan & Racker (1973).

Reconstitution of ubiquinol oxidase

Concentrated solutions of Complex III and Complex IV were mixed, with or without additional phospholipid, and reconstituted by dialysis as described by Diggins & Ragan (1982).

Assays

Ubiquinol oxidase was assayed at 30°C with 0.1 mM-ubiquinol-2 as substrate, either spectrophotometrically or polarographically (Diggins & Ragan, 1982). The former assay, measuring ubiquinol-2 oxidation, was carried out at 275 nm minus 290 nm. We take the opportunity to correct an error in our previous description of this assay. Because of partition of the ubiquinol-2 and ubiquinone-2 into the phospholipid phase of the system the absorption spectrum of both reduced and oxidized forms is quite different from that found in either organic solvents or aqueous buffers and somewhat different from that found in pure phospholipid vesicles. We have therefore determined the absorption coefficient for the spectrophotometric assay by comparison of rates with those obtained under identical conditions polarographically. The value obtained at 275 nm minus 290 nm was $3250\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. Complexes III and IV activities were assayed as described previously (Diggins & Ragan, 1982). Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as a standard. Phospholipid was determined by analysis of phosphate after digestion of samples with $HClO_4$ (Bartlett, 1969).

Kinetic analysis of cytochrome *c*-pool behaviour

The oxidation of reduced cytochrome *c* by isolated cytochrome oxidase is a pseudo-first-order

process (Smith *et al.*, 1979), despite the complex dependence of rate on total cytochrome *c* concentration (e.g. Ferguson-Miller *et al.*, 1976; Errede & Kamen, 1978). Thus, for any particular cytochrome *c*-to-oxidase ratio, the velocity of oxidation, *v*, is given by:

$$v = k_4 \cdot \text{IV} \cdot c_r \quad (1)$$

where k_4 , the rate constant for oxidation through Complex IV, is, in fact, a function of the total cytochrome *c* concentration, and IV and c_r are the concentrations of Complex IV and reduced cytochrome *c* respectively.

For reduction of cytochrome *c* by Complex III, the process is again pseudo-first-order (Smith *et al.*, 1974; Speck *et al.*, 1979). In the reconstituted system there is an appreciable antimycin-insensitive but cyanide-sensitive component of ubiquinol oxidase activity (Diggins & Ragan, 1982). This is not simply a non-enzymic reduction, since the antimycin-insensitive rate is still dependent on the presence of Complex III. We may therefore write:

$$v = (k_3 + k'_3) \cdot \text{III} \cdot c_o \quad (2)$$

where k_3 and k'_3 are respectively the rate constants for the antimycin-sensitive and -insensitive pathways, and III and c_o are respectively the concentrations of Complex III and oxidized cytochrome *c*.

Maximum rates through the oxidase (V_4) and reductase (V_3) are obtained when cytochrome *c* is fully reduced or oxidized respectively. Therefore:

$$V_4 = k_4 \cdot \text{IV} \cdot c_t$$

and

$$V_3 = (k_3 + k'_3) \cdot \text{III} \cdot c_t \quad (3)$$

where c_t is the total cytochrome *c* concentration. Thus:

$$\frac{V_4}{V_3} = \frac{k_4 \cdot \text{IV}}{(k_3 + k'_3) \cdot \text{III}} \quad (4)$$

If the cytochrome *c* pool is the obligatory intermediate between the Complex III and Complex IV and equally accessible to both enzymes, then, in the steady state:

$$v = k_4 \cdot \text{IV} \cdot c_r = (k_3 + k'_3) \cdot \text{III} \cdot c_o \quad (5)$$

These basic expressions can be further developed to test how well the reconstituted ubiquinol oxidase system conforms to the cytochrome *c*-pool model.

(a) Determination of V_3 and V_4

From eqns. (4) and (5) we obtain:

$$\frac{k_4 \cdot \text{IV}}{(k_3 + k'_3) \cdot \text{III}} = \frac{V_4}{V_3} = \frac{c_o}{c_r} \quad (6)$$

Thus V_4/V_3 can be determined directly from measurements of the steady-state level of reduction of cytochrome *c*. In the presence of antimycin, $k_3 = 0$, and, therefore:

$$\frac{k_4 \cdot \text{IV}}{k'_3 \cdot \text{III}} = \frac{c_o}{c_r}$$

Thus k'_3 can be determined as a function of k_3 and should be independent of both cytochrome *c* concentration and the molar ratio of Complex III to Complex IV.

An independent method of determining V_3 and V_4 involves measuring activity as a function of the molar ratios of the two Complexes. From eqn. (5) we can derive:

$$v = \frac{(k_3 + k'_3) \cdot \text{III} \cdot k_4 \cdot \text{IV} \cdot c_t}{[(k_3 + k'_3) \cdot \text{III}] + [k_4 \cdot \text{IV}]} \quad (7)$$

Letting $\text{III}/\text{IV} = N$, the molar ratio of Complex III to Complex IV, we obtain:

$$v = \frac{k_4 \cdot \text{IV} \cdot N \cdot c_t}{N + k_4/(k_3 + k'_3)} \quad (8)$$

Eqn. (8) shows that velocity is hyperbolically related to the molar ratio, N . The maximum velocity is given by $k_4 \cdot \text{IV} \cdot c_t (= V_4)$, whereas the ' K_m ' is given by $k_4/(k_3 + k'_3)$. We note from eqn. (4) that:

$$k_4/(k_3 + k'_3) = \frac{V_4 N}{V_3}$$

or:

$$'K_m' = \frac{V_4 N}{V_3} \quad (9)$$

Thus the V_4/V_3 value obtained from cytochrome *c* redox-state measurements can be compared with that obtained from a Complex III/IV titration. Eqn. (9) shows that the V_4/V_3 ratio and hence the c_o/c_r ratio should be inversely proportional to the molar ratio, N .

Eqn. (8) may also be recast to express velocity as a function of $1/N$, the molar ratio of Complex IV to Complex III. This gives:

$$v = \frac{(k_3 + k'_3) \cdot \text{III} \cdot (1/N) \cdot c_t}{[(k_3 + k'_3)/k_4] + (1/N)} \quad (10)$$

Again, velocity is hyperbolically related to $(1/N)$, whereas the maximum velocity is now $(k_3 + k'_3) \cdot \text{III} \cdot c_t (= V_3)$ and the ' K_m ' value is the reciprocal of that obtained by using eqn. (8).

(b) *Calculation of inhibitor-sensitivity and inhibitor titration curves*

From eqn. (8), the velocity in the presence of excess antimycin, v' , is given by:

$$v' = \frac{k_4 \cdot IV \cdot N \cdot c_i}{N + k_4/k'_3} \quad (11)$$

The degree of inhibition from eqns. (8) and (11) is:

$$\frac{v'}{v} = \frac{N + k_4/(k_3 + k'_3)}{N + k_4/k'_3} \quad (12)$$

When N is very low, v'/v tends to $k'_3/(k_3 + k'_3)$, whereas, when N is very high, v'/v tends to 1, i.e. the sensitivity to antimycin is dependent on the molar ratio in a predictable fashion.

Eqn. (12) could also be used to predict antimycin titration curves. If we assume that k_3 is linearly decreased by antimycin, we obtain:

$$\frac{v'}{v} = \frac{N + k_4/(k_3 + k'_3)}{N + k_4/[k_3(1-n) + k'_3]} \quad (13)$$

where n is the molar ratio of antimycin to Complex III. Although Kröger & Klingenberg (1973b) successfully applied expressions of this type, other workers (e.g. Berden & Slater, 1972) have found more complex dependence of Complex III activity on antimycin binding. This point is described more fully in the Results section.

Results

For the experiments in the present paper we have adopted two levels for the phospholipid-to-protein ratio. As discussed by Diggins & Ragan (1982), the two Complexes as isolated contain approx. $0.2 \mu\text{mol}$ of phospholipid P/mg of protein. Under these conditions, the rotational mobility of the two enzymes is low (Poore *et al.*, 1982; Froud & Ragan, 1984) and cytochrome oxidase activity is also low because of aggregation of the Complex IV (Diggins & Ragan, 1982). Increasing the level of phospholipid to $3 \mu\text{mol}$ of P/mg of protein maximally increases cytochrome oxidase activity by disaggregation, thereby stimulating ubiquinol oxidase 4–5-fold. In addition, rotational mobility is considerably enhanced (Froud & Ragan, 1984). We have therefore carried out tests of cytochrome c -pool behaviour at both low and high levels of phospholipid in case the changes in membrane fluidity affected the mode of interaction of the enzymes (as, for example, is found with NADH-ubiquinone reductase and Complex III; Ragan & Heron, 1978).

Determination of the steady-state reduction of cytochrome c

Fig. 1 shows the method used to determine the cytochrome c redox state in reconstituted ubiquinol oxidase. Cytochrome c reduction was monitored after addition of ubiquinol-2. The steady state was rapidly reached and the level of reduction gradually decreased as the ubiquinol was oxidized. The final position differs from the starting point because of absorption changes due to ubiquinone-2. Ubiquinol-2 also caused a small offset. To eliminate errors from these sources, the initial steady-state level (representing absorption due to ubiquinol-2 plus partial cytochrome c reduction) was compared with the level reached on final addition of dithionite (representing absorption due to ubiquinol-2 plus complete cytochrome c reduction). Separate experiments confirmed rapid reduction of ubiquinone-2 by dithionite. The difference between the two levels gives c_o , whereas c_i was determined in a separate experiment in which the ubiquinol-2 cycle was omitted. The contribution of cytochrome c_1 to the cytochrome c absorption would be considerable at very low molar ratios of cytochrome c to Complex III. However, since the redox potentials of cytochromes c and c_1 are so similar, and redox equilibration is rapid (Yu *et al.*, 1973), this is unlikely to be a source of error. A further problem is the degree of binding of cytochrome c . Under the experimental conditions of Fig. 1 we can calculate how much of the added cytochrome c is bound in high-affinity sites, low-affinity sites or free in solution (Diggins & Ragan, 1982). We find that, in the useful range of enzyme and cytochrome c concentrations (Fig. 2), the high-affinity sites are nearly fully occupied, the low-affinity sites rather little, while a considerable proportion of free cytochrome c is present at high molar ratios. Since we had no prior knowledge of whether free cytochrome c would rapidly reach a steady state of reduction and we were concerned about possible effects on k_4 (and hence V_4) of binding of cytochrome c in the low-affinity sites, we performed all experiments with a range of cytochrome c concentrations. These experiments were carried out in the presence and absence of a phospholipid supplement, the presence and absence of antimycin and at three different molar ratios of Complex III to Complex IV. The results are shown in Fig. 2 and analysed in Table 1. Determinations at low cytochrome c concentration are less accurate because of the relatively greater corrections to be made for ubiquinone/ubiquinol absorption changes. Nevertheless, the degree of reduction of cytochrome c was largely independent of cytochrome c concentration, indicating (a) that any variation in the low-

affinity binding was not strongly affecting V_4 , and (b) that the free cytochrome *c* was reaching much the same redox state as the bound cytochrome *c*. Therefore values for different cytochrome *c* con-

Fig. 1. *Measurement of cytochrome *c* redox state* The reconstituted ubiquinol oxidase contained Complexes III and IV in equimolar concentrations. The assay contained $2.31\ \mu\text{M}$ -cytochrome *c* and $0.25\ \mu\text{M}$ -Complex III (or Complex IV) in $0.25\ \text{M}$ -sucrose/ $25\ \text{mM}$ -acetate/Tris, pH 7.8, at 30°C and measurement was at $550\ \text{nm}$ minus $540\ \text{nm}$. Other additions were $0.13\ \text{mM}$ -ubiquinol-2 (UQ_2H_2) and solid sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$). The final volume was $1.3\ \text{ml}$.

centrations have been averaged and are indicated by broken lines in Fig. 2. These are entered in Table 1. In the presence of additional phospholipid, the c_0/c_r values (equal to V_4/V_3) show the predicted dependence on molar ratio, since V_4N/V_3 is approximately constant. The value for $N=3$ is less accurate, since the cytochrome *c* is nearly all reduced, leading to uncertainty in the value of c_0 . In the presence of antimycin, cytochrome *c* was much less extensively reduced. Again, V_4N/V_3 values were approximately constant, major deviation occurring at $N=0.33$, where c_r is very low. The constancy of k'_3/k_3 with molar ratio is also predicted by the kinetic model and indicates that the antimycin-insensitive pathway accounts for 5% of the electron flux through Complex III.

The results in the absence of additional phospholipid are less clear. The extent of cytochrome *c* reduction at any molar ratio was considerably more than that found in the presence of phospholipid, i.e. V_4/V_3 values are lower. Also, V_4N/V_3 was not constant and increased considerably with increasing N . Similar trends were found in the presence of antimycin, but k'_3/k_3 was constant, as predicted by the model, and in this experiment the antimycin-insensitive electron flux was 11% of the total through Complex III. The experiments in the presence or absence of additional lipid were performed with the same preparations of enzymes but a different preparation of ubiquinol-2. It is the latter which accounts for the difference in k'_3/k_3 values.

Fig. 2. *Dependence of cytochrome *c* redox state on cytochrome *c* concentration and ubiquinol oxidase composition* Ubiquinol oxidase was reconstituted with three different molar ratios of Complex III to Complex IV in the presence and absence of an additional phospholipid supplement to $3\ \mu\text{mol}$ of phospholipid P/mg of protein. Cytochrome *c* reduction in the steady state was measured, as in Fig. 1, with a variety of cytochrome *c* concentrations and, additionally, in the presence of antimycin ($1\ \mu\text{g/ml}$). The Complex -III-plus-Complex-IV concentration in the assay was $0.5\ \mu\text{M}$. (a,b) No additional phospholipid; (c,d) with a phospholipid supplement; (a,c) no antimycin; (b,d) plus antimycin. Molar ratios of Complex III to Complex IV were: $\square$, 3:1; $\bullet$, 1:1; $\circ$, 0.33:1. Broken lines show values entered into Table 1.

The variation of V_4N/V_3 with N in the absence of additional lipid can be explained by the variation of Complex IV activity with Complex III concentration (Diggens & Ragan, 1982). We found that oxidase activity was as low as 16s^{-1} after dialysis of the enzyme alone, but, in the presence of high concentrations of Complex III, could reach a value as high as 80s^{-1} . This variation in oxidase activity was attributed to disaggregation of the oxidase in the presence of Complex III, and accounted for the sigmoidal dependence of ubiquinol oxidase activity on the molar ratio, N (Diggens & Ragan, 1982). This also explains the results of Table 1. The increase in V_4N/V_3 with N is caused by an increase in V_4 , the oxidase activity. Although this complicates the analysis of the results, Complex III activity (V_3) is little affected by the presence of Complex IV, and the constancy of k'_3/k_3 with N is in accord with the predictions of the cytochrome *c*-pool model.

Dependence of ubiquinol oxidase activity on the molar ratio of the two Complexes

In the presence of additional lipid, cytochrome oxidase activity is stimulated and no longer varies with Complex III concentration (Diggens & Ragan, 1982). This is probably because phospholipid alone can disaggregate the enzyme. Thus, as

expected, V_4/V_3 values are higher than those encountered in the absence of lipid, and V_4N/V_3 is constant (Table 1). Fig. 3(a) shows the dependence of ubiquinol oxidase activity (per mg of oxidase protein) on the molar ratio, N , in the presence of additional lipid. The curve is no longer sigmoidal, but hyperbolic (eqn. 8). Fig. 3(b) shows a similar dependence of oxidase activity (per mg of Complex III protein) on $1/N$ (eqn. 10). The ' K_m ' values and maximum velocities are given in Table 2. The ' K_m ' values of the two curves are reciprocally related (eqns. 9 and 10) and give values of V_4N/V_3 which are very close to that determined from the cytochrome *c* redox-state measurements (Table 1). The maximum velocity from Fig. 3(a), expressed per mol of oxidase, is k_4 (eqn. 8), whereas the maximum velocity from Fig. 3(b), expressed per mol of Complex III, is $(k_3 + k'_3)$ (eqn. 10). The ratio of k_4 to $(k_3 + k'_3)$ is V_4N/V_3 and this value is again in good agreement with the other determinations.

The agreement between the measurements of cytochrome *c* redox state and dependence of velocity on molar ratio provide good evidence for the cytochrome *c*-pool model. However, in our previous studies we failed to detect any dependence of antimycin titration curves on the molar ratio of the two Complexes (Diggens & Ragan, 1982), which is contrary to expectations from the

Table 1. Analysis of cytochrome *c* redox-state measurements

All values were taken from Fig. 2. For explanation of symbols, see the 'Kinetic analysis of cytochrome *c*-pool behaviour' section. Further details are given in the Results section.

Complex III/Complex IV (N) . . .	0.33	1.0	3.0
Plus phospholipid supplement			
c_o/c_r	0.91	0.33	0.12
V_4N/V_3	0.30	0.33	0.36
c_o/c_r (plus antimycin)	16	7.3	3.3
V_4N/V_3 (plus antimycin)	5.3	7.3	9.9
k'_3/k_3	0.06	0.05	0.04
Minus phospholipid supplement			
c_o/c_r	0.25	0.11	0.053
V_4N/V_3	0.082	0.11	0.16
c_o/c_r (plus antimycin)	2.8	1.4	0.48
V_4N/V_3 (plus antimycin)	0.92	1.4	1.4
k'_3/k_3	0.10	0.09	0.12

Table 2. Analysis of the dependence of rate on molar ratio

The data of Fig. 3 were analysed by reciprocal plots to give values of the maximum velocities and the ' K_m ' values, i.e. the molar ratios required for one half of maximum velocity. Derivation of V_4N/V_3 from the ' K_m ' values or the maximum velocities is described in the Materials and methods section.

	From Fig. 3(a)	From Fig. 3(b)
' K_m ' (mol/mol)	0.30	2.91
V_4N/V_3 (from ' K_m ')	0.30	0.34
Maximum velocity ($\mu\text{mol/min}$ per mg of complex protein)	6.95	28.6
Maximum turnover (mol/min per mol of complex)	1960	7620
V_4N/V_3 (from ratio of turnovers)		0.26

cytochrome *c*-pool model (eqn. 13). In the light of the more detailed analyses provided by Figs. 1, 2 and 3, we have re-examined this point.

Antimycin inhibition of ubiquinol oxidase

The maximum extent of antimycin inhibition depends on the molar ratio, *N*, as suggested already by the results of Fig. 1 and predicted by eqn. (12). In Table 3, maximum extents of inhibition at various molar ratios are compared with those calculated from eqn. (12), taking $k_4/(k_3 + k'_3)$ as

Table 3. Variation of antimycin-insensitivity with molar ratio

Ubiquinol oxidase was reconstructed with the indicated molar ratios of Complex III to Complex IV (*N*). Activity was measured spectrophotometrically with 0.46 μM-cytochrome in the presence or absence of 1 μg of antimycin/ml. Eqn. (12) was used to calculate the antimycin-insensitive rate (*v'*) as a fraction of the total (*v*), taking $k_4/(k_3 + k'_3) = 0.30$ (Table 1) and $k'_3 = 0.045 k_3$. Eqn. (12) was therefore reduced to:

$$v'/v = (N + 0.30)/(N + 6.97)$$

<i>N</i>	Antimycin-insensitive rate (as fraction of total rate)	
	Measured	Calculated
0.33	0.10	0.09
0.5	0.13	0.11
1.0	0.12	0.16
2.0	0.28	0.26
4.6	0.38	0.42

0.30 (see Table 1) and assuming $k'_3 = 0.045 k_3$. Thus, in these experiments, the underlying insensitivity of the Complex-III reaction was 4.5%. Again, the variation of antimycin-sensitivity of ubiquinol oxidase with molar ratio agrees well with cytochrome *c*-pool predictions, a point which we had not considered previously (Diggens & Ragan, 1982). Prediction of the actual shape of antimycin-titration curves is a more complex problem, however. In Fig. 4(a), curves have been drawn according to eqn. (13), assuming the same values as used in Table 3, but varying *N*. To compare shapes more accurately, Fig. 4(b) shows the same curves redrawn with only the antimycin-sensitive component considered, as was done in our previous antimycin titrations. It is clear that the corrected curve shape is not, in fact, a particularly sensitive indicator of variation in $k_4/(k_3 + k'_3)$ compared with the end point of the titrations as given in Table 2. Representative titrations have been superimposed on the curves of Figs. 4(a) and 4(b) to illustrate this point, and, indeed, the fit is reasonably good. A further complexity is our obser-

Fig. 3. Dependence of ubiquinol oxidase rate on the molar ratio of the constituent complexes

Ubiquinol oxidase was reconstituted from the indicated molar ratios of the two Complexes and a phospholipid supplement to 3 μmol of phospholipid P/mg of protein. Activity was assayed spectrophotometrically with 0.15 μM-(Complex III plus Complex IV) and 1.5 μM-cytochrome *c*. In Fig. 3(a), ratios are expressed per mg of Complex IV protein and plotted against the molar ratio of Complex III to Complex IV. In Fig. 3(b) ratios are expressed per mg of Complex III protein and plotted against 1/*N*.

vation that inhibition of ubiquinol-2-cytochrome *c* reductase activity is not a linear function of antimycin concentration, as assumed by eqn. (13) (results not shown). Thus changes in sigmoidicity attributable to cytochrome *c*-pool behaviour would have to be superimposed on the underlying sigmoidicity of the Complex-III inhibition. Indeed, the actual titration curves (Fig. 4) show less inhibition of activity at low concentrations of antimycin than expected, in agreement with this suggestion. Similar findings for antimycin inhibition of Complex III have been reported by other workers (Berden & Slater, 1972; Zhu *et al.*, 1982). Thus the end points of antimycin titrations are the most reliable, and we have already shown that these conform well to prediction of cytochrome *c*-pool behaviour.

Fig. 4. Antimycin inhibition of ubiquinol oxidase

In (a), theoretical curves were drawn according to eqn. (13) with $k_4/(k_3 + k'_3) = 0.30$, $k'_3 = 0.045 k_3$ and $N = 0.33, 1.0$ or 3.0 . In (b) the curves were normalized by correction of calculated rates for the antimycin-insensitive contribution. Experimental curves were obtained as described by Diggins & Ragan (1982). Samples containing a 1:1 (●) or a 0.33:1 (○) molar ratio of Complex III to Complex III reconstituted in the presence of a lipid supplement were preincubated with the indicated concentrations of antimycin in ethanol for 2 h at 0°C before assay. The ethanol concentration in all incubations was 2.5% (v/v). Ubiquinol-2 oxidase was determined with final concentrations of $0.06 \mu\text{M}$ -Complex IV and $0.27 \mu\text{M}$ -cytochrome *c* (●) and $0.15 \mu\text{M}$ -Complex IV and $0.46 \mu\text{M}$ -cytochrome *c* (○).

Discussion

We have found that the properties of reconstituted ubiquinol oxidase can be accurately accounted for by a model based on that of Kröger & Klingenberg (1973a,b) for ubiquinone-pool behaviour. The features of this model are that oxidation of cytochrome *c* is a first-order process, that all cytochrome *c* molecules are equally accessible to the constituent Complex III and Complex IV molecules and, by inference, that the free diffusion of cytochrome *c* between Complex-III and Complex-IV molecules is an obligatory part of the electron-transfer process. In view of the complex kinetic interaction between cytochrome *c* and its oxidase, it is somewhat surprising that simple first-order behaviour should be found. The explanation may be that, in any reconstituted ubiquinol oxidase system, the total concentration of cytochrome *c* is constant. The activity of the oxidase may therefore depend purely on the proportion of oxidase molecules which have bound a reduced cytochrome *c* as opposed to an oxidized cytochrome *c*. If the two redox forms are bound with equal affinity, the velocity of oxidation is proportional to the concentration of reduced cytochrome *c*. The same may be true for Complex III and oxidized cytochrome *c*. In an elaboration of this proposal applied to ubiquinone kinetics (C. I. Ragan & I. R. Cottingham, unpublished work) it has been shown that binding and release of the substrate (i.e. ubiquinol or, in the present

context, cytochrome *c*) has to be rapid compared with electron transport for these simple kinetics to apply. In the reconstituted system described here, the maximum turnover of the oxidase is considerably lower (up to 90 s^{-1}) than that reported for cytochrome oxidase maximally activated *in vitro*, or in the mitochondrial membrane ($400\text{--}500 \text{ s}^{-1}$; Vik *et al.*, 1981), and, as mentioned in the introduction, there is some doubt as to whether the off-rate for cytochrome *c* is adequate for a free-diffusion model (Ferguson-Miller *et al.*, 1979). The lower turnover of the reconstituted system, which is unlikely to result in cytochrome *c* diffusion becoming limiting, allows description of the free diffusion, or pool model, in the simplest kinetic terms. If the turnover becomes higher, as in the mitochondrial membrane, the kinetic description of the pool model may become much more complicated, as electron transfer becomes faster compared with the on- and off-rates for cytochrome *c*. This might result in poor agreement with the simple cytochrome *c*-pool equations, but we feel it is unlikely that there would be any fundamental change of mechanism. In other words, although conditions in the reconstituted system may be considered to be rigged so as to allow simple prediction of the kinetic properties, the demonstration of free diffusion of cytochrome *c* here most probably means that this is the mechanism which applies in intact mitochondria regardless of what the rate-limiting steps may be.

A trivial explanation for cytochrome *c*-pool behaviour is that, in the reconstituted system, electron transfer takes place via soluble cytochrome *c* which, of course, could act as a freely diffusible pool. We showed previously that, at low molar ratios of cytochrome *c* to the Complexes, as found in mitochondria, electron transport by the reconstituted system was strongly in favour of membrane-bound cytochrome *c* (Diggins & Ragan, 1982). In the experiments described in the present paper we have again used conditions which promote cytochrome *c* binding and which, therefore, promote electron transport at low concentrations of cytochrome *c*. In addition, we have found little effect, if any, of cytochrome *c* concentration on the shapes of antimycin-titration curves or the dependence of ubiquinol oxidase activity on the molar ratio of the Complexes. As mentioned above, at the cytochrome *c* concentrations which we employ, high-affinity binding sites are largely occupied, low-affinity sites are occupied to a small extent, and variable amounts of soluble cytochrome *c* are present. We conclude, therefore, that soluble cytochrome *c* contributes little to the electron-transport rate and, therefore, that the cytochrome *c* pool is membrane-bound. The fact that most of the bound cytochrome *c* will, in fact, be occupying high-affinity sites on the oxidase and Complex III is not a hindrance to pool behaviour. All that is required is that the bound cytochrome *c* can dissociate at least as fast as the electron-transfer rate. Although soluble cytochrome *c* is not kinetically competent to participate in electron transport, it is, nevertheless, in redox communication with the Complexes and the bound cytochrome *c*, as shown by the experiments of Fig. 2. Since the redox state of cytochrome *c* was largely independent of the concentration of cytochrome *c*, we conclude that c_o/c_r is much the same for bound or soluble cytochrome *c*. This implies that the ratio of the velocity of oxidation to the velocity of reduction is the same as V_4/V_3 for bound cytochrome *c*. Since electron transfer through the soluble pool is slow, we conclude that the individual velocities are less than V_4 or V_3 respectively.

Pool behaviour of ubiquinone (Kröger & Klingenberg, 1973*a,b*) provides a ready explanation for how respiratory chains may branch. Since there is no branching at the cytochrome *c* level in the mitochondrial respiratory chain, one might ask why a cytochrome *c*-pool mechanism should operate in preference to an assembly containing all the components of Complexes III and IV in addition to cytochrome *c*. One possibility is that this is an evolutionary left-over, since bacterial respiratory chains can branch at the cytochrome *c* level, employing not only multiple oxidases or terminal acceptors, but also multiple cytochrome *c*

species. Homology between, for example, cytochrome oxidases from yeast, *Paracoccus denitrificans* and ox heart (Ludwig, 1980) demonstrates that the structure of respiratory proteins is strongly conserved and, therefore, that although the need for pool behaviour has been lost, the original mechanism is still used for linear electron transfer. On the other hand, although the inner-membrane respiratory pathway is unbranched, Bernardi & Azzone (1981) have shown that electron transport in the outer membrane converges with inner-membrane electron transport at the cytochrome *c* level and uses inner-membrane cytochrome oxidase as acceptor. It is apparently membrane-bound cytochrome *c* which carries electrons from Complex III (as concluded in the present paper), whereas soluble cytochrome *c* carries electrons from outer-membrane cytochrome *b₅*. The bound and soluble cytochrome *c* pools are not in rapid equilibrium, but can be fully reduced by either source of electrons. The conclusions of Bernardi & Azzone (1981) are in very good agreement with our own concerning the involvement of soluble and membrane-bound cytochrome *c* in reconstituted ubiquinol oxidase. In the following paper (Froud & Ragan, 1984) we attempt to provide more direct evidence that the cytochrome *c* pool is membrane-bound, and we consider the manner by which cytochrome *c* diffuses between Complex III and cytochrome oxidase.

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