Properties of ubiquinol oxidase reconstituted from ubiquinol-cytochrome *c* reductase, cytochrome *c* and cytochrome *c* oxidase

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Ubiquinol-cytochrome *c* reductase (Complex III), cytochrome *c* and cytochrome *c* oxidase can be combined to reconstitute antimycin-sensitive ubiquinol oxidase activity. In 25 mM-acetate/Tris, pH 7.8, cytochrome *c* binds at high-affinity sites ($K_D = 0.1 \mu\text{M}$) and low-affinity sites (K_D approx. $10 \mu\text{M}$). Quinol oxidase activity is 50% of maximal activity when cytochrome *c* is bound to only 25% of the high-affinity sites. The other 50% of activity seems to be due to cytochrome *c* bound at low-affinity sites. Reconstitution in the presence of soya-bean phospholipids prevents aggregation of cytochrome *c* oxidase and gives rise to much higher rates of quinol oxidase. The cytochrome *c* dependence was unaltered. Antimycin curves have the same shape regardless of lipid/protein ratio, Complex III/cytochrome *c* oxidase ratio or cytochrome *c* concentration. Proposals on the nature of the interaction between Complex III, cytochrome *c* and cytochrome *c* oxidase are considered in the light of these results.

The association of cytochrome *c* with cytochrome *c* oxidase (EC 1.9.3.1) or with ubiquinol-cytochrome *c* reductase (EC 1.10.2.2) has been the object of much study. Experiments with isolated oxidase have shown that there is one high-affinity binding site and one lower-affinity site for ferricytochrome *c* per cytochrome a_3 (i.e. per oxidase monomer) (Ferguson-Miller *et al.*, 1976). Similar behaviour is found for binding of cytochrome *c* to the reductase from both bovine heart (Bosshard *et al.*, 1979) and *Neurospora crassa* (Weiss & Juchs, 1978). In all cases, the dissociation constants are extremely sensitive to ionic strength and, where tested, to the redox state of the system (e.g. Weiss & Juchs, 1978). The high- and low-affinity sites appear to be intrinsically different since there is good evidence that in oxidase, the low-affinity site involves binding to phospholipid (probably cardiolipin) (Vik *et al.*, 1981), whereas the high-affinity site involves binding to protein (Bisson *et al.*, 1980).

Kinetic studies of ferrocycytochrome *c* oxidation by oxidase indicate the presence of two phases of activity (Ferguson-Miller *et al.*, 1976; Errede & Kamen, 1978; Smith *et al.*, 1979), which have been interpreted as due to separate electron transfer through the high- and low-affinity binding sites (Ferguson-Miller *et al.*, 1976). Only one phase, corresponding to the high-affinity site, is seen for

reduction of ferricytochrome *c* by the reductase, however (Speck *et al.*, 1979).

Besides the mechanistic significance of the high- and low-affinity binding of cytochrome *c* to its reductase and oxidase, a further unsettled question is how do the reductase, oxidase and cytochrome *c* interact laterally in the mitochondrial membrane. The domain of cytochrome *c* that interacts with the oxidase is the same as that which interacts with the reductase (Rieder & Bosshard, 1980), implying that a rigid ternary complex of all three proteins cannot exist. However, it is not certain whether the three component proteins of the system are freely and independently diffusible in the plane of the membrane or whether there may be a closer interaction between the reductase and oxidase between which the cytochrome *c* can oscillate. Although kinetic and other measurements on the isolated reductase and oxidase can provide information on possible rates of cytochrome *c* association and dissociation, the lateral interaction between the component proteins has mainly been studied in intact mitochondrial membranes. The experiments of Schneider *et al.* (1980) with lipid-enriched mitochondrial membranes favour separate non-interacting oxidase and reductase. On the other hand, Erecinska *et al.* (1980) find that cytochrome *c* covalently bound to mitochondrial membranes can function in rapid electron transport from succinate to O_2 . A covalent succinate-cytochrome *c* reductase–cytochrome *c* complex can also donate electrons to cytochrome

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oxidase at high rates. These experiments suggest that free diffusion of cytochrome *c* between oxidase and reductase is not required for electron transfer.

In the present paper we describe a reconstituted ubiquinol oxidase system comprising ubiquinol-cytochrome *c* reductase (Complex III of Hatefi *et al.*, 1962a), cytochrome *c* and cytochrome oxidase. This is the simplest system in which the activity of cytochrome *c* as an intermediary electron carrier can be studied. Moreover, the system is amenable to variations in the ratios of the component enzymes, the lipid composition and lipid concentration. We demonstrate that under certain circumstances, electron transfer between the reductase and oxidase is mediated exclusively by membrane-bound cytochrome *c* and describe experiments to test the role of lateral mobility in electron transfer.

Materials and methods

Preparation

Complex III and cytochrome *c* oxidase were purified from bovine heart mitochondria by the methods of Rieske (1967a) and Capaldi & Hayashi (1972) respectively. The latter method of preparation was chosen because it avoids the use of non-ionic detergents that might interfere with the reconstitution. Molarity of Complex III solutions was based on cytochrome *c*₁ content (3.6–4.0 nmol/mg of protein), assuming one cytochrome *c*₁ per molecule. Molarity of cytochrome oxidase solutions was based on haem *a* content (7–12 nmol/mg of protein), assuming two haem *a* per molecule. Horse heart cytochrome *c* (type III) was from Sigma, Poole, Dorset, U.K. Polymeric cytochrome *c* was removed by gel filtration (Brautigan *et al.*, 1978) and solutions were standardized spectrophotometrically.

Chemicals

Ubiquinone-2, 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone and duroquinone were reduced to the respective quinols by the method of Rieske (1967a). Solutions were acidified to pH 2 with HCl and kept under nitrogen. Soya-bean phospholipids were purified as described by Ragan & Racker (1973) from 'L- α -lecithin' (Sigma, Poole, Dorset, U.K.).

Reconstitution of quinol oxidase

Concentrated solutions of Complex III and cytochrome oxidase are clear because of residual cholate and deoxycholate. Such solutions, containing at least 10 mg of protein/ml of 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, were mixed in the desired molar proportions, diluted with sucrose/Tris buffer to a final total protein concentration of approx. 10 mg/ml and dialysed overnight at 4°C against 500 vol. of the same buffer. After dialysis,

the concentrations of oxidase and Complex III were determined by spectrophotometric measurement of the haem *a* and cytochrome *c*₁ contents. In experiments where phospholipids were included, stock solutions were prepared by ultrasonic dispersion of the dry phospholipids in water to give a final concentration of 40 mM. Required amounts were added to the enzymes before dialysis.

Measurement of cytochrome *c* binding

Cytochrome oxidase or Complex III/oxidase mixtures were dialysed as described above. The dialysed enzymes (total concentration approx. 10 μ M) were incubated in 25 mM-acetate/Tris, pH 7.8, containing various amounts of ferricytochrome *c* at 4°C. The samples were centrifuged for 2 h at 100 000 *g* and the supernatants were then assayed spectrophotometrically for cytochrome *c*. An uncentrifuged sample lacking cytochrome *c* was used for assay of haem *a* and cytochrome *c*₁ contents. Data were presented as Scatchard (1949) plots. Dissociation constants and numbers of binding sites were determined from intercepts (Klotz & Hunston, 1971).

Assays

Quinol oxidase was routinely assayed in 25 mM-acetate/Tris, pH 7.8, at 30°C, either by polarographic determination of O₂ uptake or by spectrophotometric determination of quinol oxidation. The former assays were conducted in 2 ml final volume containing either 0.1 mM-ubiquinol-2 or 0.75 mM-duroquinol. Both substrates gave similar rates. Spectrophotometric assays were carried out at 275 nm minus 290 nm, with 0.1 mM-ubiquinol-2 as substrate in a final volume of 1.3 ml. An absorption coefficient of 8770 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ was used. All rates were corrected for activity insensitive to 1 μ g of antimycin.

Cytochrome *c* oxidase activity was assayed polarographically at 30°C in 2 ml final volume containing 50 mM-potassium phosphate, pH 7.0, 40 mM-ascorbate, 0.1 mM-EDTA, various cytochrome *c* concentrations and enzyme. Activities were determined at $V_{\max}$ for cytochrome *c* by reciprocal plots. Turnover numbers are expressed as mol of O/s per mol of oxidase (equivalent to mol of cytochrome *c* oxidized/s per mol of haem *a*, assuming two haems per molecule).

Complex III was assayed by its quinol-cytochrome *c* reductase activity. The assay contained 50 mM-potassium phosphate, pH 7.0, 40 μ M-6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol, 0.01 mM-EDTA, 50 μ M-cytochrome *c* and enzyme in a final volume of 1 ml at 30°C. Reduction of cytochrome *c* was followed at 550 nm using an absorption coefficient of 18500 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹. Where cytochrome oxidase was also present, 1 mM-

NaN_3 was included in the assay. In later experiments, ubiquinol-2 was used as the substrate. Rates were corrected for non-enzymic reduction of cytochrome *c* by the quinol.

Molarities of Complex III and cytochrome oxidase were determined from reduced minus oxidized difference spectra of cytochrome *c*₁ (Rieske, 1967*b*) and cytochrome *a* (Van Buuren *et al.*, 1974) respectively. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V from Sigma) as a standard. Phospholipid was determined by analysis of phosphate after digestion of the sample with HClO_4 (Bartlett, 1969).

Results

Reconstitution of quinol oxidase

Reconstitution of electron transfer from one respiratory complex to another can be achieved by mixing concentrated solutions of the enzymes in detergent and then removing the detergent by dialysis or dilution (Hatefi *et al.*, 1962*b*; Fowler & Richardson, 1963; Tzagoloff *et al.*, 1967). Such methods were used to reconstitute NADH oxidase with NADH-cytochrome *c* reductase and cytochrome oxidase, and Tzagoloff *et al.* (1967) showed that a membranous aggregate was formed. The successful formation of such an active aggregate obviously depends on the relative solubilities of the constituent enzymes. In the present context it was necessary to show that electron transfer from Complex III to cytochrome oxidase took place between enzymes within the same membrane via membrane-bound cytochrome *c* and not between enzymes in separate membranes via water-soluble cytochrome *c*. The experiments of Fig. 1 show how this was done. The two enzymes were dialysed separately or together to remove detergent and the reconstituted activity was measured as a function of added cytochrome *c* concentration. The cytochrome *c* dependence of the co-dialysed enzymes was the same whether cytochrome *c* was added to the assay medium or to the solution of enzymes before dialysis. The quinol oxidase activity of the enzymes that had been dialysed separately and then mixed in the assay was very low except at much higher concentrations of cytochrome *c*. We attribute the latter behaviour principally to electron transfer between separate aggregates of reductase and oxidase mediated by soluble cytochrome *c*. Much lower concentrations of cytochrome *c* are required by the co-dialysed enzymes because the reductase and oxidase are present in the same membrane and electron transfer is mediated by membrane-bound cytochrome *c*. At concentrations that are saturating for the membrane-mediated activity, the aqueous-mediated activity is negligible. If the enzymes are not dialysed but just diluted into the assay, there is a less

Fig. 1. Demonstration of quinol oxidase mediated by membrane-bound cytochrome *c*

Complex III (112 μM), cytochrome oxidase (121 μM) or the two enzymes mixed in 1:1 molar ratio were dialysed overnight as described in the Materials and methods section. Duroquinol oxidase activity was measured as a function of cytochrome *c* added to the assay with 0.3 μM final concentration of each enzyme. (a) Enzymes dialysed together; (b) enzymes dialysed separately and mixed in the assay.

clear-cut differentiation between the two types of mixing. Probably, the enzymes diluted separately into the assay medium can still associate to some extent because of the presence of residual detergent. Whatever the reason, a convincing demonstration of membrane-mediated activity requires the use of dialysed oxidase and reductase.

Dependence of activity on Complex III and cytochrome oxidase

Fig. 2 shows the reconstituted activity as a function of Complex III concentration. The apparent stoichiometry of interaction of Complex III and oxidase does not have any particular significance. This is because the turnover numbers (catalytic-centre activity) of the individual enzymes are not constant throughout the titration. Thus, cytochrome oxidase activity after dialysis was as low as 16s^{-1} but in the presence of high Complex III concentrations, rose to as high as 80s^{-1} . These values may be contrasted with the activity of oxidase diluted directly into the assay (140s^{-1}). Even this value is much lower than that which has been reported for oxidase under optimal conditions (Vik *et al.*, 1981). The major factor involved here is the state of aggregation of the enzyme. Highest activities can only be obtained in the presence of a dispersing

Fig. 2. Reconstituted activity as a function of complex III/cytochrome oxidase ratio

Mixtures containing Complex III and cytochrome oxidase in the indicated molar ratios were dialysed and assayed for duroquinol oxidase activity as described in the Materials and methods section. The oxidase concentration in the assay was $0.14 \mu\text{M}$ and the cytochrome *c* was $5.5 \mu\text{M}$.

agent such as Tween 80 or lysophosphatidylcholine (Vik *et al.*, 1981), which of course could not be used in the experiments described here. Complex III obviously acts to some extent to prevent oxidase aggregation and in experiments to be described later we show that high concentrations of phospholipid have an activating effect. Complex III turnover varies to a much lesser extent with dialysis and oxidase concentration.

Variation of activity with pH and buffer composition

The reconstituted activity at high levels of cytochrome *c* was much the same whether assayed in 25 mM-acetate/Tris, pH 7.8, 50 mM-Tris/HCl, pH 7 or 8, or 50 mM-potassium phosphate, pH 7 or 8. However, the dependence on cytochrome *c* concentration varied markedly as expected from the known sensitivity of its binding to ionic strength and buffer composition (Ferguson-Miller *et al.*, 1976). Fig. 3 shows a comparison of the cytochrome *c* dependence assayed in acetate/Tris buffer or 50 mM-potassium phosphate, pH 7.0. Although the maximum rates are very similar, the apparent affinity for cytochrome *c* was very much lower in phosphate. In experiments of this type we routinely used 25 mM-acetate/Tris, pH 7.8, to promote cytochrome *c* binding (Dethmers *et al.*, 1979) and a Complex III/oxidase molar ratio of 1:1 to simplify the expression of the results.

Fig. 3. Effect of buffer composition on cytochrome *c* dependence

The reconstituted quinol oxidase contained a 1:1 molar ratio of Complex III to cytochrome oxidase. Dialysed samples ($0.15 \mu\text{M}$ final concentration of each enzyme) were assayed for duroquinol oxidase in 25 mM-acetate/Tris, pH 7.8 (●), or 50 mM-potassium phosphate, pH 7.0 (○) as a function of cytochrome *c* concentration.

Binding of cytochrome *c* to Complex III and oxidase

The decreased activity of aggregated oxidase has been attributed to restricted accessibility of cytochrome *c* to the active site of the enzyme (Vik *et al.*, 1981). To quantify cytochrome *c* binding in experiments such as that of Fig. 3 it was necessary to find out whether the number of cytochrome *c*-binding sites was decreased by aggregation. The measurement of cytochrome *c* binding by gel filtration (Ferguson-Miller *et al.*, 1976) was found to be unsuitable for aggregated enzyme and instead we equilibrated oxidase with cytochrome *c* and separated the bound and unbound ligand by centrifugation. As shown in Fig. 4, the binding of cytochrome *c* was very similar to that reported for dispersed oxidase (Ferguson-Miller *et al.*, 1976; Dethmers *et al.*, 1979) with one high-affinity (K_D approx. $0.1 \mu\text{M}$) and one low-affinity ($K_D = 10 \mu\text{M}$) site per molecule of oxidase. The high-affinity K_D is approximate because the concentration of unbound cytochrome *c* is very low at low molar ratios. Similar assays of binding to Complex III were complicated by the impossibility of completely centrifuging down the enzyme. This also hampered efforts to measure binding to a reconstituted quinol oxidase by this method, although we could see that both high- and low-affinity binding sites for cytochrome *c* were contributed by Complex III as well as oxidase.

The applicability of values obtained from such binding studies to the quinol oxidase system is doubtful because the binding constants seem to depend on the redox state of the system, at least for reductase (Weiss & Juchs, 1978). In view of this and

the experimental difficulties inherent in the centrifugation assays, we adopted a different approach to find an effective K_D for cytochrome *c* in the quinol oxidase system.

Fig. 4. Binding of cytochrome *c* to dialysed cytochrome oxidase

Measurements were made as described in the Materials and methods section.

Binding of cytochrome *c* to reconstituted quinol oxidase

From an experiment such as that of Fig. 3, we were not able to determine K_D directly since we did not know what the relationship was between activity and bound cytochrome *c* and could certainly not assume linearity. We performed similar cytochrome *c* titrations with different concentrations of the reconstituted quinol oxidase. As expected (Fig. 5*a*), at lower concentrations of quinol oxidase, higher cytochrome *c* concentrations were required to give the same specific activity. We then calculated, for each quinol oxidase concentration, theoretical curves for activity as a function of bound cytochrome *c*, assuming different values of K_D . This generated curves such as those of Fig. 5*b*). Looking for the moment at the part of the curve up to approx. 1 mol of cytochrome *c* bound/mol of quinol oxidase, we found that only a value for K_D of $0.1 \mu\text{M}$ generated the same curve irrespective of the concentration of quinol oxidase assayed (Fig. 5*b*). This was determined more exactly by taking the cytochrome *c* concentration that gave 40% of maximum activity for each quinol oxidase concentration, calculating the amount bound and plotting this value against the assumed K_D . As shown in Fig. 6, all lines cross over at $K_D = 0.1 \mu\text{M}$. Using this value, which is the same as that determined for binding of cytochrome *c* to oxidase (Fig. 4), we note that 50% of maximum

Fig. 5. Reconstituted activity as a function of cytochrome *c* concentration

Dialysed quinol oxidase containing a 1:1 molar ratio of Complex III to cytochrome oxidase was assayed for ubiquinol-2 oxidase activity as a function of added cytochrome *c*. The concentration of oxidase (or Complex III) in the assay was 186 nM (●), 37.2 nM (○) or 18.6 nM (▲). (a) Activity as a function of added cytochrome *c* expressed per mol of cytochrome oxidase; (b) activity as a function of bound cytochrome *c* assuming one high-affinity binding site ($K_D = 0.1 \mu\text{M}$) per oxidase and one per Complex III.

Fig. 6. *Determination of effective K_D for cytochrome c* For each curve of Fig. 5(a), curves such as those of Fig. 5(b) were calculated assuming the various indicated values of K_D . The cytochrome c concentration giving 40% of maximal activity was determined from these curves and is plotted as a function of K_D .

activity is reached with approx. 0.5 mol of cytochrome c /mol of quinol oxidase, i.e. with only 25% of the high-affinity sites occupied. The upturn of the curve at 1–2 mol of cytochrome c bound is caused by the low-affinity sites, which have not been taken into account in the calculation. The problem here is that we do not know how many low-affinity sites are present, and, for the reductase, whether any low-affinity binding contributes to activity. If we assume one low-affinity site on the reductase and one on the oxidase with $K_D = 10 \mu\text{M}$ (Fig. 3), we can generate curves that show a linear dependence of activity on bound cytochrome c between 1 and 3 mol per mol of quinol oxidase (results not shown). Whatever the true dependence on bound cytochrome c , the experiments of Figs. 5 and 6 clearly show that low-affinity binding contributes approx. 50% of quinol oxidase activity just as with isolated oxidase.

Effects of phospholipid on quinol oxidase activity

Inclusion of soya-bean phospholipids in the enzyme mixture before dialysis caused a 4-fold stimulation of activity (Fig. 7) to values greater than could be achieved in reconstitution by dilution. The phospholipid requirement was extremely high and we attribute the activation to dispersion of the cytochrome oxidase, whose turnover in the absence of Complex III was increased to 90 s^{-1} . Quinol

Fig. 7. *Effects of phospholipid on reconstituted activity* Quinol oxidase was reconstituted by dialysis from a 1:1 molar ratio of Complex III and cytochrome oxidase in the presence of soya-bean phospholipids. Ubiquinol-2 oxidase was determined with final concentrations of $0.093 \mu\text{M}$ -oxidase and $1.9 \mu\text{M}$ -cytochrome c (1:20 molar ratio). The endogenous phospholipid of the quinol oxidase was $0.20 \mu\text{mol/mg}$ of protein.

Fig. 8. *Antimycin inhibition of reconstituted quinol oxidase*

Quinol oxidase was reconstituted by dialysis with a 1:1 (●) or 1:3 (○) molar ratio of Complex III to oxidase. Samples were incubated with the indicated concentrations of antimycin dissolved in ethanol for 2 h at 0°C before assay. The ethanol concentration in the incubations was 2.5% (v/v). Ubiquinol-2 oxidase activity was determined with final concentrations of $0.092 \mu\text{M}$ -oxidase and $0.46 \mu\text{M}$ -cytochrome c (●) and $0.22 \mu\text{M}$ -oxidase and $0.7 \mu\text{M}$ -cytochrome c (○). Rates in the absence of inhibitor were 1.4 (●) and 0.87 (○) μmol of O/min per mg of oxidase.

oxidase activity was stimulated to a similar extent irrespective of the oxidase/reductase molar ratio. Moreover, although measurements of cytochrome *c* binding showed an increased number of low-affinity binding sites, the cytochrome *c* dependence of activity was exactly the same as in Fig. 3. Therefore, the high-affinity binding at least was unchanged and the cytochrome *c* dependence did not vary with the turnover of the system. Rates in the presence or absence of added phospholipid were not affected by uncoupling agents.

Antimycin inhibition of quinol oxidase

Maximal inhibition occurred at close to 1 mol of antimycin per mol of reductase (Fig. 8). The shape of the titration curve was the same at oxidase/reductase molar ratios of 1:1 or 3:1, i.e. with different turnover numbers of the reductase. Variations of the turnover of the whole system by altering the cytochrome *c* concentration or by including phospholipid also had no effect on the shape of the titration curve (results not shown).

Discussion

The reconstituted quinol oxidase exhibits activity that can be ascribed to binding of cytochrome *c* at both high- and low-affinity binding sites. The K_D values of $0.1 \mu\text{M}$ and $10 \mu\text{M}$ respectively are comparable with those reported for ferricytochrome *c* binding to detergent-solubilized oxidase and reductase under otherwise similar buffer conditions (Dethmers *et al.*, 1979; Weiss & Juchs, 1978). The dependence of activity on bound cytochrome *c* is not linear. In particular, we find that 50% of maximum activity can be achieved by binding of cytochrome *c* to only 25% of the available high-affinity sites. The remaining 50% of activity appears to be related to binding at low-affinity sites, although the exact dependence is unknown. The high-affinity binding results suggest that activity through these sites can be saturated by 1 molecule of cytochrome *c* per oxidase dimer (Robinson & Capaldi, 1977; Bisson *et al.*, 1980) and reductase dimer (von Jagow *et al.*, 1978). This value does not vary with the catalytic-centre activity of the system (varied, for example, by adding phospholipid) and suggests the possibility of a ternary reductase–cytochrome *c*–oxidase complex. This idea is supported by the antimycin titrations, which show no variation in shape with variations in the oxidase/reductase molar ratio. This behaviour contrasts with that found in the reconstituted NADH-ubiquinone reductase–Complex III system (Heron *et al.*, 1978), where the pool function of ubiquinone produces a predictable variation in antimycin titration curves with molar ratio of the two enzymes.

The stoichiometric relationships described above

depend on full availability of the high-affinity sites in the reconstituted quinol oxidase complex. We were not able to accurately determine the total number of sites from binding studies and the stoichiometry shown in Fig. 5 would, of course, be altered if only a fraction of the high-affinity sites were available for binding cytochrome *c*. We do not think that this is happening for a number of reasons. First, the activity of Complex III is not greatly affected by the presence or absence of oxidase. Secondly, activity of cytochrome oxidase increases with increasing Complex III concentration to values approaching those obtained with undialysed (i.e. disaggregated) enzyme. Neither of these findings is consistent with loss of cytochrome *c*-binding sites, which would be expected to decrease the activities of the constituent enzymes. Third, recalculation of the data of Fig. 5(a) assuming, for example, that only 25% of the high-affinity sites are available, leads to curves of the same general shape as those of Fig. 5(b). This means that saturation of activity through the high-affinity sites is still reached with less than half of the available ones occupied (i.e. less than 12.5% of the total). Thus the rather unexpected stoichiometry shown in Fig. 5(b) cannot be circumvented by assuming low availability of high-affinity binding sites.

Inclusion of phospholipid into the reconstituted system has a number of effects on the physical state of the system. First, disaggregation of oxidase results in substantially higher rates of quinol oxidase. Secondly, by analogy with the experiments of Schneider *et al.* (1980) we would expect that the average distance between active complexes in the reconstituted membrane would increase. If indeed the reductase and oxidase do not interact directly but through freely diffusible cytochrome *c*, then one might expect to find a loss of activity particularly at very low cytochrome *c* concentrations, where diffusion could become rate-limiting. Such a loss might be masked by the increase in activity due to disaggregation. However, a further prediction is that this loss of activity should be relieved by increasing the cytochrome *c* concentration. In practice, the cytochrome *c* dependence was the same in the presence or absence of added lipid.

Lastly, in the absence of added phospholipid, the rotational mobility of the oxidase and reductase is extremely low (Swanson *et al.*, 1980; V. M. Poore & C. I. Ragan, unpublished work). This is again likely to be due to protein aggregation, although e.s.r. results show a correspondingly low mobility of the phospholipid (V. M. Poore & C. I. Ragan, unpublished work). Increasing the lipid/protein ratio causes a substantial decrease in rotational correlation time (e.g. 10–100-fold for Complex III; V. M. Poore & C. I. Ragan, unpublished work). In the reconstituted NADH-ubiquinone reductase–

Complex III system, such marked changes in rotational diffusion correlate with alterations in the mode of interaction of the two enzymes. In particular, ubiquinone pool behaviour is not found when the rotational mobility of the proteins is very low (Ragan & Heron, 1978; V. M. Poore & C. I. Ragan, unpublished work). In reconstituted quinol oxidase, these extensive variations in rotational diffusion of the constituent enzymes do not seem to cause any alteration in the mode of their interaction as judged by antimycin titrations and cytochrome *c* dependence. Diffusion of cytochrome *c*, however, might be quite different from diffusion of the oxidase and reductase. The former is a peripheral protein, which might diffuse very rapidly along the membrane surface, whereas the latter are integral proteins, whose diffusion will be greatly affected by the lipid/protein ratio. To try and find conditions where quinol oxidase activity is definitely limited by rotational or lateral diffusion of the component proteins, we are using enzymes whose endogenous phosphatidylcholine and phosphatidylethanolamine have been replaced by dimyristoylglycerophosphocholine. Abrupt losses of quinol oxidase activity below the phase-transition temperature of this lipid are found. Parallel e.s.r. studies with spin-labelled Complex III, cytochrome *c* and oxidase should provide further information on the nature of their interaction.

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References

- Bartlett, G. R. (1969) *Methods Enzymol.* **14**, 485–487
- Bisson, R., Jacobs, B. & Capaldi, R. A. (1980) *Biochemistry* **19**, 4173–4178
- Bosshard, H. R., Zürcher, M., Schägger, H. & von Jagow, G. (1979) *Biochem. Biophys. Res. Commun.* **89**, 250–258
- Brautigan, D. L., Ferguson-Miller, S. & Margoliash, E. (1978) *Methods Enzymol.* **53**, 128–164
- Capaldi, R. A. & Hayashi, H. (1972) *FEBS Lett.* **26**, 261–263
- Dethmers, J. K., Ferguson-Miller, S. & Margoliash, E. (1979) *J. Biol. Chem.* **254**, 11973–11981
- Erecinska, M., Davis, J. S. & Wilson, D. F. (1980) *J. Biol. Chem.* **255**, 9653–9658
- Errede, B. & Kamen, M. D. (1978) *Biochemistry* **17**, 1015–1027
- Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1976) *J. Biol. Chem.* **251**, 1104–1115
- Fowler, L. R. & Richardson, S. W. (1963) *J. Biol. Chem.* **238**, 456–463
- Hatefi, Y., Haavik, A. G. & Griffiths, D. E. (1962a) *J. Biol. Chem.* **237**, 1681–1685
- Hatefi, Y., Haavik, A. G., Fowler, L. R. & Griffiths, D. E. (1962b) *J. Biol. Chem.* **237**, 2661–2669
- Heron, C., Ragan, C. I. & Trumpower, B. L. (1978) *Biochem. J.* **174**, 791–800
- Klotz, I. M. & Hunston, D. L. (1971) *Biochemistry* **10**, 3065–3069
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ragan, C. I. & Heron, C. (1978) *Biochem. J.* **174**, 783–790
- Ragan, C. I. & Racker, E. (1973) *J. Biol. Chem.* **248**, 2563–2569
- Rieder, R. & Bosshard, H. R. (1980) *J. Biol. Chem.* **255**, 4732–4739
- Rieske, J. S. (1967a) *Methods Enzymol.* **10**, 239–245
- Rieske, J. S. (1967b) *Methods Enzymol.* **10**, 488–493
- Robinson, N. C. & Capaldi, R. A. (1977) *Biochemistry* **16**, 375–382
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660
- Schneider, H., Lemasters, J. J., Höchli, M. & Hackenbrock, C. R. (1980) *J. Biol. Chem.* **255**, 3748–3756
- Smith, L., Davies, H. C. & Nava, M. E. (1979) *Biochemistry* **18**, 3140–3146
- Speck, S. H., Ferguson-Miller, S., Osheroff, N. & Margoliash, E. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 155–159
- Swanson, M. S., Quintanilha, A. T. & Thomas, D. D. (1980) *J. Biol. Chem.* **255**, 7494–7502
- Tzagoloff, A., MacLennan, D. H., McConnell, D. G. & Green, D. E. (1967) *J. Biol. Chem.* **242**, 2051–2061
- Van Buuren, K. J. H., Van Gelder, B. F., Wilting, J. & Braams, R. (1974) *Biochim. Biophys. Acta* **333**, 421–429
- Vik, S. B., Georgevich, G. & Capaldi, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1456–1460
- Von Jagow, G., Schägger, H., Engel, W. D., Riccio, P., Kolb, H. J. & Klingenberg, M. (1978) *Methods Enzymol.* **53**, 92–98
- Weiss, H. & Juchs, B. (1978) *Eur. J. Biochem.* **88**, 17–28