Cytochrome *c* mediates electron transfer between ubiquinol-cytochrome *c* reductase and cytochrome *c* oxidase by free diffusion along the surface of the membrane

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Ubiquinol oxidase can be reconstituted from ubiquinol-cytochrome *c* reductase (Complex III) and cytochrome *c* oxidase (Complex IV) whose endogenous phosphatidylcholine and phosphatidylethanolamine have been replaced by dimyristoylglycerophosphocholine. Phase transition of the lipid has no effect on Complex III and Complex IV activities assayed separately, but ubiquinol oxidase activity rapidly decreases as the temperature is lowered through the phase transition. A spin-labelled yeast cytochrome *c* derivative has been synthesized. Binding of the cytochrome *c* to liposomes demonstrates that only cardiolipin is involved under the conditions used for the ubiquinol oxidase experiments. In liposomes consisting of cardiolipin and dimyristoylglycerophosphocholine, e.s.r. (electron-spin-resonance) measurements show that rotational diffusion of cytochrome *c* is slowed in the gel phase of the latter lipid. We propose that the cytochrome *c* pool is bound to cardiolipin molecules, whose lateral and rotational diffusion in the bilayer is adequate to account for electron-transport rates.

In the preceding paper (Froud & Ragan, 1984) we have presented detailed kinetic evidence that cytochrome *c* acts as a mobile pool in the membrane linking ubiquinol-cytochrome *c* reductase (ubiquinol: ferricytochrome *c* oxidoreductase, EC 1.10.2.2) with cytochrome *c* oxidase (ferrocycytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1). Thus we discount models of electron transfer which involve direct association of the reductase and oxidase, and favour a mechanism involving binding of cytochrome *c* to the reductase followed by reduction, complete dissociation, diffusion on the membrane, binding to the oxidase, and so on. In the present paper we concentrate on the diffusion of cytochrome *c* on the membrane. Studies of isolated cytochrome *c* reductase or oxidase can not address this point directly, although several groups of workers have concluded that free diffusion of cytochrome *c* is the likely mechanism of electron transport (Capaldi *et al.*, 1982; Koppenol & Margoliash, 1982). In other studies, free diffusion has been inferred from the absence of evidence for direct association of the

oxidase and reductase (Höchli & Hackenbrock, 1979; Kawato *et al.*, 1981), whereas estimates of cytochrome *c* diffusion rates have indicated a strong probability that the process could be a kinetically competent step in the overall pathway (Kawato *et al.*, 1981, 1982). The manner in which cytochrome *c* interacts with phospholipid bilayers is obviously relevant to the proposed model, and results of several studies have been published. Preferential binding of cytochrome *c* to acidic phospholipids such as cardiolipin has been widely recognized (Green & Fleischer, 1963; Nicholls, 1974; Van & Griffith, 1975), and generally it seems agreed that cytochrome *c* binds to the phospholipid headgroup region without deep penetration of the bilayer (Van & Griffith, 1975; Brown & Wüthrich, 1977). Of particular interest is the evidence that cytochrome *c* binds to membranes with a preferred orientation (Vanderkooi *et al.*, 1973; Brown & Wüthrich, 1977; Teissie, 1981). It seems that the haem crevice remains accessible to the aqueous phase, and therefore that the binding domain is not the same as that involved in the association of cytochrome *c* with its oxidase or reductase. An obviously useful tool for studying orientation and molecular mobility of cytochrome *c* would be a

Abbreviations used: MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy; TMPD, *NNNN*-tetramethylphenylenediamine; τ_c , rotational correlation time.

spin-labelled derivative in which the spin probe accurately reported the motion of the whole protein. Many attempts have been made to synthesize such a derivative (Barrett *et al.*, 1968; Drott *et al.*, 1970; Azzi *et al.*, 1972; Melhorn *et al.*, 1980), but all so far produced have exhibited rotation of the spin label independent of the protein molecule, and therefore they have been of limited use. In the present paper we report partial success at synthesizing a spin-labelled molecule with the desired properties and describe its interaction with cytochrome *c* reductase, cytochrome oxidase and phospholipid bilayers. We have also exploited the flexibility of the reconstituted ubiquinol oxidase system to exchange endogenous phosphatidylcholine and phosphatidylethanolamine for a synthetic phospholipid, dimyristoylglycerophosphocholine. Thus we have been able to provide direct evidence that free diffusion of cytochrome *c* on the membrane is necessary for ubiquinol oxidase by showing inhibition of such activity in gel-phase lipid.

Materials and methods

Preparations

Complexes III and IV were purified and molarities assayed as described in the preceding paper (Froud & Ragan, 1984). Horse heart and yeast cytochrome *c* were obtained from Sigma, Poole, Dorset, U.K. Solutions were standardized as described by Ferguson-Miller *et al.* (1976) and Fuller *et al.* (1981) respectively. Reduced horse heart cytochrome *c*, free of polymeric forms, was prepared by the method of Brautigan *et al.* (1978). Solutions were stored at -20°C .

Chemicals

Dimyristoylglycerophosphocholine was from Koch-Light, Colnbrook, Bucks., U.K. Egg phosphatidylcholine (Grade 1) and egg phosphatidylethanolamine (Grade 1) were from Lipid Products, Redhill, Surrey, U.K.; ox heart cardiolipin was from Sigma, and soya-bean phospholipids were prepared by the method of Ragan & Racker (1973). Ubiquinol-2 was prepared and stored as in the preceding paper (Froud & Ragan, 1984). The spin label, (MSL) was from Molecular Probes, Plano, TX, U.S.A.

Assays of activity

Complex III was assayed in 1 ml final volume containing 25 mM-acetate/Tris, pH 7.8, 30 μM -ubiquinol-2, 0.01 mM-EDTA, 25 μM -cytochrome *c* and enzyme. Reduction of cytochrome *c* was monitored at 550 nm by using an absorption coefficient of 20000 $\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Ferguson-Miller *et al.*,

1976). Rates were corrected for non-enzymic reduction of cytochrome *c* by the ubiquinol-2.

Complex IV was assayed by three methods. The first involved polarographic measurement of O_2 uptake with Ruthenium Red as electron donor (Schwerzmann *et al.*, 1976). Commercial preparations of the stain Ruthenium Red contain a colourless impurity, $\text{Ru}(\text{NH}_3)_6^{2+}$, which is the true donor to cytochrome oxidase (Hochman *et al.*, 1981). The assay contained, in a final volume of 2 ml, 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, 0.04 mg of Ruthenium Red/ml, 2.8 mM-ascorbate and enzyme. The rate of O_2 uptake was corrected for non-enzymic reduction by subtraction of the rate obtained in the presence of 0.05 mM- NaN_3 . Calibration of the oxygen content of the buffer at different temperatures was carried out by measuring the change in signal caused by oxidation of a standard amount of NADH by submitochondrial particles from ox heart, essentially as described by Estabrook (1967).

The second assay employed ascorbate and TMPD as reductants of cytochrome *c*, O_2 uptake being monitored polarographically. The assay contained, in a final volume of 2 ml, 25 mM-acetate/Tris, pH 7.8, 17.5 mM-ascorbate, 35 μM -EDTA, 0.5 mM-TMPD, 25 μM -horse heart cytochrome *c* and enzyme. O_2 uptake was calibrated as described above. This assay is essentially similar to one described by Ferguson-Miller *et al.* (1976).

The third assay used reduced horse heart cytochrome *c* as substrate, oxidation being monitored spectrophotometrically. The assay contained, in a final volume of 1.3 ml, 25 mM-acetate/Tris, pH 7.8, 0.8 μM -reduced cytochrome *c* and enzyme. Oxidation was monitored at 418 nm minus 410 nm, an absorption coefficient of 44500 $\text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Margoliash & Frohwirt, 1959) being used. This assay was based on that described by Smith *et al.* (1979).

Ubiquinol oxidase was reconstituted as described by Diggins & Ragan (1982) and assayed by the method given in the preceding paper (Froud & Ragan, 1984). Phospholipid phosphorus was measured by the method of Bartlett (1969), and protein by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. Protein thiol groups were assayed by reaction with 5,5'-dithio-bis-(2-nitrobenzoate) as described by Habeeb (1972).

Lipid replacement of Complexes III and IV

The method was based on that of Heron *et al.* (1979). Dimyristoylglycerophosphocholine was solubilized in 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, containing 0.7% (w/v) sodium cholate by ultrasonic dispersion to give a final concentration

of 60 mM. Complex III (10 mg of protein/ml of sucrose/Tris buffer) was mixed with an equal volume of lipid solution and after 20 min at 0°C the enzyme was precipitated by addition of 100%-saturated, neutralized $(\text{NH}_4)_2\text{SO}_4$ to 53% saturation. After 10 min the enzyme was collected by centrifugation at 37 000 *g* for 20 min, dissolved in the original volume of sucrose/Tris buffer and the replacement procedure repeated. After rinsing of the final pellet with 1 vol. of buffer plus 1 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$, the enzyme was dissolved in sucrose/Tris buffer and stored at -70°C. Complex IV was treated similarly, except that incubation with lipid was for 60 min and centrifugation was at 47 000 *g* for 30 min. The progress of lipid exchange was monitored by t.l.c. as described by Heron *et al.* (1977).

Temperature-dependence of activity

To compensate for the effects of temperature on pH, buffers were made at temperature intervals of 5°C so that in any assay the buffer was used no more than 2.5°C away from its preparation temperature. Assays were carried out in a water-jacketed vessel or cuvette and temperatures were measured by a thermistor in the assay mixture.

Spin-labelling of cytochrome *c*

Freeze-dried yeast cytochrome *c* (0.8 μmol) was dissolved in 0.2 ml of 0.25 M-sucrose/25 mM-acetate/Tris, pH 7.0, and 0.12 mmol of 2-mercapto-ethanol added from a 0.3 M-stock solution in the same buffer to ensure maximum availability of free thiol groups. After 15 min the cytochrome *c* was separated by chromatography on a column (20 cm $\times$ 0.8 cm internal diam.) of Sephadex G-25 equilibrated with sucrose/acetate buffer. The cytochrome *c* (0.35–0.38 mM) was allowed to react with 1 molar equivalent of MSL, freshly prepared as a 10 mM solution in ethanol, for 1 h at room temperature. The mixture was then dialysed against 1000 vol. of sucrose/acetate buffer at 4°C overnight and the cytochrome *c* solution standardized spectrophotometrically.

Spin-labelling of Complex III and Complex IV

This followed the methods described by Swanson *et al.* (1980) and Poore *et al.* (1982). Complex III (0.08 mM) or Complex IV (0.12 mM) in 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, was allowed to react with a 5-fold molar excess of MSL for 1 h at room temperature. The sample was then dialysed against 1000 vol. of sucrose/acetate buffer at 4°C overnight. The solution was centrifuged at 48 000 *g* for 100 min to pellet the enzyme, which was then taken up in the minimal volume of sucrose/acetate buffer which allowed transfer to the e.s.r. cell. Where indicated, soya-bean phospholipid was

added to the enzyme prior to dialysis (Poore *et al.*, 1982).

Binding of cytochrome *c* to liposomes

Stock solutions of phospholipids (egg phosphatidylcholine, egg phosphatidylethanolamine or heart cardiolipin) in chloroform or ethanol were mixed in the desired proportions and the solvent removed under a stream of N_2 . The lipids were dissolved in a small volume of diethyl ether and again evaporated to dryness with a stream of N_2 and finally under vacuum for 15 min. Lipids were suspended in 25 mM-acetate/Tris, pH 7.0, by vigorous vortex-mixing under N_2 to give final phosphorus concentrations of approx. 10 mM. Yeast cytochrome *c*, either native or spin-labelled (12–30 nmol), was mixed with the liposome suspension, which was then centrifuged at 12 000 *g* for 20 min. Free cytochrome *c* in the supernatant was assayed spectrophotometrically, the pelleted liposomes being used for e.s.r. experiments. It was essential to carry out all operations under an atmosphere of N_2 to prevent destruction of both the visible and e.s.r. spectra of the cytochrome by the lipids.

E.s.r. spectroscopy

All spectra were recorded on a Bruker ER 200D spectrometer equipped with a N_2 -gas-flow- and variable-temperature-controlled unit. The spectrometer was interfaced to a Cromemco minicomputer system for accumulation and storage of spectra on disc files. A quartz flat cell was used for all samples. Detailed conditions are given in the Figure and Table legends. Saturation transfer (V'_2) spectra were obtained on this instrument as described by Poore *et al.* (1982), including the procedures for determination of the correct phase setting and the microwave field strength in the cavity. Rotational correlation times (τ_c) in the range $10^{-11} \text{ s} < \tau_c < 10^{-9} \text{ s}$ were determined from V_1 spectra from the width of the midline (ω_0) and the heights of the mid- and high-field lines (h_0 and h_{-1} respectively) according to the formula:

$$\tau_c = 6.5 \times 10^{-10} \omega_0 [(h_0/h_{-1})^{\frac{1}{2}} - 1]$$

τ_c values in the range $10^{-7} \text{ s} < \tau_c < 10^{-3} \text{ s}$ were determined from V'_2 spectra using either the heights of the low-field lines (L and L') or the heights of the high-field lines (H and H') as described by Thomas *et al.* (1976). The calibration curve of Thomas *et al.* (1976) was also used.

Results

Replacement of endogenous phospholipids with dimyristoylglycerocephosphocholine

Heron *et al.* (1977, 1979) showed that the endogenous phosphatidylcholine and phosphati-

Table 1. *Reconstitution with detergent-depleted Complexes*
Complexes III and IV were dialysed separately for 7h or together in a 1:1 molar ratio for 21h against 500 vol. of 0.67M-sucrose/50mM-Tris/HCl, pH 8.0, at 4°C. The activity of the co-dialysed enzymes was taken as 100%. The separately dialysed enzymes (protein concentrations 5mg/ml or greater) were mixed in a 1:1 molar ratio in the presence of 0.8M-KCl and various concentrations of sodium deoxycholate. After further dialysis for 14h, samples were assayed for ubiquinol oxidase. The assay contained 0.09 μ M-Complex III or -Complex IV and 0.46 μ M-cytochrome *c*.

Conditions	[Deoxycholate] (%, w/v)	Activity (% of maximal)
Separate dialysis for 21h	—	12
Separate dialysis for 7h, then co-dialysis for 14h	0	44
	0.01	94
	0.02	95
	0.05	91
	0.10	85
	0.20	65

dylethanolamine of NADH-ubiquinone reductase and Complex III could be quantitatively replaced with dimyristoylglycerophosphocholine by cholate-mediated exchange followed by precipitation with $(\text{NH}_4)_2\text{SO}_4$. Lipid-replaced Complex III was rather unstable and tended to lose activity after freezing and thawing (Heron *et al.*, 1979). The endogenous cardiolipin of either enzyme was not replaced at the low cholate concentration (0.35% [w/v]) employed in the present work. We have applied a similar method to Complex IV, as described in the Materials and methods section. Replacement of endogenous lipids was monitored qualitatively by t.l.c. (Heron *et al.*, 1977), which showed that longer incubation times (50 min) with dimyristoylglycerophosphocholine and cholate were required for exchange of Complex IV lipids compared with Complex III lipids. After two cycles of replacement, very little endogenous phosphatidylcholine or phosphatidylethanolamine remained, but cytochrome oxidase activity was largely preserved (up to 55% of the original activity).

Reconstitution of ubiquinol oxidase with lipid-replaced enzymes

Problems in reconstitution with lipid-replaced enzymes were encountered by Heron *et al.* (1979) in studies of the association of NADH-ubiquinone reductase with Complex III. This was attributed to insufficient detergent in the lipid-replaced samples prior to mixing, and the problem was overcome by adding deoxycholate and KCl. Following the same

lines, we investigated the detergent concentration required to achieve efficient reconstitution with Complexes III and IV which had been previously dialysed separately. As shown by us previously (Diggens & Ragan, 1982), ubiquinol oxidase activity in mixtures of separately dialysed enzymes is very poor as it is mediated only by soluble cytochrome *c* moving between separate membranes. The results of such experiments are shown in Table 1. Maximum activity (100%) was taken as that found in ubiquinol oxidase reconstituted from the original, detergent-sufficient, enzymes, which had then been co-dialysed. After separate dialysis for 21h, the enzymes subsequently reassociated very poorly (activity only 12%). After separate dialysis for 7h followed by co-dialysis for 14h, rather better activity was achieved (44%). However, near-maximal activity was reached if, under otherwise identical conditions, 0.8M-KCl and 0.01–0.02% (w/v) sodium deoxycholate was included before the co-dialysis step. Higher concentrations of deoxycholate were inhibitory. A concentration of 0.02% (w/v) sodium deoxycholate was therefore adopted as standard for reconstitutions involving lipid-replaced Complexes III and IV. As in the work presented in the preceding paper (Froud & Ragan, 1984), we have employed two levels of phospholipid-to-protein ratio in the reconstituted ubiquinol oxidase. The lower level, that which was present in enzymes after isolation or after lipid replacement, varied from 0.2 to 0.4 μ mol of phospholipid P/mg of protein. The higher level (3 μ mol of phospholipid P/mg of protein) was achieved by addition of soya-bean phospholipids or dimyristoylglycerophosphocholine as appropriate and gave maximal activation of the cytochrome oxidase (Diggens & Ragan, 1982). The higher lipid level might also increase the average diffusion path between Complex III and Complex IV molecules (e.g. Hackenbrock, 1981).

Temperature-dependence of Complex III activity

Fig. 1 shows that the temperature-dependence of ubiquinol-2-cytochrome *c* reductase activity was unaffected by the phospholipid-to-protein ratio, and although activation energies were somewhat different for the lipid-replaced enzyme, there was no evidence for any effect of transition of the lipid into the gel state, since there was no abrupt change of either slope or position of the Arrhenius plot below 25°C (Ladbrooke & Chapman, 1969). Lipid-replaced Complex III is rather unstable, particularly at higher temperatures (Heron *et al.*, 1979), and this may well account for the flattening of the Arrhenius plots above 26°C ($10^3/\text{temperature less than } 3.35$). That dimyristoylglycerophosphocholine associated with Complex III does indeed undergo phase transition has been amply demonstrated

Fig. 1. *Temperature-dependence of Complex III activity* Complex III or lipid-replaced Complex III was dialysed in the presence or absence of a phospholipid supplement as described previously for reconstitution of ubiquinol oxidase (Diggens & Ragan, 1982; Froud & Ragan, 1984). Assays of ubiquinol-2-cytochrome *c* reductase were carried out as described in the Materials and methods section. ○, Complex III containing natural lipids (0.30 μmol of phospholipid P/mg of protein); ●, Complex III supplemented with soya-bean phospholipids (total of 3 μmol of phospholipid P/mg of protein); △, lipid-replaced Complex III (0.36 μmol of phospholipid P/mg of protein); ▲, lipid-replaced Complex III supplemented with dimyristoylglycerophosphocholine (total of 3 μmol of phospholipid P/mg of protein).

previously (Heron *et al.*, 1979; Poore & Ragan, 1982; Poore *et al.*, 1982).

Temperature-dependence of Complex IV activity

Unlike Complex III, Complex IV can be assayed by several procedures which examine different aspects of the reaction. The simplest is the use of $\text{Ru}(\text{NH}_3)_6^{2+}$ as electron donor (Hochman *et al.*, 1981), since this does not involve cytochrome *c* at all. Arrhenius plots of this activity were linear, regardless of the nature or concentration of the lipid associated with the enzyme, and activation energies were in the range 70–80 kJ/mol (results not shown).

The second assay involved donation of electrons by cytochrome *c* maintained reduced with ascorbate and TMPD. Hill & Nichols (1980) showed that reduced TMPD reduces cytochrome *c* more rapidly when the latter is bound to cytochrome oxidase than when it is free in solution. Thus several turnovers of cytochrome oxidase can occur without dissociation of cytochrome *c* from the high-affinity binding site (Smith *et al.*, 1979) and

Fig. 2. *Temperature-dependence of Complex IV activity* Complex IV or lipid-replaced Complex IV was dialysed in the presence or absence of a phospholipid supplement and assayed for activity using reduced cytochrome *c* as substrate (see the Materials and methods section). ○, Complex IV containing natural lipids (0.20 μmol of phospholipid P/mg of protein); ●, Complex IV supplemented with soya-bean phospholipids (total of 3 μmol of phospholipid P/mg of protein); △, lipid-replaced Complex IV (0.40 μmol of phospholipid P/mg of protein); ▲, lipid-replaced Complex IV supplemented with dimyristoylglycerophosphocholine (total of 3 μmol of phospholipid P/mg of protein).

this assay measures cytochrome oxidase activity without the complication of cytochrome *c* binding and dissociation. Arrhenius plots of this activity were rather curved (for unknown reasons), but were unaffected by the nature or concentration of the phospholipid, and activation energies were similar, at lower temperatures, to those obtained with $\text{Ru}(\text{NH}_3)_6^{2+}$ as donor.

The third assay used chemically reduced cytochrome *c* as donor. Rates are lower than with ascorbate/TMPD as reductant, since an oxidized cytochrome *c* must dissociate before a new reduced cytochrome can bind (Smith *et al.*, 1979) and the former step may well be rate-limiting (e.g. Capaldi *et al.*, 1982). Even though this assay involves cytochrome *c* diffusion on and off the enzyme, Arrhenius plots were again unaffected by phase transition of the lipid or the lipid concentration (Fig. 2).

The different cytochrome oxidase assays provide some indication of the step which is involved in stimulation of cytochrome oxidase activity by disaggregation. Diggens & Ragan (1982) found that the number of cytochrome *c*-binding sites was not decreased by aggregation of the enzyme, and therefore that the stimulation of activity by

Fig. 3. Temperature-dependence of ubiquinol oxidase activity

Ubiquinol oxidase was reconstituted by dialysis in the presence or absence of a phospholipid supplement. The Complex III to IV ratio was 1:1. Activity was assayed spectrophotometrically (Diggens & Ragan, 1982; Froud & Ragan, 1984) with $0.12\mu\text{M}$ -Complex III or -Complex IV ($\circ$, $\bullet$) or $0.15\mu\text{M}$ -Complex III or -Complex IV (Δ , $\blacktriangle$) and $0.46\mu\text{M}$ -cytochrome *c* ($\circ$, $\bullet$) or $1.85\mu\text{M}$ -cytochrome *c* (Δ , $\blacktriangle$). $\circ$, Ubiquinol oxidase containing natural lipids ($0.20\mu\text{mol}$ of phospholipid P/mg of protein); $\bullet$, ubiquinol oxidase supplemented with soya-bean phospholipids (total of $3\mu\text{mol}$ of phospholipid P/mg of protein); Δ , lipid-replaced ubiquinol oxidase ($0.35\mu\text{mol}$ of phospholipid P/mg of protein); $\blacktriangle$, lipid-replaced ubiquinol oxidase supplemented with dimyristoylglycerophosphocholine (total of $3\mu\text{mol}$ of phospholipid P/mg of protein).

phospholipid must be exerted on a reaction step. In all these assays employed here, oxidase activity was increased by phospholipid. However, activity with reduced cytochrome *c* as donor was increased up to 5-fold more than activity with other donor systems (where the stimulation was only 1–3-fold). This was true whether soya-bean or synthetic lipids were used. Thus the major step involved in stimulation by disaggregation may be the off-rate for cytochrome *c*.

Temperature-dependence of ubiquinol oxidase

Since there was no evidence for effects of lipid phase transition on either Complex III or Complex

IV activities alone, it was of interest to see how reconstituted ubiquinol oxidase behaved. As shown in Fig. 3, Arrhenius plots were slightly curved for the enzyme containing natural lipids, but extremely curved with lipid-replaced enzymes, activity rapidly falling to zero below 20°C . Similar effects were seen at high or low lipid-to-protein ratios, except that the former rates were as much as 10-fold greater than the latter. This supports the idea that dissociation of cytochrome *c* is an integral step in the ubiquinol oxidase mechanism.

The experiments of Fig. 3 show that ubiquinol oxidase is not active in gel-phase lipid, and Figs. 1 and 2 show that the effect can only be exerted on the diffusion of cytochrome *c* between the two Complexes and not on oxidoreduction of cytochrome *c* by the enzymes. These results also show that diffusion must be in the membrane rather than through cytochrome *c* in solution, despite the presence of free cytochrome *c* in the ubiquinol oxidase assays.

Synthesis of spin-labelled cytochrome *c*

Previous attempts to spin-label cytochrome *c* have employed both the ox heart and yeast proteins. The former protein has been modified either at surface lysine residues (Barratt *et al.*, 1968; Melhorn *et al.*, 1980) or at methionine-65 (Azzi *et al.*, 1972), whereas the yeast protein has been modified at cysteine-103 with a spin-labelled bromoacetamide (Drott *et al.*, 1970). All such preparations showed τ_c values in the range 0.2–1.0 ns, considerably smaller than that expected if the spin-label was reporting the motion of the entire protein molecule. We encountered the same problem with a variety of commercially available spin-labelled protein modifiers, and the longest correlation times were obtained by modifying yeast cytochrome *c* with MSL. After reduction with 2-mercaptoethanol, yeast cytochrome *c* contained 0.8 mol of free thiol groups/mol of protein (from cysteine-103). After reaction with 1 mol of MSL/mol of protein, this was decreased to 0.1 mol/mol of protein and, moreover, prior reaction of the thiol groups with 5,5'-dithiobis-(2-nitrobenzoate) prevented spin-labelling by MSL. Thus spin-labelling was confined completely to the cysteine residue. The rotational correlation time in 0.25 M-sucrose (Fig. 4a) was 2 ns, slower than for other spin-labelled derivatives, but nevertheless still faster than that calculated for the cytochrome *c* molecule (5 ns; Azzi *et al.*, 1972) under the same conditions. Variation of reaction conditions, such as pH, time and concentration of MSL, did not produce any improvement. Fig. 4(b) shows that the spectrum is in fact composed of two signals with different correlation times. The spin-labelled cytochrome *c* was bound to CM-cellulose through

Fig. 4. V_1 e.s.r. spectra of MSL-labelled cytochrome *c*

(a) MSL-labelled yeast cytochrome *c* in 0.25 M-sucrose/25 mM-acetate/Tris, pH 7.0 at 20°C. Microwave frequency was 9.3 GHz, microwave power 1 mW, modulation amplitude 0.1 mT (1.0 G), modulation frequency 100 kHz, scan width 10 mT (100 G). (b) MSL-labelled yeast cytochrome *c* absorbed on to CM-cellulose in the same buffer. Conditions were as for (a), but with a modulation amplitude of 0.25 mT (2.5 G). (c) MSL-labelled yeast cytochrome *c* bound to liposomes. Liposomes containing egg phosphatidylcholine and cardiolipin (4:1 by phospholipid P) were prepared as described in the Materials and methods section at a final lipid concentration of 13.7 mM-phospholipid P. MSL-labelled cytochrome *c* was added under N_2 to give a molar ratio of cytochrome to lipid of 1:250. The liposomes were pelleted by centrifugation (12000g for 20 min) and resuspended in sucrose/acetate buffer. E.s.r. conditions were as in (a). The spectra have been scaled to have approximately the same central peak height.

its surface lysine residues, thus effectively immobilizing the protein. The resulting e.s.r. spectrum clearly reveals two components. The more immobilized component had a large hyperfine splitting at the rigid limit of 6.3–6.4 mT (63–64 G) (Freed, 1976). Thus, for reasons that are not clear, reaction at a single amino acid residue had produced two spin-label environments, one which had rotational motion independent of the protein molecule and one which did not. The presence of the former signal severely restricted the usefulness of the spin-probe and, in general, only qualitative conclusions could be drawn, as described below.

Binding of cytochrome c to Complex III and Complex IV

In Fig. 5 we compare the conventional (V_1) and saturation-transfer (V'_2) spectra of Complex IV spin-labelled with MSL (Swanson *et al.*, 1980) and MSL-labelled cytochrome *c* bound to the oxidase in the high-affinity site. The V_1 spectrum of MSL-labelled Complex IV shows a predominantly immobile spin environment with only a small contribution from a weakly immobilized signal. The V'_2 spectrum indicates a τ_c for the major component of at least 1 ms. This results from the low lipid-to-protein ratio of this sample (e.g.

Swanson *et al.*, 1980; Poore *et al.*, 1982). The spectra of MSL-labelled cytochrome *c* bound to the oxidase are complicated by the presence of the large mobile component. Nevertheless, the V_1 spectrum strongly resembles that of Fig. 4(b) for cytochrome *c* bound to CM-cellulose, whereas the low-field parameters (L, L') and, more reliably perhaps, the high-field parameters (H, H') of the V'_2 spectrum indicate that cytochrome *c* (bound in the high-affinity site) is not independently mobile of the oxidase on a millisecond time scale. This might indicate that cytochrome *c* is unlikely to mediate electron transfer between Complex III and Complex IV by rotation while still bound to the Complexes (Ferguson-Miller *et al.*, 1979). Increasing the lipid-to-protein ratio from 0.16 $\mu\text{mol/mg}$ of protein up to 2.9 $\mu\text{mol/mg}$ of protein caused a progressive decrease in τ_c for Complex IV down to a limiting value of 50 μs , thus directly demonstrating that phospholipid disaggregates the enzyme. Unfortunately, we could not monitor the effect on cytochrome *c* rotation, since high lipid-to-protein ratios rapidly destroyed the e.s.r. signal of the MSL-labelled yeast cytochrome *c*.

Comparable experiments with Complex III produced quite different results (Fig. 6). The V_1 and V'_2 spectra of MSL-labelled Complex III

Fig. 5. V_1 and V_2 e.s.r. spectra of MSL-labelled Complex IV and MSL-labelled cytochrome *c* bound to Complex IV

(a) V_1 E.s.r. spectrum of MSL-labelled Complex IV in sucrose/acetate buffer at 20°C. Conditions were as described in Fig. 4(a), except that the modulation amplitude was 0.25 mT (2.5 G). (b) V_2 spectrum of MSL-labelled Complex IV in sucrose/acetate buffer at 20°C. Microwave frequency was 9.3 GHz, microwave power 38 mW, modulation amplitude 0.5 mT (5 G), modulation frequency 50 kHz, and phase sensitive detection was at 100 kHz (second harmonic) out of phase. Rotational correlation times were estimated from the heights L , L'' , H and H'' . (c) V_1 spectrum of MSL-labelled cytochrome *c* bound to Complex IV. MSL-labelled cytochrome *c* (approx. 100 μ M) and dialysed Complex IV (approx. 100 μ M) were mixed in 2:1 molar ratio and incubated at 4°C for 15 min before centrifugation at 48 000 *g* for 45 min. The pellet was resuspended in the minimal volume of sucrose/acetate buffer for transfer to the flat cell. E.s.r. conditions were as in (a). (d) V_2 spectrum of sample from (c). E.s.r. conditions were as in (b). The spectra have been scaled for convenience of presentation.

(Poore *et al.*, 1982) again showed a predominantly immobilized spin environment with $\tau_c \geq 1$ ms. However, MSL-labelled cytochrome *c* was considerably less immobilized when bound to Complex III, as judged from the V_1 spectra, and it was not possible to estimate the τ_c value. This might imply that cytochrome *c* bound to Complex III still exhibits a measure of independent motion. On the other hand the off-rate for cytochrome *c*, even under tight-binding conditions, might be adequate to cause an apparent increase in the τ_c by averaging of spectra from slowly and rapidly mobile components, although this is unlikely.

Fig. 6. V_1 and V_2 spectra of MSL-labelled Complex III and MSL-labelled cytochrome *c* bound to Complex III

(a) V_1 E.s.r. spectrum of MSL-labelled Complex III. Conditions were the same as in Fig. 5(a). (b) V_2 spectrum of MSL-labelled Complex III. Conditions were as in Fig. 5(b). (c) V_1 spectrum of MSL-labelled cytochrome *c* bound to Complex III. MSL-labelled cytochrome *c* (approx. 100 μ M) and dialysed Complex III (approx. 100 μ M) were mixed in 1:1 molar ratio and incubated at 4°C for 15 min before centrifugation at 48 000 *g* for 100 min. The pellet was resuspended in the minimal volume of sucrose/acetate buffer for transfer to the flat cell. E.s.r. conditions were as in Fig. 5(a). Scan width was 10 mT (100 G) in all instances.

Binding of cytochrome *c* to phospholipid bilayers

A large number of studies of cytochrome *c* binding to lipid bilayers have been performed. The main features of the findings are that the protein binds strongly to acidic lipids such as cardiolipin, less strongly to phosphatidylethanolamine and not at all to phosphatidylcholine. However, because of the widely differing conditions of these experiments, it is difficult to relate them to our experiments with ubiquinol oxidase. We have therefore looked at binding of cytochrome *c* to lipid mixtures under conditions approximating to those that we have used in reconstituted ubiquinol oxidase. In these experiments the phospholipid content varied between approx. 50 and 700 mol/mol of Complex III or Complex IV. The cytochrome *c* content varied between 1 and 10 mol/mol of Complex III or Complex IV. These ranges would also cover the concentrations encountered in natural mitochondrial membranes. Birrell & Griffith (1976) measured a dissociation constant

of around 1×10^{-5} M for cytochrome *c* bound to phospholipid bilayers. We would therefore expect the phospholipid classes involved in lateral diffusion of cytochrome *c* to exhibit affinity for the protein in this range.

Initial experiments involved incubating cytochrome *c* with liposomes of various compositions and measuring binding by the loss of cytochrome *c* from solution after centrifugation. No binding of cytochrome *c* to mixtures of phosphatidylcholine and phosphatidylethanolamine was found, even under low-ionic-strength conditions and molar ratios of lipid to cytochrome *c* of up to 250:1. Van & Griffith (1975) showed that up to 1 cytochrome *c* molecule per 100 phosphatidylethanolamine molecules could be bound under suitable conditions. Had this been taking place with a K_D of approx. 1×10^{-5} M, we could have expected to see appreciable binding of cytochrome *c* even if only a small proportion of the phosphatidylethanolamine was available for binding (because of sidedness and multilayer formation). We conclude that, under ubiquinol oxidase conditions, phosphatidylethanolamine does not contribute to cytochrome *c* binding. In liposomes containing phosphatidylcholine and cardiolipin (4:1 by phospholipid P) or phosphatidylethanolamine, phosphatidylcholine and cardiolipin (2:2:1, by phospholipid P) approximating the lipid composition of mitochondria, up to 50% of the cytochrome *c* was bound. This would be expected for a K_D of 1×10^{-5} M, a binding site comprising four to five molecules of cardiolipin (Green & Fleischer, 1963) and approx. 13% of the cardiolipin molecules available for binding. Thus it is clear that, in reconstituted ubiquinol oxidase, it is cardiolipin that will be predominantly involved in binding of cytochrome *c* to the lipid phase.

Fig. 4(c) shows the spectrum of spin-labelled cytochrome *c* bound to liposomes containing phosphatidylcholine and cardiolipin (4:1, by phospholipid P). An identical spectrum was obtained with cytochrome *c* bound to liposomes containing phosphatidylethanolamine, phosphatidylcholine and cardiolipin (2:2:1, by phospholipid P). The apparent τ_c value was 2.95 ns, indicating an appreciable loss of protein mobility. Strict interpretation of the τ_c value is impossible, since there are two spin-label motions to be considered and, moreover, tumbling of cytochrome *c* is probably anisotropic (e.g. Teissie, 1981). Thus we use the apparent τ_c as an indication of changes in rotational motion. In Fig. 7 the τ_c values obtained from the liposomes of Fig. 4(c) are compared with those obtained from cytochrome *c* bound to liposomes consisting of dimyristoylphosphatidylcholine and cardiolipin (4:1, by phospholipid P). At temperatures above the phase-transition tem-

Fig. 7. Temperature-dependence of the rotational correlation time of spin-labelled cytochrome *c* bound to liposomes. MSL-labelled cytochrome *c* was bound to liposomes of egg phosphatidylcholine and cardiolipin as described in Fig. 4(c), and V_1 e.s.r. spectra were recorded at different temperatures with the conditions described in Fig. 4(a). Apparent τ_c values were calculated as described in the Materials and methods sections. Liposomes containing dimyristoylphosphatidylcholine and cardiolipin (4:1 by phospholipid P) were prepared by the same method, except that both preparation of liposomes and binding of cytochrome *c* were carried out at 27°C, above the phase-transition temperature of the lipid. O, Values obtained with liposomes containing egg phosphatidylcholine; ●, values obtained with dimyristoylphosphatidylcholine.

perature, τ_c values are very similar, but below 23°C the lines diverge, indicating an effect of the phase transition of the synthetic lipid, despite the fact that cytochrome *c* is only bound to cardiolipin. Although we cannot quantify the extent of the effect on the rotational mobility of cytochrome *c*, this experiment clearly shows that cytochrome *c* remains bound to the liposomes in the gel state of the phosphatidylcholine (otherwise τ_c would decrease) and that the rotational diffusion (and by inference, the lateral diffusion) of the protein is decreased.

Discussion

The effects of lipid phase transition on ubiquinol oxidase activity strongly suggest that lateral diffusion of cytochrome along the membrane surface is an obligatory step in overall electron transport. In our previous study (Diggens & Ragan, 1982) we

found that the cytochrome *c*-dependence of ubiquinol oxidase activity was quite unaffected by large increases in the lipid-to-protein ratio. Since this would increase the average distance between Complexes III and IV (e.g. Schneider *et al.*, 1980), one might have expected to see a decrease in rate which could be alleviated by more cytochrome *c*, by analogy with the behaviour of ubiquinone-mediated electron transfer (Schneider *et al.*, 1982). Since this was not seen, we concluded either that cytochrome *c* diffusion was not required, or that it was not rate-limiting under the conditions used. We now favour the latter explanation and propose that cytochrome *c* diffusion can only be made rate-limiting in gel-phase lipid.

We have additionally shown that the likely carrier of cytochrome *c* along the membrane is cardiolipin, a lipid which has also been implicated directly in the low-affinity phase of cytochrome *c* binding (Vik *et al.*, 1981) and may also be absolutely required, in a manner as yet not known, for activity through the high-affinity site (Fry & Green, 1981). Cardiolipin, compared with phosphatidylcholine, is preferentially bound to cytochrome oxidase (Cable & Powell, 1980; Knowles *et al.*, 1981), but the degree of preference is small and the exchange rate between the bound and bulk lipid phases will still exceed 10^4 s^{-1} (e.g. Lee, 1983). This is still considerably higher than the maximum rate of electron transfer, and in the bulk lipid phase it has already been shown that lipid lateral diffusion rates ($1 \times 10^{-8} \text{ cm}^2/\text{s}$) comfortably exceed that which would be necessary for a role in electron transfer (Kawato *et al.*, 1981; Hackenbrock, 1981). Moreover, Dixit *et al.* (1982) have shown that rotational diffusion rates of lipid-bound cytochrome *c* are similar to those of phospholipid molecules. However, some measurements of cytochrome *c* diffusion do not fit this model. Hochman *et al.* (1982) used fluorescent cytochrome *c* analogues and fluorescent recovery after photobleaching to measure a lateral diffusion coefficient of only $1.6 \times 10^{-10} \text{ cm}^2/\text{s}$, which is too slow to account for electron transport. They calculate that the free-diffusion model requires a minimum diffusion rate of $4 \times 10^{-9} \text{ cm}^2/\text{s}$ and conclude that electron transfer proceeds via aggregates of Complexes present in dynamic equilibrium with monomers. Hochman *et al.* (1982) cited the work of Kawato *et al.* (1981, 1982) as evidence for the existence of aggregated Complexes in the membrane. However, the experiments revealed only that cytochrome oxidase was aggregated, and no evidence was found for association of Complex III with the oxidase. The experiments of the present and the preceding (Froud & Ragan, 1984) paper support a free-diffusion model under conditions in which both Complexes are extensively

aggregated (low lipid-to-protein ratio) or extensively disaggregated (high lipid-to-protein ratio). We found no evidence for preferential electron transfer via cytochrome *c* from one Complex III molecule to an associated Complex IV molecule, since this would have distorted cytochrome *c*-pool kinetics. As an alternative explanation for the results of Hochman *et al.* (1982), it may be that there is some constraint to long-range lateral diffusion (as measured by fluorescence recovery after photobleaching) and that only short-range motions are necessary in the mitochondrial membrane (Cherry, 1979; Kawato *et al.*, 1981). Dixit *et al.* (1982) measured the rotational diffusion of zinc cytochrome *c* on mitochondrial membranes by using the decay of phosphorescence anisotropy. The rotational relaxation time was similar to that measured for cytochrome oxidase (Kawato *et al.*, 1981), suggesting that cytochrome *c* is associated with its oxidase *in situ*. However, if the majority of cytochrome *c* molecules were bound to the oxidase high-affinity sites, and the off-rate is slow compared with the relaxation time, as appears to be the case, then only the relaxation of oxidase-bound cytochrome *c* would have been detected.

The next point concerns the nature of cytochrome *c* binding in gel-phase lipid and the reasons for the loss of ubiquinol oxidase activity. In gel-phase dimyristoylglycerophosphocholine, mitochondrial complexes undergo lateral phase separation, being squeezed into patches, probably containing some lipid, separated by pure gel phase lipid (e.g. Höchli & Hackenbrock, 1976; Poore *et al.*, 1982). The proteins are rotationally immobile and the lipid fluidity is low. Under these conditions, cytochrome *c* will be constrained to move in the limited lipid phase surrounding the proteins and, although the diffusion distances may be small, the diffusion rate may be slow and the proteins may not be able to assume the favourable relative orientation shown to be so important by Koppenol & Margoliash (1982). In liposomes, cytochrome *c* itself can promote lateral separation of phospholipids (Birrell & Griffith, 1976), in which case the gel-phase liposomes may contain patches of cytochrome *c*-cardiolipin complexes separated by gel-phase dimyristoylglycerophosphocholine, thus accounting for the decreased rotational mobility of cytochrome *c*. However, the phase behaviour of cardiolipin/phosphatidylcholine mixtures is complicated (Berclaz & McConnell, 1981) and the actual structure of the membrane under these conditions is not important for the main argument of the present paper.

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