The Role of Phospholipids in the Reduction of Ubiquinone Analogues by the Mitochondrial Reduced Nicotinamide–Adenine Dinucleotide–Ubiquinone Oxidoreductase Complex

By C. IAN RAGAN

*Department of Biochemistry, School of Biochemical and Physiological Sciences,
University of Southampton, Southampton SO9 3TU, U.K.*

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The isolated NADH–ubiquinone oxidoreductase complex of bovine heart mitochondria reduces ubiquinone analogues by two pathways. One pathway is inhibited by rotenone, and reduction of quinones takes place in the lipid phase of the system. The other pathway is insensitive to rotenone and reduction takes place in the aqueous phase. The variation of rates of electron transport with the chemical nature of the quinone analogue and the concentrations of both quinone and phospholipid can be rationalized in terms of partition of the quinone between the aqueous and lipid phases of the system. Thus one function of phospholipid associated with the enzyme appears to be to act as solvent for ubiquinone reduced by the rotenone-sensitive pathway. This proposal is supported by the kinetic behaviour of enzyme whose endogenous lipids have been replaced by (1,2)-dimyristoyl-*sn*-glycero-3-phosphocholine. Thus, under certain circumstances, the rotenone-sensitive reduction of ubiquinone-1 exhibited a substantial increase in activation energy below the phase-transition temperature of the synthetic lipid, whereas the reduction of other acceptors was unaffected.

The catalytic activity of several membrane enzymes has been shown to depend on the chemical and physical properties of the surrounding phospholipid. It has been proposed that the shell of phospholipid molecules surrounding the protein (the 'annulus') serves to maintain the enzyme in an active conformation (Warren *et al.*, 1975). Transition from the liquid-crystalline state to the gel state of the lipid is frequently accompanied by loss of enzyme activity (Warren *et al.*, 1974). Conversely, the protein exerts an effect on the structure of the phospholipid in the annulus, since the annular lipid exchanges relatively slowly with bulk-phase lipid, and the transition between the gel and liquid-crystalline states of the annular lipid frequently occurs at a lower temperature than that of bulk-phase lipid, and less sharply (Lee, 1977).

The enzymes of the mitochondrial respiratory chain also appear to be dependent on phospholipids for their activity (e.g. Fleischer & Fleischer, 1967). The respiratory chain may be fractionated into large lipoprotein complexes (Hatefi, 1963), which retain the properties of the intact membrane with regard to electron transport (Hatefi, 1963) and, under suitable conditions, oxidative phosphorylation (Racker, 1975). These complexes contain a number of redox groups and polypeptides whose association is probably through protein–protein interactions. The manner in which one complex interacts with another is less clear. It has been proposed that relatively small mobile

lipid-soluble molecules (e.g. ubiquinone-10) act as mediators of hydrogen or electron transfer between the complexes. In particular, the mobile-ubiquinone-pool hypothesis (Kröger & Klingenberg, 1967) accounts adequately for the kinetic and redox behaviour of ubiquinone (Kröger & Klingenberg, 1973) and the relatively high concentration of ubiquinone relative to other respiratory carriers, and allows flexible stoichiometry between complexes. On the other hand, some evidence suggests that Complex I (NADH–ubiquinone oxidoreductase) and Complex III (ubiquinol–cytochrome *c* oxidoreductase) interact to form a 1:1 binary complex (Hatefi *et al.*, 1962; Fowler & Richardson, 1963). If the latter is correct, then these complexes may associate through protein–protein interaction, and mobility of ubiquinone through the lipid phase may be restricted to the interface between the Complexes. The Q-cycle hypothesis (Mitchell, 1976) suggests that ubiquinone is reduced by electrons from both the dehydrogenase (e.g. Complex I or succinate dehydrogenase) and Complex III at some site on the M-side (Mitchell, 1968) of the membrane. This proposal required a more ordered interaction between Complexes I and III than is suggested by the ubiquinone-pool idea, but is consistent with stoichiometric interaction between the complexes. Nevertheless, ubiquinone/ubiquinol mobility is still a necessary feature of Mitchell's (1976) hypothesis. These proposals for

ubiquinone function suggest that the membrane phospholipid may in addition act as a solvent for the ubiquinone. Phospholipid-dependence of Complex I or III activity may therefore merely reflect the fact that one of the substrates is extremely hydrophobic and oxidoreduction takes place in the lipid phase.

The present paper describes experiments designed to determine the role of phospholipids in the reduction of ubiquinone analogues by Complex I. The results suggest that phospholipids act as solvent for the ubiquinone, in addition to a possible role in maintaining the enzyme conformation.

Materials and Methods

Biological preparations

Complex I (NADH-ubiquinone oxidoreductase, EC 1.6.99.3) was purified from bovine heart mitochondria by the method of Hatefi & Rieske (1967). The purity was within the range previously reported (Ragan, 1976a). The phospholipid content of the enzyme was determined by phosphate analysis (Bartlett, 1969) to be 0.21–0.23 $\mu\text{mol}/\text{mg}$ of protein.

Chemicals

Soya-bean phosphatidylcholine and phosphatidylethanolamine were purified as described by Ragan & Racker (1973). (1,2)-Dimyristoyl-*sn*-glycero-3-phosphocholine was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Assays

NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ oxidoreductase and NADH-juglone (5-hydroxy-1,4-naphthoquinone) reductase were measured as described by Ragan & Bloxham (1977). NADH-ubiquinone oxidoreductase was measured by the decrease in A_{340} of a solution containing 20 μmol of potassium phosphate, pH 8, 0.1 μmol of NADH, 5–50 μg of Complex-I protein (depending on conditions) and the amounts of ubiquinone and phospholipid detailed in the text. Rotenone-insensitive rates were measured in the presence of 5 μg of rotenone added as a methanolic solution (1 mg/ml). The reaction was routinely initiated by addition of enzyme, but the order of reagent addition was not important. Stock solutions of ubiquinone analogues were prepared in methanol or ethanol and standardized by the change in u.v. absorption on reduction with NaBH_4 . The absorption coefficients and wavelengths used were as follows: ubiquinone-0, 13.2 $\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 263 nm (Morton, 1965); ubiquinone-1, 12.0 $\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 275 nm (Morton, 1965); 2,3-methoxy-5-methyl-6-pentyl-1,4-benzoquinone, 12.0 $\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 275 nm (assumed the same as ubiquinone-1); 2,3-

methoxy-5-methyl-6-decyl-1,4-benzoquinone, 11.7 $\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at 275 nm (assumed the same as ubiquinone-2; Morton, 1965). The concentration of the stock solutions was such that, when diluted into the assay, the final methanol or ethanol concentration did not exceed 2% (v/v). Stock solutions of soya-bean phosphatidylcholine were prepared by ultrasonic dispersion of the dry lipid in aqueous solutions containing 0.67 M-sucrose, 50 mM-Tris/HCl, pH 8, and 2% (w/v) cholate, to give final concentrations of approx. 20 mM. Ultrasonic irradiation was performed in a bath-type sonicator (Megason) until solutions were no longer turbid (usually 1–2 min). Solutions were standardized by determining the phosphate content (Bartlett, 1969). All rate assays were conducted in a water-jacketed cell holder whose temperature was varied by external heating or cooling units. The temperature of the assay was measured directly with a thermistor in the cuvette to within 0.2°C. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin [fraction V from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] as a standard.

Measurement of the solubility of quinones in water

The quinones were emulsified by vortex-mixing with approx. 2 ml of 20 mM-potassium phosphate, pH 8, and incubated at 30°C for 6–24 h. At the end of this period, residual turbidity was removed by centrifugation at 10000g for 15 min at 30°C and the clear liquid assayed for ubiquinone content as described above.

Results

Complex I is conventionally assayed by the rate of reduction of ubiquinone-1 by NADH (Hatefi & Rieske, 1967). This rate is stimulated by the addition of phospholipid (usually from soya beans) to the assay medium (Hatefi & Rieske, 1967). However, as shown in Table 1, the effect of a lipid supplement is not only to stimulate the rotenone-sensitive rate of ubiquinone-1 reduction but also to inhibit the rotenone-insensitive pathway. Moreover, the phospholipid-concentration-dependences of these two effects are different. Similar results were obtained with purified soya-bean phosphatidylethanolamine and if lipids were ultrasonically dispersed in the absence of cholate.

Effect of phospholipids on the rotenone-insensitive reduction of ubiquinones by NADH

The rotenone-insensitive reduction of ubiquinone-1 is not linked to ATP synthesis in submitochondrial particles (Schatz & Racker, 1966) and is unaffected

Table 1. Effect of phospholipid on rotenone-sensitive and rotenone-insensitive NADH-ubiquinone-1 oxidoreductase activity

Enzyme activities were measured at 24°C as described in the Materials and Methods section. The ubiquinone-1 concentration was 35 μM.

Phospholipid added to assay (μM)	Activity (μmol of NADH/min per mg of Complex-I protein)	
	Rotenone-sensitive	Rotenone-insensitive
0	0.69	0.19
1.6	1.15	0.19
10.5	1.70	0.19
200	2.78	0.15
600	—	0.10
1000	—	0.08

by removal of phospholipids from the enzyme (Machinist & Singer, 1965). This and other evidence is held to indicate that there are two sites for ubiquinone-1 reduction on the enzyme. As shown below, the rate of rotenone-insensitive reduction of ubiquinone analogues increases with increasing solubility of the analogue in water. It seems reasonable to propose that this site is exposed to the aqueous phase and that only that portion of the ubiquinone analogue dissolved in the aqueous phase can be reduced through the rotenone-insensitive pathway. On addition of phospholipid, the aqueous concentration of ubiquinone will be decreased, causing a decrease in the rate of rotenone-insensitive reduction.

The distribution of the analogue between the aqueous and lipid phases is determined by *P*, the partition coefficient:

$$P = \frac{Q_L/L}{Q_w/W}$$

where *Q_L* and *Q_w* are the mol of quinone in the lipid and aqueous phases respectively, *L* is the mol of lipid and *W* the mol of water. Since *Q_L*+*Q_w* = *Q*, the total amount of quinone present,

$$Q_w = \frac{Q}{1 + \frac{PL}{W}}$$

Since the volume of the lipid phase is negligible compared with the volume of the aqueous phase in the experiments described, *W* has the constant value of 55.5 mol if a 1-litre system is considered, and *Q_w* may be regarded as the molarity of the quinone in the aqueous phase. *Q* is also given in units of molarity for convenience, but strictly this is the total molecules of quinone in both phases per litre of the total system.

Assuming that the rotenone-insensitive reaction occurs exclusively in the aqueous phase and follows

Fig. 1. Variation of the rate of rotenone-insensitive ubiquinone-1 reduction with the concentration of added phospholipid and ubiquinone-1

Enzyme activities were measured at 30°C as described in the Materials and Methods section. The enzyme concentration was 44 μg of protein/ml, which contributed 10 μM-phospholipid to the total (0.23 μmol of phospholipid P/mg of protein). Open symbols denote measurements made in the absence of added phospholipid. The ubiquinone-1 concentrations used were: 189 μM (●, ○); 94.3 μM (▲, △); 47.1 μM (■, □); 31.4 μM (▼, ▽).

Michaelis–Menten kinetics with respect to quinone, the velocity *v* is given by:

$$v = \frac{V_{max} \cdot Q_w}{K_m + Q_w}$$

Substituting for *Q_w*:

$$v = \frac{V_{max} \cdot \frac{Q}{1 + 0.018 PL}}{K_m + \frac{Q}{1 + 0.018 PL}}$$

or

$$v = \frac{Q V_{max}}{0.018 L K_m P + K_m + Q}$$

This equation implies that 1/*v* should vary linearly with *L*, the lipid concentration, and that all such plots with different *Q* concentrations should intersect where:

$$0.018 L K_m P + K_m = 0$$

or

$$L = \frac{-55.5}{P}$$

Fig. 1 shows that this was the case for ubiquinone-1 as substrate, and the common point of intersection gave a value for *P* of 1.01 × 10⁵. According to Hill (1974), the partition coefficient *P* is related to the solubility of the substance in water, *S*, by the expression:

$$PS = 2$$

The value of S for ubiquinone-1, determined as described in the Materials and Methods section, was $15.2 \mu\text{mol/mol}$ of water. From this and Fig. 1:

$$PS = 1.54$$

which is in reasonable agreement with the theory.

It should be noted that the points closest to the $1/v$ axis in Fig. 1 (open symbols) were obtained without addition of phospholipid to the assay. Therefore,

Fig. 2. Variation of the rate of rotenone-insensitive reduction of ubiquinone analogues as a function of the aqueous concentration of the analogue

Rates were measured at 30°C as described in the Materials and Methods section. Reduction of each analogue was measured with the phospholipid concentrations shown in Table 2 and at total quinone concentrations of 50 and $100 \mu\text{M}$. Open symbols denote measurements obtained in the absence of added lipid. Analogues used were: ubiquinone-0 ($\bullet$, $\circ$); ubiquinone-1 ($\blacktriangle$, $\triangle$); 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone ($\blacksquare$, $\square$).

within the limitations of the experiment, the endogenous phospholipid of Complex I behaves no differently from the added phospholipid.

In the experiment of Fig. 2, rates of rotenone-insensitive reduction of a number of ubiquinone analogues were determined under different conditions. From the water-solubilities, values of the partition coefficients were calculated and used to determine the appropriate concentrations of each analogue in the aqueous phase under each set of conditions (Table 2). As shown in Fig. 2, the reciprocal velocities were linearly related to the reciprocal of the aqueous ubiquinone concentration for each of the ubiquinone analogues used, although the values for K_m and $V_{\max}$ were generally different. Points obtained without added phospholipid are shown as open symbols and did not deviate significantly from points obtained in the presence of added phospholipid. The water-solubility of the decylbenzoquinone analogue is extremely low, and under the experimental conditions of Fig. 2 and Table 2 undissolved quinone was present. Since the analysis assumes equilibrium between quinone dissolved in the aqueous and lipid phases, no attempt was made to fit results obtained with this analogue to the theory. However, as expected, this extremely hydrophobic analogue was reduced by the rotenone-insensitive pathway at very low rates.

Effect of phospholipids on the rotenone-sensitive reduction of ubiquinone by NADH

The rotenone-sensitive rate of ubiquinone reduction is stimulated by addition of phospholipid to the assay (Table 1). The degree of stimulation depended on the nature of the ubiquinone analogue used. Thus, in one experiment, 0.4 mM -phosphatidylcholine stimulated the rates of reduction of ubiquinone-0,

Table 2. Calculation of aqueous ubiquinone concentrations in the presence of various concentrations of phospholipid Solubility was determined as described in the Materials and Methods section. The solubility of DB was extremely low and the estimate is not considered reliable. Partition coefficients were calculated from the Hill (1974) expression and the percentage of aqueous quinone calculated from $Q_w = Q/(0.018\text{PL} + 1)$. The phospholipid concentration of $10 \mu\text{M}$ applies to the unsupplemented enzyme as in Fig. 1. Abbreviations: PB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone.

Analogue	$10^5 \times$ Solubility (mol/mol of H_2O)	$10^{-5} \times$ Partition coefficient	$10^5 \times$ Phospholipid concentration (M)	Percentage of total quinone in the aqueous phase
Ubiquinone-0	78.4	0.0255	1	100
			41	98.2
			101	95.6
Ubiquinone-1	1.52	1.32	1	97.7
			21	66.7
			81	34.1
PB	0.65	3.08	1	94.7
			11	62.1
			41	30.5
DB	~ 0.03	~ 70	1	~ 47

ubiquinone-1, the pentybenzoquinone analogue and the decylbenzoquinone analogue (all at 50 μM) by 70, 270, 230 and 750% respectively. The final rates obtained with the last three compounds were similar. If the phospholipid supplement was re-activating phospholipid-depleted enzyme molecules, activity should increase towards all ubiquinone analogues to the same extent. Alternatively, the observation that the degree of stimulation increased with the hydrophobicity of the analogue suggests that the effect of the lipid may be on the partition of ubiquinone between the aqueous and lipid phases. From the previous section, it also follows that:

$$Q_L = \frac{QPL}{W + PL}$$

In the experiment of Fig. 3, the rotenone-sensitive reduction of ubiquinone-1 was measured as a function of the total phospholipid present (endogenous plus added) and the total ubiquinone-1 concentration. From the above equation, values of Q_L were calculated for each set of conditions, and the reciprocals of these are plotted in Fig. 3 against the reciprocal velocity. For all values of Q and L , including measurements made in the absence of added lipid, the points lie in a straight line, with a break to a steeper slope at high values of Q_L .

Although Fig. 3 provides strong evidence that the effect of phospholipid on the partition of quinone is responsible for the dependence of the velocity of the reaction, it is surprising that the velocity appears to be hyperbolically related to Q_L and not to the concentration of quinone in the lipid phase, Q_L/L . Furthermore, the biphasic nature of the reciprocal plot remains unexplained, unless there are two classes of rotenone-sensitive quinone-reduction sites which differ in K_m and V_{max} for ubiquinone-1. The part of the line obtained at low values of Q_L gave a K_m for ubiquinone-1 in the lipid phase of 0.044 μmol per litre of the total system. The enzyme concentration was 5.5 $\mu\text{g}/\text{ml}$ or 6.1 nM (based on the FMN content) (Ragan, 1976c). Therefore the K_m value above may be expressed as approx. 7 molecules of ubiquinone-1 in the lipid phase per molecule of Complex I. This value is comparable with the ubiquinone-10 concentration of purified electron-transport particles (Blair, 1967), which is 11 molecules per molecule of acid-extractable FMN (here equated with the Complex-I content). This would therefore be sufficient to operate Complex I at near to V_{max} , especially as the physiological oxidant may well have a lower K_m than the analogue, ubiquinone-1.

Extrapolation of the curve of Fig. 3 to infinite Q_L gave a V_{max} of 3.5 μmol of NADH/min per mg of protein at 25°C, which would correspond to a rate of 15 $\mu\text{mol}/\text{min}$ per mg at 38°C (see below), which is comparable with V_{max} reported elsewhere (Hatefi & Stempel, 1969; Ragan, 1976a).

Fig. 3. Variation of the rate of rotenone-sensitive ubiquinone-1 reduction with the concentration of added phospholipid and ubiquinone-1

Enzyme activities were measured at 25°C as described in the Materials and Methods section. The enzyme concentration was 5.5 μg of protein/ml which contributed 1.26 μM -phospholipid to the total. Open symbols denote measurements made in the absence of added phospholipid. The ubiquinone-1 concentrations used were: 5.64 μM ($\bullet$, $\circ$); 9.4 μM (Δ , $\triangle$); 18.8 μM ($\blacksquare$, $\square$); 36.7 μM ($\blacktriangledown$, $\triangledown$). At each of these concentrations rates were measured in the presence of the following total (endogenous plus added) phospholipid concentrations: 1.26 μM (i.e. no added lipid); 1.78 μM ; 2.84 μM ; 3.88 μM ; 5.46 μM ; 11.8 μM ; 42.2 μM . Values of Q_L were calculated as described in the text by using $P = 1.01 \times 10^5$. Inset shows a replot on expanded abscissa for high values of Q_L .

Rates obtained with no added phospholipid (open symbols) also fitted the lines of Fig. 3. Therefore the endogenous lipid appears to behave no differently from added lipid, and it follows that one function of lipid associated with the enzyme is to act as solvent for the quinone substrate.

Replacement of endogenous phospholipids by dimyristoyl-*sn*-glycero-3-phosphocholine

Complex-I phospholipids consist primarily of phosphatidylcholine, phosphatidylethanolamine and cardiolipin. Heron *et al.* (1977) described a procedure by which the endogenous lipids, apart from cardiolipin, could be quantitatively replaced by dimyristoyl-*sn*-glycero-3-phosphocholine with complete retention of activity. Since dimyristoyl-*sn*-glycero-3-phospho-

choline freezes at 25°C (Ladbrooke & Chapman, 1969), lipid-substituted Complex I was used in the following experiments to determine the effect of phase transition in the lipid phase on the kinetics of ubiquinone reduction.

In Table 3, the activation energies for the reduction of a number of acceptors by NADH are listed. These were determined from Arrhenius plots, which were linear between 2 and 40°C. The rate of reduction of $K_3Fe(CN)_6$ was almost independent of temperature, whereas rotenone-insensitive reduction of ubiquinone-1 or reduction of juglone (5-hydroxy-1,4-naphthoquinone) had activation energies of around 50 kJ/mol. The rotenone-sensitive reduction of ubiquinone-1 had a much higher activation energy of 80 kJ/mol. These values were unaffected by the presence of phospholipid in the assay.

Replacement of endogenous phospholipids with dimyristoyl-*sn*-glycero-3-phosphocholine was performed as described by Heron *et al.* (1977), and the material dialysed for 16 h against 200 vol. of sucrose/Tris buffer at 4°C to remove cholate. Over the temperature range 5–35°C, Arrhenius plots were linear for all the electron acceptors of Table 3, and the activation energies were unchanged. The phospholipid content of such preparations was a little higher (0.28–0.30 μ mol/mg of protein) than that of native Complex I (0.21–0.23 μ mol/mg of protein). As discussed by Heron *et al.* (1977), phospholipid concentrations of this magnitude appear to constitute the lipid annulus of the enzyme. Thus phase transition, if any, of the dimyristoyl-*sn*-glycero-3-phosphocholine in the annulus did not have any effect on the activity of the enzyme towards the electron acceptors tested.

An alternative method of lipid substitution is to equilibrate the enzyme with a large excess of dimyristoyl-*sn*-glycero-3-phosphocholine in the presence of detergent and dialyse to remove the detergent, in

this case cholate. By this procedure, annular and extra-annular lipid are present, most of which is the synthetic lipid. As shown in Fig. 4, the Arrhenius plot of the rotenone-sensitive reduction of ubiquinone-1 was characterized by a break at around 20°C. The break occurred gradually between 22 and 17°C, outside which range the activation energies were 84 kJ/mol above 22°C and approx. 290 kJ/mol below 17°C. This behaviour can be attributed to transition of the dimyristoyl-*sn*-glycero-3-phosphocholine from the liquid-crystalline to the gel state, which occurs at 25°C for the pure lipid (Ladbrooke & Chapman, 1969). Breaks in Arrhenius plots at temperatures below the transition temperature of the pure lipid (as in Fig. 4) are usually attributed to disordering of the annular lipid by the protein, resulting in a lowering of the transition temperature (Warren *et al.*, 1974). An

Fig. 4. Arrhenius plots for the rotenone-sensitive (●) and rotenone-insensitive (▲) ubiquinone-1 reductase activities of Complex I substituted with an excess of dimyristoyl-glycerophosphocholine

Complex I (1.25 mg of protein in 0.25 ml of 0.67 M-sucrose/50 mM-Tris/HCl, pH 8) was mixed with 1.25 mg of cholate and 2.5 mg of dimyristoylglycerophosphocholine dispersed by sonication in 0.25 ml of sucrose/Tris buffer at 4°C. The mixture was dialysed for 16 h against 150 ml of sucrose/Tris buffer at 4°C. Enzyme assays were performed as described in the Materials and Methods section, but in the absence of a phospholipid supplement to the assay. The enzyme concentration used was 39 μ g of protein/ml, contributing approx. 100 μ M-phospholipid, and the ubiquinone-1 concentration was 70 μ M.

Table 3. Activation energies for NADH-acceptor oxidoreductase activities of Complex I

Rates were determined as described in the Materials and Methods section over the temperature range 4–40°C. Activation energies were calculated from the slopes of plots of log rate versus the reciprocal of the absolute temperature.

Acceptor and other additions	Activation energy (kJ/mol)
$K_3Fe(CN)_6$	≤ 1
Juglone	50
Ubiquinone-1 (rotenone-insensitive)	46
Ubiquinone-1 (rotenone-insensitive) plus 0.4 mM-phosphatidylcholine	48
Ubiquinone-1 (rotenone-sensitive)	80
Ubiquinone-1 (rotenone-sensitive) plus 0.4 mM-phosphatidylcholine	84

alternative explanation is that the dissolved ubiquinone-1 lowers the freezing point of the lipid. Hill (1974) derived a relationship between the lowering of the freezing point and the fractional saturation of the solute in the aqueous phase. Application of this to the conditions of Fig. 4 (70 μ M-ubiquinone-1 and 100 μ M-phospholipid) leads to a calculated depression of the freezing point of 3.5°C, which agrees well with the results obtained. A break in the Arrhenius plot was obtained only with the rotenone-sensitive reduction of ubiquinone-1 and not with other substrates, such as K₃Fe(CN)₆, juglone and ubiquinone-1 (rotenone-insensitive).

Despite the presence of a phospholipid excess in the substituted enzyme, the rate of reduction of ubiquinone-1 was still stimulated by the addition of soya-bean phosphatidylcholine to the assay. Above the transition temperature, the stimulation was effectively instantaneous, as for the native enzyme. Below the transition temperature the rate of reduction of ubiquinone was initially unaffected by a phospholipid supplement, but the rate gradually increased with time to a final steady value. At 7°C, the final rate was attained only some 4 min after addition of the phospholipid, whereas at temperatures closer to the transition point the difference between initial and final rates became smaller and the final rate was attained more quickly. In Fig. 5, the Arrhenius plot shows both initial and final rates measured in the presence of phospholipid in the assay. Below 17°C, the initial and final rates gave rise to activation energies of 270 and 84 kJ/mol respectively, the latter being the same as was obtained above the transition temperature. It appears therefore that the added phospholipid effectively displaces the endogenous dimyristoyl-*sn*-glycero-3-phosphocholine from the enzyme, so that no transition is obtained. Above the transition temperature of the synthetic lipid this process was effectively instantaneous, whereas below the transition there was a marked delay, depending on the temperature. Only when fusion of the endogenous and added lipid takes place is the rate of ubiquinone-1 reduction stimulated by added lipid. The experiment of Table 4 shows this effect more clearly. Rates of rotenone-sensitive and -insensitive ubiquinone-1 reduction were measured at 10°C with the dimyristoyl-*sn*-glycero-3-phosphocholine-substituted enzyme. Addition of soya-bean phosphatidylcholine had no effect initially on the rotenone-sensitive activity, but eventually a substantial stimulation was obtained. On the other hand, the rotenone-insensitive rate was immediately inhibited by phospholipid and the rate was unchanged during the fusion process. This was entirely expected, as reduction at the rotenone-insensitive site takes place in the aqueous phase. As little as 0.02 % cholate in the assay totally abolished the delay in stimulation of the rotenone-sensitive activity at 10°C, presumably by

Fig. 5. Arrhenius plot for the rotenone-sensitive ubiquinone-1 reductase activity of Complex I substituted with an excess of dimyristoylglycerophosphocholine and measured in the presence of soya-bean phosphatidylcholine. Conditions were as described in the legend to Fig. 4, except that the assays were performed in the presence of 0.4 mM-soya-bean phosphatidylcholine. The phospholipid supplement was prepared in cholate-free buffer. ●, Initial rates; ▲, final rates.

Table 4. Effect of phospholipid on NADH-ubiquinone-1 oxidoreductase activity of dimyristoylglycerophosphocholine-substituted Complex I. Complex I (5 mg of protein/ml of 0.67 M-sucrose/50 mM-Tris/HCl, pH 8 at 0°C) was mixed with an equal volume of a solution containing 10 mg of dimyristoylglycerophosphocholine and 5 mg of cholate/ml of sucrose/Tris buffer. Dialysis was performed for 16 h at 4°C against 150 vol. of sucrose/Tris buffer. Assays were performed at 10°C as described in the Materials and Methods section. The phospholipid supplement was prepared in cholate-free buffer.

Additions to assay	Activity (μmol of NADH/min per mg of protein)	
	Rotenone-sensitive	Rotenone-insensitive
None	0.026	0.064
Phosphatidylcholine (1 mM)	0.026 (initial) 0.098 (final)	0.026 (initial) 0.026 (final)

interfering with the gel structure of the endogenous lipid and effectively lowering the transition temperature to below 10°C.

The observation that a break in the Arrhenius plot was obtained only in the presence of extra-annular dimyristoyl-*sn*-glycero-3-phosphocholine suggests that conformational immobility of the protein is not responsible for the change in activation energy, since this should only be affected by transitions in the annular lipid. This conclusion is supported by the finding that reduction of electron acceptors other than ubiquinone-1 was unaffected by freezing of the lipid phase, whether annular or extra-annular. Therefore the most likely explanation for the breaks in Figs. 4 and 5 is that freezing of the bulk-phase lipid prevents access of ubiquinone-1 to the rotenone-sensitive site, either by lowering the solubility of ubiquinone-1 in the lipid phase or by restricting the mobility of the ubiquinone-1 so that the rate becomes diffusion-limited. The absence of a break, in the presence of annular lipid only, could be due to a number of contributing causes. Firstly, depression of freezing point by the ubiquinone-1 was calculated to be 4°C. Secondly, the annular lipid may be disordered by the presence of both the protein and cardiolipin such that the transition temperature is lowered considerably. Thirdly, although freezing of the annular lipid would decrease both the concentration of ubiquinone-1 in the lipid phase and its diffusional mobility, neither of these effects might be sufficient to lower the enzyme activity, especially as the annular lipid may not form a perfect gel phase. Only in the presence of an excess of frozen lipid would diffusion become a rate-controlling process.

Discussion

Evidence has been presented that the rotenone-sensitive ubiquinone reductase activity of Complex I has a requirement for two classes of phospholipid, namely phosphatidylcholine (or phosphatidylethanolamine) and the acidic lipid cardiolipin (Heron *et al.*, 1977). Neither class of lipid alone, in concentrations comparable with the natural lipid content, was capable of supporting high activity. In the present paper it has been proposed that one function of phospholipid associated with the enzyme is to act as solvent for the hydrophobic substrate, ubiquinone. Thus the variation of activity with both ubiquinone and lipid concentrations can be rationalized in terms of partition of the quinone between aqueous and lipid phases. In support of this, it has been shown that stimulation of activity by added lipid requires fusion with the endogenous lipid of the enzyme. The observation that Complex I depleted of phosphatidylcholine and phosphatidylethanolamine (and inactive) cannot be reactivated by added lipid (Heron *et al.*, 1977) suggests that added lipid cannot fuse with the depleted enzyme, but only with the annular lipid. Restoration of the annulus and hence activity under

special conditions has been described (Ragan & Racker, 1973).

Any type of lipid should support the solvent function, assuming that it interacts correctly with the enzyme protein. Since two classes of lipid are required for activity it is clear that the solvent role is not the only function of the lipid. Thus there may be specialized roles for both phosphatidylcholine and phosphatidylethanolamine on the one hand and cardiolipin on the other. Obvious possibilities are that such lipid-protein interactions are required for the conformational stability of Complex I. It has been shown that lipid depletion leads to changes in the midpoint potential of the non-haem-iron centre 2 (Ohnishi *et al.*, 1974) and to greater susceptibility of the enzyme to proteolytic digestion (Ragan, 1976b; Crowder & Ragan, 1977). The experiments with dimyristoyl-*sn*-glycero-3-phosphocholine did not provide any evidence that freezing the annular and extra-annular lipid directly affected activity through the protein conformation. Effects of lipid freezing were interpreted in terms of quinone mobility in the lipid phase. Although conditions have been described for replacement of endogenous cardiolipin with exogenous cardiolipin (Heron *et al.*, 1977), the present unavailability of synthetic cardiolipins with phase transitions in the physiological temperature range prevents direct experimentation on the function of this lipid.

A soluble lipid-free NADH dehydrogenase has been purified that catalyses the rapid rotenone-sensitive reduction of ubiquinones and is capable of linking to a purified cytochrome *b-c*₁ particle in the presence of ubiquinone-6 to reconstitute an active NADH-cytochrome *c* oxidoreductase (Baugh & King, 1972, 1974). These observations are difficult to reconcile with those reported in the present and other papers (Machinist & Singer, 1965; Fleischer & Fleischer, 1967; Ragan & Racker, 1973; Heron *et al.*, 1977). However, the enzyme is extremely unstable compared with Complex I, and has very high *K_m* values for ubiquinone-1 and higher homologues. Rotenone-sensitivity was only reported for reduction of ubiquinone-6. It is possible that, under suitable conditions of isolation, lipid depletion might occur with retention of the native conformation of the enzyme. Owing to the extremely hydrophobic nature of the higher homologues of ubiquinone, these might still be reduced via the rotenone-sensitive pathway, even in the absence of phospholipids, especially at high concentrations of the ubiquinone. The failure to observe any stimulation of this activity by phospholipid is in agreement with the observation by Heron *et al.* (1977) that Complex I depleted of phospholipid cannot be re-activated by phospholipid in the assay. In addition, the interaction of the soluble enzyme with the cytochrome *b-c*₁ particle would not be inconsistent with the proposal that protein-protein

interaction may be significant in the functional linking of respiratory Complexes (Heron *et al.*, 1977).

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