The Effects of Lipid Phase Transitions on the Interaction of Mitochondrial NADH–Ubiquinone Oxidoreductase with Ubiquinol–Cytochrome *c* Oxidoreductase

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1. The endogenous phosphatidylcholine and phosphatidylethanolamine of Complexes I and III from bovine heart mitochondria may be completely replaced with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine with at least partial retention of activity. 2. The lipid-replaced enzymes associate in a 1:1 molar ratio to give a Complex I–III unit catalysing NADH–cytochrome *c* oxidoreductase activity. 3. On increasing the concentration of ubiquinone-10 and the synthetic phospholipid, the lipid-replaced Complexes appear to operate independently of each other as in the natural membrane. Thus the lipid-replaced enzymes associate in exactly the same ways as the enzymes containing natural phospholipids. 4. Arrhenius plots of NADH–cytochrome *c* oxidoreductase activity reconstituted from lipid-replaced Complexes I and III exhibit changes in slope at 24°C. When the concentrations of phospholipid and ubiquinone-10 are increased, the Arrhenius plots show discontinuities at 24°C as well as changes in slope. 5. The kinetics of cytochrome *b* reduction by NADH were measured in mixtures containing 2 mol of Complex III/mol of Complex I. When the enzymes contained natural phospholipids, the reduction kinetics were biphasic. When the enzymes had been supplemented with further phospholipid and ubiquinone-10 the kinetics were monophasic. When lipid-replaced enzymes were supplemented with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine and ubiquinone-10, reduction of cytochrome *b* was monophasic above the phase-transition temperature of the lipid but biphasic below it. 6. These findings are interpreted in terms of the model for the interaction of Complexes in the natural membrane proposed by Heron, Ragan & Trumppower [(1978) *Biochem. J.* 174, 791–800].

The respiratory Complexes of the inner mitochondrial membrane are lipoproteins (Hatefi, 1963) and require phospholipid for enzymic activity (Fleischer & Fleischer, 1967). An approach that has proved useful for the study of such lipid–protein interactions is substitution of the endogenous phospholipids by single synthetic phospholipids. This enables one to look at the lipid head group and fatty acid specificity of the enzyme and, perhaps most importantly, to study the effects on activity of the physical structure of the phospholipid, since some synthetic phospholipids have phase-transition temperatures in the physiological temperature range. We have previously shown that 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine can replace the phosphatidylcholine and phosphatidylethanolamine of Complex I (NADH–ubiquinone oxidoreductase; Hatefi *et al.*, 1962*a*) from bovine heart mitochondria (Heron *et al.*, 1977). With the lipid-substitution technique of Warren *et al.* (1974), little loss of ubiquinone reductase activity was incurred. Lipid-

substituted Complex I was used to investigate the effects of phospholipids on the reduction of ubiquinone analogues. It was concluded that effects on activity of phase transition of the phospholipid were exerted not directly on the protein but on the mobility and concentration of ubiquinone in the lipid phase of the system (Ragan, 1978).

Arising from studies on the interaction between purified Complex I and Complex III (ubiquinol–cytochrome *c* oxidoreductase; Hatefi *et al.*, 1962*b*) we have proposed that in the natural membrane, rapidly reversible association of these enzymes into stoichiometric Complex I–III units takes place (Ragan & Heron, 1978; Heron *et al.*, 1978). Electron transfer from Complex I to Complex III takes place through these units, and the relative mobility of the separate Complexes in the dissociated state is the basis of ‘Q-pool’ behaviour as originally described by Kröger & Klingenberg (1973*a,b*). The model is described in more detail in the discussion.

The present paper describes experiments similar to those reported previously (Ragan & Heron, 1978; Heron *et al.*, 1978), except that endogenous phospho-

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lipids were replaced by 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine. The effects of phase transition of the lipid on the NADH-cytochrome *c* oxidoreductase activity of Complex I and III mixtures and the kinetics of reduction of Complex III cytochrome *b* are consistent with the above model and indicate that lipid-protein segregation takes place when the lipid is in the gel state.

Materials and Methods

Enzyme preparations

Complex I (EC 1.6.99.3) and Complex III were prepared from bovine heart mitochondria as described by Hatefi & Rieske (1967) and Rieske (1967*a*) respectively. Molarities of Complex-I and Complex-III solutions were determined respectively from the acid-extractable flavin content (1.0–1.2 nmol/mg of Complex-I protein) and the cytochrome *c*₁ content (3.4–3.8 nmol/mg of Complex-III protein). Complex-III preparations did not contain any detectable Complex-I activity, and Complex-I preparations contained approx. 0.1 mol of Complex III/mol of Complex I. Where molar ratios are given, these have been corrected for the Complex-III content of Complex I.

Chemicals

Ubiquinone-1 was a gift from Hoffman-La Roche, Basle, Switzerland. The ubiquinone-2 analogue, 6-decyl-2,3-methoxy-5-methyl-1,4-benzoquinone, was kindly provided by Dr. B. Trumpower, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, U.S.A. This compound was reduced to the benzoquinol as described by Rieske (1967*a*). Soya-bean phospholipids were purified as described by Ragan & Racker (1973) from 'L- α -lecithin' [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.]. 1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine was obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K.

Assays

NADH-ubiquinone-1 oxidoreductase was measured as described by Ragan (1976*a*) and NADH-cytochrome *c* oxidoreductase as described by Hatefi & Rieske (1967), except that the phospholipid supplement to the assay was omitted. The oxidation of 6-decyl-2,3-methoxy-5-methyl-1,4-benzoquinol by cytochrome *c* was measured as described by Ragan & Heron (1978). The following were assayed as indicated: acid-extractable flavin (Ragan, 1976*a*); cytochrome *c*₁ (Rieske, 1967*b*); phosphate (Bartlett, 1969); protein (Lowry *et al.*, 1951; with fraction V bovine serum albumin from Sigma as a standard).

Lipid replacement

Solutions of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine were prepared in 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, containing 0.7% (w/v) sodium cholate by ultrasonic dispersion in a bath-type sonicator until optically clear. The final lipid concentration was 60 mM. Complex I or Complex III (10 mg of protein/ml of sucrose/Tris buffer) was mixed with an equal volume of lipid solution at 4°C. After 15 min, the enzyme was precipitated by addition of cold, saturated and neutralized (NH₄)₂SO₄ solution to 46% saturation (for Complex I) or 56% saturation (for Complex III). After a further 10 min, the sample was centrifuged at 100 000g for 20 min at 4°C in the 10×10 ml rotor of the MSE 65 centrifuge. The supernatant was discarded and the enzyme redissolved in sucrose/Tris buffer to the original volume. Lipid solution was then added exactly as before, and the enzyme recovered by (NH₄)₂SO₄ precipitation. The final pellet and the walls of the centrifuge tube were carefully rinsed several times with a mixture of 1 vol. of sucrose/Tris buffer and 1.5 vol. of saturated (NH₄)₂SO₄ solution to remove excess lipid. The pellet was finally redissolved in sucrose/Tris buffer, and solutions were stored in liquid N₂.

Lipid analyses

Total phospholipid was determined by analysis of phosphate after digestion of protein samples with HClO₄ (Bartlett, 1969). Different phospholipids were separated by t.l.c. on silica-gel plates as described by Rouser & Fleischer (1967). The solvent system was chloroform/methanol/water (65:25:4, by vol.) and the phospholipids were detected with I₂ vapour. Relative proportions of the different lipids were determined by scraping off the appropriate area of the plate and assaying the material directly for phosphorus.

T.l.c. was also used qualitatively to follow the lipid replacement, and since 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine is stained very weakly with I₂ vapour, replacement of endogenous phosphatidylcholine could also be followed (Heron *et al.*, 1977). Quantitative analyses of the results of lipid replacement were obtained by g.l.c. of the fatty acid methyl esters (Heron *et al.*, 1977). After two cycles of replacement with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine the only endogenous phospholipid remaining was cardiolipin, which was measured from the residual linoleic acid content as described by Heron *et al.* (1977).

Restoration of 'Q-pool' behaviour

Supplementation of mixtures of Complex I and Complex III with soya-bean phospholipid and ubiquinone-10 was performed exactly as described by Heron *et al.* (1978). Lipid-replaced Complexes

were supplemented with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine and ubiquinone-10 under identical conditions.

Analysis of 'Q-pool' behaviour

Titration of Complex I with Complex III (Figs. 2 and 3) were fitted to hyperbolas by unweighted least-squares regression analysis (Wilkinson, 1961), thus giving values for the K_m for Complex III and V_1 , the maximum activity (Heron *et al.*, 1978). Theoretical K_m values were calculated from V_1 and V_3 , the maximum activity of the Complex III, as described by Heron *et al.* (1978), and compared with the experimental value. This treatment was based on the original description of 'Q-pool' behaviour by Kröger & Klingenberg (1973a,b).

Stopped-flow measurements

Cytochrome *b* reduction was monitored at 430 nm by using a stopped-flow apparatus built by M.G.G. The mixing ratio was 1:1 and the dead time approx. 2 ms. The light-source was a 150 W tungsten halogen lamp, and the required wavelength was selected by a Spectrolab monochromator (Spectrolab Ltd., Newbury, Berks., U.K.). The band-width was 2 nm. Changes in absorbance were recorded simultaneously on a storage oscilloscope and a Biomac 1000 Data Averaging Device (Data Laboratories Ltd., Mitcham, Surrey, U.K.). Recorded traces were transcribed from

the Biomac memory on to a chart recorder for analysis. Usually four separate runs were summed for each final recording.

Results

Replacement of endogenous phospholipids with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine

The endogenous phosphatidylcholine and phosphatidylethanolamine of Complex I may be replaced with synthetic 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine by equilibration of the enzyme with this lipid in 0.35% cholate (Heron *et al.*, 1977). Little loss of activity occurs unless higher concentrations of cholate are used, under which conditions replacement of endogenous cardiolipin also takes place. Similar results were obtained for Complex III. Thus, after two cycles of equilibration with the synthetic lipid followed by $(\text{NH}_4)_2\text{SO}_4$ fractionation, the total phospholipid content of the enzyme was virtually unchanged, but 75% of this was 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine, and of the remainder, 19% was cardiolipin (Table 1). Thus the cardiolipin content was unchanged, but very nearly all the endogenous phosphatidylcholine and phosphatidylethanolamine had been replaced. Table 2 shows the enzyme activities of both Complex I and Complex III during the course of lipid replacement. Complex I lost a little activity when the synthetic lipid was added

Table 1. *Phospholipid analysis of Complexes before and after lipid replacement*

Analyses were performed as described in the Materials and Methods section. Concentrations of cardiolipin are expressed per phosphate residue or per two fatty acid side chains, i.e. the true molar concentrations are half those indicated.

	Total phospholipid	Content ($\mu\text{mol}/\text{mg}$ of protein)		
		Cardiolipin	Phosphatidylethanolamine	Phosphatidylcholine
Complex I	0.23	0.049	0.073	0.080
Lipid-replaced Complex I	0.28	0.045	0.00	0.22*
Complex III	0.23	0.052	0.080	0.076
Lipid-replaced Complex III	0.24	0.046	0.00	0.18*

* All the phosphatidylcholine was 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine.

Table 2. *Effects of lipid replacement on the activity of Complexes I and III*

Complexes I and III were subjected to two cycles of lipid replacement as described in the Materials and Methods section. Assays of activity at 20°C were performed before and after addition of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine in cholate and on the material obtained after $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Treatment	Complex-I activity (μmol of NADH/min per mg of protein)	Complex-III activity (μmol of cytochrome <i>c</i> / min per mg of protein)
None	1.04	56.4
First addition of lipid/cholate	0.96	8.8
First $(\text{NH}_4)_2\text{SO}_4$ precipitate	0.90	47.5
Second addition of lipid/cholate	0.87	4.8
Second $(\text{NH}_4)_2\text{SO}_4$ precipitate	0.80	40.2

and lost approx. 6% activity during each $(\text{NH}_4)_2\text{SO}_4$ precipitation. Complex III lost most of its activity on addition of the synthetic lipid and cholate, 85% of which returned after $(\text{NH}_4)_2\text{SO}_4$ precipitation. The same occurred during the second cycle of lipid replacement. Lipid-replaced Complex I may be stored in liquid N_2 and frozen and thawed several times with very little loss of activity. The lipid-replaced Complex III, however, was much less stable and activity declined as much as 2-fold after freezing and thawing.

Reconstitution of NADH-cytochrome c oxidoreductase activity from lipid-replaced Complex I and Complex III

A prerequisite for reconstitution of NADH-cytochrome c oxidoreductase activity is that the constituent Complexes are solubilized by detergent before mixing (Hatefi *et al.*, 1962b). The procedure for lipid replacement results in somewhat turbid solutions of Complex I and occasionally Complex III, probably because the detergent/protein ratio is too low for complete solubilization. Mixing of these solutions, followed by dilution, gives rise to poor rates of NADH-cytochrome c oxidoreductase activity. The same happened if solutions of native Complex I and Complex III were dialysed before mixing and dilution. Considerable enhancement of reconstitution could be achieved if deoxycholate and KCl were added to the concentrated mixture of Complexes before dilution (Fig. 1). Neither cholate nor deoxycholate alone was particularly effective, whereas 2% (w/v) cholate in the presence of 1M- $(\text{NH}_4)_2\text{SO}_4$ was 80% as effective as 0.1% (w/v) deoxycholate in the presence of 0.8M-KCl. The latter conditions were routinely used for all reconstitutions with lipid-replaced Complexes.

Fig. 2 shows the reconstitution of NADH-cytochrome c oxidoreductase with lipid-replaced Complex I and Complex III as a function of Complex-III concentration. The shape of the curve was very similar to that found for Complex I and Complex III containing natural lipids (Ragan & Heron, 1978) and was consistent with stoichiometric association of the Complexes in a 1:1 molar ratio. Restoration of 'Q-pool' behaviour was performed by increasing the phospholipid and ubiquinone-10 contents of the system as described by Heron *et al.* (1978), except that 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine was used instead of mixed soya-bean phospholipids. The titration curve under these conditions was hyperbolic (Heron *et al.*, 1978), with a K_m value for Complex III of 0.48 mol/mol of Complex I. The theoretical K_m value obtained from the maximum activities of the Complex I and Complex III was 0.45 mol/mol of Complex III. This value is rather higher than that observed for Complex I and Complex III containing natural phospholipids (Heron *et al.*,

Fig. 1. Effect of solubilization by deoxycholate on reconstitution of NADH-cytochrome c oxidoreductase. Complex I and complex III at protein concentrations of 10 mg/ml were dialysed overnight against 100 vol. of 0.67M-sucrose/50 mM-Tris/HCl, pH 8.0, at 4°C. Complex I (0.25 mg of protein in 40 μl) was mixed at 4°C with 10 μl of 4M-KCl and various amounts of 1% (w/v) sodium deoxycholate to give the indicated final concentrations. Complex III (0.25 mg of protein) was then added and the samples were diluted with sucrose/Tris buffer to 0.5 ml at 0°C. Samples were then assayed after 30 min.

Fig. 2. Reconstitution of NADH-cytochrome c oxidoreductase with lipid-replaced Complexes. Lipid-replaced Complex I (10 μl of a 14.3 μM solution) was mixed at 0°C with various amounts of 27.4 μM-lipid-replaced Complex III to give the indicated molar ratios. KCl (2.5 μl of a 4M solution) and 1.2 μl of 1% (w/v) sodium deoxycholate were then added to all samples together with 25 nmol of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine and 2 nmol of ubiquinone-10 where indicated. Samples were then diluted with sucrose/Tris buffer to 200 μl before assay. ●, No lipid and ubiquinone-10 added; O, with additional lipid and ubiquinone-10.

1978) and can be attributed to the low activity of the Complex III in this experiment ($23\mu\text{mol}$ of cytochrome *c*/min per mg of protein). The considerable overall enhancement of activity by addition of phospholipid and ubiquinone-10 can also be explained by low Complex-III activity. The activity of the stoichiometric Complex I-III unit is decreased by lower activity of either constituent Complex (Fowler & Richardson, 1963). However, the maximum activity during 'Q-pool' behaviour is that of the Complex-I part alone, which is relatively less affected by lipid replacement than the Complex III.

Since the two curves of Fig. 2 do not have very different shapes (for the reasons given above), clearer evidence for restoration of 'Q-pool' behaviour was obtained from titrations performed in the presence of rotenone. As shown by Ragan & Heron (1978) and Heron *et al.* (1978), rotenone decreases the K_m for Complex III during 'Q-pool' behaviour, but has no effect in the shape of titration curve obtained in the absence of 'Q-pool' behaviour. This is clearly shown in the experiments of Fig. 3, where in the presence of additional lipid and ubiquinone-10, rotenone decreased the K_m for Complex III to 0.10mol/mol while having no effect on the line shape obtained in the absence of any supplements.

Thus it is clear that the two types of behaviour of the Complex I-III system can be reproduced after replacement of endogenous phospholipids by 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine.

Fig. 3. Effects of rotenone on reconstitution of NADH-cytochrome *c* oxidoreductase with lipid-replaced Complexes. Lipid-replaced Complex I ($17.7\mu\text{M}$) was treated with rotenone (0.4mol/mol of Complex I) for 1 h at 0°C before $10\mu\text{l}$ samples were mixed at 0°C with various amounts of $27.4\mu\text{M}$ -Complex III in the indicated molar ratios. KCl, deoxycholate, lipid and ubiquinone-10 were added as in Fig. 2 before dilution to $100\mu\text{l}$ with sucrose/Tris buffer. ●, No lipid and ubiquinone-10 added; ○, with lipid and ubiquinone-10.

Since the presence of detergents has marked effects on phase transitions of phospholipids (e.g. Ragan, 1978), it was important to establish that the behaviour of the Complex I-III system was maintained after removal of endogenous detergents by dialysis. In the experiment of Table 3, presence or absence of 'Q-pool' behaviour was assessed by the ratio of the NADH-cytochrome *c* oxidoreductase activity obtained at a low Complex-III concentration to that obtained at a high concentration (Heron *et al.*, 1978). It is clear that whatever behaviour was present before dialysis was maintained after dialysis.

Effects of temperature on the NADH-cytochrome *c* oxidoreductase activity of Complex I-III

Arrhenius plots of the NADH-cytochrome *c* oxidoreductase activity of Complex I-III containing natural phospholipids were linear between 2 and 35°C as found previously (Heron *et al.*, 1978). The activation energy of the stoichiometric unit (i.e. in the absence of 'Q-pool' behaviour) increased slightly but reproducibly with increasing Complex-III/Complex-I ratio (Table 4). As discussed by Ragan & Heron (1978), in any mixture of Complex I and Complex III there is incomplete formation of the Complex I-III unit, i.e. there appears to be a finite dissociation constant for Complex I-III. Any variation of this constant with temperature would change the proportion of the Complex I-III unit in any given mixture and hence the NADH-cytochrome

Table 3. Effects of dialysis on the behaviour of Complex I-III

Complex I (36.5mg of protein/ml, containing 1.0nmol of FMN/mg of protein) and Complex III (12.9mg of protein/ml, containing 3.8nmol of cytochrome *c*₁/mg of protein) were mixed in the indicated molar ratios. Where indicated, 13nmol of ubiquinone-10 and 160nmol of soya-bean phospholipid were added per mg of Complex-I protein. Samples were diluted to a Complex-I concentration of 4mg of protein/ml with 0.67M -sucrose/ 50mM -Tris/HCl, pH8.0, and portions were either left on ice or dialysed overnight at 4°C against 100vol. of sucrose/Tris buffer. Samples were then diluted a further 100-fold and assayed for NADH-cytochrome *c* oxidoreductase activity at 30°C .

Molar ratio of Complex III to Complex I	Additions	NADH-cytochrome <i>c</i> oxidoreductase activity (μmol of cytochrome <i>c</i> /min per mg of Complex-I protein)	
		Undialysed	Dialysed
0.1:1	None	0.20	0.16
2:1	None	1.74	1.32
0.1:1	Lipid/ubiquinone	1.05	1.15
2:1	Lipid/ubiquinone	2.19	2.14

c oxidoreductase activity. The magnitude of this effect would depend on the relative concentrations of Complexes I and III present. We suggest therefore that the small variation in activation energy with Complex-III/Complex-I ratio is due to changes in the apparent dissociation constant of the stoichiometric Complex I-III unit with temperature.

Restoration of 'Q-pool' behaviour caused a very slight increase in activation energy (Table 4, and Heron *et al.*, 1978), which, however, was not dependent on the ratio of Complex III to Complex I, consistent with the explanation given above.

Replacement of endogenous lipids by 1,2-ditetradecanoyl-*sn*-glycero 3-phosphocholine produced marked changes in the Arrhenius plots. In the absence of 'Q-pool' behaviour, activation energies above 24°C were the same as found with natural phospholipids (Table 4). Below 24°C, the activation energies were higher (Fig. 4) and somewhat more dependent on the ratio of Complex III to Complex I (Table 4). These effects of phase transition of the lipid on the activation energy have been reported previously (Ragan & Heron, 1978) by using a different method of lipid replacement. The activation energies obtained by the other method were the same as those of Table 4.

In the presence of 'Q-pool' behaviour, activation energies above the phase-transition temperature were the same as with natural phospholipids, whereas below the phase-transition temperature, values were very similar to those obtained in the absence of 'Q-pool' behaviour. The most striking observation was that the Arrhenius plots exhibited a discontinuity at

20–24°C (Fig. 4). The magnitude of the discontinuity was dependent on the ratio of Complex III to Complex I, being greater at low Complex-III/Complex-I ratios (Fig. 4) than at high ratios (Fig. 5). From

Fig. 4. Arrhenius plots of NADH-cytochrome *c* oxidoreductase reconstituted with lipid-replaced Complexes: low Complex-III/Complex-I ratio

Reconstitution was achieved exactly as described in Fig. 2 with a molar ratio of Complex III to Complex I of 0.2:1. Samples were then dialysed overnight at 4°C against 500 vol. of sucrose/Tris buffer ○, No lipid and ubiquinone-10 added; ●, with additional lipid and ubiquinone-10.

Table 4. Activation energies (E_A) of NADH-cytochrome *c* oxidoreductase activities reconstituted under different conditions
NADH-cytochrome *c* oxidoreductase was reconstituted with Complexes I and III in the indicated molar ratios. Where indicated, lipid-replaced Complexes were used. 'Q-pool' behaviour was restored with soya-bean phospholipid or 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine as appropriate. All samples were dialysed as described in Table 3. Activation energies were obtained from Arrhenius plots covering the temperature range indicated. All other conditions were as described in other Figure and Table legends.

Molar ratio Complex III to Complex I	Phospholipid	Type of behaviour	Temperature range (°C)	E_A (kJ/mol)
0.1:1	Natural	Stoichiometric	2–35	56
2:1	Natural	Stoichiometric	2–35	63
0.2:1	Synthetic	Stoichiometric	2–24	74
0.2:1	Synthetic	Stoichiometric	24–34	56
2:1	Synthetic	Stoichiometric	2–24	100
2:1	Synthetic	Stoichiometric	24–34	60
0.1:1	Natural	'Q-pool'	2–35	68
2:1	Natural	'Q-pool'	2–35	70
0.2:1	Synthetic	'Q-pool'	2–19*	74
0.2:1	Synthetic	'Q-pool'	26–34*	68
0.2:1 (plus rotenone)	Synthetic	'Q-pool'	2–19*	68
0.2:1 (plus rotenone)	Synthetic	'Q-pool'	26–34*	68
2:1	Synthetic	'Q-pool'	2–19*	99
2:1	Synthetic	'Q-pool'	26–34*	72

* Temperature ranges are for linear portions of the Arrhenius plots above and below the discontinuity.

Fig. 5. Arrhenius plots of NADH-cytochrome *c* oxidoreductase reconstituted with lipid-replaced Complexes: high Complex-III/Complex-I ratio

Conditions were exactly as in Fig. 4, except that the molar ratio of Complex III to Complex I was 2:1. ○, No lipid and ubiquinone-10 added; ●, with additional lipid and ubiquinone-10.

extrapolation of the linear portions of Figs. 4 and 5, it was estimated that rate changes of 2.8- and 1.8-fold respectively accompanied the lipid phase transition.

This behaviour can be explained by inspection of Fig. 2. We propose that below the phase-transition temperature of the synthetic phospholipid, protein is excluded from the gel-phase lipid and lipid-protein separation takes place. Such behaviour has been observed in natural mitochondrial membranes (Hackenbrock & Höchli, 1977). It is likely that the protein takes with it some associated or annular phospholipid (Warren *et al.*, 1975), since both component Complexes depend on phospholipid for enzyme activity. However, any phospholipid in excess of this minimal amount, such as is present during 'Q-pool' behaviour, will separate out and the Complexes will therefore associate in 1:1 molar stoichiometry and be unable to dissociate. The discontinuity in the Arrhenius plot is therefore due to a change from 'Q-pool' behaviour to irreversible stoichiometric association. The decrease in activity after such a change would be expected to be greater at a low Complex-III/Complex-I ratio than at a high ratio, as shown by Fig. 2 and as found experimentally. Fig. 3 shows that the changes in rate due to transition between 'Q-pool' behaviour and stoichiometric association should be augmented by partial inhibition of the system with rotenone. Fig. 6 shows that this was

the case. At a molar ratio of Complex III to Complex I of 0.2:1, the change of rate at the phase-transition temperature was 6.9-fold when rotenone (0.4 nmol/mg of Complex-I protein) was present.

Kinetics of cytochrome *b* reduction

Ragan & Heron (1978) and Heron *et al.* (1978) showed that in mixtures of Complex I and Complex III inhibited by antimycin, the reduction of cytochrome *b* by NADH was biphasic. The fast phase corresponded to reduction of Complex III stoichiometrically associated with Complex I and which therefore never exceeded 1 mol of Complex III/mol of Complex I. The slow phase was due to reduction of unassociated Complex III. In accordance with the predictions of 'Q-pool' behaviour, the biphasicity was apparently abolished when such behaviour was restored to the system (i.e. by adding phospholipid). To substantiate these experiments further and to enable the kinetics to be resolved at higher temperatures we have used stopped-flow techniques. Fig. 7 shows the A_{430} changes after addition of NADH to mixtures containing 2 mol of Complex III/mol of Complex I. The inset shows the analysis of the curves as semilogarithmic plots and reveals two phases of reduction of equal magnitude with first-order rate constants of 0.52 s^{-1} and 0.041 s^{-1} at 15°C and 2.1 s^{-1} and 0.38 s^{-1} at 27°C .

Fig. 6. Arrhenius plots of NADH-cytochrome *c* oxidoreductase reconstituted with lipid-replaced Complexes: effect of rotenone

Conditions were the same as in Fig. 4, with additional lipid and ubiquinone-10. After dialysis, rotenone was added (0.4 mol/mol of Complex I) and the sample incubated 1 h at 0°C before assay. Activity at 30°C was $0.20\text{ }\mu\text{mol}$ of cytochrome *c*/min per mg of Complex-I protein.

The total A_{430} change is due to reduction of approx. 50% of the total cytochrome *b* present as previously reported (Ragan & Heron, 1978). Cytochrome c_1 is completely reduced by NADH in the presence of antimycin (Rieske, 1976) and accounts for approx. 20% of the A_{430} change. However, other experiments showed that the rate of cytochrome c_1 reduction was the same as that of cytochrome *b* (presumably because a previous step was rate-limiting). Consequently the contribution of cytochrome c_1 reduction to the A_{430} change did not interfere with the kinetic analysis.

The two phases of reduction of cytochrome *b* are due to reduction of Complex-III molecules stoichiometrically associated with Complex I (the fast phase) and those in excess of the Complex-I molecules and unassociated (the slow phase) (Ragan & Heron, 1978). Since the molar ratio of Complex III to Complex I was 2:1, the phases are of equal magnitude.

Restoration of 'Q-pool' behaviour with soya-bean

phospholipids and ubiquinone-10 abolished the biphasic response (Fig. 8). Thus, even though in molar excess over Complex I, all the Complex-III molecules were kinetically indistinguishable. The first-order rate constant for 'Q-pool' behaviour (1.06s^{-1} at 15°C) was greater than that obtained for the fast phase of Fig. 7. Experiments at higher temperatures were unreliable because of the temperature sensitivity of the system. Thus at 27°C , the extent of cytochrome *b* reduction, although monophasic, was decreased and continued to decrease with time of incubation. The trace of Fig. 8 reveals a delay in the onset of cytochrome *b* reduction, which was not found in Fig. 7. This was routinely observed and may be caused by prior reduction of the increased pool of ubiquinone-10 molecules present in such experiments. On the other hand, the delay may be due in some way to partial denaturation, since the lag was more prominent at higher temperature.

Fig. 7. Biphasic reduction of cytochrome *b*

Complex I (10nmol) and Complex III (20nmol) were mixed and diluted to 2.5ml at 4°C with 0.2M-sucrose/50mM-Tris/HCl, pH8.0. The mixture was dialysed overnight against 500ml of 0.2M-sucrose/Tris buffer at 4°C and then diluted to 10ml with the same buffer which had been filtered and degassed under vacuum. Antimycin ($10\mu\text{g}$) was then added. This mixture was loaded into one syringe of the stopped-flow apparatus. The other syringe contained 0.1mM-NADH in filtered and degassed sucrose/Tris buffer. Each trace is the sum of four separate stopped-flow experiments. The arrows indicate the initial and final absorbances for both traces. The inset shows plots of $\log(100 - \% \Delta A)$ against time. For these graphs, several traces such as those shown were obtained on different time scales and values of ΔA at any particular time were averaged. Temperatures: (a) 27°C ; (b) 15°C .

Fig. 8. Monophasic reduction of cytochrome *b*

Complex I (8 nmol), Complex III (16 nmol), soya-bean phospholipids (2.55 μ mol) and ubiquinone-10 (0.12 μ mol) were mixed and diluted to 2.0 ml with 0.2 M-sucrose/50 mM-Tris/HCl, pH 8.0, at 4°C. Dialysis, dilution to 9 ml and stopped-flow measurements proceeded as in Fig. 7. Temperature was 15°C.

The experiment of Fig. 9 shows the kinetics of cytochrome *b* reduction in mixtures of lipid-replaced Complex I and Complex III. 'Q-pool' behaviour had been restored by addition of 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine and ubiquinone-10. At 27°C, the reduction was predominantly monophasic and no delay in the onset of reduction was observed. The semilogarithmic plot of the results was slightly curved, indicating some heterogeneity of reduction.

At 15°C, on the other hand, reduction was extremely biphasic. The first-order rate constants were 0.48 s^{-1} and approx. 0.01 s^{-1} , compared with 0.68 s^{-1} at 27°C. The extents of the fast and slow phases were 44 and 56% of the total respectively.

The total extent of reduction in the experiments of Fig. 9 was smaller than expected from the known cytochrome *b* concentration. Further analysis showed that only 28% of the total cytochrome *b* was reducible by NADH (as opposed to 50% under the conditions of Figs. 7 and 8) and that the absorption maximum of the dithionite-reduced cytochrome *b* had shifted to approx. 560 nm, indicating denaturation. Thus it is likely that the loss of Complex-III activity observed after lipid replacement is due to denaturation of cytochrome *b*. In view of this added complexity to the system, it is perhaps not surprising that the kinetics of reduction of cytochrome *b* at 27°C are not pure first-order, and that the extents of slow and fast phases at 15°C are not exactly equal. In addition, 27°C is only just above the transition temperature

range and it is possible that fluidization was not quite complete. Unfortunately we were unable to get reliable results at higher temperatures, because of the instability of the lipid-replaced Complex III.

The rate constant of 2.1 s^{-1} obtained for the fast phase of Fig. 7 at 27°C may be used to obtain an initial rate of electron transfer. This rate constant applies to the reduction of only 1 mol of Complex III/mol of Complex I (i.e. 1 nmol of Complex III/mg of Complex-I protein). Only 50% of the total cytochrome *b* in the Complex III was reduced (i.e. 1 mol of cytochrome *b*/mol of Complex III). Therefore the initial rate of cytochrome *b* reduction was 2.1 nmol/s per mg of Complex-I protein. If one assumes that reduction of cytochrome *b* is accompanied by reduction of cytochrome *c*₁ (Mitchell, 1976), the rate of electron transfer is 4.2 nequiv./s per mg of Complex-I protein. This rate is considerably less than the steady-state rate of NADH-cytochrome *c* oxidoreductase (2.4 μ mol of cytochrome *c*/min per mg of Complex-I protein at 27°C). Thus the initial rate of electron transfer to antimycin-inhibited Complex III is one-tenth of the steady-state turnover.

Mitchell (1975) has proposed a 'Q-cycle' to account for electron-transfer events in Complex III. The cyclical nature of the scheme in its simplest form does not allow changes in the redox state of ubiquinone, and modifications are required for behaviour other than steady-state turnover (Mitchell, 1976). The experiments of Figs. 7, 8 and 9 proceed from fully

Fig. 9. Effects of lipid-phase transition on cytochrome *b* kinetics

Lipid-replaced Complexes I (8 nmol) and III (16 nmol) were mixed with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine (2.55 μ mol) and ubiquinone-10 (0.12 μ mol) at 4°C, and diluted to 2.0 ml with 0.2 M-sucrose/50 mM-Tris/HCl, pH 8.0. Dialysis, dilution to 8 ml and stopped-flow measurements were performed as in Fig. 7. Temperature (a) 27°C; (b) 15°C.

oxidized to fully reduced in the presence of antimycin. Thus not even one operation of the 'Q-cycle' is completed. The slower reduction kinetics may therefore be due to limitation by an intermediate reductant, e.g. ubisemiquinone, whose concentration under non-inhibited conditions would build up, perhaps after several cycles, to a value capable of sustaining the steady-state electron-transfer rates observed. Although these considerations do not affect the explanations proposed for biphasic and monophasic behaviour, they may well be important for the relative rates of the fast and slow phases of Figs. 7 and 9. If the fast phase is limited by ubisemiquinone concentration, the slow phase may be limited by something else, such as the dissociation of the Complex I-III unit. Thus it may be more correct to compare the low rate of electron transfer (0.046 μ equiv./min per mg of Complex-I protein at 27°C from Fig. 7) with the steady-state turnover (2.4 μ equiv./min per mg of Complex-I protein) to give a measure of the ability of Complex I to transfer reducing equivalents to un-associated Complex III.

Discussion

The results presented above show that the way in which the respiratory Complexes I and III interact in the mitochondrial membrane (i.e. 'Q-pool' behaviour) can be reproduced with the purified enzymes and a single pure phospholipid, 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine. The Complexes still contain their complement of cardiolipin, which is essential for the activity of Complex I at least (Heron *et al.*, 1977).

We have suggested that in the natural membrane, electron transfer from Complex I to Complex III takes place via a stoichiometric Complex I-III unit which contains 2 or 3 molecules of ubiquinone-10 in a special environment (Ragan & Heron, 1978; Heron *et al.*, 1978). These units dissociate and reassociate randomly at rates exceeding the electron-transfer rates, thereby mixing the associated and unassociated ubiquinone-10 and leading to so-called 'Q-pool' behaviour (Kröger & Klingenberg, 1973*a,b*). Irreversible formation of the Complex I-III unit takes place

when the lipid/protein ratio is low, i.e. as found in the isolated Complexes, which may only contain a single annulus of phospholipid (Heron *et al.*, 1977; Ragan, 1978; Ragan & Heron, 1978). This is illustrated in Fig. 10(a). The Complexes are drawn approximately to size on the basis of mol.wts. [250 000 for Complex III (Rieske, 1976) and 850 000 for Complex I (Ragan, 1976b)], although the molecular dimensions of Complex I are unknown. The ubiquinone-containing interface need not contain phospholipid if the approach of the two Complexes is close. When the phospholipid and ubiquinone-10 contents are raised (Fig. 10b), the Complexes are permitted to associate reversibly, leading to 'Q-pool' behaviour. Ubiquinone-10 is shown occupying both the interface and the bulk lipid. The configuration of ubiquinone-10 in the membrane is unknown, although its side chain is certainly long enough to span the membrane entirely (Trumpower & Katki, 1978).

When the Complexes are irreversibly associated (Fig. 10a), lowering the temperature to below the phase-transition temperature of the lipid had a relatively small effect on activity, the activation energy rising between 20 and 40 kJ/mol depending on conditions. When Complex I was assayed with exogenous ubiquinone-1 as substrate, phase transition had no effect on activity unless a large excess of phospholipid was present (Ragan, 1978). Complex-III activity with ubiquinol-2 as substrate was not affected by phase transitions whatever the lipid content. Thus effects of phase transitions on the activity of the stoichiometric Complex I-III unit do not appear to be related to effects on protein conformation, but probably reflect changes in the mobility and/or concentration of ubiquinone-10 in the interface between the Complexes.

Fig. 10. Postulated organization of lipid and protein in Complexes interacting under different conditions (a) Interaction of Complexes as isolated; (b) 'Q-pool' behaviour; (c) below the phase-transition temperature of the lipid. I, Complex I; III, Complex III; Q, ubiquinone-10.

It is possible that when phospholipid is added to restore 'Q-pool' behaviour, the effect is not simply due to a raising of the lipid/protein ratio, but to some much larger-scale changes in the physical state of the system. The experiments with the lipid-replaced system argue against this possibility. The simplest explanation for the discontinuities of the Arrhenius plots of Figs. 4, 5 and 6 is that lipid-protein separation is occurring during transition to the gel state, and this, in effect, lowers the lipid/protein ratio, so that the Complexes are irreversibly bound in Complex I-III units as illustrated in Fig. 10(c). The transition is reversible and rapid, implying that all events are taking place within the same membrane, as illustrated in Fig. 10.

Finally, we wish to add that we have referred to Arrhenius plots such as that of Fig. 6 as 'discontinuous' to distinguish them from plots that exhibit a simple change in slope. Strictly, the transition of Fig. 6 is not discontinuous, since it takes place over approx. 5°C, rather than, e.g., 0.1°C. This is a rather larger temperature range than might be expected even for phospholipids whose transitions are continuous in the thermodynamic sense (Lee, 1977). The fact that fluidization appears to start at approx. 20°C may indicate some effect of the protein in lowering the phase-transition temperature and decreasing the cooperativity of the transition.

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