The Interaction between Mitochondrial NADH-Ubiquinone Oxidoreductase and Ubiquinol-Cytochrome *c* Oxidoreductase

RESTORATION OF UBIQUINONE-POOL BEHAVIOUR

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1. In the inner mitochondrial membrane, dehydrogenases and cytochromes appear to act independently of each other, and electron transport has been proposed to occur through a mobile pool of ubiquinone-10 molecules [Kröger & Klingenberg (1973) *Eur. J. Biochem.* **34**, 358–368]. 2. Such behaviour can be restored to the interaction between purified Complex I and Complex III by addition of phospholipid and ubiquinone-10 to a concentrated mixture of the Complexes before dilution. 3. A model is proposed for the interaction of Complex I with Complex III in the natural membrane that emphasizes relative mobility of the Complexes rather than ubiquinone-10. Electron transfer occurs only through stoichiometric Complex I–Complex III units, which, however, are formed and re-formed at rates higher than the rate of electron transfer.

In the preceding paper (Ragan & Heron, 1978) we presented evidence for a stoichiometric association between NADH-ubiquinone oxidoreductase (Complex I) and ubiquinol-cytochrome *c* oxidoreductase (Complex III). A serious shortcoming of these observations was that Kröger & Klingenberg (1973*a,b*) had clearly shown that the kinetics of the respiratory chain and the redox and kinetic behaviour of ubiquinone-10 were apparently incompatible with the idea that dehydrogenases and cytochromes associated in fixed stoichiometric complexes. In the present paper, we show that the behaviour of the natural inner mitochondrial membrane can be restored to the purified Complex I–Complex III system, i.e. that the two Complexes appear to be linked by a mobile pool of ubiquinone-10 molecules. However, our interpretation of the mechanism of ‘Q-pool’ behaviour differs from that of Kröger & Klingenberg (1973*a,b*) and specifically takes account of stoichiometric association of Complexes.

Materials and Methods

Biological preparations

Complex I (EC 1.6.99.3) and Complex III were prepared by the methods of Hatefi & Rieske (1967) and Rieske (1967) respectively. Molarities of solutions of these enzymes were based on analyses of acid-extractable flavin and cytochrome *c*, respectively, as described in the preceding paper (Ragan & Heron, 1978).

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Chemicals

Phospholipids were obtained or purified as described in the preceding paper (Ragan & Heron, 1978). A purified phospholipid fraction was obtained from crude soya-bean lipids (‘L- α -lecithin’, from Sigma, Poole, Dorset, U.K.) as described by Ragan & Racker (1973). This preparation is referred to simply as ‘phospholipid’ below.

Assays

Enzyme assays and analyses of phosphate, protein, ubiquinone-10, acid-extractable flavin and cytochrome *c*₁ were as described in the preceding paper (Ragan & Heron, 1978). Rates of NADH-cytochrome *c* oxidoreductase activity were measured at 30°C in the absence of a phospholipid supplement in the assay, unless otherwise stated.

Supplementation of complexes with phospholipid and ubiquinone-10

Stock solutions of phospholipids were prepared by ultrasonic dispersion of the dry lipid in water at room temperature to give a final concentration of 5 mM phospholipid phosphorus. Dispersion was performed in a bath-type sonicator until the solutions were essentially clear, as described by Ragan (1978). Such solutions could be kept at 4°C for a few days, but not frozen. Ubiquinone-10 was dissolved in ethanol by warming to approx. 50°C to give a 5 mM stock solution that was subsequently kept at –20°C. As

required, the ubiquinone-10 was redissolved by warming and the solution diluted with warm (40°C) ethanol as necessary. Solutions of Complexes I and III, containing at least 10mg of protein/ml, were mixed at 0°C in the desired proportions. Phospholipid solution was then added, followed immediately by the ubiquinone-10 solution. The amount of ethanol added with the ubiquinone-10 did not exceed 5% of the total sample volume. The sample was mixed rapidly and allowed to stand for 10min at 0°C before dilution with 0.67M-sucrose/50mM-Tris/HCl, pH8, as described by Ragan & Heron (1978).

Theoretical analysis of Q-pool behaviour

Kröger & Klingenberg (1973a) showed that the rate of reduction of endogenous ubiquinone and the rate of oxidation of endogenous ubiquinol by submitochondrial particles were first-order with respect to the concentration of ubiquinone and ubiquinol respectively. They defined $V_{red.}$ as the maximum velocity of the ubiquinone-reducing system (i.e. when the endogenous ubiquinone was 100% oxidized) and $V_{ox.}$ as the maximum velocity of the ubiquinol-oxidizing system (i.e. when the endogenous ubiquinone was 100% reduced). The overall oxidase activity (V) was then related to $V_{ox.}$ and $V_{red.}$ as follows:

$$V = \frac{V_{ox.} \times V_{red.}}{V_{ox.} + V_{red.}} \quad (1)$$

Thus when $V_{ox.}$ is infinite (giving 100% oxidized ubiquinone), $V = V_{red.}$, and when $V_{red.}$ is infinite (giving 100% reduced ubiquinone), $V = V_{ox.}$. Other estimates of $V_{ox.}$ could be obtained from the initial rate of oxidation of endogenous ubiquinol after addition of O_2 to anaerobic particles or alternatively from the initial rates of oxidation of saturating concentrations of duroquinol (Kröger & Klingenberg, 1973a). This equation implies that the complete activities of the reducing and oxidizing systems, irrespective of the numbers of molecules present, determine the overall activity. Thus ubiquinone must effectively act as a mobile carrier linking any number of dehydrogenases with any other number of cytochrome chains. The total ubiquinone molecules must function as a homogeneous population, and ubiquinone must therefore fulfil a pool function if this equation is obeyed by the system under investigation.

We have adapted the pool function eqn. (1) to the titration of Complex I with Complex III. Rates of NADH-cytochrome *c* oxidoreductase (V) are expressed as μmol of cytochrome *c*/min per mg of Complex I protein. The maximum rate of this activity (V_1), at saturating concentrations of Complex III, is equal to $V_{red.}$ (since $V_{ox.}$ is infinite). Complex III activities were measured from the initial rates of

oxidation of saturating concentrations of 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol by cytochrome *c*. These rates (V_3) are expressed as μmol of cytochrome *c*/min per mg of Complex III protein, and are assumed to be a measure of $V_{ox.}$. The validity of this assumption is shown in the Results section.

If x_1 and x_3 are the nmol of flavin and cytochrome *c*₁ per mg of Complex I and Complex III protein respectively, a molar ratio of Complex III to Complex I of N corresponds to x_1N/x_3 g of Complex III. Thus $V_{ox.}$ is given by $V_3x_1N/x_3 \mu\text{mol}$ of cytochrome *c*/min per mg of Complex I. The rate of NADH-cytochrome *c* oxidoreductase (V) is therefore given by:

$$V = \frac{V_1 V_3 \frac{x_1 N}{x_3}}{V_1 + \frac{V_3 x_1 N}{x_3}} = \frac{V_1 N}{\frac{x_3 V_1}{x_1 V_3} + N} \quad (2)$$

Thus V varies hyperbolically with N , and the curve has a K_m value for Complex III of $x_3 V_1/(x_1 V_3)$. V_1 is determined by extrapolation of V to infinite N , and V_3 from the quinol-cytochrome *c* oxidoreductase rates. Thus the K_m can be predicted and compared with that obtained by direct titration.

For any given preparations of Complex I and Complex III the K_m value may be decreased by decreasing V_1 (e.g. with rotenone) and increased by decreasing V_3 (e.g. with antimycin). On the other hand, if the Q pool does not operate, and Complexes I and III associate in 1:1 stoichiometry, the titration curve is not hyperbolic and is unaffected by the absolute values of V_1 and V_3 , as shown in the preceding paper (Ragan & Heron, 1978). Half-maximal velocity is attained at a molar ratio somewhat greater than 0.5mol of Complex III per mol of Complex I. We wish to make the point that, although eqn. (2) was devised from the redox behaviour of ubiquinone in submitochondrial particles, we have not yet determined the dependence of the ubiquinone redox state on V_1 and V_3 in the Complex I-Complex III system.

Analysis of titration data

Results from titration experiments were corrected for the Complex III already present in the Complex I as described in the preceding paper (Ragan & Heron, 1978). The data were fitted to a hyperbolic curve by using an unweighted least-squares regression analysis (Wilkinson, 1961), thus giving values for K_m and V_1 .

Results

Restoration of Q-pool behaviour with ubiquinone-10 and phospholipids

In Fig. 1, Complex I was titrated with Complex III in the presence and absence of additional ubiquinone-

Fig. 1. Titration of Complex I with Complex III in the presence and absence of phospholipid and ubiquinone-10

Complex I ($19.2\mu\text{M}$ or 19.0mg of protein/ml) was mixed at 4°C with various amounts of Complex III ($49.0\mu\text{M}$ or 12.9mg of protein/ml) to give the indicated molar ratios of Complex III to Complex I after correction for the Complex III contamination of the Complex I ($0.14\mu\text{mol}$ of Complex III/mg of Complex I protein). In curve (b), phospholipid ($0.16\mu\text{mol}$ /mg of Complex I protein) and ubiquinone-10 (10.4nmol /mg of Complex I protein) were also added to each Complex I-Complex III mixture as described in the Materials and Methods section. After 10 min, samples were diluted at 4°C with 0.67M -sucrose/ 50mM -Tris/HCl, pH 8.0, to give a final concentration of 0.38mg of Complex I protein/ml. After at least 10 min at 4°C , NADH-cytochrome *c* oxidoreductase activity was measured as described in the Materials and Methods section. In curve (b), V_1 was determined by extrapolation to infinite Complex III concentration [6.0 ± 0.4 (S.E.M., $n = 6$) μmol of cytochrome *c*/min per mg of Complex I protein]. V_3 was determined from the rate of reduction of cytochrome *c* by $40\mu\text{M}$ -6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol to be $91.8\mu\text{mol}$ of cytochrome *c*/min per mg of Complex III. The theoretical K_m was therefore 0.25 ± 0.02 (S.E.M., $n = 6$) mol of Complex III/mol of Complex I.

10 and phospholipid. In the absence of ubiquinone-10 and phospholipid (Fig. 1a), the titration curve was as described in the preceding paper (Ragan & Heron, 1978). However, in the presence of ubiquinone-10 and phospholipid (Fig. 1b), the shape of the titration curve was hyperbolic, with a K_m for Complex III of 0.23 ± 0.05 (S.E.M., $n = 6$) mol/mol of Complex I. The theoretical K_m for Q-pool behaviour, calculated as described in the Materials and Methods section, was 0.25 ± 0.02 (S.E.M., $n = 6$) mol/mol of Complex I. Thus it appears that Q-pool behaviour can be restored to the Complex I-Complex III system by increasing the concentration of phospholipid and ubiquinone-10. The agreement in this and other experiments

Fig. 2. Effects of antimycin and rotenone on the K_m for Complex III

(a) Complex I ($19.2\mu\text{M}$) was mixed with a $750\mu\text{M}$ solution of rotenone in ethanol to give a final concentration of 1 mol of rotenone/mol of Complex I. After 15 min at 4°C , portions were mixed with Complex III, phospholipid and ubiquinone-10 exactly as described in the legend to Fig. 1. V_1 was $1.08\mu\text{mol}$ of cytochrome *c*/min per mg of Complex I and V_3 was $89.4\mu\text{mol}$ of cytochrome *c*/min per mg of Complex III. (b) Complex III ($49.6\mu\text{M}$) was mixed with a $400\mu\text{M}$ solution of antimycin in ethanol to give a final concentration of 0.8 mol of antimycin/mol of Complex III. Complex I was treated similarly to inhibit the endogenous Complex III to the same extent. After 15 min at 4°C , the Complex I, Complex III, phospholipid and ubiquinone-10 were mixed and diluted as described in Fig. 1. V_1 was $4.53\mu\text{mol}$ of cytochrome *c*/min per mg of Complex I and V_3 was $10.1\mu\text{mol}$ of cytochrome *c*/min per mg of Complex III.

between experimental and theoretical K_m values establishes the validity of the methods used for determination of V_1 and V_3 .

Effects of rotenone and antimycin

The K_m for Complex III was not constant, but depended on the relative activity of the Complex I and Complex III. Thus in the presence of rotenone (Fig. 2a) the K_m for Complex III was decreased to 0.06 ± 0.015 (S.E.M., $n = 6$) mol/mol of Complex I, compared with a theoretical value of 0.045 ± 0.003 (S.E.M., $n = 6$) mol/mol. The contamination of the Complex I by Complex III (0.14mol /mol of Complex I) was sufficient to give nearly maximal rates of NADH-cytochrome *c* oxidoreductase in this experiment, which accounts for the large standard error of the experimental K_m value.

Antimycin, on the other hand, increased the K_m value (Fig. 2b). The maximum velocity in the presence of antimycin ($4.53 \mu\text{mol/min}$ per mg of Complex I) was the same as that achieved in the absence of antimycin, as expected from Q-pool behaviour, but the K_m value increased to 2.1 ± 0.4 (S.E.M., $n = 6$) mol of Complex III per mol of Complex I [theoretical value 1.7 ± 0.2 (S.E.M., $n = 6$) mol/mol]. This behaviour contrasts very clearly with that obtained in the preceding paper, where inhibition by antimycin caused a percentage decrease in NADH-cytochrome *c* oxidoreductase activity that was independent of the ratio of Complex I to Complex III. In other words, when the Q-pool is not operating, titration of Complex I with antimycin-inhibited Complex III reaches a lower maximum activity without a change in the apparent K_m . This effect was confirmed (results not shown).

Restoration of Q-pool behaviour as a function of ubiquinone-10 and phospholipid concentrations

The experiment of Fig. 1 shows that the greatest increase in rate after restoration of Q-pool behaviour occurs at the lowest concentration of Complex III, i.e. when no Complex III is added to that already present in the Complex I. Restoration of Q-pool behaviour can therefore be assessed from the increase in the NADH-cytochrome *c* oxidoreductase activity of the Complex I. Since the maximum velocity also changes, the rate in the absence of Complex III expressed as a percentage of the rate in the presence

of excess Complex III is a more reliable measure of Q-pool restoration. The data of Table 1 show that ubiquinone-10 alone did not cause restoration of Q-pool behaviour and, indeed, higher concentrations seemed to decrease the ability of the Complex III to interact with the Complex I. Phospholipid alone did restore Q-pool behaviour, but not quite as well as when ubiquinone-10 was also present. Examination of the absolute rates revealed that ubiquinone-10 stimulated activities, whereas phospholipid alone tended to decrease the rates obtained in the presence of Complex III, i.e. the maximum rates of the system. Broadly, therefore, phospholipid alone restores the Q-pool function, but extra ubiquinone is required to maintain high rates of electron transport. The amounts of ubiquinone-10 and phospholipid required for optimum behaviour were approx. 25 nmol of phospholipid and 2 nmol of ubiquinone-10, although considerable variation about these values did not cause great variation in rates. Complex I and Complex III both contain about $0.20\text{--}0.24 \mu\text{mol}$ of phospholipid per mg of protein, and in the experiments of Table 1 the endogenous phospholipid content of the Complex I was 32 nmol. The endogenous ubiquinone-10 content of the Complex I was 2.1 nmol per mg of protein, i.e. 0.34 nmol per experiment. Thus restoration of the Q-pool required a 2-fold increase in phospholipid content and approx. a 6-fold increase in ubiquinone-10 content. Since Complex III contains phospholipid and ubiquinone-10 ($1.5\text{--}4.0 \text{ nmol/mg}$ of protein), then titration with Complex III alone introduces more ubiquinone-10 and phospholipid

Table 1. *Restoration of Q-pool behaviour as a function of phospholipid and ubiquinone-10 concentrations*
Complex I (0.154 nmol), or Complex I (0.154 nmol) mixed with Complex III (0.347 nmol), was mixed with the indicated amounts of ubiquinone-10 or phospholipid as described in the Materials and Methods section and diluted to a final volume of $400 \mu\text{l}$ with sucrose/Tris buffer. Samples were then assayed for NADH-cytochrome *c* oxidoreductase activity. The activity of Complex I alone was due to Complex III present as a contaminant of the Complex I (0.15 mol/mol of Complex I). Thus in the absence of Q-pool behaviour, the activity should have been approx. 15% of that obtained in the presence of excess Complex III (as found experimentally).

Additions	Amount (nmol)	NADH-cytochrome <i>c</i> oxidoreductase ($\mu\text{mol/min}$ per mg of Complex I)		Ratio of activity —Complex III to activity + Complex III
		—Complex III	+Complex III	
None		0.41	2.95	0.14
Ubiquinone-10	1	0.63	4.24	0.15
	2	0.67	4.95	0.14
	3	0.39	4.90	0.08
Phospholipid	10	0.84	2.77	0.30
	25	0.79	2.43	0.33
	50	0.76	2.00	0.38
Ubiquinone-10 plus phospholipid	2	2.09	4.27	0.49
	25			

Table 2. *Restoration of Q-pool behaviour with different phospholipids*

Experimental conditions were exactly as in Table 1. All samples received 25 nmol of the indicated phospholipid. Where phospholipid was added, 2 nmol of ubiquinone-10 was also present.

Phospholipid added	NADH-cytochrome <i>c</i> oxidoreductase ($\mu\text{mol/min per mg}$ of Complex I)		Ratio of activity –Complex III to activity+ Complex III
	–Complex III	+Complex III	
None	0.47	2.70	0.17
Soya-bean phospholipid	1.82	4.33	0.42
Soya-bean phosphatidylcholine	1.44	3.61	0.40
Soya-bean phosphatidylethanolamine	1.00	2.13	0.47
1,2-Ditetradecanoyl- <i>sn</i> -glycero-3-phosphocholine	1.67	3.64	0.46
Bovine heart cardiolipin	0.61	1.67	0.37

into the system. The failure to observe gradual restoration of Q-pool behaviour in this case suggests that the concentrations of ubiquinone-10 and phospholipid must be raised relative to the protein concentration of the system.

In the experiments so far described, the ubiquinone-10 and phospholipid were added separately as ethanolic solutions and aqueous dispersions respectively. We have not detected any effects on rates of the order of addition of these supplements, or if the ubiquinone-10 and phospholipid were co-dispersed by sonication in water before addition to the complexes.

Dependence of Q-pool behaviour on the chemical nature of the phospholipid

In the experiment of Table 2, restoration of Q-pool behaviour by a number of different phospholipids is shown. All the phospholipids used were effective, but the absolute rates varied considerably. In particular, bovine heart cardiolipin supported relatively low rates of electron transport. Possible explanations are direct inhibition of electron transport and effects on the availability of the ubiquinone-10 present for electron transfer between the complexes. However, since all lipids restored Q-pool behaviour we have not investigated these effects further.

Time course of Q-pool restoration

After addition of phospholipid and ubiquinone-10 to a Complex I–Complex III mixture, Q-pool behaviour was restored at the earliest times at which measurements could be made, i.e. approx. 30 s after addition of the supplements.

Effects of a phospholipid supplement in the assay

Rates of NADH-cytochrome *c* oxidoreductase are generally stimulated by addition of phospholipid

Fig. 3. *Effects of phospholipid in the assay on restorations of Q-pool behaviour*

Complex I ($19.4\mu\text{M}$) was treated with rotenone to a final concentration of 0.8 mol/mol of Complex I. In curve (b), the inhibited Complex I was mixed with Complex III, phospholipid and ubiquinone-10 as described in Fig. 1. In curve (a), the phospholipid was omitted. After dilution, samples were assayed for NADH-cytochrome *c* oxidoreductase activities in an assay mixture that was supplemented with 0.4 mM-phospholipid as described in the preceding paper (Ragan & Heron, 1978). K_m values were (a) 0.22 mol of Complex III/mol of Complex I and (b) 0.05 mol of Complex III/mol of Complex I. V_1 and V_3 were the same in both (a) and (b).

after dilution of the Complex I–Complex III mixture into the assay as described in the preceding paper (Ragan & Heron, 1978). Restoration of Q-pool behaviour, however, does not occur. If Complex I–Complex III is preincubated with ubiquinone-10 alone, restoration of Q-pool behaviour takes place to a considerable extent when phospholipid is subsequently present in the assay. This is shown in Fig. 3, which compares the titration of rotenone-inhibited

Complex I and Complex III preincubated with ubiquinone-10 (*a*) or ubiquinone-10 and phospholipid (*b*) and assayed in the presence of a phospholipid supplement. The system preincubated with ubiquinone-10 alone was not capable of reaching very low K_m values, as shown in Fig. 3. However, for higher K_m values, for example those in the absence of rotenone, preincubation with ubiquinone-10 and assay in the presence of phospholipid was sufficient for complete restoration of Q-pool behaviour. The different behaviour of phospholipid under these circumstances appears to be due to the lower concentration of the components involved. Thus, restoration of Q-pool behaviour by preincubation with phospholipid (as in Fig. 1) only occurred when the protein and detergent concentrations were high and the complexes were still dispersed. A 5-fold dilution of such mixtures before phospholipid addition was sufficient to prevent the restoration of Q-pool behaviour.

Restoration of hyperbolic antimycin titration curves

One of the characteristic features of intact mitochondrial membranes is the hyperbolic relationship between NADH oxidase or succinate oxidase

Fig. 4. Antimycin titration of Complex I-Complex III. In (*a*) Complex I (0.115 nmol) and Complex III (0.223 nmol) were mixed and diluted to 3.0 ml with sucrose/Tris buffer at 4°C. In (*b*) 15 nmol of ubiquinone-10 and 187 nmol of phospholipid were added before dilution of the Complex I-Complex III mixture. Samples were then incubated at 4°C for 1 h with antimycin (30 μ M in ethanol) to give the indicated molar ratios of antimycin to Complex III (after correction for the Complex III in the Complex I). The final ethanol concentration did not exceed 3% (v/v). Samples were then assayed for NADH-cytochrome *c* oxidoreductase activity. Uninhibited rates were: (*a*) 3.8 μ mol of cytochrome *c*/min per mg of Complex I protein; (*b*) 5.2 μ mol of cytochrome *c*/min per mg of Complex I protein.

activity and antimycin concentration. The shape of this curve has been exactly accounted for by Kröger & Klingenberg (1973*b*) on the basis of the Q-pool function. Fig. 4 shows antimycin titrations of the NADH-cytochrome *c* oxidoreductase activity of a mixture containing 2 mol of Complex III/mol of Complex I. Curve (*a*) was obtained in the absence of a ubiquinone-10 and phospholipid supplement, and is similar to those reported elsewhere (Fowler & Richardson, 1963; Ragan & Heron, 1978). Curve (*b*) was obtained in the presence of ubiquinone-10 and phospholipid and clearly the degree of inhibition by antimycin, particularly at intermediate concentrations, had decreased. However, the antimycin titre was unaffected. This behaviour is that expected if the Q-pool is operating (Kröger & Klingenberg, 1973*b*).

Abolition of biphasic cytochrome *b* reduction

In the preceding paper it was shown that at low temperatures reduction of cytochrome *b* in Complex I and Complex III mixtures by NADH was biphasic.

Fig. 5. Effects of phospholipid and ubiquinone-10 on the kinetics of cytochrome *b* reduction at 3°C

Mixtures containing 0.384 nmol of Complex I and 1.12 nmol of Complex III were: (*a*) diluted to a final volume of 3.0 ml with sucrose/Tris buffer; (*b*) mixed with 5 nmol of ubiquinone-10 before dilution; (*c*) mixed with 62.5 nmol of phospholipid before dilution; (*d*) mixed with both ubiquinone-10 and phospholipid before dilution. Each sample was treated with 10 nmol of antimycin. Cytochrome *b* reduction was measured at 430 nm minus 450 nm after addition of 5 μ l of 20 mM-NADH (indicated by an arrow). Temperature was 3°C.

This was attributed to rapid reduction of specifically associated Complex III and slow reduction of Complex III not present in a Complex I-Complex III unit. Fig. 5 shows the effects of ubiquinone-10 and phospholipid on cytochrome *b* reduction. Fig. 5(a) shows the biphasic reduction of the cytochrome *b* of a mixture of 2.9 mol of Complex III/mol of Complex I. Supplementation with ubiquinone-10 (Fig. 5b) caused a small increase in the rate of the slow phase, whereas phospholipid (Fig. 5c) stimulated the rate to such an extent that no biphasic behaviour could be detected. Thus within the limitations of the experiment, it appears that all the cytochrome *b* was reduced in a single phase, in accordance with the predictions of Q-pool behaviour.

Activation energy of NADH-cytochrome *c* oxidoreductase activity

In the preceding paper (Ragan & Heron, 1978) it was shown that the activation energy of NADH-cytochrome *c* oxidoreductase activity was 63 kJ/mol, somewhat lower than that of ubiquinone reduction by Complex I. As Fig. 6 shows, the activation energy of this reaction in the presence and absence of Q-pool behaviour was approximately the same (69 and 63 kJ/mol respectively). The activation energy was altered to only a small extent by wide variation in the ratio of Complex I to Complex III. The effects of replacement of the endogenous phospholipids by synthetic 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-

choline, and of phase transition of this phospholipid on the enzymic and physical properties of the system, still require investigation. However, we wish to point out at this stage that the method of lipid replacement described in the preceding paper (Ragan & Heron, 1978) does not lead to restoration of Q-pool behaviour despite the high phospholipid-to-protein ratio. Thus the previously described effects of phase transitions of the lipid on activity are effects on the stoichiometric Complex I-Complex III unit only.

Discussion

In the preceding paper (Ragan & Heron, 1978), it was suggested that Complex I and Complex III can specifically associate in a 1:1 molar ratio to give a Complex I-Complex III unit. This unit contained less than 3 mol of ubiquinone-10/mol that was not free to act as hydrogen carrier between different Complex I-Complex III units. In the present paper we have shown that the behaviour of the natural mitochondrial membrane, in which ubiquinone-10 apparently acts as a mobile pool between any dehydrogenases and cytochrome chains, can be restored to the Complex I-Complex III system by raising the concentrations of ubiquinone-10 and phospholipid.

The work of Kröger & Klingenberg (1973a,b) showed that specific association of dehydrogenases with cytochrome chains was not necessary to explain the kinetic and steady-state redox behaviour of ubiquinone-10, and offered a ready explanation for how electron transfer was catalysed in mitochondria that appeared to have an excess of cytochrome chains (i.e. cytochrome *c*₁) over dehydrogenases (i.e. acid-extractable FMN or peptidyl-FAD) (Kröger & Klingenberg, 1967). On the other hand, work with more purified systems has consistently suggested that dehydrogenases and cytochromes directly associate with each other. The evidence is particularly strong for succinate dehydrogenase, and discussions of the interaction of soluble succinate dehydrogenase with the cytochromes generally assume that specific association occurs (e.g. Yu *et al.*, 1974). Evidence that Complex I and Complex III associate has been discussed in the preceding paper (Ragan & Heron, 1978).

The stoichiometry of dehydrogenases to cytochromes, particularly of NADH dehydrogenase, appears to be considerably less than 1:1 in intact mitochondria (Kröger & Klingenberg, 1967). Interestingly though, as the inner membrane is purified, the molar stoichiometry between acid-extractable FMN, trypsin-solubilized FAD and cytochrome *c*₁ does appear to approach 1:1:1, e.g. in phosphorylating submitochondrial particles (ETP_H) and the so-called 'elementary particle' (Blair *et al.*, 1963; Green

Fig. 6. Arrhenius plots of NADH-cytochrome *c* oxidoreductase activity of Complex I-Complex III

Mixtures containing 2 mol of Complex III per mol of Complex I were prepared as in the preceding Figures. In (b), the mixture was also supplemented with phospholipid and ubiquinone-10. Rates of NADH-cytochrome *c* oxidoreductase activity were measured as a function of temperature.

& Wharton, 1963). From detergent-fractionated membranes, succinate-cytochrome *c* and NADH-cytochrome *c* oxidoreductases can be isolated, both of which contain equimolar flavin and cytochrome *c*₁. The experiments described in the present paper show that theoretical Q-pool behaviour can be observed when either Complex I or Complex III is in molar excess (e.g. Fig. 2). Thus even if specific association of complexes does occur, those that are in excess can participate in electron transfer at the same rate as the associated complexes.

The endogenous phospholipid of Complexes I or III appears to constitute an annulus around the protein (Warren *et al.*, 1975; Ragan, 1978). The lipid content of inner mitochondrial membranes (approx. 25–30%, w/w, or approx. 0.5 μ mol/mg of protein), is twice that of the complexes, whereas ubiquinone-10 in mitochondria is generally present in approx. 10-fold excess over cytochrome *c*₁ (Kröger & Klingenberg, 1967). Thus the amounts of ubiquinone-10 and phospholipid required to restore Q-pool behaviour bring the totals of these components up to the amounts present in the natural membrane. Thus the supplemented system and the natural membrane contain enough phospholipid for two annuli. This estimate agrees broadly with estimates of the occupancy of the inner mitochondrial membrane by intrinsic proteins (Vanderkooi, 1974; Hackenbrock & Höchli, 1977).

We propose that the principal difference between the systems described in the preceding paper (Ragan & Heron, 1978) and the present paper lies in the relative mobility of the constituent complexes. Hackenbrock & Höchli (1977) have proposed that the mobility of intrinsic mitochondrial membrane proteins is extremely rapid, despite the low lipid-to-protein ratio. We have observed theoretical Q-pool behaviour in the Complex I–Complex III system when the lipid-to-protein ratio is raised to that of the natural membrane, so we suggest that the relative mobility of the two complexes is high under these circumstances. Theoretical Q-pool behaviour is found even when one or other of the complexes is in molar excess. Thus, at any one time, only a fraction of one of the complexes could be in a Complex I–Complex III unit. If the Complex I–Complex III unit is a fixed entity, then it can confer no advantage for electron transfer over separate complexes or else Q-pool behaviour would not be observed when the molar stoichiometry of the complexes is not 1:1.

We therefore propose that electron transfer from NADH to cytochrome *c* takes place only through Complex I–Complex III units that, however, are formed and re-formed at rates that exceed the rates of electron transfer from Complex I to Complex III. This proposal accounts for Q-pool behaviour and flexible stoichiometry of dehydrogenases to cytochromes, but still assigns a special function to the

association of Complex I with Complex III. In support of this we have shown that the low activation energy of NADH-cytochrome *c* oxidoreductase compared with NADH-ubiquinone oxidoreductase, an effect ascribed to specific interaction of Complex I with Complex III (Ragan & Heron, 1978), is still found under conditions of Q-pool behaviour (Fig. 6).

The rapid formation and dissociation of Complex I–Complex III units require phospholipid in excess of that needed to form an annulus. When this is absent, relative mobility is lost and the complexes are in effect frozen in their Complex I–Complex III units. This proposal accounts very well for the observation that, during fractionation of mitochondria with salt and solvents or detergent, both succinate-cytochrome *c* oxidoreductase and NADH-cytochrome *c* oxidoreductase (Hatefi & Rieske, 1967; Tisdale, 1967) containing equimolar proportions of dehydrogenase flavin and cytochrome *c*₁ may be separated. However, when NADH-cytochrome *c* oxidoreductase (Complex I–Complex III) is precipitated, a considerable amount of Complex III remains in the supernatant. This Complex III is indistinguishable from the Complex III precipitated with the Complex I. We propose that, as the detergent/salt fractionation decreases the lipid-to-protein ratio, relative mobility of complexes is lost and all Complex I molecules pair up with Complex III molecules. The latter are, however, in molar excess, and consequently some of these remain unpaired. The physical properties of the Complex I–Complex III unit are sufficiently different from unpaired Complex III to allow their separation.

The Complex I–Complex III unit contains nearly enough ubiquinone-10 for maximal activity. Supplementation with ubiquinone-10 stimulates activity without restoring Q-pool behaviour. Restoration of Q-pool behaviour by preincubation with phospholipid causes some decrease in the maximum activity of the system, which can be restored by extra ubiquinone-10. Thus it is likely that endogenous ubiquinone-10 leaks out of the Complex I–Complex III unit when extra phospholipid is present, causing a decrease in activity that can be alleviated by adding more ubiquinone-10. Direct evidence for extraction of endogenous ubiquinone-10 by phospholipid was obtained from experiments where Complex I or Complex III was incubated with a large excess of phospholipid dispersed in cholate, and then precipitated with (NH₄)₂SO₄ (Heron *et al.*, 1977). In one such experiment, Complex III was incubated with 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine in 20-fold molar excess over the endogenous phospholipid. After precipitation with (NH₄)₂SO₄, the enzyme contained almost the same amount of phospholipid, but the ubiquinone-10 content had decreased from 4.0 to 1.4 nmol per mg of protein.

The function of the large amount of ubiquinone-10 in the natural membrane may be, therefore, to maintain the ubiquinone-10 content of the Complex I-Complex III unit when it is formed. During formation and dissociation of Complex I-Complex III units, randomization of ubiquinone-10 molecules would occur and all the ubiquinone-10 would behave as a single homogeneous pool, as observed by Kröger & Klingenberg (1973a).

The effects of phospholipid added to the assay medium are difficult to explain. No restoration of Q-pool behaviour occurs, and all activities are increased slightly (Ragan & Heron, 1978). Rapid fusion of the added lipid with endogenous lipid certainly takes place, since restoration of Q-pool behaviour is observed to an extent if the Complexes have been preincubated with ubiquinone-10. Such rapid fusion has also been demonstrated for Complex I alone (Ragan, 1978). The stimulatory effect of lipid in the assay may be exerted in some other way. For example, the added phospholipids could remove bound detergent that might be inhibitory to the NADH-cytochrome *c* oxidoreductase activity. An interesting possibility is that the Complexes are better dispersed by surface adsorption to the added phospholipid vesicles, so that the activity is somewhat enhanced. The failure to observe restoration of Q-pool behaviour under these circumstances must be related to the physical state of the system, which could well be quite different from that obtained when the phospholipid is mixed with the Complexes before dilution into the assay. In particular the phospholipid-to-protein ratio is approx. 2×10^5 higher when phospholipid is added to the assay. Complex I-Complex III units adsorbed on the surface of phospholipid vesicles would presumably not be so free to dissociate and associate, resulting in failure to observe Q-pool behaviour. A contributing factor might be relatively slow exchange of ubiquinone-10 between the complexes, which may explain the partial restoration of Q-pool behaviour by preincubation with ubiquinone-10 alone.

The suggestions given above for the Complex I-Complex III system may also apply to the interaction of succinate dehydrogenase with the cytochromes. Kröger & Klingenberg (1973a,b) showed that Q-pool behaviour was exhibited by succinate oxidase activity. However, the separation of succinate-cytochrome *c* oxidoreductase into constituent lipo-protein Complexes (succinate-ubiquinone oxidoreductase or Complex II, and Complex III) is less readily achieved. First, Complex II contains some polypeptide subunits in common with Complex III, and, secondly, Complex III is contaminated with succinate dehydrogenase (albeit latent) (Trumpower & Katki, 1978). Moreover, the conditions used during purification of Complex II are unusually severe (Ziegler & Rieske, 1967). An alternative

experimental system involves the binding of water-soluble succinate dehydrogenase to a purified cytochrome *b-c*₁ particle (Yu *et al.*, 1974). The noteworthy features of this system are the obvious strength of the interaction, the inability of succinate dehydrogenase to catalyse succinate-ubiquinone oxidoreductase activity until bound to the cytochrome particle, and the final stoichiometry of less than 1 mol of dehydrogenase per mol of cytochrome *c*₁. Yu *et al.* (1977a,b) may have provided the key to the problem by purifying from the cytochrome *b-c*₁ particle a protein that reacts with succinate dehydrogenase to produce a fully active succinate-ubiquinone oxidoreductase. Complex II may be an impure form of this entity. Capaldi *et al.* (1977) have shown that antibodies against purified succinate dehydrogenase co-precipitate several low-molecular-weight polypeptides in addition to the dehydrogenase. It is possible that one of these dehydrogenase-associated peptides is the 'Q-binding protein' of Yu *et al.* (1977a,b). Obviously the stoichiometry of reconstituted succinate-cytochrome *c* oxidoreductase will depend on the amount of this so called 'Q-binding protein' that remains with the cytochrome *b-c*₁ particle after purification. It is conceivable that succinate dehydrogenase plus the Q-binding protein constitutes a mobile entity in the natural membrane. When the phospholipid content is decreased, a composite succinate-cytochrome oxidoreductase is formed, from which succinate dehydrogenase may be cleaved by alkali (Yu *et al.*, 1974) or chaotropic agents (Baginsky & Hatefi, 1969), leaving the 'Q-binding protein' behind.

Since we are proposing that the stoichiometric Complex I-Complex III unit is the state of the Complexes that is active in electron transfer, efforts should be concentrated on Complex I-Complex III to determine the nature of this interaction.

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