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

EVIDENCE FOR STOICHEIOMETRIC ASSOCIATION

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1. The NADH–ubiquinone oxidoreductase complex (Complex I) and the ubiquinol–cytochrome *c* oxidoreductase complex (Complex III) combine in a 1:1 molar ratio to give NADH–cytochrome *c* oxidoreductase (Complex I–Complex III). 2. Experiments on the inhibition of the NADH–cytochrome *c* oxidoreductase activity of mixtures of Complexes I and III by rotenone and antimycin indicate that electron transfer between a unit of Complex I–Complex III and extra molecules of Complexes I or III does not contribute to the overall rate of cytochrome *c* reduction. 3. The reduction by NADH of the cytochrome *b* of mixtures of Complexes I and III is biphasic. The extents of the fast and slow phases of reduction are determined by the proportion of the total Complex III specifically associated with Complex I. 4. Activation-energy measurements suggest that the structural features of the Complex I–Complex III unit promote oxidoreduction of endogenous ubiquinone-10.

Interest in the functioning of ubiquinone in mitochondrial respiration has been stimulated by the 'Q-cycle' hypothesis of Mitchell (1976). Kröger & Klingenberg (1967, 1973*a,b*) have provided evidence that ubiquinone is mobile in the plane of the membrane and serves as a redox pool linking dehydrogenases with cytochrome chains. Thus, there was no requirement for direct association of dehydrogenase molecules with cytochrome chains; the segments of the respiratory chain occurred independently in the same membrane and were linked by the mobile hydrogen carrier, ubiquinone. The 'pool function' of the ubiquinone is essentially an extension of Green's original postulate that ubiquinone functions as a 'mobile carrier' (Green, 1962). The 'Q-cycle' hypothesis proposed, among other things, that ubiquinone is co-ordinately reduced by electrons from both the dehydrogenase and the cytochrome *b*–*c*₁ segment of the electron-transport sequence at a site on one side of the membrane. It is possible therefore that the association of dehydrogenases and cytochromes may be closer than is suggested by the work of Kröger & Klingenberg (1973*a,b*). Several other lines of evidence support this idea. Firstly, a succinate–cytochrome *c* oxidoreductase complex has been described that may be reversibly resolved into soluble succinate dehydrogenase and a cytochrome segment by alkaline treatment (Yu *et al.*, 1974). Thus it seems likely that the association of succinate

dehydrogenase with the cytochromes is in part determined by protein–protein interactions.

Secondly, NADH–ubiquinone oxidoreductase and ubiquinol–cytochrome *c* oxidoreductase (Complexes I and III respectively) apparently interact in a 1:1 molar ratio to give NADH–cytochrome *c* oxidoreductase (Complex I–Complex III) that contains equimolar FMN and cytochrome *c*₁ (Hatefi *et al.*, 1962*a*; Fowler & Richardson, 1963). Furthermore, NADH–cytochrome *c* oxidoreductase, like succinate–cytochrome *c* oxidoreductase, may be isolated as a single entity by detergent and salt fractionation of mitochondria (Hatefi *et al.*, 1961).

We decided to investigate the interaction between NADH dehydrogenase and the cytochromes by using the purified lipoprotein Complexes I and III (Hatefi *et al.*, 1962*b,c*). In previous papers we showed that the phospholipid of Complex I is essential for rotenone-sensitive ubiquinone-1 reduction (Heron *et al.*, 1977). Although phospholipids are required for the correct functioning of the enzyme protein, effects of freezing of the lipid on enzyme activity are caused by lowering of the concentration and mobility of ubiquinone in the lipid phase (Ragan, 1978). We have now extended these observations to the interaction of Complexes I and III. In the present paper we provide evidence that these complexes associate in a 1:1 molar ratio to give a Complex I–Complex III unit. The ubiquinone associated with this unit does not equilibrate rapidly with other such units, or free Complex I and Complex III. In the following paper (Heron *et al.*,

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1978), restoration of the 'Q-pool' function to this system will be described.

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*b*) and Rieske (1967*a*) respectively. Complex I preparations contained 1.0–1.2 nmol of flavin/mg of protein and Complex III preparations contained 3.4–4.5 nmol of cytochrome c_1 /mg of protein.

Chemicals

Ubiquinone-1 was generously given by Hoffman-La Roche, Basle, Switzerland. Dr. B. Trumpower kindly provided the ubiquinone-2 analogue, 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone. This compound was reduced to the benzoquinol by the method of Rieske (1967*a*). Solutions in ethanol, acidified to pH 2 with HCl, were stable at -20°C for several weeks. 1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Soya-bean phosphatidylcholine was purified from crude soya-bean lipids ('L- α -lecithin', from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K.) as described by Ragan & Racker (1973).

Assays

NADH-ubiquinone-1 oxidoreductase activity was measured as previously described (Ragan, 1976). NADH-cytochrome c oxidoreductase was measured essentially as described by Hatefi & Rieske (1967*a*), except that the phospholipid supplement was either omitted or replaced by soya-bean phosphatidylcholine (0.4 mM as phosphorus) (Ragan, 1976). The oxidation of 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol by cytochrome c was measured exactly as for NADH-cytochrome c oxidoreductase, except that 40 μM -benzoquinol replaced the NADH, and phospholipid was omitted. All rates were corrected for non-enzymic reduction of cytochrome c . Acid-extractable FMN was assayed as described by Ragan (1976). Ubiquinone-10 was extracted and assayed by the method of Kröger & Klingenberg (1966). Cytochrome c_1 was determined essentially as described by Rieske (1967*b*). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V from Sigma) as a standard.

Determination of activation energies

Activation energies were obtained from measurements of activity as a function of temperature.

Spectrophotometric measurements were made in a water-jacketed cell holder whose temperature was varied by external heating or cooling units. Assay temperatures were determined with a thermistor in the cuvette to within 0.2°C .

Formation of Complex I-Complex III

Concentrated solutions of Complex I and Complex III are optically clear owing to cholate and deoxycholate associated with the enzymes. As shown by Hatefi *et al.* (1962*a*), Complex I-Complex III is formed after mixing the two Complexes as concentrated solutions, and diluting out the detergent. In the experiments described in the present paper, solutions of Complexes I and III (containing at least 10 mg of protein/ml) were mixed and diluted with 0.67 M-sucrose/50 mM-Tris/HCl, pH 8, at 0°C until the Complex I concentration was less than 0.5 mg of protein/ml. The diluted samples were incubated at 0°C for at least 30 min to allow full association (Fowler & Richardson, 1963). Portions containing approx. 3–10 μg of protein were then taken for assay of NADH-cytochrome c oxidoreductase activity.

Results

Dependence of NADH-cytochrome c oxidoreductase activity on Complex III concentration

In the experiment of Fig. 1, Complex I was mixed with increasing amounts of Complex III, and NADH-cytochrome c oxidoreductase activity was measured after dilution of the mixtures (Hatefi *et al.*, 1962*a*). The molar stoichiometry of Complex III to Complex I was determined from the concentration of cytochrome c_1 (in Complex III) and acid-extractable flavin (in Complex I) assuming that these are present in concentrations of 1 mol per mol of Complex (Rieske, 1976; Ragan, 1976). Complex I preparations always contained some Complex III (Hatefi *et al.*, 1962*a*). Estimates of the extent of this contamination on the basis of either the cytochrome c_1 content or the 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol-cytochrome c oxidoreductase activity of the Complex I gave identical results. In the preparation used in Fig. 1, the Complex I contained 0.10 mol of Complex III per mol of Complex-I flavin.

Extrapolation of the initial linear part of the curve of Fig. 1 gave an apparent stoichiometry of interaction of close to 1 mol of Complex III per mol of Complex I, in agreement with previous findings (Hatefi *et al.*, 1962*a*; Fowler & Richardson, 1963). The curve of Fig. 1 was not hyperbolic (as shown by a reciprocal plot), but the data agreed well with the assumption that the two Complexes combined reversibly in a 1:1 molar ratio (Table 1). In Table 1, values of the ratio of the estimated Complex I-

Complex III concentration to the product of the concentrations of free Complex I and free Complex III were calculated from the data of Fig. 1 and shown to be reasonably constant over a range of Complex III concentrations. If a phospholipid supplement was included in the assay, all rates were stimulated to the same extent (approx. 30%). Thus the shape of the

Fig. 1. Titration of Complex III against Complex I. Complex I (8 μ l of a solution containing 22 mg of protein/ml or 24.2 μ M based on FMN content) was mixed at 4°C with various volumes of Complex III (10.2 mg of protein/ml or 34.7 μ M based on cytochrome c_1 content) to give the indicated molar ratios, and diluted to a final volume of 800 μ l with 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, at 4°C. After incubation for 1 h on ice, samples were assayed for NADH-cytochrome c oxidoreductase activity at 30°C in the absence of a phospholipid supplement to the assay. The curve was drawn according to the analysis of Table 1 by using a value for $b/(c \times d)$ averaged from the first six lines of Table 1, i.e. 7.36.

curve of Fig. 1 and the apparent stoichiometry were unchanged.

The turnover number of Complex III is the same whether the enzyme is in isolation or in Complex I-Complex III and is considerably greater than that of Complex I. Therefore the apparent 1:1 molar stoichiometry cannot be explained on a kinetic basis. This point is considered in greater detail in the following paper (Heron *et al.*, 1978). Thus Fig. 1 provides evidence that a binary Complex can be formed that contains 1 mol of each of the constituent Complexes. Tests of this proposal are described below.

Inhibition of NADH-cytochrome c oxidoreductase by rotenone and antimycin

In Fig. 2, Complex III was mixed with Complex I in molar ratios of 4.05:1 and 0.347:1 so that Complex I and Complex III respectively were rate-limiting for NADH-cytochrome c oxidoreductase activity (cf. Fig. 1). Both NADH-ubiquinone-1 and NADH-cytochrome c oxidoreductase activities were then measured as a function of rotenone concentration. The dependence of activity on rotenone concentration expressed per mol of Complex I was the same for either activity of either mixture of complexes. Thus, even when Complex III was rate-limiting, the degree of inhibition of NADH-cytochrome c oxidoreductase was the same as that of NADH-ubiquinone oxidoreductase. If the two complexes in the mixture had been operating independently, inhibition of cytochrome c reduction should have been less than that of ubiquinone-1 reduction under these circumstances.

A further noteworthy feature in Fig. 2 is the stoichiometry of rotenone inhibition. Thus 50% inhibition was obtained at less than 0.2 nmol of rotenone per mg of protein or approx. 0.2 mol per mol

Table 1. Association of Complex I with Complex III

The total amount (mol) of Complex III present in the experiment of Fig. 1 was calculated from amount added plus that already present in Complex I. Column b is the fraction of maximum activity obtained at each Complex III concentration and is therefore the amount (mol) of Complex I-Complex III per mol of total Complex I. Column c is given by $1-b$, and is the amount (mol) of free Complex I per mol of total Complex I. Column d is given by $a-b$ and is the mol of free Complex III per mol of total Complex I. The maximum activity in Fig. 1 was assumed to be 3.06 μ mol of cytochrome c /min per mg of Complex I protein.

Amount (mol) of Complex III added*	a Total amount (mol) of Complex III	b Amount (mol) of Complex I + III	c Amount (mol) of free Complex I	d Amount (mol) of free Complex III	$\frac{b}{c \times d}$
0	0.10	0.087	0.913	0.013	7.33
0.38	0.48	0.393	0.607	0.087	7.44
0.73	0.83	0.601	0.399	0.229	6.58
1.07	1.17	0.743	0.257	0.427	6.77
1.48	1.58	0.869	0.131	0.711	9.33
1.86	1.96	0.879	0.121	1.081	6.72
2.56	2.66	0.971	0.029	1.66	20.2
3.60	3.70	0.971	0.029	2.70	12.3

* All values in this and other columns are per mol of total Complex I.

Fig. 2. Rotenone inhibition of Complex I and Complex III mixtures

Complex I (32 mg of protein/ml or 35 μ M) was mixed at 4°C with Complex III (40.9 mg of protein/ml or 139 μ M) to give molar ratios of either 4.05 (closed symbols) or 0.347 (open symbols) mol of Complex III/mol of Complex I (including Complex III already present in the Complex I). Portions containing 0.256 mg of Complex I were then treated with the indicated amounts of rotenone and diluted with sucrose/Tris buffer to a final volume of 100 μ l. After 1 h on ice, samples were assayed for NADH-ubiquinone-1 ($\blacktriangle$, $\triangle$) and NADH-cytochrome *c* oxidoreductase ($\bullet$, $\circ$) activities at 30°C. The phospholipid supplement was omitted from the latter assay. Rates are expressed as percentages of the rates in the absence of rotenone, which were: 3.27 μ mol of cytochrome *c*/min per mg of Complex I protein ($\bullet$); 0.95 μ mol of cytochrome *c*/min per mg of Complex I ($\circ$); 2.25 μ mol of ubiquinone-1/min per mg of Complex I ($\blacktriangle$); 2.38 μ mol of ubiquinone-1/min per mg of Complex I ($\triangle$).

of Complex I. This behaviour is reminiscent of that of the covalent modifier diphenyleneiodonium (Ragan & Bloxham, 1977), which shows a similar relationship between inhibition and the extent of incorporation of the inhibitor.

Titration of mixtures of Complex I and Complex III with antimycin produced similar results to those of Fig. 2. Thus in Fig. 3 the extent of inhibition by antimycin of NADH-cytochrome *c* oxidoreductase activity was the same for mixtures containing 0.271 or 2.18 mol of Complex I per mol of Complex III. The shape of the curve of Fig. 3 and the antimycin titre (1 mol per mol of Complex III) are in agreement with results in the literature (Fowler & Richardson, 1963). Fowler & Richardson (1963) inhibited a 1:1 mixture of Complex I and Complex III with 1 mol of antimycin per mol of Complex III and found considerable restoration of NADH-cytochrome *c* oxidoreductase

Fig. 3. Antimycin inhibition of Complex I and Complex III mixtures

Complex III (139 μ M) was mixed at 4°C with Complex I (24 μ M) to give molar ratios of 2.18 mol (closed symbols) and 0.271 mol (open symbols) of Complex I per mol of Complex III. Portions containing 0.178 mol of Complex III were treated with the indicated concentrations of antimycin and diluted to 800 μ l with sucrose/Tris buffer. After 1 h at 4°C, samples were assayed for NADH-cytochrome *c* oxidoreductase activity at 30°C without a phospholipid supplement. Rates are expressed as the percentage of the rates in the absence of antimycin, which were: 16.1 μ mol of cytochrome *c*/min per mg of Complex III ($\bullet$); 4.1 μ mol of cytochrome *c*/min per mg of Complex III ($\circ$). A correction was applied for contamination of Complex I by Complex III.

activity when they added another molar equivalent of Complex III. They suggested that the added Complex III was acting as an electron acceptor from the original Complex I. They stated that the restoration of activity expected from redistribution of the antimycin between the original and added Complex III molecules was insufficient to account for the result obtained, although inspection of Fig. 3 shows that, if antimycin redistribution had occurred, some 70% restoration of activity would have been expected. We have repeated this experiment under somewhat different conditions, which enable a clearer distinction to be made between the two possible explanations. In the experiment of Fig. 4, Complex I was mixed with 3.7 mol.equiv. of Complex III, and 3.9 mol.equiv. of antimycin was added to give 93% inhibition of NADH-cytochrome *c* oxidoreductase activity. The restoration of activity as a function of added Complex III was then determined. If the added Complex III were acting as acceptor from the Complex I then approx. 1 mol.equiv. of Complex III should have substantially restored activity (curve *a*). However, if restoration were due to redis-

Fig. 4. Effect of additional Complex III on the activity of antimycin-inhibited mixtures of Complexes I and III. Complex I (0.48 nmol) and Complex III (1.78 nmol) were mixed and diluted to 4.0 ml with sucrose/Tris buffer at 4°C. To 2 ml, 1 nmol of antimycin was added in 10 μ l of ethanol. Portions (0.4 ml), with (○) and without (●) antimycin, were then mixed with the indicated amounts of additional Complex III at 4°C and assayed for NADH-cytochrome *c* oxidoreductase activity at 18°C. Curve (a) was obtained from a control experiment in which Complex I alone was mixed with Complex III in the absence of antimycin (i.e. as in Fig. 1). Curve (b) was calculated from Fig. 3 assuming that the antimycin redistributed itself amongst all the Complex III molecules present. Rates are expressed as the percentage of the rate obtained in the absence of antimycin and with saturating amounts of Complex III (1.50 μ mol of cytochrome *c*/min per mg of Complex I). All calculations include a correction for Complex III contamination of the Complex I.

tribution of antimycin, approx. 5 mol. equiv. of Complex III would be needed to reduce the antimycin bound to the Complex I-Complex III to such an amount that 80% restoration of activity was obtained. The curve labelled (b) was calculated from Fig. 3 on this basis and the observed activities closely follow this line. Thus we conclude that restoration of activity by added Complex III is due to redistribution of antimycin and not to diversion of the pathway of hydrogen or electron transfer through the added Complex III.

The redistribution of antimycin could in principle take place in two ways. The first way would not in fact involve migration of antimycin molecules, but instead a slow substitution of inhibited Complex I-Complex III units by uninhibited Complex III. This process would be compatible with the available evidence as long as its rate was much lower than the rate of electron transport. Alternatively, redistribution of anti-

mycin molecules between all Complex III molecules seems reasonable in view of the finite dissociation constant of antimycin (Kröger & Klingenberg, 1973b).

Reduction of Complex III cytochrome *b* by Complex I

The results of Figs. 2, 3 and 4 suggested that each Complex I molecule can transfer reducing equivalents to only one Complex III molecule. This proposition was further tested by measuring the rate at which cytochrome *b* in Complex III was reduced by Complex I. In the experiment of Fig. 5, Complex I was mixed with various molar proportions of Complex III in the presence of antimycin, and reduction of cytochrome *b* was followed at 430 nm minus 450 nm after addition of NADH. At 25°C, the cytochrome *b* was rapidly reduced to a maximum extent of 50% of the total present in Complex III, in agreement with Rieske (1976). However, at 3°C the reduction was clearly biphasic. The relative extents of the slow and fast phases depended on the molar ratio of Complex I to Complex III (Fig. 5, inset). The fast phase showed saturation with increasing Complex III concentration and the curve was very similar to that of Fig. 1. The sum of the fast and slow phases increased linearly with Complex III concentration. It is clear that the fast phase was due to reduction of no more than 1 mol of Complex III per mol of Complex I, and the slow phase was due to reduction of Complex III not specifically associated with Complex I. These results are therefore consistent with those of Figs. 2, 3 and 4, since reduction of the unassociated Complex III molecules is so much slower than reduction of the specifically associated molecules that the former pathway could not contribute significantly to the rate of NADH-cytochrome *c* oxidoreductase activity.

The total extent of reduction at 25°C was not affected by partial inhibition by rotenone (Table 2), but the fast phase of reduction at 3°C was decreased in proportion to the inhibition of the overall rate of NADH oxidation. Thus, in mixtures containing Complex I and two molar equivalents of Complex III, the fast phase of cytochrome *b* reduction was one-half of the total, as expected. Inhibition of NADH-cytochrome *c* oxidoreductase to the extent of 40 and 80% respectively produced decreases in the extent of the fast phase of 42 and 83% respectively (Table 2).

Activation energies of reactions of Complexes I and III

In mixtures of Complexes I and III, the activation energy for the reduction of exogenous ubiquinone-1 was the same as in Complex I alone (approx. 80 kJ/mol; Ragan, 1978). The activation energy for the oxidation of 6-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinol by cytochrome *c* was approx. 40 kJ/mol, whether measured with Complex III alone or in

Fig. 5. Reduction of cytochrome *b* in mixtures of Complex I and Complex III

Complex I (26.4 μM) and Complex III (139 μM) were mixed to give the indicated molar ratios. Portions containing 1.58 nmol of Complex I were diluted to 5.0 ml with sucrose/Tris buffer at 4°C. After 1 h at 4°C, 5 μg of antimycin was added to each sample. Reduction of cytochrome *b* was measured at $A_{430} - A_{450}$ after addition of 0.1 μmol of NADH, and at either 25 or 3°C. Curves (a)–(f) were obtained at 3°C, and curve (g) at 25°C. Molar ratios of Complex III to Complex I were: (a) 0.1; (b) 0.6; (c) 1.1; (d) 1.6; (e) 2.1; (f) 2.6; (g) 2.6. The inset shows the extent of fast (●) and fast-plus-slow phases (▲) at 3°C, and total extents at 25°C (■) as functions of the molar ratios of Complex III to Complex I. Correction was applied for the Complex III content of the Complex I.

mixtures of Complexes I and III. The Arrhenius plot for the NADH–cytochrome *c* oxidoreductase activity of a mixture of Complexes I and III was linear between 40 and 5°C and gave an activation energy of 63 kJ/mol.

The value of 63 kJ/mol for Complex I–Complex III lies between the values of 80 and 40 kJ/mol obtained for exogenous quinone reduction and oxidation respectively. This suggests that the oxidoreduction of endogenous ubiquinone-10 proceeds somewhat differently from the oxidation and, particularly, the reduction of exogenous quinones. We do not feel that the lower activation energy for ubiquinone-10 oxidoreduction can be attributed to the different chemical nature of the exogenous quinones, since we have obtained an activation energy of 80 kJ/mol for the reduction of 6-decyl-2,3-dimethoxy-5-methoxy-1,4-benzoquinone, which differs greatly from ubiquinone-1 in hydrophobicity (Ragan, 1978). We propose that the lower activation energy for endogenous ubiquinone-10 reduction may be due to the environment in which the quinone is situated. Orientation of the site of reduction of ubiquinone-10 with respect to the site of oxidation might cause a lowering of the activation energy to that observed for the overall process.

The ubiquinone-10 content of the Complex I and Complex III used in these experiments was 2.2 and 2.6 $\mu\text{mol}/\text{min}$ per mg of protein respectively. Thus in an equimolar mixture of Complex I and Complex III there were 2.8 mol of ubiquinone-10 per mol of Complex I or III. This concentration is sufficient for nearly maximal rates of electron transport, as shown in the following paper (Heron *et al.*, 1978). It is reasonable that concentrations of this order of magnitude might be specifically associated with one or both Complexes and might have quite limited mobility within the interface between the Complexes. Such limited mobility is also supported by the structure of ubiquinone as revealed in molecular models (Trumppower & Landeen, 1977).

We have also found that the phospholipids of Complex I–Complex III can be substituted by 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine with

Table 2. Effect of rotenone on reduction of Complex III cytochrome *b*

Complex I (3.56 mg of protein) and Complex III (2.31 mg of protein) were mixed, and diluted to 15 ml with 0.67 M-sucrose/50 mM-Tris/HCl, pH 8.0, at 4°C. The molar ratio of Complex III to Complex I was 2:1. Portions (5 ml) of the mixture were incubated with 0.12 nmol or 0.72 nmol of rotenone for 1 h at 4°C before assay of NADH–cytochrome *c* oxidoreductase activity. Cytochrome *b* reduction at 25 and 3°C was measured as described in Fig. 5.

Rotenone added (nmol/mg of Complex I protein)	Percentage inhibition of NADH–cyto- chrome <i>c</i> oxido- reductase activity	$(A_{430} - A_{450})$ after NADH addition		Percentage inhibition of the fast phase
		Fast phase	Fast+slow phases	
0.00	0	0.026	0.050	0
0.10	40	0.015	0.049	42
0.60	80	0.0045	0.051	83

retention of activity. Below the phase-transition temperature of this phospholipid, the activation energy for the reduction of exogenous ubiquinone-1 by NADH was greatly increased from 80 kJ/mol to 290 kJ/mol (Ragan, 1978). On the other hand the activation energy for cytochrome *c* reduction by NADH was only slightly increased to 92 kJ/mol. These experiments support the idea of a close association of Complexes I and III and show that movement of ubiquinone-10 molecules through the bulk lipid phase is not absolutely necessary for NADH-cytochrome *c* oxidoreductase activity.

Discussion

The experiments reported in the present paper provide evidence that Complex I and Complex III interact specifically in a 1:1 molar ratio to form a Complex I-Complex III unit. Transfer of reducing equivalents to or from this unit by extra Complex I or Complex III molecules is slow and does not contribute to the overall rate of electron transfer from NADH to cytochrome *c*. One trivial explanation of this latter finding is that the extra Complex I or Complex III molecules are not aggregated with the Complex I-Complex III units in the assay. Therefore, the transfer of reducing equivalents to or from unassociated Complexes (presumably via ubiquinone-10) would have to take place through the aqueous phase, which is most improbable. In the following paper (Heron *et al.*, 1978) it is shown that under certain circumstances (e.g. Fig. 3 of Heron *et al.*, 1978) transfer of reducing equivalents to or from other Complexes can be greatly stimulated by increasing the ubiquinone-10 concentration in the mixture. Since small amounts of ubiquinone-10 would not alter the physical state of the Complexes, we consider that physical separation is not the explanation for the inability of such transfer to occur in the systems described in the present paper. Hatefi *et al.* (1962a) demonstrated that Complex I-Complex III could be separated from excess of Complex III by differential centrifugation under special conditions. These conditions involved dialysis of the Complex I and Complex III mixture for a short period, which was sufficient to make the Complex I-Complex III unit insoluble, but insufficient to precipitate free Complex III. These conditions are quite different from those described in the present paper, where both complexes are insoluble after extensive dilution. Indeed, if the experiment of Hatefi *et al.* (1962a) is repeated with a prolonged, e.g. 16 h, dialysis period, then separation of Complex I-Complex III from Complex III can no longer be achieved by centrifugation, suggesting that the complexes are aggregated.

The results of the activation-energy measurements support the idea that the mobility of ubiquinone-10

in the Complex I-Complex III unit is highly restricted. In a previous paper (Ragan, 1978) it was shown that the rate of reduction of ubiquinone analogues by Complex I depended only on the amount of the quinone in the lipid phase, regardless of whether the lipid was only annular (Warren *et al.*, 1975) or consisted of a large bulk phase. Thus movement of quinone molecules between water, bulk and annular-lipid phases was never rate-limiting (except when the bulk lipid was frozen). This does not appear to be the case for Complex I-Complex III. Thus the interaction of the complexes imposes a restriction on the diffusion of ubiquinone-10 between the protein and its associated annular lipid, and any other protein and lipid.

We suggest that the association of Complex I with Complex III is determined to a considerable extent by protein-protein interactions. Ubiquinone-10 is effectively trapped between the component complexes in an environment that may be partly protein and partly derived from the annuli of those complexes. This environment promotes oxidoreduction of the ubiquinone-10 by lowering the activation energy of ubiquinone reduction.

The amount of ubiquinone-10 trapped in this environment appears to be less than 3 molecules per molecule of Complex I-Complex III, and this concentration is sufficient for near maximum rates of electron transport to cytochrome *c* [see the following paper, Heron *et al.* (1978)]. These results are consistent with the evidence for an oriented pair of ubiquinone molecules in the respiratory chain of intact mitochondria (Salerno *et al.*, 1977). Stabilization of the semiquinones formed from this pair of molecules was ascribed to a ubiquinone-binding protein, whose existence has been postulated by Yu *et al.* (1977). The present results are fully consistent with the involvement of such proteins in the Complex I-Complex III interaction. We have shown that ubiquinone mobility is restricted. However, ubiquinone may equally well be totally immobilized by binding to protein. As yet there is no direct evidence for such specific proteins, and it is possible that phospholipids may be important in ubiquinone binding. In particular, Heron *et al.* (1977) have shown an essential requirement for cardiolipin in NADH-ubiquinone-1 oxidoreductase activity. This observation and the presence of cardiolipin almost exclusively in mitochondria still require explanation.

The extensive investigations by Kröger & Klingenberg (1973a,b) of the kinetic and steady-state behaviour of ubiquinone in submitochondrial particles have shown that ubiquinone behaves as a single homogeneous pool whose redox state is determined by the relative activities of the donor to and acceptor from this pool. They propose that, as well as acting as a 'serial' mediator of electron transfer from dehydrogenases to cytochromes, ubiquinone must also act as 'lateral' mediator between the cytochrome chains,

i.e. a molecule of ubiquinone can be reduced by any dehydrogenase and oxidized by any cytochrome chain. It is clear that this behaviour does not hold for the system described here. In the following paper (Heron *et al.*, 1978), restoration of theoretical 'Q-pool' behaviour to the Complex I-Complex III system will be described. This can be achieved by increasing the ubiquinone-10 and phospholipid concentrations to values more closely approaching those found in mitochondrial membranes. The demonstration that the Complex I-Complex III system can be made to operate exactly as in the natural membrane provides strong evidence that the conclusions of this paper do not relate to an artifact produced *in vitro*. The relationship between the system described here and the behaviour *in vivo* is discussed at greater length in the following paper (Heron *et al.*, 1978).

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