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THE EFFECTS OF LIPID FLUIDITY ON THE ROTATIONAL DIFFUSION OF COMPLEX I AND COMPLEX III IN RECONSTITUTED NADH-CYTOCHROME *c* OXIDOREDUCTASE

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NADH-ubiquinone oxidoreductase (Complex I) can be recombined with ubiquinol-cytochrome *c* oxidoreductase (Complex III) to reconstitute NADH-cytochrome *c* oxidoreductase. Two modes of interaction have been found. In one, the Complexes interact stoichiometrically in one to one molar ratios to give a binary Complex I-III unit. In the other, the kinetics of NADH-cytochrome *c* oxidoreductase are characteristic of 'Q-pool' behaviour seen in intact mitochondria and submitochondrial particles in which the Complexes need not interact directly but can do so via a pool of mobile ubiquinone. Stoichiometric behaviour is found when only boundary layer or annular lipid is present or the lipid is in the gel phase. The lipid is immobile on the ESR time scale and protein rotational diffusion, measured by saturation transfer ESR, is very slow. Q-pool behaviour is found when mobile extra-annular lipid phase is also present. Protein rotational diffusion is rapid and characteristic of a fully disaggregated state. We have also used freeze-fracture electron microscopy of reconstituted NADH-cytochrome *c* oxidoreductase to monitor protein aggregation and lateral phase separation of lipids and proteins under various conditions. We discuss our findings in relation to models for lateral interactions between respiratory chain enzymes.

Introduction

Mitochondrial NADH-ubiquinone reductase (Complex I [1]) and ubiquinol-cytochrome *c* reductase (Complex III [2]) can be combined to reconstitute NADH-cytochrome *c* reductase [3,4], an activity mediated by endogenous ubiquinone-10. Two modes of interaction have been seen in this [4–6] and a similar system [7]. Firstly, the two complexes can appear to interact stoichiometrically to give a binary complex containing one molecule of each. The evidence for this includes the isolation of such a complex [8], the dependence of NADH-cytochrome *c* reductase activity on the molar ratio of the constituent enzymes, the effects of inhibitors and the kinetics of Complex III reduction by Complex I [4]. Even when all Complex I and Complex III molecules are present in the same membraneous aggregate, electron transfer only takes place through the binary complex and electron transfer through molecules in stoichiometric excess is extremely slow [6]. Secondly, the two complexes can appear to interact through a mobile pool of ubiquinone-10 molecules, as in the natural membrane [5,9,10], and there does not seem to be any direct association between the two complexes.

Abbreviations: 12-SASL, 12-stearic acid spin label (12-(4',4'-dimethyloxazolidinyl-*N*-oxy)stearic acid; MSL, maleimide spin label (3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl); PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; PE, phosphatidylethanolamine.

This so-called 'Q-pool' behaviour [9,10] arises when the lipid-to-protein ratio of the reconstituted NADH-cytochrome *c* reductase is raised from that found in the isolated enzymes to a value close to that of the inner mitochondrial membrane [5]. Stoichiometric association is found at low lipid-to-protein ratios or in enzymes containing dimyristoylphosphatidylcholine (DMPC) in the gel state [6]. The latter observation suggested that lipid fluidity rather than lipid concentration was the determining factor and this was supported by measurements of the fluorescence depolarisation of the probe, diphenylhexatriene [11]. To take account of the stoichiometric association seen at low lipid fluidity, we proposed that such association also took place during Q-pool behaviour but that the interaction was transient. This would lead to the same kinetic behaviour as a system in which ubiquinone-10 diffused between independent Complex I and Complex III molecules as long as association and dissociation were more rapid than overall electron transfer [6].

In the present paper we have used ESR to probe the lipid and protein mobility of reconstituted Complex I–III. We show that Q-pool behaviour is only encountered when there is an appreciable mobile bulk lipid phase while stoichiometric association is found when only boundary layer lipid [12,13] or gel phase lipid is present. Measurements of protein rotational mobility by saturation transfer ESR reveal substantial differences in protein mobility between the two modes of behaviour, in support of the proposed model.

Materials and Methods

Complex I [14] and Complex III [5] were prepared as described in the references. Reaction of the enzymes with the maleimide spin label, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (MSL) was done before liquid exchange or supplementation. The enzymes dissolved in 0.67 M sucrose/50 mM Tris-HCl 9pH 8.0), at concentrations of approx. 20 μM (Complex I) or 40 μM (Complex III), were incubated with a 10-fold molar excess of MSL for 1 h at 4°C. The MSL was used as a stock solution of 2.11 mM in Tris/sucrose containing 10% methanol. The samples were then dialysed against several hundred volumes of Tris-sucrose buffer at 4°C for 16 h in the dark with two changes of buffer. The dialysed enzymes were then centrifuged at $100\,000 \times g$ for 30 min at 4°C, resuspended in Tris/sucrose buffer, centrifuged again and then resuspended in buffer to a protein concentration of 30 mg/ml. Unlabelled enzyme, to be used in reconstitution, was dialysed and centrifuged as for the labelled enzyme.

Lipid exchanges were performed as described in the preceding paper [16]. Reconstitution of NADH-cytochrome *c* oxidoreductase activity followed the procedure of Heron et al. [6]. Labelled and unlabelled complexes containing native lipids or lipid-replaced with dimyristoylphosphatidylcholine (DMPC) and suspended in Tris/sucrose buffer, were mixed in the desired molar ratio and resolubilised by addition of 4 M KCl and 10% (w/v) sodium deoxycholate of final concentrations of 0.8 M and 0.1% (w/v), respectively. When required, purified soyabean phospholipids [6] or DMPC were added from a stock dispersion of 60 mM in 0.35% cholate to give the desired lipid to protein ratio. The mixtures were then dialysed and centrifuged as described above using Tris/sucrose buffer, resuspended in the same buffer to a protein concentration of 30 mg/ml and stored at −70°C.

Complex I-III samples were also prepared containing the fatty acid spin label 12-SASL. The label was added as a stock solution of 2.6 mM in methanol to give a final concentration not exceeding 2 mol% of the total lipid content. The addition was made during the reconstitution prior to the dialysis and centrifugation.

Analyses of phospholipid phosphorus and protein were carried out as in the preceding paper [16]. Molarities of Complex I and Complex III were determined from the FMN content [18] and cytochrome c_1 content [19], respectively.

For freeze-fracture studies the samples used in the ESR experiments were transferred to gold-nickel specimen holders. After equilibration at the required temperature for 30 min these samples were rapidly quenched in a slurry of solid and liquid N_2. Fracturing and platinum-carbon replication were performed at −112°C and $2 \cdot 10^{-6}$ Torr using a Polaron freeze-fracture module (Polaron Ltd., Watford, Herts, U.K.). Micrographs were obtained with a Hitashi HU-12 electron microscope operated at 75 kV.

Conventional (V_1) ESR spectra were obtained with a Bruker ER 200D spectrometer equipped with a variable temperature-controlled unit as described in the preceding paper [16]. All samples were run in a quartz flat cell. Saturation transfer esr spectra in the V_2' mode were obtained under instrumental conditions comparable with those described by Thomas et al. [20]. A field modulation of 5 G and frequency 50 kHz was employed with phase-sensitive detection at 100 kHz (second harmonic) out of phase. Since we were unaware at the beginning of these studies of any published saturation transfer experiments using the Bruker spectrometer, our procedures for phase-nulling and determination of the microwave field strength, H_1, are described here in detail. Determination of the phase setting followed the self-null procedure described by Johnson and Hyde [21]. Samples of lyophilised MSL-labelled haemoglobin [22], weak pitch, or a nitrogen-saturated solution of Fremy's salt were used. Second harmonic spectra were obtained at a non-saturating power of 0.019 mW at phase settings either side of the approximate null point. The peak to peak amplitude was then plotted as a function of phase angle and the phase null point was determined as the intersection of the lines fitted by least squares analysis. The null point did not vary with microwave power below approx. 0.07 mW or with the chemical nature and concentration of the sample or with the type of sample container. Thus, it was not necessary to recalibrate the null point for each experimental sample and no day to day variation was noticed.

The microwave field strength was determined using Fremy's salt [20,23]. Stock solutions (90 mM) were prepared in N_2-saturated 50 mM K_2CO_3. This was added to either water, or suspensions of Complex I and III in Tris/sucrose buffer to give final concentrations of 0.24 mM. V_1 spectra were obtained as a function of microwave power (12.8 to 128 mW) and temperature (2 to 35°C). The peak to peak line width of the first derivative spectrum (ΔHpp) was measured and ΔHpp^2 plotted as a function of microwave power (P_{inc}). The slopes of such lines (S) can then be used to relate the microwave field strength (H_1) to P_{inc} according to the equation:

$$H_1^2 = 0.75S \times P_{inc}$$

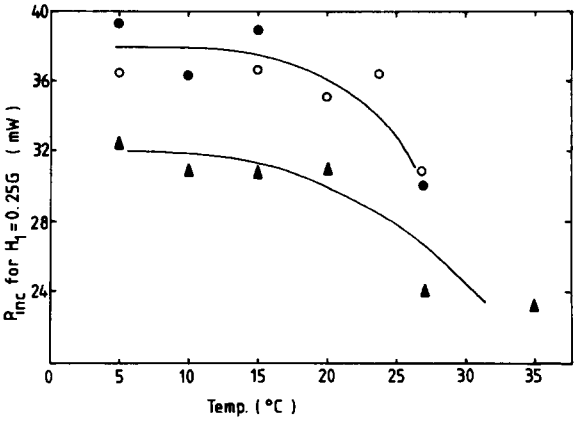


Fig. 1. Variation with temperature and sample conditions of incident microwave power required to give $H_1 = 0.25$ G. The microwave field strength of 0.25 G at the sample (H_1) was determined as a function of incident microwave power (P_{inc}) using Fremy's salt. ●, Fremy's salt (0.24 mM) in 0.67 M sucrose/50 mM Tris-HCl (pH 8.0), containing Complex I and Complex III in 1:4 molar ratio and at a final protein concentration of 30 mg/ml; ○, the same except that the Complex I and III mixture had been supplemented with soyabean phospholipid (0.5 μmol of lipid P/mg of protein); ▲, Fremy's salt (0.24 mM) in water. The V_1 spectra from which these values were derived were measured using a field modulation intensity of 0.4 G and frequency 100 kHz. The microwave power ranged from 2–12 dB (12.8 mW) and the temperature ranged from 5 to 35°C.

In Fig. 1 we show the microwave power required to give $H_1 = 0.25$ G under different conditions. This microwave field strength was established as a standard by Thomas et al. [20]. Under this condition the spin system is moderately saturated and absorption spectra approximate to maximum intensity. As noted by Kusumi et al. [24], the required power varies with temperature and the nature of the sample. The additional presence of soyabean phospholipids did not, however, make any difference to the curve for proteins in Tris/sucrose buffer. The actual power setting used for protein samples was therefore either 7 dB attenuation (38 mW) or 8 dB attenuation (30 mW) depending on the temperature of measurement. An alternative method to check on the appropriate phase and power settings was to compare V_2' spectra of lyophilised MSL-labelled haemoglobin with those published by Thomas et al. [20]. Gratifyingly, the closest fit to the published spectrum for $H_1 = 0.25$ G was obtained using 7 dB attenuation

at 21°C. We note that this power level is within the range found by users of Varian spectrometers.

It has been noted that oxygen-induced relaxation can cause significant changes in saturation-transfer ESR spectra [21]. Such changes were found to occur with our protein system. The major effect was to cause a deepening of the centre field dip of the V_2' spectrum giving the appearance of a greater mobility. Precautions were therefore taken to exclude oxygen from the solutions used in this work. All solutions were degassed and then saturated with nitrogen prior to replacement in the spectrometer cavity. A flow of nitrogen gas was maintained throughout the cavity and dewar.

V_2' spectra were obtained for the protein samples at various temperatures. Using the interfaced computer system all spectra were accumulated, averaged and stored on disc file as described in the preceding paper [16]. Approximate rotational correlation times (τ_2) were obtained from the ratio of the low-field lines (L''/L) using the calibration curve of Thomas et al. [20] derived from isotropic tumbling of MSL-labelled haemoglobin.

Results

As described in the previous paper [16] Complex I as isolated contains approx. 0.2 μmol of lipid P/mg of protein. This is rather less than one boundary layer or annulus which requires 0.32 μmol of lipid P/mg of protein. The lipid content of Complex III is very similar [6] and, in either Complex I alone or in reconstituted Complex I-III, the lipid is all annular and of low mobility as revealed by ESR. If the lipid-to-protein ratio is increased, the additional lipid is mainly extra-annular and mobile. Thus, even small increases in lipid content have very marked effects on the lipid environments surrounding the proteins. This is shown in Fig. 2 where the lipid environment of reconstituted Complex I-III was monitored at 27°C with the fatty acid spin probe, 12-SASL. Using enzymes as isolated, the total lipid content was 0.26 μmol/mg of protein and the spectrum (a) shows mainly a highly immobilised component with a broad outer splitting and very little mobile component. On raising the phospholipid content with soyabean PC to 0.50 μmol/mg of protein (b) the spectrum is substantially different. Now a large

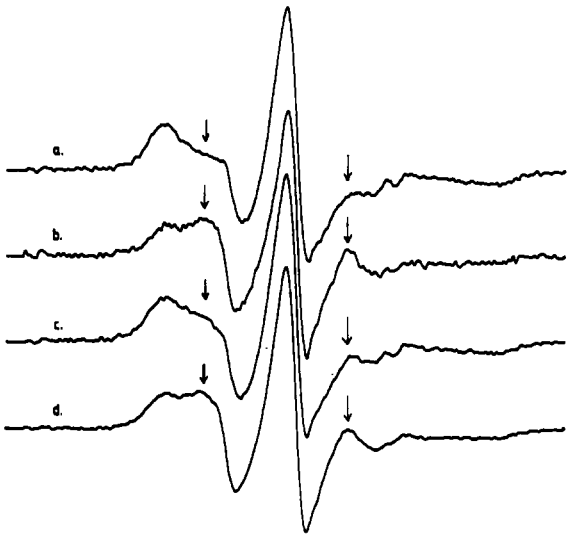


Fig. 2. V_1 spectra of 12-SASL in Complex I and III mixtures at 27°C. In all these spectra the molar ratio of Complex I to III was 1:4. The enzymes were reconstituted to give NADH-cytochrome *c* oxidoreductase activity and the spin label, 12-SASL, added. (a) enzymes containing natural lipids (0.26 μmol/mg of protein); (b) enzymes containing natural lipids supplemented with soyabean phospholipids (final lipid content, 0.50 μmol/mg of protein); (c) DMPC-replaced enzymes (final lipid content, 0.31 μmol/mg of protein); (d) DMPC-replaced enzymes supplemented with DMPC (final lipid content, 0.50 μmol/mg of protein). Protein concentrations were all 30 mg/ml. Spectra were measured using a field modulation intensity of 3.2 G, frequency 100 kHz and a microwave power of 1 mW (22 dB). Arrows mark the positions of the high and low field peaks of the mobile spin component. Scan width was 100 G.

mobile component is also present arising from extra-annular lipid. Similar behaviour was found in Complex I-III whose endogenous PC and PE had been replaced by DMPC. Spectrum (c) is of a sample with a lipid content of 0.31 μmol/mg of protein which again shows a predominantly immobile lipid phase. On adding DMPC to raise the lipid content to 0.50 μmol/mg of protein (spectrum d) the mobile component is substantially increased. Since the size of the lipid annulus (expressed per mg of protein) is similar for Complex I or Complex III, the spectra of Fig. 2 are largely independent of the molar ratio of the two constituent enzymes.

Corresponding spectra at 5°C are shown in Fig. 3. In complex I, the apparent size of the boundary layer increases to 0.40 μmol/mg of pro-

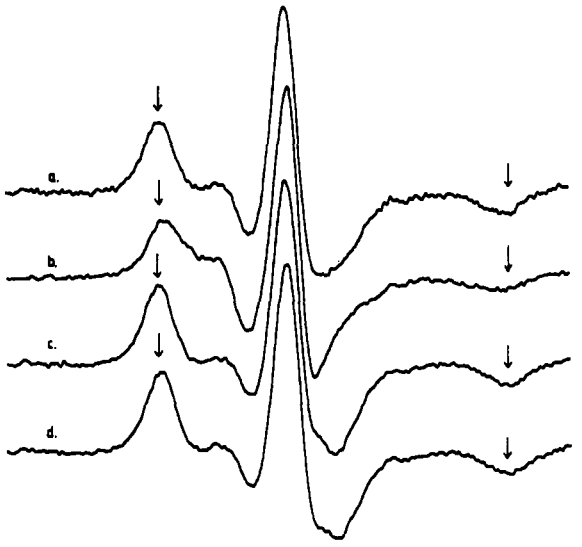


Fig. 3. V_1 spectra of 12-SASL in Complex I and III mixtures at 5°C. The samples were exactly the same as those of Fig. 2. Instrumental conditions were identical apart from the sample temperature. Arrows mark the positions of the high and low field peaks of the immobile spin component. Scan width was 100 G.

tein at 5°C [16] and Complex III may behave similarly. Such an effect can be seen by comparison of Figs. 2 and 3. Thus in Fig. 3, spectrum (a) shows no mobile component at all whereas some was visible at 27°C. The mobile spectrum at 5°C is broader than at 27°C and the two components present in spectrum (b) can only properly be separated by a computer subtraction. Nevertheless, the presence of a mobile component is clearly indicated by the decrease in the relative intensities of the high and low field extrema attributed to the immobile phase. Spectra (c) and (d) of DMPC-replaced enzymes both show totally immobilised spin environments. This was expected even for spectrum (d) where extra-annular lipid was present in the sample, because DMPC is in the gel phase at 5°C [25].

Freeze-fracture electron micrographs of the samples used in Figs. 2 and 3 are shown in Figs. 4 and 5. At 27°C (Fig. 4) vesicular (and occasionally multilayered) membranes can be seen in all samples. The distribution of the protein particles appears to be quite random and there is no evidence for lateral separation of lipid and protein except in the DMPC-replaced sample containing 0.50 μmol of lipid/mg of protein (Fig. 4d). In this, there are some areas of particle-free membrane which show the rippled surface characteristic of gel phase DMPC [26]. Since 27°C is above the phase transition temperature, this is most likely due to insufficiently rapid freezing from 27°C (see, for example, Ref. 27). At 5°C, the appearance of samples containing native or soyabean lipid is indistinguishable from that found at 27°C. In the sample containing only annular DMPC (Fig. 5c) the distribution of protein particles still seems to be random and no separation of protein and lipid appears to have taken place. In the sample containing additional DMPC (Fig. 5d), the characteristic pattern of gel-phase DMPC can be seen with protein-rich areas found at the edges of the membrane sheets or at the interface between gel-phase DMPC areas of different orientations (arrow). The failure to see this kind of behaviour in Fig. 5c can be attributed to the effect of proteins on the annular lipid in preventing the regular packing of the DMPC molecules. In addition, the lipid-replaced enzymes still contain their original cardiolipin (0.04 μmol of lipid P/mg of protein) which might also play a part in preventing the formation of a regular DMPC gel phase.

The samples used in Figs. 2 to 5 illustrate the range of conditions under which stoichiometric association or Q-pool behaviour occur. These are summarised in Table I. At low lipid-to-protein ratios, stoichiometric association is always found. At higher ratios, Q-pool behaviour occurs except when the lipid is in the gel phase. Thus, the latter behaviour only occurs when a fluid bulk lipid phase is present. This agrees with our findings using diphenylhexatriene fluorescence [11] except that with this probe it was not possible to distinguish bulk and annular lipid phase.

To monitor the rotational mobility of the protein components, Complex I and Complex III were derivatized with a spin-labelled maleimide (MSL). By double integration we found that under the conditions of labelling described in Materials and Methods, approximately one molecule of MSL was incorporated per molecule of Complex I FMN or Complex III cytochrome c_1 and activities were largely unaffected. Conventional (V_1) spectra of such samples are shown in Fig. 6. The spectrum of MSL-Complex III is that of a completely immobi-

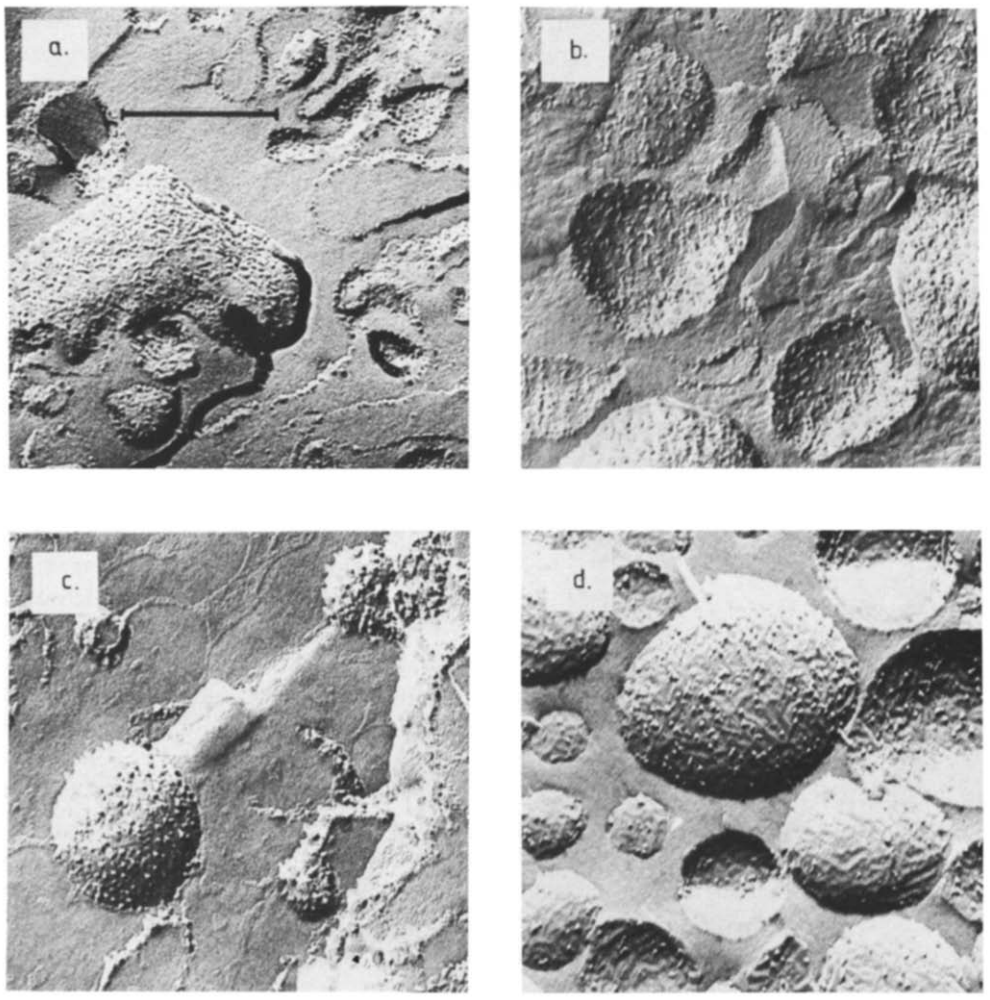


Fig. 4. Freeze-fracture micrographs of native and lipid-replaced Complex I-III samples equilibrated at 27°C. The samples were exactly the same as those of Fig. 2: (a) Complex I-III containing natural lipids; (b) supplemented with soyabean PC; (c) lipid-replaced Complex I-III; (d) supplemented with DMPC. Samples were equilibrated at 27°C before rapid freezing. The bar in (a) indicates 0.4 μm.

lised spin probe, while with Complex I, a small contribution from a mobile component is also present. This is decreased by centrifugation and washing but not entirely eliminated. Treatment of the sample with cysteine and $K_3Fe(CN)_6$ did not cause a relative decrease in the mobile component as found for cytochrome oxidase [28]. While the mobile component can be removed by spectral subtraction, we have preferred to use MSL-Complex III in most experiments because it shows a single spectrum.

Saturation-transfer esr spectra in the V_2' mode [20] are shown in Figs. 7 and 8. These correspond to the samples used in previous figures. At 27°C, the effects of increased lipid-to-protein ratio (Fig. 7b and d) are clearly evident in that the spectra show a much enhanced mobility characterised by a decrease in the ratio L''/L, a deepening of the centre-field dip and a more prominent peak at the high-field end. At 5°C (Fig. 8) the protein mobility is only enhanced by increase of the lipid-to-protein ratio when natural and soyabean lipids are present (Fig. 8B). Both DMPC-containing samples have very low rotational mobility. Rotational cor-

TABLE I

KEY TO SAMPLES OF COMPLEX I-III [a] TO ILLUSTRATE THE RANGE OF CONDITIONS UNDER WHICH STOICHIOMETRIC ASSOCIATION OR Q-POOL BEHAVIOUR OCCUR

a, enzymes as isolated; b, enzymes supplemented with soybean lipids; c, DMPC-replaced enzymes; d, DMPC-replaced enzymes supplemented with DMPC.

Sample	Lipid content (μmol·mg^{-1})	Mode of interaction	
		5°C	27°C
a	0.26	Stoichiometric	Stoichiometric
b	0.50	Q-pool	Q-pool
c	0.31	Stoichiometric	Stoichiometric
d	0.50	Stoichiometric	Q-pool

[a] The complex I to III ratio was 1:4 in this instance, but the mode of interaction is not dependent on the ratio.

relation times (τ_2) were estimated from the ratio L''/L [20] and are given in Table II for the samples of Figs. 7 and 8 as well as a variety of others containing MSL-Complex III. The most striking finding is the enormous change in τ_2 attendant on the presence of extra-annular lipid. In most instances, τ_2 is less by a factor of 10 when a mobile, extra-annular lipid phase is present. The presence of Complex I also has an effect on the rotational correlation time of Complex III. At higher lipid to protein ratios, the effect is small and τ_2 increases from 20 μs at 27°C for Complex III alone to only 80 μs when a 2-fold molar excess of Complex I is present. Complex I had a much greater effect when the samples contained only annular lipid. Thus, at 27°C, τ_2 increases from 90 μs for Complex III alone to 900 μs for a 2:1 molar ratio of Complex I to Complex III. Lowering the temperature increases τ_2. For samples containing DMPC at 5°C, the spectra resemble those obtained with a completely immobilised protein and L''/L for example, has the limiting value of 1.2 [20]. Attainment of such a value indicates that probe-protein motion is not significant. In native Complex I-III samples containing no extra-annular lipid, L''/L is between 1.1 and 1.2 at 5°C and the precision of the τ_2 values is low. However, in these samples, the Complex III is not completely immobilised on the saturation transfer time scale.

The complete absence of a mobile signal in MSL-Complex III enabled us to test whether such a signal affects estimates of τ_2. To do this, low

TABLE II

ROTATIONAL CORRELATION TIMES (τ_2) OBTAINED FROM ST-ESR SPECTRA OF VARIOUS COMPLEX I-III SAMPLES, WHERE THE COMPLEX III WAS LABELLED WITH MSL

	Molar ratio I:III	Lipid content (μmol·mg^{-1} protein)	τ_2 (μs) at		Mode of interaction
			5°C	27°C	
Complex I-III					
Native	1:4	0.26	1500	700	Stoichiometric
	2:1	0.20	1500	900	Stoichiometric
	1:4	0.50	100	40	Q-pool
	2:1	0.50	150	80	Q-pool
Lipid-replaced	1:4	0.31	v. high [a]	150	Stoichiometric
	1:4	0.50	v. high	30	Stoichiometric (5°C) Q-pool (27°C)
Complex III alone					
Native	–	0.25	350	90	–
	–	0.50	100	20	–
Lipid-replaced	–	0.20	v. high	20	–
	–	0.50	v. high	20	–

[a] Refers to values of L''/L at the limit of approx. 1.2 as described in the text.

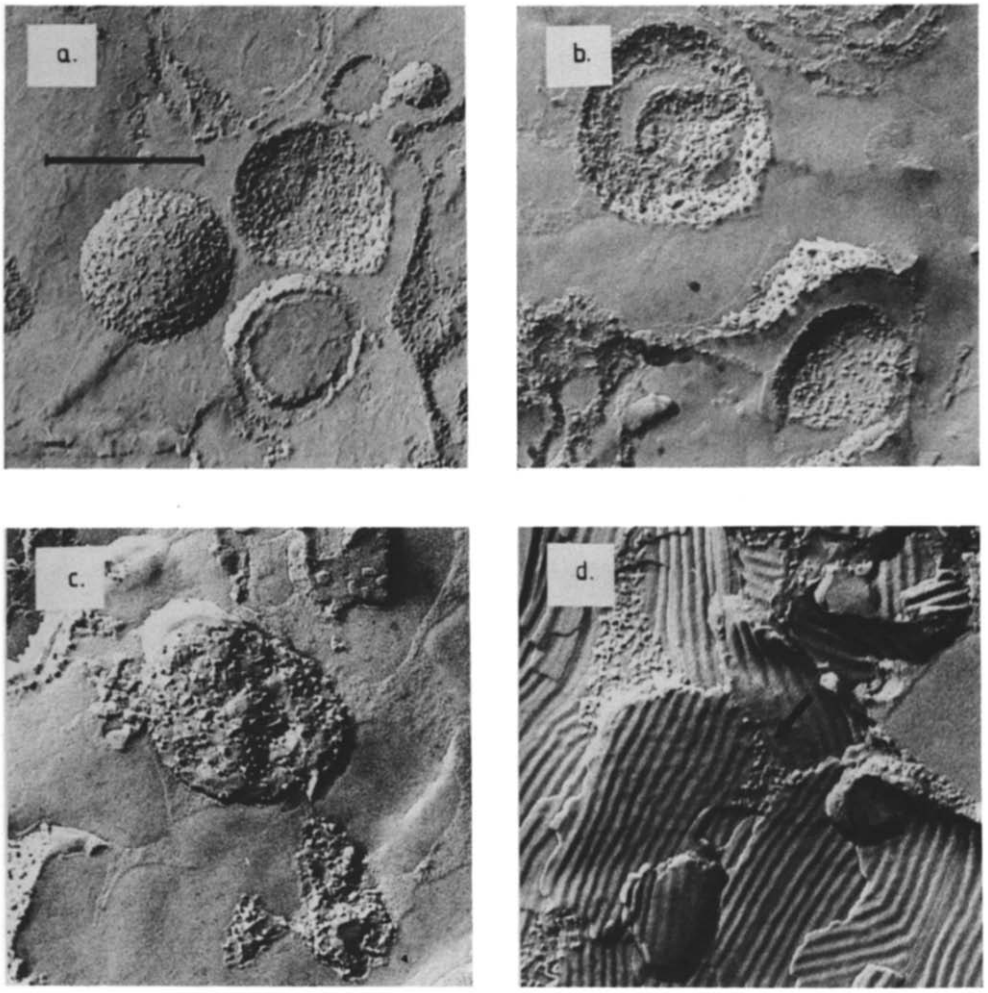
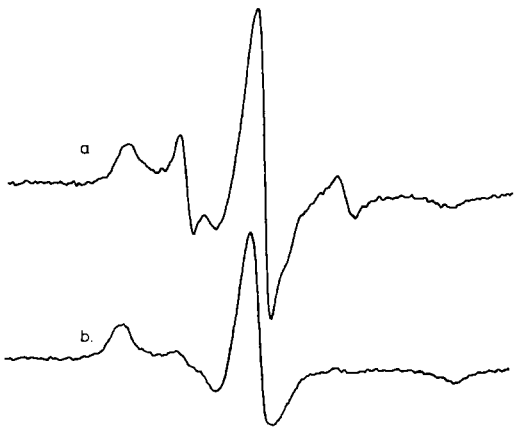


Fig. 5. Freeze-fracture micrographs of native and lipid-replaced Complex I-III samples produced at 5°C. The samples were exactly the same as those of Fig. 4 except that they were incubated at 5°C prior to freezing.



concentrations of free MSL were introduced into MSL-Complex III samples and the V_2' spectra were compared. While the line shape shows considerable change when a mobile signal is present (see, for example, Ref. 29), we could show that values of L''/L were unaffected at concentrations of mobile spin label such as those found in MSL-

Fig. 6. V_1 spectra of MSL-labelled Complex I and Complex III. V_1 spectra were obtained at 27°C using a field modulation intensity of 3.2 G, frequency 100 kHz and a microwave power of 1 mW (22 dB). (a) Complex I containing natural lipid (0.22 μmol/mg of protein); (b) Complex III containing natural lipid (0.24 μmol/mg of protein). Scan width was 100 G.

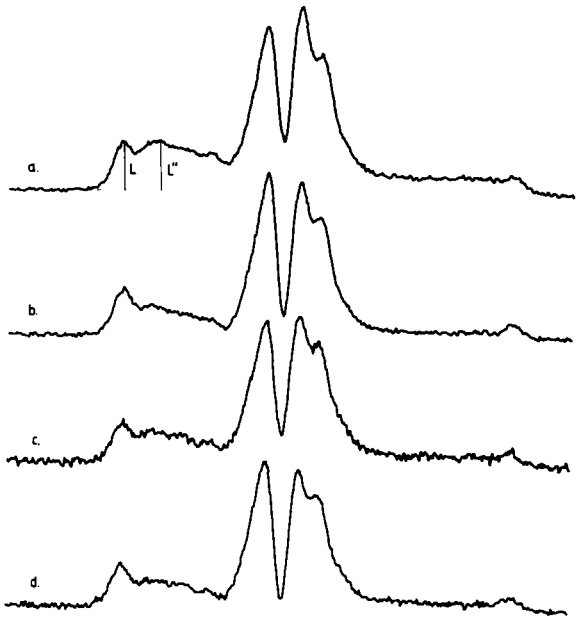


Fig. 7. Saturation transfer spectra of MSL-labelled native and lipid-replaced Complex I-III samples measured at 27°C. In all these spectra, Complex III was labelled with MSL and the molar ratio of Complex I to III was 1:4. The enzymes were reconstituted to give NADH-cytochrome *c* oxidoreductase activity. (a) Enzymes containing natural lipids (0.26 μmol/mg of protein); (b) enzymes containing natural lipids supplemented with soyabean phospholipids (final lipid content 0.50 μmol/mg of protein); (c) DMPC-replaced enzymes (final lipid content 0.31 μmol/mg of protein); (d) DMPC-replaced enzymes supplemented with DMPC (final lipid content 0.50 μmol/mg of protein). Protein concentrations were all 30 mg/ml. Spectra were measured with a modulation intensity of 5 G, a modulation frequency of 50 kHz and phase sensitive detection at 100 kHz. Scan width was 100 G.

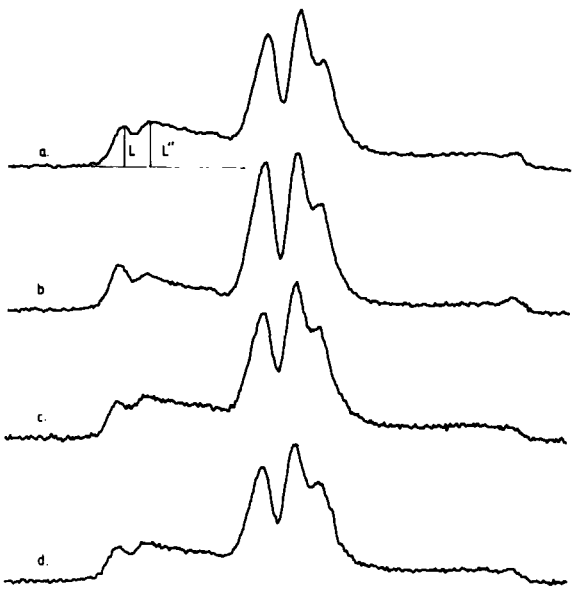


Fig. 8. Saturation transfer spectra of MSL-labelled native and lipid-replaced Complex I-III samples measured at 5°C. The samples were exactly the same as those in Fig. 7 except that spectra were taken at 5°C. Scan width was 100 G.

Complex I samples (Fig. 6). In preliminary experiments, we find that MSL-Complex I containing native lipids is less mobile than equivalent samples of MSL-Complex III and rotational correlation times in the presence of extra-annular lipid were approximately 5-fold higher. These values did not change appreciably when Complex III was included.

Discussion

Relatively small differences in lipid to protein ratio determine whether stoichiometric or Q-pool behaviour is exhibited by reconstituted Complex I-III. The results of this paper and the preceding one [16] show that it is not the total lipid concentration which is important but the amount of mobile extra-annular lipid. This is only found when the lipid content exceeds approx. 0.3 μmol/mg of protein.

At concentrations of lipid below this value, or in gel phase lipid, the Complexes interact stoichiometrically. In the gel phase, the proteins and extra-annular lipid segregate (Fig. 5d) as found in other systems [30], and the protein environment is presumably the same regardless of the lipid to protein ratio. When only annular DMPC is present, segregation cannot be seen at 5°C and the pattern characteristic of pure gel phase DMPC is absent (Fig. 5c). It is likely, therefore, that even when segregation does occur, the protein still retains its boundary layer of lipid and only extra-annular lipid separates out. Indeed, the annular lipid structure in DMPC-replaced Complex I-III at low temperature may be quite similar to that found in non-replaced enzymes, as suggested by diphenylhexatriene fluorescence [11]. Thus, under all condi-

tions leading to stoichiometric association, the protein environment is likely to be very similar and characterised by a close packing of the protein molecules and a lipid phase with a low rate and amplitude of motion.

Aggregation of membrane proteins is a common phenomenon. Bacteriorhodopsin [30] and the Ca^{2+} ATPase of sarcoplasmic reticulum [31] are both aggregated in gel phase dipalmitoylphosphatidylcholine, but show rapid rotational diffusion in the liquid crystalline phase. The former protein, and cytochrome oxidase [32] aggregate into ordered arrays under suitable circumstances. However, it does appear that such aggregates can retain phospholipid around each molecule [33] as suggested here for Complex I-III in the gel phase. Direct protein-protein interaction can also be found. For example, in rod outer segments, illumination causes aggregation of rhodopsin which cannot be reversed by phospholipids or detergents [29] and appears to be caused by crosslinking through exposed cysteine residues. Delipidation also gives rise to aggregation [29] which is reversed by detergents but not phospholipids, and again this is attributed to direct protein-protein contact. This kind of behaviour does not seem to apply to the Complex I-III system. Where extra DMPC is available, the aggregation induced by lowering the temperature is freely and rapidly reversible showing that phospholipids can indeed separate the closely packed protein molecules.

The fact that, in the aggregated state, NADH-cytochrome *c* reductase activity is still found, implies that the relative orientation of Complex I and Complex III molecules is not random. Although diffusion of ubiquinone and ubiquinol between randomly oriented Complexes would allow NADH-cytochrome *c* reductase activity, the kinetics of such a process would differ greatly from those found in practice, i.e. so-called stoichiometric association [4]. If ubiquinone and ubiquinol diffusion does take place, it must be from a Complex I molecule to only one other Complex III molecule (and vice versa). The relative orientation of Complex I and III molecules must therefore be specific although individual Complex I-III units and any enzymes in stoichiometric excess may be quite randomly distributed in the aggregate. Recognition of Complex I molecules by Complex III molecules could occur in several ways. The presence of annular lipid does not seem to prevent ordered packing of membrane proteins (e.g. bacteriorhodopsin [33]) and it is not necessary therefore to invoke direct protein-protein interaction within the membrane. Alternatively, it could be the parts of the protein molecules which extend beyond the bilayer which are involved.

The long rotational correlation times characteristic of stoichiometric association may apply to rotational diffusion of large aggregates (and ultimately whole membranes) rather than of individual molecules within the aggregate. The relative rotation of individual Complexes may therefore be even slower. In Complex I-III containing natural lipids, Complex III activities of 100 μmol of cytochrome *c* reduced/min per mg of protein have been found at 30°C [5] and even higher values (300–600 μmol/min per mg of protein at 37°C) have been reported [15]. These numbers correspond to catalytic centre activities of the order of 10^3/s, i.e. one catalytic event per ms. Comparison of this with the values given in Table II shows that stoichiometric association is found when the rotational correlation time is of the same order of magnitude or greater than the shortest time for one electron transfer through Complex III. Maximum Complex I activities are approx. 10-fold slower than Complex III. The large effect of Complex I on the rotational diffusion of Complex III cannot be interpreted as evidence for the formation of the Complex I-III unit. From measurements of diphenylhexatriene fluorescence, we found that the apparent viscosity of the annular lipid of Complex I is considerably higher than that of Complex III [11]. Thus, in Complex I and III mixtures, the average lipid viscosity increases with increasing Complex I content, and thus must affect the rotational diffusion of Complex III proportionately. Alternatively, if the long rotational correlation times are indicative of rotational diffusion of large aggregates, then it is quite possible that these vary in size depending on the proportion of Complex I.

When a mobile lipid phase is present, rotational correlation times are much shorter. The values obtained for Complex III alone (20 μs at 27°C and 100 μs at 5°C) are consistent with a completely dispersed state (probably as a dimer [34])

and agree with values obtained for other membrane proteins such as cytochrome oxidase (40 μs at 4°C [28]), rhodopsin (20 μs at 20°C [29]), and Complex III (70 μs at 4°C [35]). Again, Complex I inclusion has an effect on τ_2 for Complex III, but this is less marked than when there is no fluid lipid phase. This may be explained, as before, by the effect of Complex I on the overall fluidity of the lipid phase [11]. It is unlikely that the increase in τ_2 is due to the formation of Complex I-III dimers or aggregates since Complex I alone under similar conditions has an even longer rotational correlation time.

The rotational correlation times obtained from saturation-transfer ESR are necessarily approximate since they are based on calibration curves derived for isotropic motion of the spin label [20]. Nevertheless, where comparison has been made with other methods, e.g. flash photolysis, there is reasonably good agreement [36]. The relaxation times obtained from flash photolysis experiments (ϕ) are in fact, not directly comparable with τ_2 since ϕ is defined as the reciprocal of the anisotropic rotational diffusion coefficient, $D_{\|}$ [36], while τ_2 is the reciprocal of $6D_R$, where D_R is the isotropic rotational diffusion coefficient [36]. Even if $D_{\|}$ and D_R can be equated (which is doubtful) there is still the factor of 6 to be considered. One might therefore expect τ_2 values to be rather smaller than the corresponding ϕ values as found, for example, for cytochrome oxidase [28,37].

Despite these uncertainties, it appears that Q-pool behaviour is characterised by a rapid rotational diffusion of the constituent protein molecules. The theory proposed by us [5,6] to explain Q-pool behaviour in terms of transient association and dissociation of Complex I and Complex III would require such rapid diffusion and the results presented in this paper are quite consistent with this viewpoint. They are also, of course, perfectly consistent with rapid diffusion of ubiquinone and ubiquinol between separate Complex I and III molecules [9,10]. However, this model fails to explain the stoichiometric association of Complex I and III. It is interesting that stoichiometric association is found when rotational diffusion is comparable to or slower than the potential turnover of Complex III. A characteristic feature of stoichiometric association is the limitation of the maximum turnover of Complex III to a value dictated by the slower enzyme, Complex I [5,6] or L-3-glycerophosphate dehydrogenase [7]. We see this effect as the inability of an oxidised Complex III molecule to transfer electrons from more than one donor enzyme. As the rotational and lateral mobility of the enzymes is increased, a Complex III molecule may interact with several donors within a time comparable with overall electron transfer. In the limit, the kinetics would be those of 'Q-pool' behaviour where all donor molecules are equally effective in electron transfer to any Complex III molecule. As expected, behaviour intermediate between the two extremes has been seen [7].

Lastly, in comparing our findings with other studies on mitochondrial complexes (see, for example, Refs. 28 and 35), we note that reconstitution of the protein into a membrane to obtain high rotational mobility is usually done at very high lipid-to-protein ratios, e.g. 12.5 : 1 by weight in [35]. Our work shows that this is unnecessary (as long as the protein does not exhibit aggregation which is difficult to reverse). At these high lipid-to-protein ratios, it is, of course, rather more difficult to see the immobile boundary layer lipid by ESR and it has been concluded [28,35] that proteins are rotationally mobile only when all surrounding lipid is mobile on the ESR timescale. Using much lower lipid to protein ratios we can clearly demonstrate rapid rotational motion of proteins in the presence of an immobile boundary layer. The prerequisite is, in fact, a mobile extra-annular lipid phase. Our results also demonstrate that protein aggregation is not necessary for immobilisation of the lipid phase and that the latter phenomenon is therefore a property of monomeric membrane proteins as originally proposed [12,13]. We are, however, aware that the relationship between lipid and protein mobility might be system-dependant.

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