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A SPIN LABEL STUDY OF THE LIPID BOUNDARY LAYER OF MITOCHONDRIAL NADH-UBIQUINONE OXIDOREDUCTASE

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Mitochondrial NADH-ubiquinone oxidoreductase (Complex I) is a lipoprotein enzyme containing phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin. Enzyme preparations containing endogenous cardiolipin and a range of either soyabean PC or dimyristoylphosphatidylcholine (DMPC) concentrations have been made. Using a spin-labelled fatty acid, two probe environments differing in mobility have been shown to be present. The fatty acid probe has a relative binding constant (or partition coefficient between lipid and protein) of unity. The boundary layer or lipid annulus reported by the probe has a value of approx. 300 lipid molecules per molecule of enzyme FMN in preparations containing soyabean PC, or DMPC above the phase transition temperature of the latter. In soyabean PC-replaced enzyme the apparent size of the boundary layer is independent of temperature between 30°C and 14°C but shows a modest increase to about 400 lipid molecules per molecule of FMN between 14°C and 2°C. Complex I replaced with high concentrations of DMPC gives non-linear Arrhenius plots of NADH-ubiquinone oxidoreductase activity. The results of the ESR experiments show that both boundary layer and bulk lipid must be motionally restricted for this to occur. Thus, the change in activity is probably not caused by an effect exerted directly on the catalytic activity of the enzyme but is more likely due to restriction of free diffusion of ubiquinone to its site of reduction.

Introduction

The mitochondrial NADH-ubiquinone oxidoreductase complex (Complex I, [1]) is a lipoprotein enzyme containing phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin. Lipid-depletion and replacement studies have shown that both cardiolipin [2,3] and either phosphatidylcholine or phosphatidylethanolamine [2] are essential for ubiquinone reductase activity but not for reduction of other electron acceptors such as $K_3Fe(CN)_6$. More specifically, it is the rotenone-sensitive pathway of ubiquinone reduction which is lipid-dependent, as expected from the determination [4] that reduction by this pathway takes place in the lipid phase while rotenone-insensitive ubiquinone reduction, like that of $K_3Fe(CN)_6$ takes place in the aqueous phase.

Using Complex I whose endogenous PC and PE had been replaced by dimyristoylphosphatidylcholine (DMPC), we showed [4,5] that, at high lipid-to-protein ratios, Arrhenius plots of rotenone-sensitive NADH-UQ-1 or UQ-2 reductase activity showed a break to greater slope as the temperature was lowered through the phase transition temperature of DMPC. Activities with

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; UQ-1, ubiquinone-1; UQ-2, ubiquinone-2; 12-SASL; 12-stearic acid spin label (12-(4′,4′-dimethyloxazolidinyl-*N*-oxy)stearic acid).

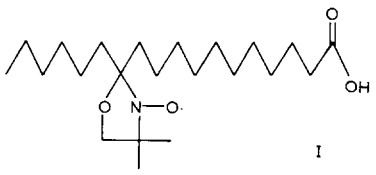
other acceptors did not show this behaviour. Thus the physical state of the lipid appeared to specifically affect the interaction of ubiquinone analogues with the enzyme.

Fluorescence depolarisation of the probe 1,6-diphenyl-1,3,5-hexatriene was used [5] to monitor the physical state of Complex I phospholipid and give some idea of variations in lipid 'fluidity'. This work demonstrated that increasing the lipid-to-protein ratio increased the overall fluidity which was low in native Complex I containing 0.20—0.25 μmol of phospholipid P/mg of protein. However, only when the lipid content was higher and of low fluidity (as in gel phase DMPC) was the Arrhenius behaviour affected. We concluded that the effect of lipid was not on the catalytic activity of the enzyme per se, but on the accessibility of ubiquinone analogues to their site of reduction, i.e. diffusion of ubiquinone could become rate limiting.

In the present paper, we have extended these studies by use of a spin-labelled fatty acid probe of the lipid environment. Our purpose was to investigate the boundary layer [6] or lipid annulus [7] around this enzyme and to determine how the size and fluidity of the boundary and bulk lipid phases influenced the ability of the enzyme to reduce ubiquinone analogues. In the following paper, we extend this work to the interaction of Complex I with ubiquinol-cytochrome *c* oxidoreductase (Complex III) and describe measurements of protein rotational diffusion by saturation transfer ESR.

Materials and Methods

Complex I was purified from bovine heart mitochondria by the method of Hatefi and Rieske [8]. The preparation contained approx. 1.0 nmol of flavin per mg of protein. NADH-ubiquinone-1 oxidoreductase activity at 20°C was measured as previously described [4]. Dimyristoylphosphatidylcholine was obtained from Koch-Light. Soyabean phosphatidylcholine was purified by the method of Ragan and Racker [9]. The fatty acid spin label (I), 12-(4′,4′-dimethyloxazolidinyl-*N*-oxy)stearic acid, (12-SASL), was purchased from Syva Associates, Palo Alto, CA and used as a stock solution of 2.6 mM in 100% methanol.



I

The buffer used throughout this study was 0.67 M sucrose, 50 mM Tris-HCl (pH 8.0) at 4°C.

The endogenous PC and PE of Complex I preparations were exchanged for DMPC in the presence of 0.35% sodium cholate followed by ammonium sulphate precipitation according to the method of Heron et al. [2]. Samples of Complex I containing increased lipid-to-protein ratios were prepared by incubating the native or DMPC-replaced enzyme with a range of soyabean PC or DMPC concentrations respectively, in the presence of 0.35% cholate. Lipid-depleted Complex I was prepared by incubating the native enzyme with 2% cholate followed by ammonium sulphate precipitation. The spin label 12-SASL was added during each sample incubation, at a concentration not exceeding 2 mol% of the total lipid content. Cholate and excess label were removed by dialysis against several changes of buffer for 30 h at 4°C, in the dark. The samples were then centrifuged at $100\,000 \times g$ for 20 min at 4°C. After the supernatant was discarded the walls of each centrifuge tube were carefully rinsed with buffer to remove excess lipid and any remaining supernatant and the enzyme resuspended in sucrose/Tris buffer to the original volume. After further centrifugation each sample pellet was finally redispersed in sucrose/Tris buffer to a protein concentration of 30 mg/ml. This procedure served to remove protein-free liposomes which were formed at the highest lipid-to-protein ratios employed. The solutions were stored in liquid nitrogen. For comparative purposes the spin label was incubated with lipid solutions (DMPC or PC) in the absence of protein and then dialysed to remove cholate.

Total phospholipid was determined by analysis of phosphate after digestion of protein samples with perchloric acid [10]. Protein was measured by the method of Lowry et al. [11] with bovine serum albumin (fraction V from Sigma) as a standard.

ESR spectra were recorded on a Bruker ER 200D spectrometer equipped with a nitrogen gas-flow variable temperature-controlled unit. Spectra

were recorded as the first derivative of the absorption curves over a range of 100 G with a field modulation frequency of 100 kHz and amplitude 3.2 G. The microwave power was kept at 1 mW to avoid signal saturation or sample heating. A quartz aqueous flat cell was used to enable the maximum amount of sample to be placed in the cylindrical cavity with maximum sensitivity. On each insertion the plane of the flat cell was set to a fixed position by means of a marker system.

The spectrometer was interfaced with a Cromemco minicomputer system by which means spectra were accumulated on disc files, averaged, smoothed, shifted horizontally to correct for drift, subtracted and integrated. The criteria for the end point of subtraction included values of zero at the start and end of the first integral, the absence of a negative slope in the double integral and the absence of a negative contribution at the low-field shoulder of the fluid component spectrum.

Results

ESR spectra were recorded for samples of Complex I containing the freely-diffusable lipid spin label 12-SASL. Spectra were measured as a function of lipid content and temperature. Using the computer each spectrum was repeatedly scanned at a slow sweep speed and with a long time constant to produce an average spectrum of maximum signal to noise ratio.

Corrected spectra were produced by computer subtraction of unwanted contributory signals from each of the accumulated spectra. Firstly a 'background' signal was subtracted from each spectrum. This signal comprises a base line shift that occurs at the centrefield setting for nitroxide resonance absorption. It would appear to originate from the quartz glassware and was found to vary with the thickness of the glass container placed in the cavity. The signal was constant for a given container and could therefore be accumulated and stored on disc file ready for spectral subtraction. This effect has not been reported by other workers in the field who in the main have employed Varian spectrometers. It is possible that the mode of tuning peculiar to the Varian may mean that such an effect is not obvious or eliminated.

A second signal which was removed where necessary was the 'liquid line spectrum' corresponding to the spin label in aqueous solution. This contribution was only found to be significant in samples containing low lipid to protein ratios and was removed by subtraction of the appropriate fraction of a standard spectrum of a solution of the spin label in buffer.

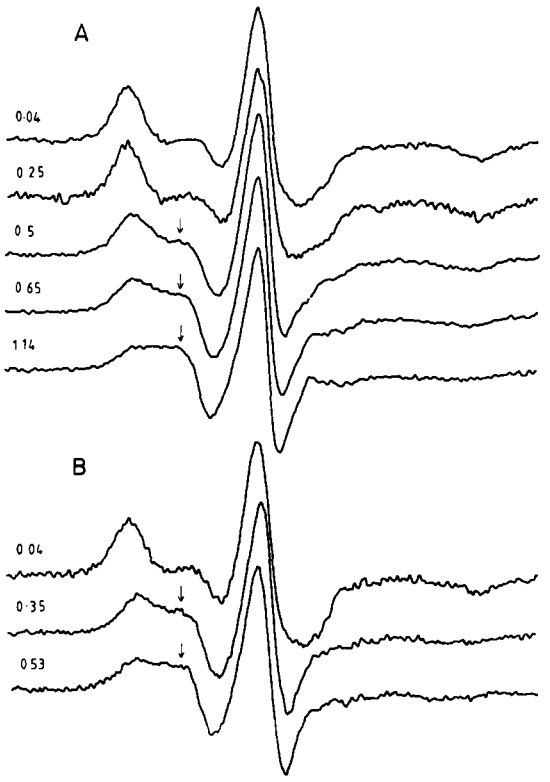


Fig. 1. ESR spectra of 12-SASL in Complex I samples of differing lipid-to-protein ratio and at either 5°C (A) or 15°C (B). The lipid content is indicated on each trace in μmol of lipid P/mg of protein. Lipid-depleted and native Complex I samples contained 0.04 and 0.25 μmol of lipid P/mg of protein, respectively. All spectra have been normalised to the same centre field peak height and the appearance of a low field peak attributed to a mobile lipid phase is shown by the arrows. Total scan width in this and subsequent figures was 100 G.

Typical corrected spectra for native Complex I samples with a varied lipid to protein ratio are shown in Fig. 1. It can be seen that in the lipid-depleted sample containing 40 nmol of lipid P/mg of protein, a single immobilised component was present with a broad outer splitting. As the lipid content was increased a second, mobile, component appeared concomitant with a decrease in the

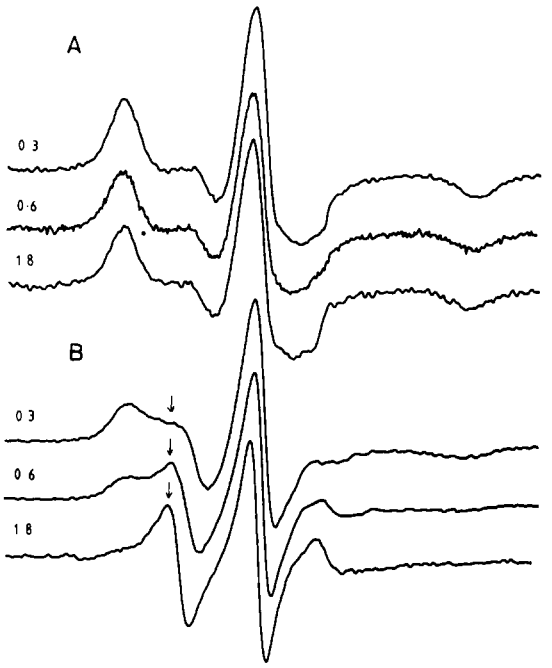


Fig. 2. ESR spectra of 12-SASL in DMPC-replaced Complex I samples of differing lipid-to-protein ratio and at either 5°C (A) or 27°C (B). Lipid content is given as in Fig. 1 and the arrows show the low-field peak of mobile spin probe.

outer splitting which eventually became ill-defined. The appearance of a second, mobile component at higher lipid to protein ratios was achieved with native Complex I samples whether the spectra were measured at 5°C or 15°C. However the results with the lipid-replaced enzyme were quite different. As shown in Fig. 2, a mobile component was only present at the higher temperature of 27°C. At 5°C the spin label was totally immobilised irrespective of the lipid to protein ratio of the sample. This result is explained by phase transition of the DMPC (21–24°C for the pure lipid [12]) such that at 5°C all the lipid may be assumed to be in the gel phase. At 27°C an immobilised component was still present as part of the spectrum even though in the pure state the lipid phase would have been totally liquid crystalline. This implies that in native or lipid-replaced Complex I samples, the immobilisation of the lipid spin label is dependent on the presence of the protein.'

Further information may only be obtained from such spectra by use of computer subtraction to reveal the separate components. It was assumed that the spectra were composed of only two possible components and therefore the lineshape of the immobilised component would be constant at a given temperature. The lipid-depleted Complex I sample exhibited an outer-splitting of 63.3 G at 5°C which is within the range (63–64 G) attributed to a rigidly immobilised spin label [13]. The appropriate fraction of this spectrum was therefore subtracted from each composite spectrum measured at 5°C to reveal the mobile component. The outer splitting of the immobilised component was temperature dependent decreasing to 62.7 G at 15°C and 61.5 G at 27°C. This effect has been noted for the 14-doxyl stearic acid spin label [14] and it is to be expected that the whole system may become more mobile with increasing temperature. To allow for this effect, the immobilised spectrum used for special subraction was always measured at the same temperature as the spectrum to be

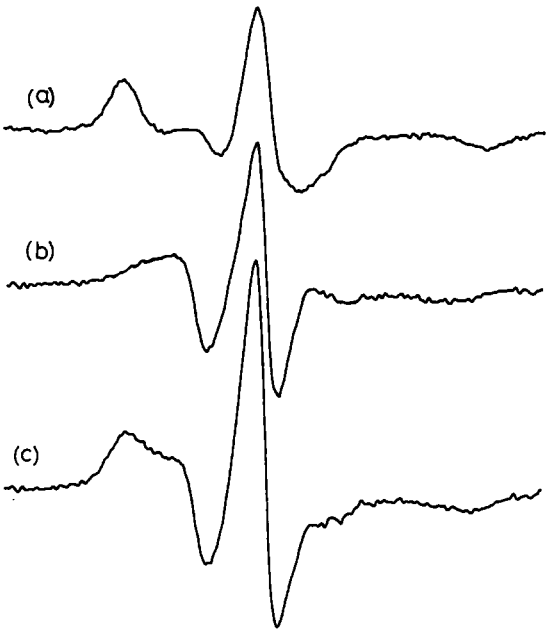


Fig. 3. Resolution of the composite ESR spectrum into the immobile and mobile components. Spectrum (c) was obtained from 12-SASL in native Complex I supplemented with soyabean PC to give a final lipid content of 0.65 μmol/mg of protein. Temperature was 5°C. The immobilised component (spectrum a) was obtained from a lipid-depleted sample at 5°C. This was subtracted from spectrum (c) in the appropriate proportion to give the mobile component (b).

analysed. However, at a given temperature the outer splitting of each resolved immobile component did not change with increasing lipid to protein ratio. Fig. 3 shows the results of a typical subtraction of an immobile spectrum (a) from the composite spectrum (c), leaving the spectrum of the mobile component (b). The line shape of the resolved mobile spectrum was independent of lipid to protein ratio, but was temperature dependent indicating increased probe motion at higher temperatures.

The amounts of mobile and immobilised probe in each sample were obtained by double integration of the component spectra. In Fig. 4 the ratio of mobile to immobile probe has been plotted against the lipid to protein ratio as described by Griffiths and Jost [15]. Error bars indicate extreme values beyond which the spectral subtraction clearly fails to meet the ccriteria listed in the Materials and Methods section. The x-axis intercept gives the size of the boundary layer (i.e. the number of probe binding sites) while the y-axis intercept gives the relative binding constant for the spin label. At two temperatures (5°C and 15°C) this intercept is close to one indicating no preference or a lack of preference for binding of the fatty acid spin label to the protein compared with the phospholipid. However, there is a small temperature dependence of the number of binding sites which increases from 0.32 μmol/mg of protein at 15°C to 0.40 μmol/mg of protein at 5°C.

The theory only applies to full occupancy of all boundary layer sites (i.e. for lipid to protein ratios greater than 0.3–0.4 μmol/mg of protein). At lower values than this, one would not expect to see a mobile lipid phase at all, as reported by Jost et al. [16] for bovine heart cytochrome oxidase. However, in Complex I we do find mobile lipid at low lipid to protein ratios. This is shown in Fig. 5, which gives the amount of immobilised lipid as a function of lipid to protein ratio, determined from

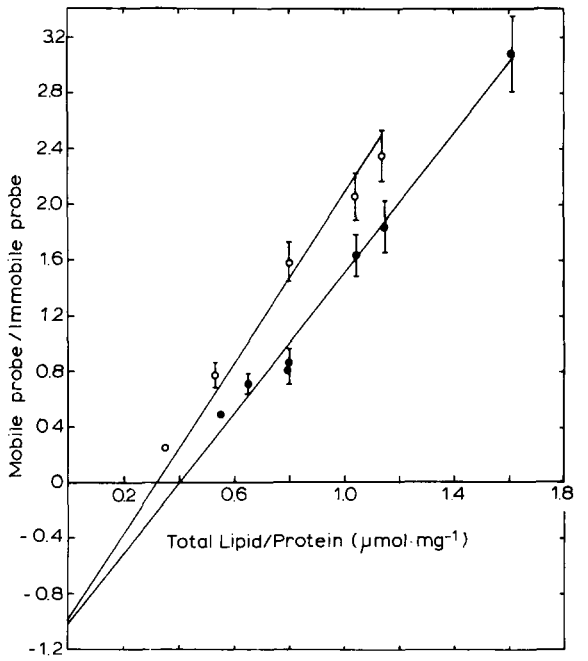


Fig. 4. Graphical estimation of the relative binding constant and number of probe binding sites for 12-SASL in samples of Complex I. Spectra of samples such as those of Fig. 1 were resolved into the constituent mobile and immobile components, and the amount of probe in each environment was obtained by double integration. The error bars indicate the limits beyond which the spectral subtraction clearly failed to meet the listed criteria. ○, 15°C; ●, 5°C.

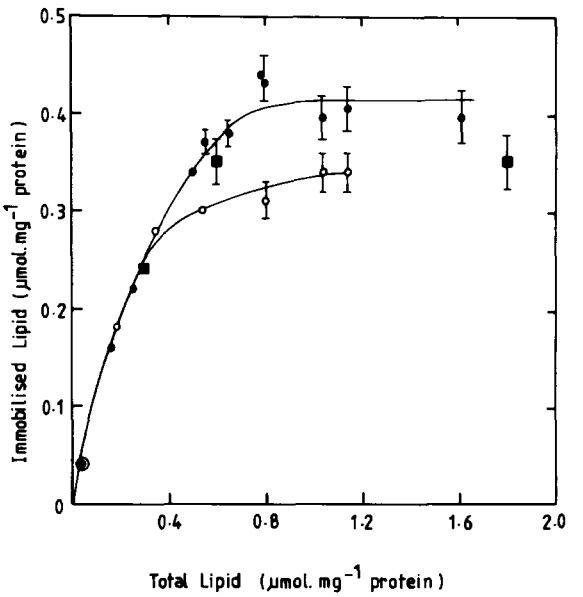


Fig. 5. The variation of immobilised lipid content with total lipid content. The amount of immobilised lipid was obtained from the amount of immobilised spin probe assuming a relative binding constant for the latter of one. Error bars denote the subtraction limits as in Fig. 4. ●, Complex I supplemented with soyabean PC at 5°C; ○, same at 15°C; ■, DMPC-replaced Complex I supplemented with DMPC at 27°C.

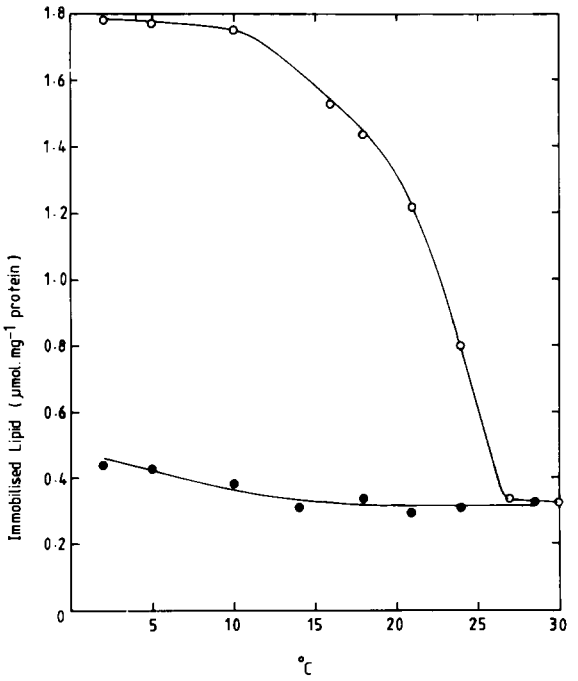


Fig. 6. Temperature dependence of the magnitude of the immobilised lipid phase. The extent of immobilisation of 12-SASL was measured: ●, in native Complex I supplemented with soyabean PC (final lipid content, 0.8 μmol/mg of protein); or ○, in DMPC-replaced Complex I supplemented with DMPC (final lipid content, 1.8 μmol/mg of protein).

the data of Fig. 4. At low levels, up to 0.2 μmol/mg of protein, all lipid is immobilised and the slope of the line is unity, while at high concentrations the amount of immobilised lipid reaches a constant value. However, the annulus is not filled until approx. 0.6 μmol of lipid/mg of protein have been added, indicating that both mobile and immobile lipid are found even when the boundary layer is apparently incomplete. Similar behaviour has been reported by Marsh et al. [17] for yeast cytochrome oxidase. This can be explained by the effect of random protein-protein contacts which diminish the available lipid-binding sites.

Fig. 5 also shows that at 27°C, DMPC-replaced Complex I behaves very similarly and contains a boundary layer of much the same magnitude. The temperature dependence of the magnitude of the boundary layer (Fig. 4) was investigated in more detail since there has been controversy over the existence of such an effect [18,19]. Fig. 6 is a phase diagram for native and DMPC-replaced samples of relatively high lipid content (i.e. with a complete boundary layer). In the native enzyme, the apparent magnitude of the boundary layer decreases from 0.45 μmol/mg of protein at 2°C to a constant value of 0.32 μmol/mg of protein at 14°C or higher. Thus, over a wide range of temperature there is no variation in apparent boundary layer magnitude but this does begin to increase at lower temperatures. DMPC-replaced enzyme gives a very similar value at 30°C but the magnitude of the immobilised lipid phase increases rapidly below 25°C due to phase transition of the lipid, until all phospholipid is immobilised at 10°C or below. The shape of the curve obtained with DMPC-replaced enzyme will probably nor permit a simple interpretation since, around the phase transition temperature of the lipid, preferential segregation of the spin-label into liquid-crystalline lipid may well take place [20].

Discussion

Despite much argument in recent years, the concept of a boundary layer or lipid annulus around integral membrane proteins is now widely accepted and there seems to be no major disagreement between conclusions drawn from ESR studies or other methods such as deuterium NMR. Much of the interpretation of the ESR spectra presented here depends on accurate and appropriate separation of the two contributory signals present in the spectra. The use of spectra of lipid-depleted samples to give the immobile component is widespread (see, for example, Refs. 6 and 21) and has been supported by recent work of Brotherus et al. [19] using an entirely independent method. This relied on the use of differently charged spin probes with different relative binding constants. Computer manipulation of composite spectra obtained with the two probes enabled these workers to derive the underlying mobile and immobile components without resorting to lipid-depleted samples. The results obtained with either method were, however, identical. These authors also showed that inappropriate choice of the immobile spectrum could lead to extensive errors. In particular, they showed that inattention to the variation of the immobile spectrum with tempera-

ture could lead to a fairly large apparent increase in boundary layer size with decreasing temperature. Use of the correct spectrum showed that, for $(Na^+ + K^+)$-ATPase at least, the boundary layer size was only slightly temperature dependent. In the Complex I studies reported here, we find a similar small temperature dependence using the appropriate immobile spectrum for subtractions. Although the immobile spectrum did vary with temperature, the effect was not great and in fact it made little difference to our results if the immobile spectrum obtained at say 5°C was subtracted from the composite obtained at 25°C. It is possible that the apparent increase in boundary layer lipid with decreasing temperature may be due to our use of 12-SASL instead of the 14- or 16-SASL used by other workers. Spectra from 12-SASL in lipid bilayers are less mobile overall because of the closer proximity of the nitroxide to the relatively fixed polar end [22]. We assume that the temperature effect is not an actual increase in the size of the boundary layer but an immobilisation of lipid extending beyond the first layer surrounding the protein. This extra lipid is much less rigidly immobilised than the boundary layer lipid [21] but may appear to be completely immobile using 12-SASL rather than 16-SASL for example, particularly at low temperatures.

It has been proposed in the past that boundary layer lipid arises from protein-protein aggregation (see, for example, Ref. 23), while more recently, aggregation has been invoked to explain any increase in the apparent size of the boundary layer with decreasing temperature [18]. Aggregation and the rotational diffusion of Complex I are considered in detail in the following paper. In the present context, the explanation for this behaviour is not important. If aggregation occurs, it does not seem to have any effect on Complex I activity, since Arrhenius plots of NADH-ubiquinone oxidoreductase are linear down to 2°C [4,5]. Changes in slope, such as those found in DMPC-replaced Complex I at high lipid-to-protein ratios [4,5] are not therefore produced by immobilisation of the annular lipid alone. It is only when both the boundary layer and an extensive bulk phase are immobilised that Arrhenius plots become non-linear. This is presumably due to restriction of the mobility of ubiquinone diffusion in the lipid phase or possibly on its partition between aqueous and lipid phases as we concluded previously [4,5].

Based on the FMN content of our Complex I preparations (0.9–1.1 nmol/mg of protein) we estimate that the Complex I boundary layer contains approximately 300 lipid molecules. Marsh et al. [17] have found that for several membrane proteins, the boundary layer size is proportional to the square root of the molecular weight, as might be expected as long as all proteins protrude from the membrane to the same extent. Using their average value for the proportionality constant and a molecular weight of 800 000 for Complex I [24], we estimate that the boundary layer should contain only 100 lipid molecules. Moreover, using the recently determined molecular dimensions of Complex I [25], we calculate that the monomer could accommodate approx. 130 molecules of lipid around its perimeter, in reasonable agreement with the above calculation, but not with the experimental result.

Cardiolipin probably forms an important part of this boundary layer since lipid-depletion by cholate or lipid exchange in low concentrations of cholate does not remove cardiolipin from the enzyme [2]. Thus, the lipid-depleted enzyme with 0.04 μmol of lipid P/mg of protein contains only cardiolipin (20 molecules/molecule of enzyme). For this reason we have preferred to use a less specific hydrophobic spin probe, 12-SASL, rather than a phospholipid spin label since we are uncertain how it would partition into cardiolipin binding sites. Preferential binding of cardiolipin has been found in both bovine heart [26,27] and yeast [28] cytochrome oxidase although it is not totally clear as yet whether this preferential association represents a small number of specific sites with high affinity or a general preference of all boundary layer sites for cardiolipin. The former seems more likely in view of the absolute requirement of cardiolipin for both cytochrome oxidase [29] and Complex I activity [2].

Acknowledgements

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