THE PHOSPHOLIPID ANNULUS OF MITOCHONDRIAL NADH–UBIQUINONE REDUCTASE

A dual phospholipid requirement for enzyme activity

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1. Introduction

A phospholipid requirement for the activity of several membrane-bound enzymes has been clearly established, e.g. [1,2] but the evidence for a phospholipid dependence of mitochondrial NADH dehydrogenase is conflicting at present. Reversible loss of NADH–ubiquinone-1 oxidoreductase activity following removal of phospholipids by phospholipase A treatment [3,4] or cholate and ammonium sulphate extraction [5] has been reported, but a water-soluble, lipid-free NADH dehydrogenase catalyzing ubiquinone reduction has been isolated by Baugh and King [6]. An approach to the study of lipid–protein interactions which avoids the potential problem of irreversible denaturation on removal of lipids is the lipid-substitution technique of Warren et al. [7]. We have applied this method to purified NADH–ubiquinone oxidoreductase (Complex I) [8] which is isolated as a lipoprotein complex. We are interested in investigating the role of lipids in mitochondrial electron transport for reasons other than the conflicting views on NADH dehydrogenase. Firstly, the respiratory chain complexes contain relatively high levels of cardiolipin whose specialized function (if any) is unknown. Secondly, the means by which respiratory complexes interact in the membrane is unclear. For example although ubiquinone-10 behaves as a kinetically homogeneous pool mediating hydrogen transfer from (separate) Complex I to Complex III [9], this picture is difficult to reconcile with the formation of a 1:1 binary complex from these two segments (Complex I–III) [10]. Thirdly, since ubiquinone and ubiquinol are so hydrophobic the function of phospholipid in the electron transfer processes of Complexes I and III may be to provide a suitable environment for these hydrophobic substrates rather than to preserve an active conformation of the protein.

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2. Materials and methods

Complex I was purified from bovine heart mitochondria by the method of Hatefi and Rieske [11]. NADH–ubiquinone-1 oxidoreductase activity at 20°C was measured as previously described [5]. Dimyristoyl phosphatidylcholine (DMPC) was obtained from Koch-Light. Soyabean phosphatidylcholine was purified by the method of Ragan and Racker [12]. Bovine heart cardiolipin was obtained from Sigma Chemical Co. and cardiolipin from *Saccharomyces cerevisiae* was generously given by Drs M. Virji and P. Knowles.

Phospholipid concentrations were determined by the phosphate content after digestion with perchloric acid [13]. In this context, all concentrations of cardiolipin are also expressed as phosphate and therefore the true molar concentrations of cardiolipin are one-half those indicated. Phospholipid was quantitatively extracted from protein and analysed by thin-layer chromatography on silica gel as described by Rouser and Fleischer [14]. The solvent system used was $CHCl_3$/methanol/H_2O (65/25/4, by vol.). Fatty acid composition of lipids was determined by gas chromatography of the methyl esters prepared by

transesterification. Separation was achieved on a 10% DEGS column at 180°C.

3. Results and discussion

In the experiment of fig.1, Complex I was incubated with various concentrations of cholate or soyabean phosphatidylcholine and precipitated with ammonium sulphate to produce samples of the enzyme with phospholipid contents lower or higher respectively than the native enzyme. Complex I as isolated could not be activated by increasing the phospholipid content but decreased in activity linearly with decreasing phospholipid content. The profile of activity versus phospholipid content was similar when NADH–ubiquinone-1 oxidoreductase was measured without a phospholipid supplement in the assay, although absolute activities were lower and the dependence not linear. This experiment and others (Ragan and Heron, unpublished observations) have established that stimulation of activity by phospholipid in the assay is due to more efficient delivery of the hydrophobic substrate, ubiquinone-1, to its site of reduction, and not to reactivation of phospholipid-depleted Complex I molecules. It is clear from fig.1 that Complex I as isolated has only just enough phospholipid associated with it to maintain maximum activity. The changes in activity shown in fig.1 could not be attributed to inactivation by cholate, since in the presence of excess phospholipid, cholate and ammonium sulphate treatment did not cause loss of activity (or loss of phospholipid). There was no effect of lipid depletion on NADH–$K_3Fe(CN)_6$ oxidoreductase and rotenone-insensitive NADH–ubiquinone-1 oxidoreductase activities.

The enzyme was completely inactive when the phospholipid content had been reduced to approx. 0.05 μmol/mg protein. Further treatment with cholate did not decrease this value and thin-layer chromatography established that the residual lipid was almost entirely cardiolipin. The cardiolipin content of Complex I was determined by assaying spots on thin-layer chromatograms for phosphate and found to be 0.05 μmol/mg protein.

In experiments of the type shown in table 1, Complex I was first treated with DMPC in 30-fold molar excess over the endogenous phospholipid. The

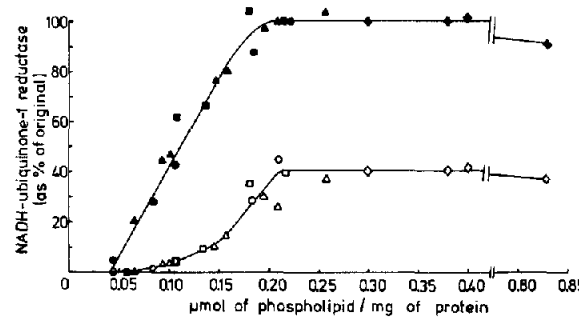

Fig.1. Dependence of Complex I activity on phospholipid content. The phospholipid content of Complex I (0.21–0.23 μmol/mg protein) was decreased by treating Complex I (5 mg protein/ml 0.67 M sucrose/50 mM Tris/HCl, pH 8) at 4°C with various concentrations of cholate between 0.1% and 2.5% for 30 min followed by precipitation with 46% saturated ammonium sulphate. The enzyme was collected by centrifugation at 100 000 × *g* for 20 min at 4°C and redissolved in the original volume of sucrose–Tris buffer. The phospholipid content was increased by treating Complex I (5 mg protein/ml) with soyabean phosphatidylcholine dispersed by sonication in sucrose–Tris buffer at concentrations ranging from 1–16 mM for 30 min at 4°C followed by ammonium sulphate precipitation as above. Samples were assayed for phospholipid phosphorus and for rotenone-sensitive NADH–ubiquinone-1 reductase activity in the presence (closed symbols) and absence (open symbols) of a phospholipid supplement in the assay. Results from four separate experiments are shown.

cholate concentration was 0.35% (w/v). After precipitation with ammonium sulphate the activity was unaffected and the total phospholipid content had increased somewhat to around 0.28 μmol/mg protein. Analysis of the fatty acid composition of this phospholipid revealed that the myristic acid content was 76%, which was increased to a maximum of 80% after a second treatment with DMPC. The maximum extent of exchange was reached within 10 min and did not increase over a 24 h period. Of the remaining fatty acids, linoleic acid (18 : 2) accounted for a further 12–14%. Linoleic acid comprises 70% of the fatty acids of bovine heart cardiolipin, implying that the 20% of unexchangeable fatty acids were mostly from cardiolipin. This was confirmed by thin-layer chromatography (fig.2).

Some exchange of endogenous cardiolipin for DMPC could be shown at higher cholate concentrations, although this was accompanied by loss of activity

Table 1
Exchange of Complex I phospholipids for DMPC and yeast cardiolipin

Lipid added	Cholate concn. (% w/v)	Total P lipid content (μmol/mg protein)	DMPC content	Endogenous CL content	Yeast CL content	Total CL content	Rotenone-sensitive NADH–ubiquinone-1 reductase activity (μmol/min/mg protein)
None	–	0.23	–	0.049	–	0.049	1.04
DMPC	0.35	0.28	0.22	0.045	–	0.045	0.98
DMPC	2.5	0.31	0.26	0.019	–	0.019	0.32
DMPC + yeast CL	0.35	0.37	0.23	0.023	0.092	0.115	1.10
DMPC + yeast CL	1.0	0.30	0.22	0.012	0.048	0.060	1.00
DMPC + yeast CL	2.5	0.30	0.22	0.009	0.033	0.042	0.86
Yeast CL	2.5	0.17	0.04	0.017	0.072	0.089	0.20

Complex I (10 mg protein/ml of sucrose–Tris buffer) was mixed at 4°C with an equal volume of a 60 mM solution of DMPC in 0.7% (w/v) Na-cholate. After 15 min the enzyme was precipitated with 46% saturated ammonium sulphate and collected by centrifugation at 100 000 × *g* for 20 min at 4°C. The pellet was carefully rinsed with 50% saturated ammonium sulphate and dissolved in sucrose–Tris buffer to a final concentration of 10 mg protein/ml. Portions (0.2 ml) were then treated with 0.2 ml of solutions containing either 60 mM DMPC, 2.5 mM yeast cardiolipin or both, dispersed by sonication in twice the cholate concentrations indicated. After 3 h at 4°C, the enzyme in each sample was collected by ammonium sulphate precipitation as above. The pellets were carefully washed with 50% saturated ammonium sulphate and each was redissolved in 0.4 ml sucrose–Tris buffer. Assays and phospholipid analysis were performed as described in the text.

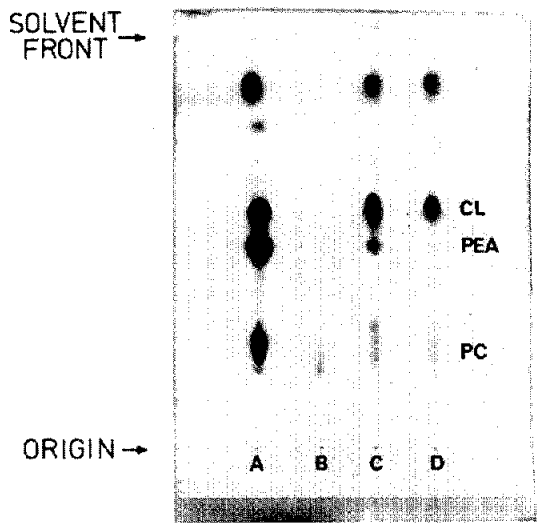

Fig.2. Thin-layer chromatography of the phospholipids of Complex I after exchange with DMPC. Complex I lipids were exchanged with DMPC in 0.35% cholate for 1 h as described in table 1. A portion of the exchanged enzyme was treated with DMPC again under identical conditions. (A) Phospholipid from 0.5 mg Complex I; (B) 0.13 μmol DMPC; (C) phospholipid from 0.45 mg once-exchanged Complex I; (D) phospholipid from 0.37 mg twice-exchanged Complex I. *Abbreviations:* CL, cardiolipin; PEA, phosphatidylethanolamine; PC, phosphatidylcholine. Assignments of spots was by comparison with authentic markers.

even though the DMPC content was unchanged (table 1). More successful were attempts to exchange the endogenous cardiolipin for exogenous cardiolipin. To follow this process, we used yeast cardiolipin whose fatty acid composition (42% palmitoleic acid and 56% oleic acid) is quite different from bovine heart cardiolipin. In table 1, Complex I which had already been exchanged with DMPC in 0.35% cholate to remove endogenous palmitoleic (16 : 1) and oleic (18 : 1) acids was further treated with yeast cardiolipin or DMPC and yeast cardiolipin. The concentrations of the two types of cardiolipins were calculated from the linoleic, palmitoleic and oleic acid contents. In the presence of both DMPC and yeast cardiolipin, activity was largely maintained even at high cholate concentrations. At low cholate concentrations exchange of cardiolipins was incomplete and the incorporation of yeast cardiolipin was considerably greater than the loss of endogenous cardiolipin. As the cholate concentration was increased, the incorporation of yeast cardiolipin more closely matched the loss of endogenous cardiolipin, and the extent of exchange was close to that expected for equilibration of the endogenous cardiolipin with the five-fold greater concentration of added cardiolipin. When yeast cardiolipin alone was presented at a high cholate concentration, most of the DMPC was removed with loss of activity. In addition, the incorporation of yeast cardiolipin was greater than the loss of endogenous cardiolipin showing that more cardiolipin could be taken up when the enzyme was deficient in DMPC.

The results show the presence of two classes of lipid-binding sites in Complex I. Phosphatidylethanolamine and phosphatidylcholine bind at one class of site. These lipids are readily removed by cholate with loss of enzyme activity. Enzyme activity may be restored by dialysis with either type of lipid in cholate under special conditions [5]. In addition, lipids bound at these sites rapidly exchange with DMPC at low cholate concentrations but have low affinity for cardiolipin (table 1). Cardiolipin binds preferentially at the second class of site, and is not removed by cholate treatment (fig.1). Exchange with DMPC occurs only at high cholate concentrations and with loss of activity (table 1). Exchange with added cardiolipin takes place more readily (i.e., at lower cholate concentrations) and with retention of activity. It is clear that appropriate phospholipids bound at both classes of sites are required for enzyme activity.

We have also shown that the phospholipid content of Complex I is only just sufficient to maintain maximum activity. The physical properties of this lipid (Ragan and Heron, unpublished observations) suggest that it may form a single shell or 'annulus' [15] around the protein of the complex. If this is true of other complexes, e.g., Complex III, then when Complex I and Complex III interact they may be separated by no more than a single layer of phospholipid molecules, allowing protein–protein interaction to occur. The relative mobility of the two Complexes may be very slow or non-existent in which case there is no reason why stoicheiometric interaction should not occur [10]. Ubiquinone and ubiquinol could still act as mobile hydrogen transfer agents through the annular lipid around and between the complexes. We are currently using complexes of defined lipid composition to investigate the importance of ubi-

quinone mobility in electron transport and to study the interaction between Complexes I and III.

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