Interactions between Phospholipids and NADH:Ubiquinone Oxidoreductase (Complex I) from Bovine Mitochondria†

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ABSTRACT: NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria is a highly complicated, energy transducing, membrane-bound enzyme. It contains 46 different subunits and nine redox cofactors: a noncovalently bound flavin mononucleotide and eight iron−sulfur clusters. The mechanism of complex I is not known. Mechanistic studies using the bovine enzyme, a model for human complex I, have been precluded by the difficulty of preparing complex I which is pure, monodisperse, and fully catalytically active. Here, we describe and characterize a preparation of bovine complex I which fulfills all of these criteria. The catalytic activity is strongly dependent on the phospholipid content of the preparation, and three classes of phospholipid interactions with complex I have been identified. First, complex I contains tightly bound cardiolipin. Cardiolipin may be required for the structural integrity of the complex or play a functional role. Second, the catalytic activity is determined by the amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) which are bound to the complex. They are more weakly bound than cardiolipin, exchange with PC and PE in solution, and can substitute for one another. However, their nontransitory loss leads to irreversible functional impairment. Third, phospholipids are also required in the assay buffer for the purified enzyme to exhibit its full activity. It is likely that they are required for solubilization and presentation of the hydrophobic ubiquinone substrate.

NADH:ubiquinone oxidoreductase (complex I) is the first enzyme of the mitochondrial electron transport chain (*1*, *2*). It catalyzes the oxidation of NADH to NAD^+ in the mitochondrial matrix and the reduction of ubiquinone to ubiquinol in the inner mitochondrial membrane. The redox reaction is coupled to proton translocation across the membrane (*3*), contributing to the proton motive force. In addition, complex I is a significant source of reactive oxygen species in the mitochondrion (*4*), and complex I dysfunction has been implicated in a number of neuromuscular diseases (*5*).

Complex I from bovine heart mitochondria is a highly complicated, membrane-bound enzyme. It has a molecular mass of ∼980 kDa, and the sequences of 45 different subunits have been determined (*6*−*8*); 14 of them are the conserved "core" subunits, which are sufficient for energy transduction. The enzyme is L-shaped, with one arm extending into the mitochondrial matrix and the other in the membrane plane (*9*). Recently, an electron density map of the hydrophilic (membrane-extrinsic) arm of *Thermus thermophilus* complex I showed that a "chain" of iron−sulfur (FeS)[1] clusters (two [2Fe-2S] and six [4Fe-4S] clusters in bovine complex I) extends through the protein matrix (*10*). They connect the noncovalently bound flavin mononucleotide (FMN, the direct oxidant of NADH) to the ubiquinone binding site(s) in the membrane domain. The mechanism of coupling between the redox reaction and proton translocation is unknown: proposals include Q-cycle mechanisms and both directly and indirectly coupled mechanisms (reviewed in ref *11*). The FeS clusters, and flavin and quinone radicals, have been characterized by electron paramagnetic resonance (EPR) spectroscopy (*12*), and redox-linked protonation and conformational changes are being investigated by Fourier transform infrared (FTIR) spectroscopy (*13*). Although the lack of accessible spectroscopic features has restricted many studies to determining rates of steady-state catalytic turnover, the scope of this approach has been augmented significantly by model systems [from *Escherichia coli* (*14*) and *Yarrowia lipolytica* (*15*)] which allow the study of site-directed mutants.

For bovine complex I, functional studies have been precluded by the lack of a method for preparing pure, monodisperse, stable, *and* fully catalytically active enzyme. As prepared first by Hatefi and co-workers, "Hatefi's" complex I retains significant NADH:ubiquinone oxidoreductase activity [7 μmol of e^- min^{-1} mg^{-1} of inhibitor-sensitive ubiquinone-2 activity (*16*)], but it is polydisperse, impure, and has proved difficult to reproduce (*17*). Subsequently, Walker and co-workers developed a series of three chromatographic procedures for preparing bovine complex I (*18*−*20*). These methods (referred to by first author) provide

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[1] Abbreviations: CL or cardiolipin, diphosphatidylglycerol; CI, complex I; DDM, *n*-dodecyl-β-D-maltoside; decylubiquinone or DBQ, ubiquinone with a straight, saturated, 10-carbon chain; DMPC, dimyristoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EPR, electron paramagnetic resonance; FeS, iron−sulfur cluster; FMN, flavin mononucleotide; ubiquinone-1, ubiquinone with one isoprene unit; ubiquinone-2, ubiquinone with two isoprene units; ubiquinone-10, ubiquinone with ten isoprene units.

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highly pure complex I in high yield and were used in extensive studies of the enzyme's subunit composition (*8*). However, they are not suitable for mechanistic studies. The Finel (*18*) and Sazanov (*20*) preparations displayed only low NADH:ubiquinone-1 oxidoreductase activity (2−3 μmol of e^- min^{-1} mg^{-1}), and the activity was not sensitive to rotenone: the ubiquinone-1 reacts adventitiously, not at the physiological binding site. The Buchanan preparation gave low NADH:decylubiquinone oxidoreductase activity, reported to be inhibitor sensitive (2.4 μmol of e^- min^{-1} mg^{-1}) (*19*). Finally, Okun and co-workers reported a new protocol, but their enzyme is less pure and has only very low activity (0.9 μmol of e^- min^{-1} mg^{-1} with nonylubiquinone, rotenone sensitive) (*21*). Clearly, a reproducible method of preparing fully active bovine complex I that is also pure, monodisperse, and stable, with a full complement of subunits and cofactors, is required for functional and structural studies which are both fundamental and relevant to the human enzyme.

Here we describe work which fulfills two objectives. (i) We describe a method of preparing complex I from bovine heart mitochondria which satisfies all of these criteria, based on previously described (*20*) chromatographic procedures. (ii) We characterize the fully active enzyme and define three classes of interactions between phospholipids and complex I as the determinants of the catalytic activity. Finally, we compare our results with those of previous studies.

EXPERIMENTAL PROCEDURES

Preparation of Complex I from Bovine Heart Mitochondria. Mitochondria were isolated from bovine hearts (*22*); then mitochondrial membranes were prepared by disruption with a Waring blender in the presence of KCl (*17*), resuspended in 20 mM Tris-HCl (pH 7.4), 10% v/v glycerol, and 1 mM EDTA, and stored as aliquots at −20 °C (~12 mg mL^{-1}). Ten milliliters were thawed slowly on ice, then solubilized by 1% *n*-dodecyl-β-D-maltoside (DDM, Anatrace), stirred for 20 min, and centrifuged (23 min, 34500*g*). The supernatant was filtered and then applied to a pre-equilibrated 16 mL Q-Sepharose HP column (Amersham Pharmacia Biotech). The Q-Sepharose buffers (A and B) contained 20 mM Tris-HCl (pH 7.4), 10% (v/v) ethylene glycol, 0.1% (w/v) DDM, and 0.005% (w/v) phenylmethanesulfonyl fluoride (PMSF); buffer B contained 1 M NaCl. When present, phospholipids (Avanti Phospholipids) were added to 0.005% (w/v) from a 1% stock in 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; Calbiochem) and 20 mM Tris-HCl (pH 7.5). Asolectin (Fluka) was partially purified under nitrogen by recrystallization (*23*). The Q-Sepharose column was washed with 16 mL of buffer A and 75 mL of 0.25 M NaCl; then complex I was eluted either by a step or a linear gradient (30 mL) to 0.35 M NaCl (at 2 mL min^{-1}). Complex I containing fractions were pooled, concentrated (100 kDa polyethersulfone Vivaspin centrifugal concentrator), and applied to a Superose 6 10/300 GL gel-filtration column (Amersham Pharmacia Biotech). Monomeric complex I elutes after 11 mL [20 mM Tris-HCl, 0.15 M NaCl, 10% (v/v) ethylene glycol, 0.02% (w/v) DDM, pH 7.4, 0.3 mL min^{-1}]. Complex I containing fractions were pooled and concentrated to ~2 mg mL^{-1}, determined using the Pierce bicinchoninic acid (BCA) assay. The purification was carried out at 4 °C.

Determination of the NADH:Decylubiquinone Oxidoreductase Activity. NADH:decylubiquinone oxidoreductase activities were determined in 20 mM Tris-HCl (pH 7.5) and 0.5 mg mL^{-1} asolectin at 30 °C. The reaction was initiated by 100 μM decylubiquinone (Sigma, 20 mM stock in ethanol), following a 3 min preincubation of complex I (~5 μg mL^{-1}) with 100 μM NADH (Sigma) in the assay buffer. NaN_3 (2 mM) (BDH) and 300 nM antimycin A (Sigma) were included when other respiratory chain enzymes were present. The reaction was monitored via the NADH concentration (340−380 nm; $\epsilon = 4.81$ mM^{-1} cm^{-1}); catalytic activities reported are initial rates. Sensitivity to inhibition was determined using 2.5 μM rotenone (Sigma; ~100 × IC_{50}); less than 3% of the activity is insensitive, so all activities reported here refer to quinone reduction at the physiological binding site. Note that all of our catalytic activities are reported per milligram of complex I, including those which refer to complex I before purification: these values were normalized using the FMN content and a molecular mass of 980 kDa to facilitate direct comparison.

HPLC Analysis. All HPLC solvents were CHROMASOLV grade (Sigma-Aldrich). Analyses were carried out on an Agilent 1100 series HPLC system equipped with a manual injector (Rheodyne injection valve and 20 μL loop), column thermostat (30 °C), and multiple wavelength detector and controlled by an Agilent ChemStation.

Determination of the FMN, Iron, and Quinone Contents. Flavin mononucleotide (FMN) concentrations were analyzed fluorometrically using the method of Burch (*24*) modified for a 96-well plate reader (Spectra Max Gemini XS microplate spectrofluorometer). FMN (73−79% fluorometric grade; Sigma) was used to make "everyday" standards; then these were normalized using a pure FMN sample (approximately 95% by HPLC; Sigma).

HPLC analysis [based on the method of Gliszczyńska-Świgło and Koziołowa (*25*)] was used to confirm which flavin species were present. Samples were heated at 80 °C for 30 min or digested with proteinase-K and then eluted isocratically from a Luna C18 column (25 cm × 3.0 mm i.d., 5 μm; Phenomenex) in 0.05 M ammonium acetate (Merck) pH 5.5/methanol (3:1 v/v) at 0.4 mL min^{-1}.

The iron contents of complex I samples were determined using the method of Doeg and Ziegler (*26*).

Ubiquinone-10 was extracted from complex I samples using a hexane/ethanol mixture and quantified, against standards of ubiquinone-10 (Sigma), by HPLC analysis (*27*). It was eluted from a Nucleosil C18 column (25 cm × 3.2 mm i.d., 5 μm; Hichrom) with a mobile phase of 50 mM sodium perchlorate (Fluka) in 70:30:0.1 ethanol/methanol/70% perchloric acid (Aldrich), at 0.5 mL min^{-1}, with detection at 275 nm. Ubiquinone elutes at ~13 min and ubiquinol (not present) at ~9 min.

Analysis of Phospholipids by Thin-Layer Chromatography (TLC). Phospholipids were extracted into chloroform by the method of Bligh and Dyer (*28*) and separated on silica plates (HPTLC, 20 × 10 cm, silica gel 60 GLP; Merck) using a mobile phase of 50:40:8:4 (v/v/v/v) chloroform/methanol/water/acetic acid (*29*). Chloroform extracts from the complex I samples, asolectin, and mitochondrial membranes were applied to the plate using Drummond microcaps; the standard mixture contained 0.5 μg of cardiolipin, PC, and PE. The plate was developed for 30 min with the mobile phase and

then air-dried. Phospholipids were detected by heating to 120 °C for 17 min, after spraying with cupric acetate spray reagent [20.8% phosphoric acid, 2.6% cupric acetate (Alltech)], and showed up as gray spots on a white background.

Analysis of Phospholipids by HPLC. Phospholipids were extracted by the method of Bligh and Dyer (*28*). The chloroform was evaporated, and the residue was dissolved in the appropriate HPLC mobile phase and filtered (0.20 μm minisart RC-4 filter; Sartorius) before being injected (20 μL) onto the column. Cardiolipin was quantified by normal-phase separation using a silica column which separates PE and cardiolipin but does not elute PC. PC and PE were quantified using an aminopropyl-bonded stationary phase, with typical reversed-phase HPLC solvents; it separates PC and PE, but cardiolipin does not elute.

Cardiolipin was eluted from a Waters Resolve silica column (15 cm × 3.9 mm i.d., 5 μm) with a mobile phase of 50:50:2.5 (v/v/v) cyclohexane/2-propanol/5 mM phosphoric acid (Fluka), at 1 mL min^{-1}, with detection at 208 nm (*30*). Cardiolipin elutes at ∼7 min. Bovine heart cardiolipin was used to prepare standard solutions; a new standard line was generated each day. Molar concentrations were calculated using the molecular mass of tetralinoleoyl (18:2) cardiolipin (1494 Da), the predominant form of cardiolipin in bovine heart mitochondria (*31*).

PC and PE were eluted from a Zorbax NH_2 column (25 cm × 4.6 mm i.d., 5 μm; Hichrom) with a mobile phase of 70:20:5 (v/v/v) acetonitrile/methanol/50 mM ammonium acetate (Merck) pH 4.8, 1 mL min^{-1}, with detection at 208 nm (based on the method in ref *32*). PC and PE elute at ∼7 and ∼17 min, respectively. PC and PE from total bovine heart extract were used as standards, and a new standard line was generated each day. Molar concentrations were calculated using molecular masses of 758.7 Da for PC and 768.06 Da for PE, calculated from the most common species in bovine hearts [palmitoyl-linoleoyl (16:0-18:2) PC and steroyl-arachidonyl (18:0-20:4) PE] (*33*).

RESULTS

The Correlation between Catalytic Activity and Phospholipid Content. Our initial method of preparing complex I from bovine heart mitochondrial membranes comprised the solubilization of mitochondrial membranes with 1% DDM, ion-exchange chromatography in 0.1% DDM, and gel-filtration chromatography in 0.02% DDM.[2] The enzyme is pure, and it elutes from the gel-filtration column in a single (monodisperse) peak with the retention time of monomeric enzyme (see Figures S1 and S2 in Supporting Information). But, in comparison to complex I at the start of the preparation, it has only low NADH:decylubiquinone oxidoreductase activity. The purified enzyme is fully active in the NADH:ferricyanide oxidoreductase assay, FMN and iron analyses demonstrated the presence of 1 FMN per complex I and 22−26 irons per FMN, and EPR spectra comprised signals from 5 iron−sulfur clusters. These observations suggest that the NADH:decylubiquinone oxidoreductase activity is lost because the complex is unable to bind or reduce decylubiquinone.

[2] The method is based on that of Sazanov and co-workers, but we have omitted the second ion-exchange column and the ammonium sulfate precipitation.

Table 1: Average Phospholipid Contents and Catalytic Activities of Complex I Prepared with and without Asolectin, PC, and PE[a]

preparation	average activity (μmol of e$^-$ min^{-1} mg^{-1})	average no. of phospholipids per complex I (mol/mol)		
		PE	PC	cardiolipin
0.005% asolectin ($n = 6$)	8.1 ± 1.4	7.4 ± 1.8	11.8 ± 4.0	9.4 ± 0.9
no phospholipid ($n = 7$)	3.6 ± 1.2	2.3 ± 0.7	3.2 ± 0.5	10.4 ± 0.8
0.005% PC ($n = 2$)	7.6 ± 1.0	3.5 ± 0.1	25.2 ± 3.8	10.2 ± 0.3
0.005% PE ($n = 1$)	8.2	21.5	4.2	10.2

[a] The two PC experiments used bovine and soybean PC separately. The results were essentially identical, and so they are combined. Confidence intervals cannot be reported for the single PE point. ±, 95% confidence intervals.

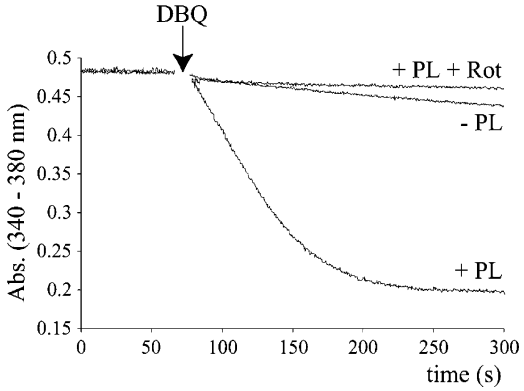

FIGURE 1: NADH:decylubiquinone oxidoreductase activity measurements on complex I. High activity is observed in the presence of asolectin phospholipids (+PL) but not in their absence (−PL). Negligible activity remains in the presence of rotenone (+PL +Rot). Conditions: 20 mM Tris-HCl (pH 7.5), 0.5 mg mL^{-1} asolectin, 30 °C; 100 μM NADH, 100 μM decylubiquinone, 2.5 μM rotenone; ∼5 μg mL^{-1} complex I.

The NADH:decylubiquinone oxidoreductase activity was measured at each stage of the preparation, showing that the activity is lost during ion-exchange chromatography. Consequently, two methods of retaining the activity were developed, which suggest that activity loss occurs because phospholipids are washed from the complex while it is bound to the column: decreasing the concentration of DDM in the ion-exchange buffers or adding phospholipids to them. Lower detergent concentrations led to increased contamination by cytochrome *c* oxidase, and so this alternative was not pursued. Preparations carried out with different concentrations of asolectin showed that 0.005% (w/v) provided both optimal purity, monodispersity, and activity. Therefore, several preparations were carried out in the presence and absence of 0.005% asolectin, and Table 1 presents the results of activity measurements carried out immediately afterward. It is clear that samples prepared with asolectin have significantly higher activities than those prepared without. Figures S1 and S2 show that neither purity or monodispersity are compromised by the inclusion of asolectin.

The values reported in Table 1 (and throughout) were measured in assay buffers which contained 0.5 mg mL^{-1} asolectin. Figure 1 shows that the activity of complex I prepared using asolectin is abolished by rotenone (a complex

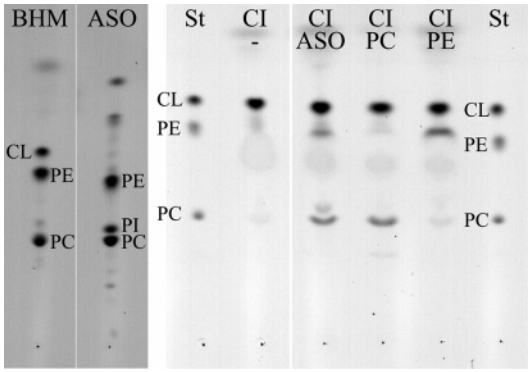

FIGURE 2: Thin-layer chromatography (TLC) plates showing the phospholipids which are present in bovine heart membranes (BHM) and asolectin (ASO) (left-hand plate) and in complex I (CI) samples prepared without phospholipids (−), with asolectin (ASO), or with PE or PC (right-hand plate). St: standards of 0.5 μg of cardiolipin (CL), PE, and PC.

I inhibitor which prevents quinone reduction) and is absent unless asolectin is present in the assay buffer. As the asolectin concentration in the assay is increased, the activity increases and then becomes constant; the amount of asolectin required to reach the "plateau" is independent of the activity of the preparation, and samples prepared without using asolectin could not be reactivated by the addition of extra phospholipids at a later stage. Therefore, phospholipids must be maintained on complex I during its preparation but also provided during the assay.

Importantly, the activity of freshly prepared solubilized membranes (the complex I preparation immediately before application to the ion-exchange column) was typically 8 μmol of e^- min^{-1} (mg of CI)$^{-1}$, equal to the average activity of complex I prepared using asolectin.[3] Therefore, this is the first report of complex I isolated from bovine heart mitochondria in a pure, monodisperse, *and* fully catalytically active form.

Thin-layer Chromatography (TLC) Analysis of the Phospholipid Content. The inner membrane of bovine heart mitochondria contains predominantly three phospholipids: phosphatidylcholine (PC, 40%), phosphatidylethanolamine (PE, 35%), and cardiolipin (CL, 20%); minor amounts of phosphatidylinositol (PI) have also been detected (*34*, *35*). Here, TLC was used semiquantitatively to confirm which phospholipids were present in different mixtures and samples of complex I. Figure 2 confirms that (i) the three major phospholipids present in bovine heart mitochondrial membranes are PC, PE, and cardiolipin and (ii) asolectin comprises a complex mixture which does not include cardiolipin, but it does contain significant amounts of PC and PE. Figure 2 shows also that purified complex I contains PC, PE, and cardiolipin. Inspection of the spot densities suggests that the amounts of PC and PE vary according to whether the preparation contained asolectin (or PC or PE; see below) but that the amount of cardiolipin remains approximately constant. There is no evidence for phosphatidic acid, a product of phospholipid degradation which runs just below PE.

[3] The activity of the solubilized membranes is reported per milligram of complex I (not per milligram of total protein) for comparison with the activity of the purified enzyme. Complex I concentration was determined by the FMN concentration and normalized using a molecular mass of 980 kDa.

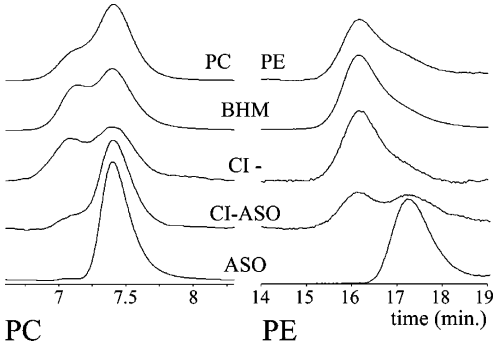

FIGURE 3: HPLC traces showing the elution of PC and PE from different sources. Note that the *x*-axis differs between the two sets and that the PC and PE peaks are each normalized to the same peak area. Key: PC and PE, commercial bovine heart PC and PE standards; BHM, bovine heart mitochondrial membranes; CI −, complex I purified without phospholipids; CI-ASO, complex I purified using asolectin; ASO, asolectin. Conditions: Zorbax NH_2 column, mobile phase of 70:20:5 v/v/v acetonitrile/methanol/50 mM ammonium acetate, pH 4.8, 1 mL min^{-1}, and detection at 208 nm.

Quantitative HPLC Analysis of PC, PE, and Cardiolipin. The HPLC retention times for the different phospholipids are determined primarily by the phospholipid class (PC, PE, or cardiolipin), but they are modulated by the fatty acid composition. Bovine heart cardiolipin elutes in a single peak as it is composed predominantly of a single fatty acid species, 18:2 (*31*), but the elution profiles of PC and PE are more complex (Figure 3): the fatty acid composition varies according to species, tissue type, and subcellular fraction (*36*), and unsaturated fatty acids absorb more strongly at 208 nm (*37*). This has two consequences: (i) The origin of the PC and PE in a complex I sample (endogenous or exogenous) can be identified. (ii) A systematic error may arise in the quantification because the commercially available "total bovine heart" standards differ in composition from the samples. This error does not arise for cardiolipin, because cardiolipin derives only from mitochondria. However, correction for the error in PC and PE quantification, by comparison of absorption intensities and elution profiles, led only to small changes in the absolute number of PC and PE molecules present and did not affect the relationships between different preparations or the conclusions drawn. Therefore, we have chosen to present only the uncorrected data. Importantly, the quantitative HPLC results were consistent with the semiquantitative information available from TLC.

PC and PE Determine the Catalytic Activity of Purified Complex I. Table 1 presents the average amount of each phospholipid present per complex I (mole per mole) in samples prepared with and without asolectin. Phospholipids were not included in the final stage of the preparation so only bound phospholipids were present. There is a clear correlation between the catalytic activity and the PC and PE contents: typically ~12 PC and ~7 PE molecules are present in preparations of the purified enzyme which display native levels of activity. In contrast, cardiolipin is always present at the same level (~10 cardiolipins per complex I): it does not determine the catalytic activity, and, as the purified enzyme is fully active, it is unlikely that functionally required cardiolipins are being lost. Cardiolipin may bind more tightly because it has four acyl chains, or cardiolipins may be integral to the structure.

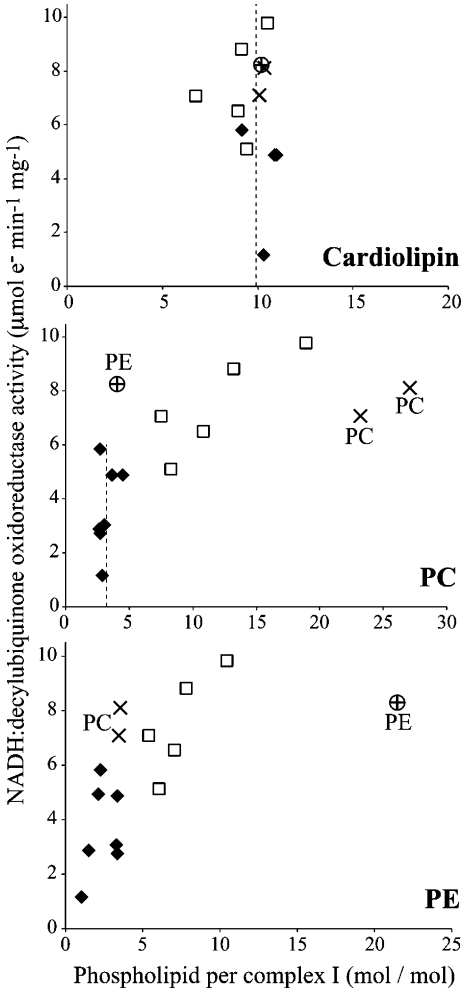

FIGURE 4: Variation of the NADH:decylubiquinone oxidoreductase activity with the number of moles of PC, PE, and cardiolipin retained per mole of complex I. PC, PE, and cardiolipin were determined by HPLC analysis. Approximately ten cardiolipins are retained in all preparations, and there is no correlation with the activity. PC and PE vary significantly, and they are correlated strongly to the activity. Key: (◆) prepared without phospholipids; (□) prepared using asolectin; (⊕) prepared using PE only; (×) prepared using PC only.

Figure 4 presents the catalytic activity of purified complex I as a function of the cardiolipin, PC, and PE contents. It confirms that the activity is independent of the cardiolipin content: of the 11 preparations (activities 1−10 μmol of e^- min^{-1} mg^{-1}) 10 contained 9−11 cardiolipins. Considering first only the asolectin and phospholipid-free preparations, Figure 4 confirms also that the activity is dependent on the PC and PE contents. Every preparation contains at least 3 PC molecules: they may be equivalent to the 10 cardiolipins, and they do not determine the activity. No preparation is completely devoid of PE, but the data do not strongly support the idea of tightly bound PE molecules because the amount of PE varies for even the least active preparations. Interestingly, using higher amounts of asolectin gave neither higher activity or increased amounts of PE and PC, suggesting that a "limit" in the PC and PE that can be retained has been reached.

Exchange between PC and PE. HPLC elution profiles allow the bound PC and PE to be identified as either exogenous or endogenous. Figure 3 shows clearly that samples prepared using asolectin contain both endogenous and exogenous PC and PE (so phospholipids exchange between the bound and solution phases), whereas those prepared without asolectin have the same composition as solubilized membranes. Note that exchange does not reach completion during the ion-exchange separation, since some bovine lipids are always retained.

To establish whether PC and PE are functionally interchangeable, or whether one of them is the major determinant of catalytic activity, further purifications used either 0.005% PC or PE. Their activities were similar to those achieved with asolectin (see Table 1). The phospholipid contents were analyzed by TLC (Figure 2) and by HPLC (Table 1 and Figure 4). As expected, if PC was included in the ion-exchange buffers, it was present in significantly increased amounts in the purified enzyme, and PE was present in approximately the same amount as in phospholipid-free preparations (and vice versa). The cardiolipin content is the same in all cases. These results suggest that PC and PE substitute for each other in the function of complex I and that their binding sites accept either PC or PE, depending on availability. PC and PE do not compete for the cardiolipin binding sites, consistent with the dissimilarity of the headgroups and the different number of acyl chains.

Characterization of Purified Complex I with Activity Equal to That of the Native Enzyme. Although catalytic activity is strongly correlated to the PC and PE contents, it is possible that PC and PE act only indirectly. For example, they may retain a tightly bound ubiquinone in the complex or protect the ubiquinone binding site or one of the clusters. Consequently, complex I samples purified with and without asolectin (with high and low catalytic activity) were compared as follows:

(i) SDS−PAGE analysis did not reveal any differences between high and low activity preparations.[4] However, purified complex I contains 46 different subunits, so further analysis is required to confirm that there are no extra or missing small or hydrophobic proteins. The purity of all preparations was comparable to that of previous preparations (Figure S2).

(ii) HPLC confirmed that the only flavin present is FMN (*38*). The FMN/protein ratio (mol/mol) varied from 1.0 to 1.1 for all preparations (assuming a mass of 980 kDa for complex I): the FMN content does not determine the catalytic activity. Protein was quantified using the BCA assay and confirmed, for a single typical sample, by amino acid analysis; the results differed by less than 10%. Therefore, there is only one FMN per complex I. Note that our interpretation conflicts with that of Albracht and co-workers, who observed 1.3−1.6 FMN per complex I and proposed that it contains two FMNs per complex (*39*).

(iii) EPR spectra from complex I prepared with asolectin and reduced by NADH showed signals from five iron−sulfur clusters [four [4Fe-4S] clusters (N2, N3, N4, and N5) and one [2Fe-2S] cluster (N1b)], and the spectra and measured *g*-values are very similar to those that have been described previously (see Figure S3) (*12*). In contrast to the results of Sinegina and co-workers on complex I from *E. coli* (*40*), no differences could be observed between these spectra and

[4] In contrast to highly characterized previous preparations (*8*, *18*) the 42 kDa subunit is present in apparently stoichiometric amounts in all of our preparations.

spectra from less active samples prepared without asolectin. Previously, Ohnishi and co-workers observed significant broadening of the N2 signal upon delipidation (*41*). However, no variation was observed here, and all of our spectra resemble their "reconstitutively active" enzyme: it is possible that Ohnishi's "reconstitutively inactive" enzyme was more stringently delipidated and perhaps partially denatured. Thus, our data do not support modification of an FeS cluster, or its environment, as a determinant of the catalytic activity.

(iv) The amount of ubiquinone-10 present was quantified by HPLC analysis. Ubiquinone was present in the same, substoichiometric amount, regardless of the catalytic activity: 0.3 ± 0.05 per complex I. Therefore, the activity is not determined by the ubiquinone content, and because our preparations are fully active, the data do not support the idea that a tightly bound, nonexchangeable, ubiquinone cofactor is required for catalysis. The observed stoichiometry is higher than that of Okun's purified complex I (ubiquinone not detectable) (*21*), similar to that of purified *Y. lipolytica* complex I (0.2−0.4) (*42*) but significantly less than that of Hatefi's complex I (4.2−4.5) (*16*). It is likely that ubiquinone content is related to phospholipid content: Hatefi's complex I contains ∼20% phospholipid (w/w) (*43*), whereas our preparations contain only ∼3%.

In summary, no differences between complex I of high and low activity could be detected other than the phospholipid content: the effects of the phospholipids are direct and unmediated.

DISCUSSION

It is becoming increasingly evident that specific interactions between membrane proteins and phospholipids do exist and that these may be important determinants of function (*44*−*46*). Tightly bound phospholipids have been located in structural models of membrane proteins, and in some cases they have proved to be crucial for crystallization (*47*, *48*). Phospholipids are known to interact specifically with the major energy-transducing complexes and to have important influences on their structure and function. Cardiolipin is important for electron transport activity and subunit−subunit interactions in cytochrome *c* oxidase (*49*, *50*) and for the structural integrity of the cytochrome bc_1 complex (*51*, *52*). It may participate in proton uptake at the Q_i site (*53*) and modulate the properties of Q_A in the bacterial photoreaction center (*54*). Here we have shown that 10 tightly bound cardiolipins support the native activity of complex I, but our experiments do not address their structural or functional role. Interestingly, cardiolipin oxidation is a possible cause of decreased complex I activity in submitochondrial particles following treatment with reactive oxygen species (*55*). Therefore, understanding the role of cardiolipin in complex I is relevant to understanding pathologies in which complex I function is impaired, as well as to understanding the enzyme's mechanism.

Here, we have identified three classes of phospholipid interaction for bovine complex I. (i) Cardiolipin is tightly and specifically bound. (ii) PE and PC are less tightly bound, exchange slowly with PE and PC in solution, and can substitute for one another. However, they are required to maintain the complex in an active state, and their sustained and progressive depletion leads to irreversible loss of the NADH:decylubiquinone oxidoreductase activity. (iii) Phospholipids are required in solution during the assay. It is likely that they partition and solubilize the hydrophobic decylubiquinone, facilitate formation of the enzyme:decylubiquinone complex, and perhaps help to disperse the enzyme in the assay buffer.

In 1973 Ragan and Racker used cholate/ammonium sulfate precipitation to delipidate Hatefi's complex I (from ∼240 to ∼110 phospholipids per complex) (*56*). Reactivation of the delipidated enzyme required an additional ∼500 phospholipids. Heron and co-workers concluded that ∼200 phospholipid molecules per enzyme were required to maintain the activity of bovine complex I (*57*), and Fry and Green required ∼360 phospholipids per complex for reconstitution, following delipidation using high concentrations of detergent (5% Triton X-100) (*58*). Thus, it is likely that these experiments primarily addressed the dispersive role of the phospholipid annulus (*58*), not specifically bound phospholipids. Correspondingly, spin label studies demonstrated that the phospholipid annulus of complex I comprises 300−400 molecules (*59*).

There are several clear distinctions between the earlier work described above (*56*−*58*) and that described here. The absolute number of phospholipids discussed is very different, suggesting that different classes of phospholipid are being addressed. The methodologies are also distinct: our chromatographic purification requires maintenance of the phospholipid level, rather than exhaustive delipidation of the isolated enzyme. Indeed, the delipidation methods applied led to significant loss of activity even in control samples [for example, the control enzyme of Heron and co-workers retained only 2 μmol of e^- min^{-1} mg^{-1} of rotenone-sensitive ubiquinone-1 reductase activity (*57*)]. Despite these differences, some observations are common to both approaches: Heron and co-workers observed that decreasing the PC and PE content decreased the enzyme activity, and they could replace PC and PE by DMPC without significant effect (*57*). They noted also that large amounts of DMPC and cholate were required to decrease the cardiolipin content (so decreasing the activity), and Fry and Green described a specific cardiolipin requirement during reconstitution (*58*). Finally, Ragan used ubiquinone analogues of different hydrophobicities to propose that phospholipids in the assay buffer exert their effect by partitioning the quinone substrate into the hydrophobic phase, as also suggested here (*60*).

Recently, native phospholipids have been used in the assay buffer to stimulate the activity of complex I from *E. coli* (*61*). Assuming that the *E. coli* enzyme retains specific lipids during its preparation, these phospholipids probably play the same role as those added to our assay buffer. *Y. lipolytica* complex I retains ∼50 phospholipids per complex I during preparation (similar to the "active" enzyme described here) (*42*). However, it did not exhibit activity unless preincubated with additional phospholipids before addition to the assay buffer. Again, it is likely that most of these phospholipids fulfill the same role as those added to our assay buffer. For the bovine enzyme, similar results were obtained whether the phospholipids were added directly to the assay buffer or in a preincubation step. Preincubation with phospholipids also effected a 2-fold activation of *E. coli* complex I (*40*). Interestingly, activation did not affect the observed K_m of the substrate quinone, but it did affect the EPR signals from

two [4Fe-4S] clusters, perhaps consistent with a modification of the enzyme's conformation. Similar changes were not observed here for the bovine complex.

Finally, direct comparison of the activities of our preparation and previous preparations is difficult because of the different ubiquinone substrates and assay conditions. However, the Sazanov preparation [upon which our method is based (*20*)] and the Finel preparation (*18*) both display only rotenone-insensitive activity, demonstrating that quinone reduction does not occur at the physiological site. Conversely, all of the activities reported here are rotenone sensitive. This allows decylubiquinone to be employed as the substrate: it partitions effectively into the hydrophobic phase (*62*) and is effective in proton pumping (*63*, *64*). Buchanan's preparation had ~25% of the inhibitor-sensitive activity reported here, but surprisingly, phospholipids could not be detected in this preparation (*19*). Conversely, Okun's preparation had only low activity but contained ~100 phospholipids per complex (*21*), indicating that its activity is decreased for a different, unknown, reason. Hatefi's preparation is the only one which possesses comparable catalytic activity to the enzyme described here (*16*). However, its very high phospholipid content [0.22 mg (mg of CI)$^{-1}$] is incompatible with contemporary purification methods, and it is impure, polydisperse, and difficult to reproduce (*17*). Therefore, this paper describes the first method for preparing complex I from bovine heart mitochondria in a pure, monodisperse, *and* fully catalytically active state. Consequently, bovine complex I, which is closely related to the human enzyme, can now be exploited for structural *and* mechanistic studies.

SUPPORTING INFORMATION AVAILABLE

Figure S1, elution of complex I from the gel-filtration column; Figure S2, SDS−PAGE analysis of complex I preparations; Figure S3, electron paramagnetic resonance spectra of complex I prepared using asolectin. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Saraste, M. (1999) Oxidative phosphorylation at the *fin de siècle*, *Science 283*, 1488−1493.
2. Schultz, B. E., and Chan, S. I. (2001) Structures and proton-pumping strategies of mitochondrial respiratory enzymes, *Annu. Rev. Biophys. Biomol. Struct. 30*, 23−65.
3. Wikström, M. (1984) Two protons are pumped from the mitochondrial matrix per electron transferred between NADH and ubiquinone, *FEBS Lett. 169*, 300−304.
4. Turrens, J. F., and Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria, *Biochem. J. 191*, 421−427.
5. Smeitink, J., van den Heuvel, L., and DiMauro, S. (2001) The genetics and pathology of oxidative phosphorylation, *Nat. Rev. Genet. 2*, 342−352.
6. Walker, J. E. (1992) The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains, *Q. Rev. Biophys. 25*, 253−324.
7. Hirst, J., Carroll, J., Fearnley, I. M., Shannon, R. J., and Walker, J. E. (2003) The nuclear encoded subunits of complex I from bovine heart mitochondria, *Biochim. Biophys. Acta 1604*, 135−150.
8. Carroll, J., Fearnley, I. M., Shannon, R. J., Hirst, J., and Walker, J. E. (2003) Analysis of the subunit composition of complex I from bovine heart mitochondria, *Mol. Cell. Proteomics 2*, 117−126.
9. Guénebaut, V., Schlitt, A., Weiss, H., Leonard, K., and Friedrich, T. (1998) Consistent structure between bacterial and mitochondrial NADH:ubiquinone oxidoreductase (complex I), *J. Mol. Biol. 276*, 105−112.
10. Hinchliffe, P., and Sazanov, L. A. (2005) Organization of iron−sulfur clusters in respiratory complex I, *Science 309*, 771−774.
11. Hirst, J. (2005) Energy transduction by respiratory complex I—an evaluation of current knowledge, *Biochem. Soc. Trans.* 525−529.
12. Ohnishi, T. (1998) Iron−sulfur clusters/semiquinones in complex I, *Biochim. Biophys. Acta 1364*, 186−206.
13. Hellwig, P., Scheide, D., Bungert, S., Mäntele, W., and Friedrich, T. (2000) FT-IR spectroscopic characterization of NADH: ubiquinone oxidoreductase (complex I) from *Escherichia coli*: oxidation of FeS cluster N2 is coupled with the protonation of an aspartate or glutamate side chain, *Biochemistry 39*, 10884−10891.
14. Spehr, V., Schlitt, A., Scheide, D., Guénebaut, V., and Friedrich, T. (1999) Overexpression of the *Escherichia coli nuo*-operon and isolation of the overproduced NADH:ubiquinone oxidoreductase (complex I), *Biochemistry 38*, 16261−16267.
15. Kerscher, S., Dröse, S., Zwicker, K., Zickermann, V., and Brandt, U. (2002) *Yarrowia lipolytica*, a yeast genetic system to study mitochondrial complex I, *Biochim. Biophys. Acta 1555*, 83−91.
16. Hatefi, Y., Haavik, A. G., and Griffiths, D. E. (1962) Studies on the electron-transfer system, *J. Biol. Chem. 237*, 1676−1680.
17. Walker, J. E., Skehel, J. M., and Buchanan, S. K. (1995) Structural analysis of NADH:ubiquinone oxidoreductase from bovine heart mitochondria, *Methods Enzymol. 260*, 14−34.
18. Finel, M., Skehel, J. M., Albracht, S. P. J., Fearnley, I. M., and Walker, J. E. (1992) Resolution of NADH-ubiquinone oxidoreductase from bovine heart mitochondria into two subcomplexes, one of which contains the redox centers of the enzyme, *Biochemistry 31*, 11425−11434.
19. Buchanan, S. K., and Walker, J. E. (1996) Large-scale chromatographic purification of F_1F_0-ATPase and complex I from bovine heart mitochondria, *Biochem. J. 318*, 343−349.
20. Sazanov, L. A., Peak-Chew, S. Y., Fearnley, I. M., and Walker, J. E. (2000) Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme, *Biochemistry 39*, 7229−7235.
21. Okun, J. G., Zickermann, V., Zwicker, K., Schägger, H., and Brandt, U. (2000) Binding of detergents and inhibitors to bovine complex I—a novel purification procedure for bovine complex I retaining full inhibitor sensitivity, *Biochim. Biophys. Acta 1459*, 77−87.
22. Smith, A. L. (1967) Preparation, properties and conditions for assay of mitochondria: slaughterhouse material, small scale, *Methods Enzymol. 10*, 81−86.
23. Darley-Usmar, V. M., Capaldi, R. A., Takamiya, S., Millett, F., Wilson, M. T., Malatesta, F., and Sarti, P. (1987) in *Mitochondria, A Practical Approach* (Darley-Usmar, V. M., Rickwood, D., and Wilson, M. T., Eds.) pp 143−144, Oxford University Press, Oxford.
24. Burch, H. B. (1957) Fluorimetric assay of FAD, FMN, and riboflavin, *Methods Enzymol. 3*, 960−962.
25. Gliszczyńska-Świgło, A., and Koziołowa, A. (2000) Chromatographic determination of riboflavin and its derivatives in food, *J. Chromatogr. A 881*, 285−297.
26. Doeg, K. A., and Ziegler, D. M. (1962) Simplified methods for the estimation of iron in mitochondria and submitochondrial fractions, *Arch. Biochem. Biophys. 97*, 37−40.
27. Takada, M., Ikenoya, S., Yuzuriha, T., and Katayama, K. (1982) Studies on reduced and oxidized coenzyme Q (ubiquinones), *Biochim. Biophys. Acta 679*, 308−314.
28. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol. 37*, 911−917.
29. Skipski, V. P., Peterson, R. F., and Barclay, M. (1964) Quantitative analysis of phospholipids by thin-layer chromatography, *Biochem. J. 90*, 374−378.
30. Gomez, B., and Robinson, N. C. (1999) Quantitative determination of cardiolipin in mitochondrial electron transferring complexes by silicic acid high-performance liquid chromatography, *Anal. Biochem. 267*, 212−216.
31. Schlame, M., Brody, S., and Hostetler, K. Y. (1993) Mitochondrial cardiolipin in diverse eukaryotes, *Eur. J. Biochem. 212*, 727−735.

32. Grit, M., Crommelin, D. J. A., and Lang, J. (1991) Determination of phosphatidylcholine, phosphatidylglycerol and their lyso forms from liposome dispersions by high-performance liquid chromatography using high-sensitivity refractive index detection, *J. Chromatogr. A 585*, 239−246.
33. Schlame, M., Beyer, K., Hayer-Hartl, M., and Klingenberg, M. (1991) Molecular species of cardiolipin in relation to other mitochondrial phospholipids, *Eur. J. Biochem. 199*, 459−466.
34. Daum, G. (1985) Lipids of mitochondria, *Biochim. Biophys. Acta 822*, 1−42.
35. Fleischer, S., Rouser, G., Fleischer, B., Casu, A., and Kritchevsky, G. (1967) Lipid composition of mitochondria from bovine heart, liver, and kidney, *J. Lipid Res. 8*, 170−180.
36. Holub, B. J., and Kuksis, A. (1978) Metabolism of molecular species of diacylglycerophospholipids, *Adv. Lipid Res. 16*, 1−125.
37. McHowat, J., Jones, J. H., and Creer, M. H. (1996) Quantitation of individual phospholipid molecular species by UV absorption measurements, *J. Lipid Res. 37*, 2450−2460.
38. Rao, N. A., Felton, S. P., Huennekens, F. M., and Mackler, B. (1963) Flavin mononucleotide: the coenzyme of reduced diphosphopyridine nucleotide dehydrogenase, *J. Biol. Chem. 238*, 449−455.
39. Albracht, S. P. J., van der Linden, E., and Faber, B. W. (2003) Quantitative amino acid analysis of bovine NADH:ubiquinone oxidoreductase (complex I) and related enzymes. Consequences for the number of prosthetic groups, *Biochim. Biophys. Acta 1557*, 41−49.
40. Sinegina, L., Wikström, M., Verkhovsky, M. I., and Verkhovskaya, M. L. (2005) Activation of isolated NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli* by detergent and phospholipids. Recovery of ubiquinone reductase activity and changes in EPR signals of iron-sulfur clusters, *Biochemistry 44*, 8500−8506.
41. Ohnishi, T., Leigh, J. S., Ragan, C. I., and Racker, E. (1974) Low-temperature electron paramagnetic resonance studies of iron-sulfur centers in cardiac NADH dehydrogenase, *Biochem. Biophys. Res. Commun. 56*, 775−782.
42. Dröse, S., Zwicker, K., and Brandt, U. (2002) Full recovery of the NADH:ubiquinone activity of complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica* by the addition of phospholipids, *Biochim. Biophys. Acta 1556*, 65−72.
43. Ragan, C. I. (1976) NADH-ubiquinone oxidoreductase, *Biochim. Biophys. Acta 456*, 249−290.
44. Dowhan, W., Mileykovskaya, E., and Bogdanov, M. (2004) Diversity and versatility of lipid−protein interactions revealed by molecular genetic approaches, *Biochim. Biophys. Acta 1666*, 19−39.
45. Lee, A. G. (2004) How lipids affect the activities of integral membrane proteins, *Biochim. Biophys. Acta 1666*, 62−87.
46. Schlame, M., Rua, D., and Greenberg, M. L. (2000) The biosynthesis and functional role of cardiolipin, *Prog. Lipid Res. 39*, 257−288.
47. Palsdottir, H., and Hunte, C. (2004) Lipids in membrane protein structures, *Biochim. Biophys. Acta 1666*, 2−18.
48. Fyfe, P. K., McAuley, K. E., Roszak, A. W., Isaacs, N. W., Cogdell, R. J., and Jones, M. R. (2001) Probing the interface between membrane proteins and membrane lipids by X-ray crystallography, *Trends Biochem. Sci. 26*, 106−112.
49. Robinson, N. C. (1982) Specificity and binding affinity of phospholipids to the high-affinity cardiolipin sites of beef heart cytochrome *c* oxidase, *Biochemistry 21*, 184−188.
50. Sedlák, E., and Robinson, N. C. (1999) Phospholipase A_2 digestion of cardiolipin bound to bovine cytochrome *c* oxidase alters both activity and quaternary structure, *Biochemistry 38*, 14966−14972.
51. Schägger, H., Hagen, T., Roth, B., Brandt, U., Link, T. A., and von Jagow, G. (1990) Phospholipid specificity of bovine heart bc_1 complex, *Eur. J. Biochem. 190*, 123−130.
52. Hayer-Hartl, M., Schägger, H., von Jagow, G., and Beyer, K. (1992) Interactions of phospholipids with the mitochondrial cytochrome *c* reductase studied by spin-label ESR and NMR spectroscopy, *Eur. J. Biochem. 209*, 423−430.
53. Lange, C., Nett, J. H., Trumpower, B. L., and Hunte, C. (2001) Specific roles of protein-phospholipid interactions in the yeast cytochrome bc_1 complex structure, *EMBO J. 20*, 6591−6600.
54. Rinyu, L., Martin, E. W., Takahashi, E., Maróti, P., and Wraight, C. A. (2004) Modulation of the free energy of the primary quinone acceptor (Q_A) in reaction centers from *Rhodobacter sphaeroides*: contributions from the protein and protein−lipid(cardiolipin) interactions, *Biochim. Biophys. Acta 1655*, 93−101.
55. Paradies, G., Petrosillo, G., Pistolese, M., and Ruggiero, F. M. (2002) Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage, *Gene 286*, 135−141.
56. Ragan, C. I., and Racker, E. (1973) Resolution and reconstitution of the mitochondrial electron transport system, *J. Biol. Chem. 248*, 6876−6884.
57. Heron, C., Corina, D., and Ragan, C. I. (1977) The phospholipid annulus of mitochondrial NADH-ubiquinone reductase, *FEBS Lett. 79*, 399−403.
58. Fry, M., and Green, D. E. (1981) Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain, *J. Biol. Chem. 256*, 1874−1880.
59. Poore, V. M., and Ragan, C. I. (1982) A spin label study of the lipid boundary layer of mitochondrial NADH-ubiquinone oxidoreductase, *Biochim. Biophys. Acta 693*, 105−112.
60. Ragan, C. I. (1978) The role of phospholipids in the reduction of ubiquinone analogues by the mitochondrial reduced nicotinamide-adenine dinucleotide-ubiquinone oxidoreductase complex, *Biochem. J. 172*, 539−547.
61. Sazanov, L. A., Carroll, J., Holt, P., Toime, L., and Fearnley, I. M. (2003) A role for native lipids in the stabilization and two-dimensional crystallization of the *Escherichia coli* NADH: ubiquinone oxidoreductase (complex I), *J. Biol. Chem. 278*, 19483−19491.
62. Rich, P. R., and Harper, R. (1990) Partition coefficients of quinones and hydroquinones and their relation to biochemical reactivity, *FEBS Lett. 269*, 139−144.
63. Degli Esposti, M., Ngo, A., McMullen, G. L., Ghelli, A., Sparla, F., Benelli, B., Ratta, M., and Linnane, A. W. (1996) The specificity of mitochondrial complex I for ubiquinones, *Biochem. J. 313*, 327−334.
64. Lenaz, G. (1998) Quinone specificity of complex I, *Biochim. Biophys. Acta 1364*, 207−221.

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