Biochimica et Biophysica Acta 821 (1985) 121–129
Elsevier

BBA 72786

Location of ubiquinone-10 (CoQ-10) in phospholipid vesicles

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(Received July 2nd, 1985)

Key words: Ubiquinone; Phospholipid vesicle; Liposome; NMR; Electron microscopy

Egg yolk phosphatidylcholine (PC) liposomes were prepared by ultrasonic irradiation. At least 25 mol% of coenzyme Q-10 (CoQ-10) can be incorporated nonstoichiometrically into PC liposomes. Electron microscopy showed no visible influence of CoQ-10 on the membrane structure. Nuclear magnetic resonance spectra of sonicated PC liposomes containing CoQ-10 showed two peaks (3.82 and 3.98 ppm) due to CoQ-10 methoxyl protons and a 'high-field component' (1.52 and 1.58 ppm). The areas of these peaks were inversely related and influenced by the time of ultrasonic irradiation. After short sonication the low-field positions (3.98 / 1.58 ppm) are favoured, after long sonication the high-field positions (3.82 / 1.52 ppm). No gradual shift of the two peaks is observed. The 'critical' liposome diameter was found to be between 500 to 700 Å. Lanthanide induced pseudocontact shift on CoQ-10 resonances ('high-field component' and methoxyl) does not lead to a split of the peaks and the difference between them remains constant. It is concluded that CoQ-10 is incorporated into the membrane core, beyond C-2 of the PC acyl chains, with two bilayer curvature-dependent resonance positions.

Introduction

The involvement of ubiquinone (coenzyme Q, CoQ) as a redox carrier of the respiratory chain in the inner mitochondrial membrane is today well established on the basis of reconstitution [1] and kinetic studies [2,3].

The coenzyme is an essential redox component between dehydrogenase and the cytochrome *b*-c_1 complex. It may act as a mobile carrier, as in Mitchell's [4] postulated protonmotive ubiquinone cycle, given in Fig. 1. He assumes a second interaction site of ubiquinone on the side of oxygen/cytochrome *b*. The concept implies that the reduced ubiquinone (ubiquinol, $CoQH_2$) is the species that reduces cytochrome *b*-566, the reduced semiquinone radical thus being formed and a proton released outside the membrane. The semiquinone radical reduces cytochrome c_1 and a second proton is released outside the membrane. Oxidized ubiquinone migrates across the membrane and receives one electron from cytochrome *b*-562 and a proton is taken up from the matrix space. The second electron is received from the dehydrogenase and a second proton taken up again

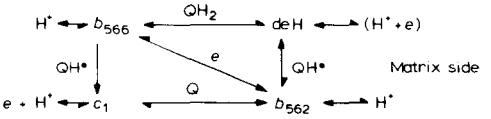

Fig. 1. Flow diagram of ubiquinone cycle in cytochrome $b-c_1$-dehydrogenase complexes. deH, dehydrogenase; QH_2, reduced ubiquinone; QH·, semiquinone radical.

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from the matrix space. Cytochrome *b*-562 is reduced by cytochrome *b*-566. The reduced ubiquinone so-formed migrates across the membrane completing the cycle. It appears that ubiquinone acts as mobile species, crossing the membrane by transverse diffusion or by 'flip-flop' movements.

A detailed discussion of the various aspects of proton translocation reactions in the respiratory chain may be found in a review by Papa [5] as well as in papers mentioned there. On the basis of calorimetric and fluorescence polarisation experiments Alonso et al. [6] argue that ubiquinone-10 is most probably located between the two half-bilayers of small unilamellar phospholipid vesicles; ubiquinone-3, however, is nearer the polar phospholipid headgroups. Hauska [7] reported that only plastoquinones and ubiquinones with long isoprenoid side chains (nine and 10 isoprenoid units, respectively) are efficient translocators of electrons and protons. Using proton NMR spectroscopy Kingsley and Feigenson [8] concluded that the quinol rings (or reduced ubiquinone) are closer to the membrane surface than the ubiquinone (oxidized form). As the length of the side chain increases beyond 1 isoprenoid unit the quinone/quinol ring tends to be deeper in the outer monolayer but little or no change is observed in the inner monolayer. Quinn and Esfahani [9] have shown that with increasing surface pressure on ubiquinone-10 containing monolamellar phospholipid films the quinone is progressively squeezed out from the phospholipid molecules. They conclude that ubiquinone/ol forms a separate phase overlying the phospholipid monolayer.

Stidham et al. [27] have reported that CoQ-10 does position into the bilayer but an appreciable fraction of the coenzyme forms a separate phase located outside the lipid bilayer. More recently Ulrich et al. [28] argue against transmembrane 'flip-flop' of long chain quinones and propose that one essential property of long chain ubiquinones for transfer of electrons and protons across the bilayer is their residence in the hydrophobic core.

All the above-mentioned experiments were carried out using synthetic or semisynthetic phospholipids with saturated and well defined fatty acyl chains such as dipalmitoylphosphatidylcholine (DPPC). We used highly purified egg yolk phospholipid liposomes (with poly-unsaturated fatty acyl chains of varying lengths) as model membrane systems to investigate some of the aspects of incorporation, location and motion of CoQ-10 in a biomembrane component.

Materials and Methods

Preparation of phosphatidyl choline (PC). PC was extracted from fresh egg yolks by a method based on Ref. 10. Purification was achieved by column chromatography on aluminium oxide (Brockmann activity 1, chloroform/ methanol (1 : 1, v/v)) to produce crude egg phospholipids, which were applied to a silicic acid column (Mallinckrodt CC4 Special, chloroform/ methanol (2 : 1, v/v)). Homogeneity of the preparation was checked by thin-layer chromatography on SIlicar Gel G plates (chloroform/ methanol/ water (65 : 25 : 4, v/v)). Phospholipids were assayed qualitatively using Dittmer and Lester spray [11] and quantitatively by digestion of dried samples with 72% perchloric acid, followed by the determination of inorganic phosphate by the method of Gee and Deitz [12]. The yellow colour due to the ammonium phosphomolybdovanadate was assayed in a spectrophotometer at 390 nm. The extend of oxidation of the phospholipid preparations and stock solutions was determined by the method of Klein [13]. Fractions showing a ratio greater than 0.2 for oxidation (approx. 1%) were rejected. All preparations were stored in 1% ethanol stabilized chloroform under nitrogen atmosphere at $-20°C$. After each sampling the containers were flushed with nitrogen. Loss of chloroform by evaporation led to an increase in concentration with time. This increase was monitored by regular lipid phosphorus assay.

Preparation of ubiquinone-10 (CoQ-10). CoQ-10 was either bought (BDH Chemicals Ltd., Poole, U.K.) or extracted by a method based on the methods of Crane et al. [14] and Crane and Dilley [15].

Saponification of pig- or bovine-heart mitochondria was followed by column chromatography with neutral alumina (Brockmann 1). Light petroleum fractions containing increasing volumes of diethyl ether (2 to 15%) were collected, concentrated by rotary evaporation and checked for

homogeneity on Silica Gel G plates (chloroform/benzene (1 : 1, v/v)) impregnated with Rhodamine 6 G. CoQ-10 was assayed quantitatively by the method of Crane et al. [16]. All preparations were stored in an appropriate solvent under nitrogen atmosphere at −20°C and kept in the dark.

Reduction of CoQ-10. CoQ-10 was reduced by adding small amounts of solid sodium borohydride (or sodium borodeuteride for NMR experiments) to the samples which were kept under inert gas atmosphere until hydrogen effervescence had ceased.

Lanthanide salts. Lanthanide nitrates and chlorides were prepared from the dried oxides according to a method described by Cotton and Wilkinson [17]:

$$M_2O_3 + 6\,NH_4Cl \rightarrow 2\,MCl_3 + 3\,H_2O + 6\,NH_3$$

$$M_2O_3 + 6\,HNO_3 \rightarrow 2\,M(NO_3)_3 + 3\,H_2O$$

M = lanthanide

The salts were stored in a desiccator over phosphorpentoxide until further use. Residual water was azeotropically removed with dry carbon tetrachloride under dry nitrogen atmosphere using a rotary evaporator. At least four evaporations were carried out for each preparation. A volume of ^2H_2O (99.8 atom% ^2H) was added to the dried lanthanide salts to make a convenient stock solution (usually 0.1 M). A nominal pH of 5.6 was measured for these solutions.

Preparation of liposomes. A volume of PC stock solution was taken to dryness in vacuo on a rotary evaporator, at temperature less than 35°C. After all traces of solvent had been removed the sample was allowed to cool to room temperature and nitrogen at atmospheric pressure was admitted. Carbon tetrachloride was added (30 ml) to remove ethanol traces azeotropically. This step was repeated three times. Residual carbon tetrachloride was blown off with dry nitrogen. A volume of buffer or ^2H_2O was then added (usually 2 ml) and the flask was shaken mechanically until all the lipid adhering to the bottom and the walls had been dispersed. The dispersion was transferred to a flat-bottomed capped tube and sonicated under nitrogen for any length of time or until the contents became opalescent or waterclear.

A 100 W ultrasonic water bath (Kerry Ultrasonic Ltd., Hitchin, Herts) filled with destilled water and a drop of Triton X-405 was used. Temperature of the sonication bath was controlled and kept at 20 ± 2°C.

Incorporation of CoQ-10 into liposomes. Volumes of PC stock solution and CoQ-10 stock solution were mixed in a round bottom flask and taken to dryness in vacuo on a rotary evaporator, at temperature less than 35°C. The washing procedure with dry carbon tetrachloride was repeated until a homogeneous film adhering to the walls of the flask was obtained, and no CoQ-10 crystals were visible. Then the procedure was the same as described for phosphatidylcholine alone.

All sonicated liposome samples were kept in the dark at room temperature for not more than 24 h.

Electron microscopy. Mechanically shaken sonicated PC liposomes containing upto 25 mol% of CoQ-10 were prepared in freshly made 0.5% ammonium molybdate solution to a concentration of 1 mM of phospholipid. Copper grids (200 to 300 mesh) were coated with a film of vacuum-evaporated carbon [18]. Three to five microlitres of liposome suspensions were applied to the carbon grids by using a micropipette. The grids were examined in a J.E.M. 100 C electron microscope (JEOL Ltd., Tokyo, Japan).

NMR-spectroscopy. Preliminary experiments were carried out on a 60 MHz ^1H-NMR continu-

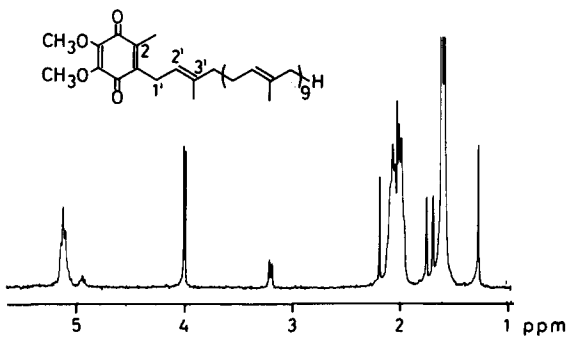
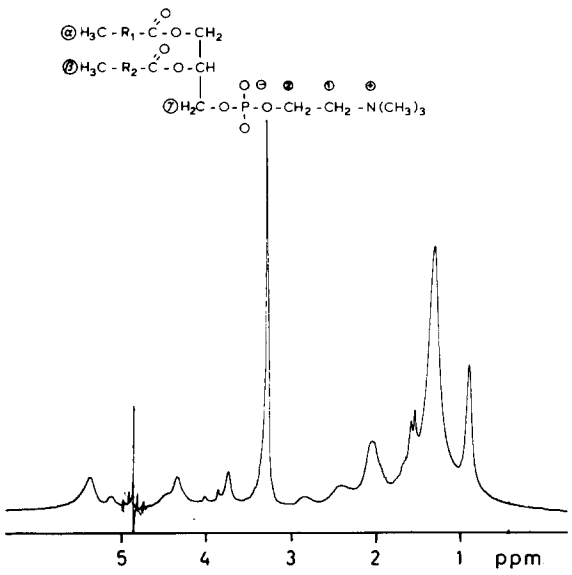


Fig. 2. 300 MHz ^1H-NMR spectrum of CoQ-10 in deuterated chloroform (6 mg/ml), $T = 22$°C, 10 transients. Assignment of ^1H-resonances: Impurity 1.28; isoprenoid methyl groups 1.61; terminal methyl 1.69; methyl on C-3 1.75; isoprenoid methylenes 1.94 to 2.10; methyl on C-2 2.18; proton on C-1 3.20; methoxyl protons 4.00; isoprenoid vinyl 5.14.



ous wave spectrometer at the Department of Chemistry, The University of Hull, but all results reported here were obtained on the a 300 MHz ^{1}H-NMR Fourier Transform spectrometer (Varian SC 300) at the Department of Chemistry, The University of Manchester. Chemical shifts are given

Fig. 3. 300 MHz ^{1}H-NMR spectrum of sonicated PC liposomes containing 10 mol% CoQ-10 in ^2H_2O, $T = 22°C$, 10 transients. Assignment of ^{1}H-resonances: PC terminal methyl 0.88; $(CH_2)_n$ of acyl chains 1.27; CoQ-10 'high-field component' 1.52 and 1.56; isoprenoid methylenes and PC $CH_2C=C$ 1.80 to 2.20; PC acyl C-2 protons 2.36; acyl-CH_2-(C=C) 1.80 to 2.20; PC acyl C-2 protons 2.36; acyl-CH_2-$(C=C)_2$ 2.80; choline methyl 3.26; choline 1 (-CH_2N) 3.70; CoQ-10 methoxyl protons 3.82 and 3.98; choline 2 (-$POCH_2$-) 4.29; isoprenoid vinyl 5.08; PC glycerol and vinyl 5.31. R_1 and R_2, aliphatic hydrocarbon C_{16}–C_{22} containing double bonds.

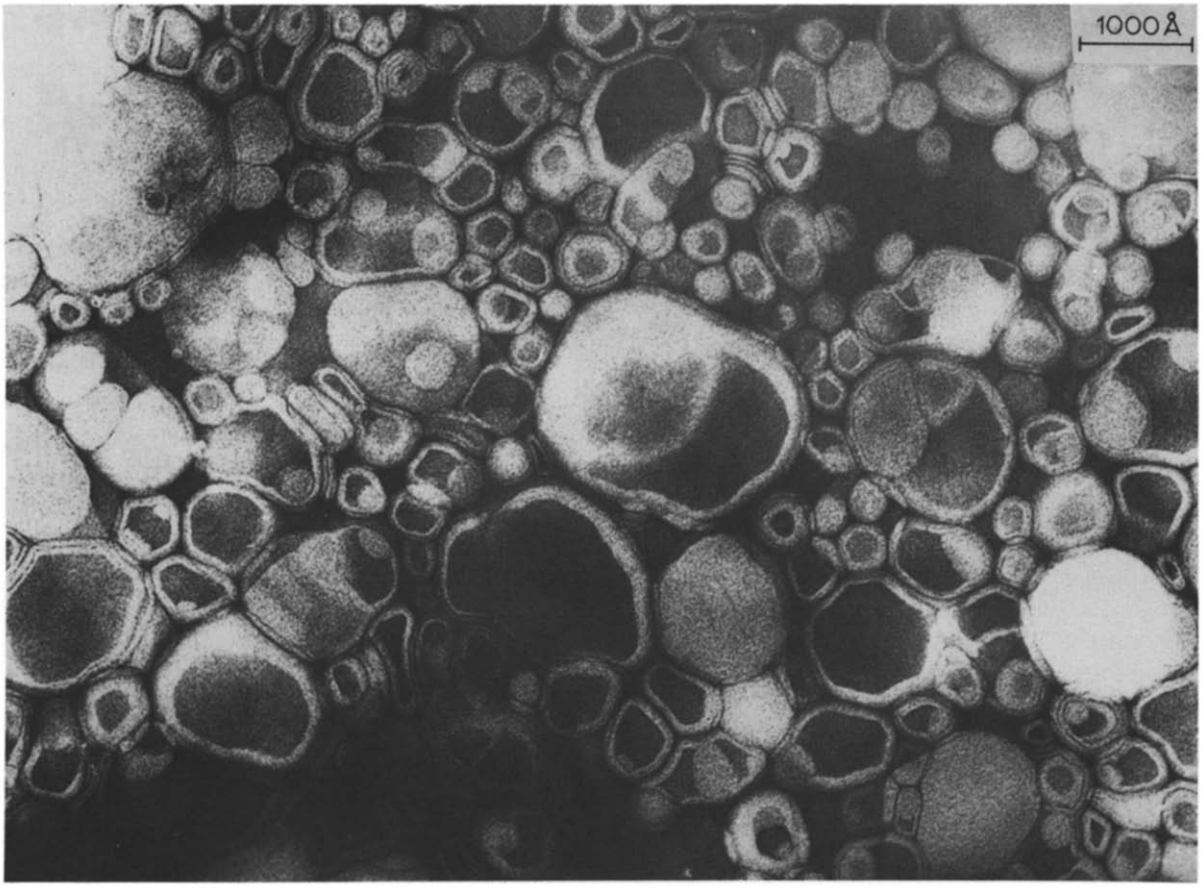


Fig. 4. Electron microscopy. Sonicated phosphatidylcholine liposomes containing 10 mol% CoQ-10, negatively stained with 0.5% ammonium molybdate solution on carbon-coated copper grids. Sonication (water bath) for 20 min at 20 ± 2°C, total magnification 147000×.

in ppm (parts per million) relative to TMS (tetramethylsilane) or DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate), respectively.

Results

Fig. 2 shows the 300 MHz spectrum of CoQ-10 in deuterated chloroform with resonance assignments according to Ref. 19. The resonance at 1.28 is not assignable to CoQ-10 and taken to be a minor impurity of unknown source and nature. The 300 MHz spectrum of sonicated PC liposomes containing 10 mol% CoQ-10 is given in Fig. 3 together with the necessary structural formula. Assignments of the resonances were made in accordance with Refs. 20 and 21. Relevant peaks for this work are PC terminal methyl (of acyl chains, six protons) at 0.88, CoQ-10 'high-field component' (origine not entirely clear) at 1.52 and 1.58 adjacent to $(CH_2)_n$ of PC acyl chains at 1.27, choline methyl (nine protons) at 3.26, choline 1 and 2 according to structural formula at 3.70 and 4.29, CoQ-10 methoxyl protons between choline 1 and 2 at 3.82 and 3.98.

The electron microscopy photographs (Figs. 4 and 5) were obtained from liposome preparations in negative stain (0.1% ammonium molybdate) with PC and CoQ-10 concentrations equivalent to those used in NMR spectroscopy (20–54 mM PC) and dilution to a final concentration of 1 mM.

Fig. 6 was obtained from peak area integration of the CoQ-10 methoxyl resonances at 3.98 and 3.82 compared to PC terminal methyl (0.88), choline methyl (3.26) and choline 1 (3.70). Several integration techniques were compared and minimum as well as maximum baseline calculations carried out.

Increasing amounts of CoQ-10 were incorpo-

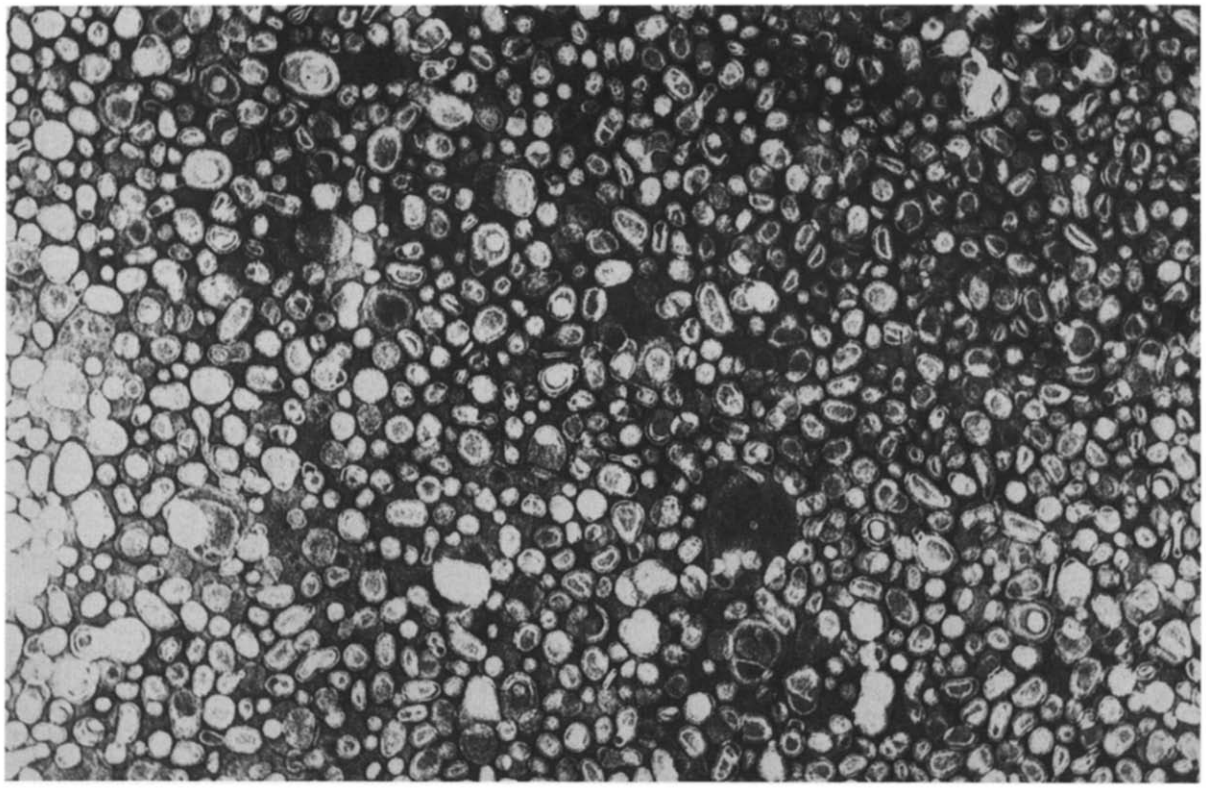


Fig. 5. Electron microscopy. All details as for Fig. 4 but with a sonication (water bath) time of 60 min and total magnification 93000×.

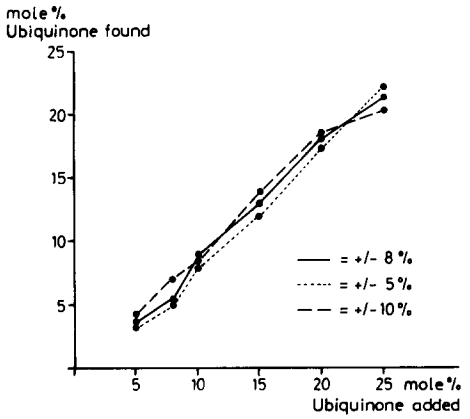


Fig. 6. Recovery of CoQ-10 incorporated into PC liposomes. Peak area integration of the two CoQ-10 methoxyl resonances at 3.98 and 3.82 ppm compared to PC terminal methyl (————), the choline methyl (------) and the choline 1 peak (— — —).

rated into PC liposomes and the recovery rate by peak area integration was always less (2 to 5%) than the CoQ-10 added. When the liposomes were reextracted (after the NMR experiments) to check on oxidation and degradation of PC and CoQ-10 by thin-layer chromatography and ultraviolet spectroscopy (see Methods) the recovery rate for CoQ-10 was 100% (within experimental errors) without measurable degradation or oxidation of both CoQ-10 and PC.

The high-field and low-field components of CoQ-10 (1.52/1.58 and 3.82/3.98) incorporated into PC liposomes are given in Fig. 7. The peaks are depicted from typical spectra obtained on the 300 MHz spectrometer. When 'shift reagents' (such as Pr^{2+} or Eu^{3+}) are added to the liposome suspensions after sonication all resonances in the outer monolayer are shifted towards lower magnetic field (for Pr^{3+}) which is demonstrated by Fig. 8. Thus in this particular spectrum the choline methyl membrane outside contribution to the peak is shifted from 3.23 to 3.38, the choline 1 from 3.68 to 3.88 and the choline 2 from 4.29 to 4.63.

The lanthanide titration experiments were carried out by quickly adding volumes of the salt solution (10 μl of 0.1 M) directly to the NMR tubes. An inert gas atmosphere was found to be an unnecessary precaution.

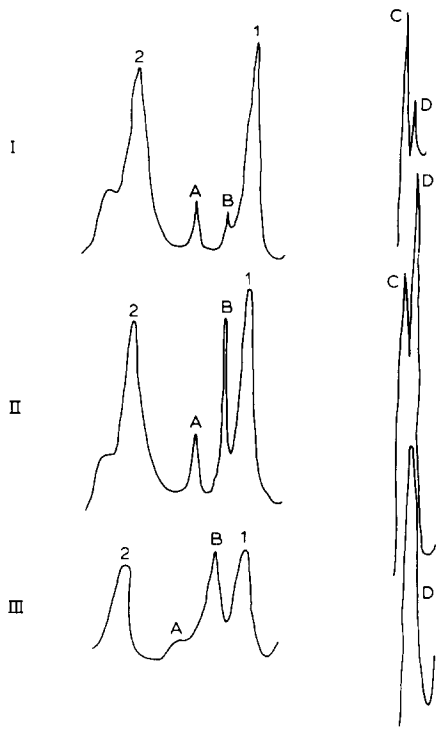


Fig. 7. High-field and low-field components of CoQ-10 incorporated into phosphatidylcholine liposomes (54 mM): depicted from typical spectra obtained on the 300 MHz spectrometer. Assignments: (1) phosphatidylcholine 2 (-$POCH_2$) 4.32; (2) phosphatidylcholine 1 (-CH_2N) 3.70; A and B = CoQ-10 methoxyl protons 3.98 and 3.82; C and D = 'high-field component' 1.58 and 1.52. I, sonication time 20 min; II, sonication time 60 min; III, sonication time 150 min.

Discussion

The results of Fig. 6 show that CoQ-10 can be incorporated nonstoichiometrically into sonicated phosphatidylcholine liposomes upto at least 25 mol%. The amount of CoQ-10 recovered was always less (minus 2 to 5%) than that added with the stock solution. This suggests: (a) a highly unlikely but still possible systematical error or (b) certain amounts of CoQ-10 do not exhibit detectable NMR signals. An explanation for (b) could be that small amounts of the coenzyme are in domains which do not exhibit NMR signals because CoQ-10 is not freely mobile, as it is in solvent or in bilayer dispersion. Alternatively, certain amounts of CoQ-10 are not incorporated into the membrane, but are present in the aqueous phase as aggregates.

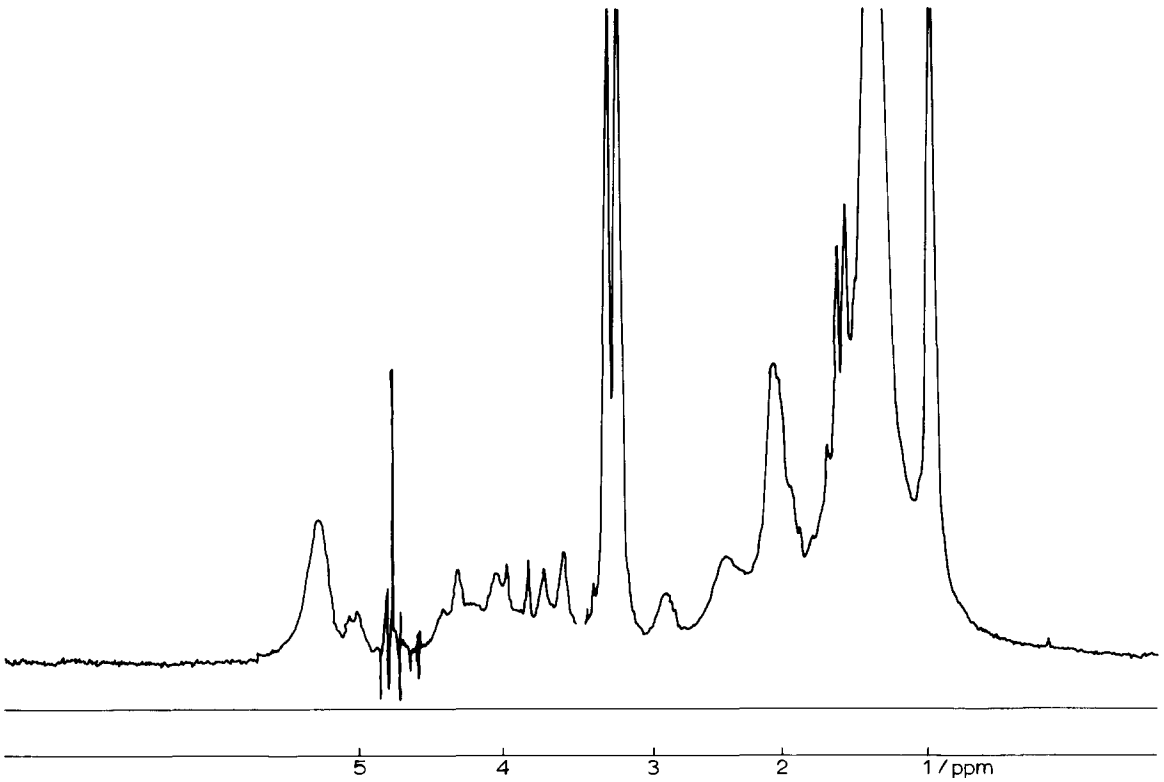


Fig. 8. 300 MHz ^{1}H-NMR spectrum of down-field (lower magnetic field) shift of Pr^{3+} ions on sonicated PC liposomes containing 10 mol% CoQ-10 in ^2H_2O, $T = 22°C$, 10 transients. Assignments of ^{1}H resonances: PC terminal methyl was set at 0.88; $(CH_2)_n$ of acyl chains 1.27; CoQ-10 'high-field component' 1.52 and 1.56; isoprenoid methylenes and PC acyl $-CH_2C{=}C$ 1.80 to 2.20; PC acyl C-2 2.36; acyl-$CH_2(C{=}C)$ 2.80; choline methyl membrane inside 3.23 and outside 3.38; choline 1 membrane inside 3.68 and outside 3.88; CoQ-10 methoxyl 3.81 and 3.95; choline 2 membrane inside 4.29 and outside 4.63; isoprenoid vinyl 5.09; glycerol β and PC vinyl 5.33.

CoQ-10 alone sonicated in ^2H_2O does not exhibit an NMR signal (it is practically water-insoluble).

The bulk of CoQ-10 (95–98%) is, however, incorporated into the membrane and the CoQ-10 methoxyl as well as the 'high-field component' resonances at 3.98/3.82 and 1.56/1.52 (Fig. 3) are considerably narrower than the PC glycerol peak at 5.31, the most rigid part in the membrane. This provides evidence that CoQ-10 can move fairly freely in the bilayer and is therefore dispersed. Another argument that CoQ-10 is dispersed in the membrane and does not exist in domains can be deduced from the fact that the melting point of CoQ-10 (48–52°C) is well above the experimental temperature (20–35°C). It is then unlikely that CoQ-10 existing in domains would exhibit fluid behaviour.

A direct comparison of sonication times for liposomes negatively stained with ammonium molybdate (for electron microscopy) and those of liposomes in ^2H_2O (for NMR spectroscopy) is possible when sufficient care is taken to keep experimental conditions constant (concentration of lipid and coenzyme, volume, temperature of sonication bath, sonication time).

(1) After short sonication (about 20 min) the electron microscopy photographs show smallest liposomes between 500 and 700 Å diameter, but the bulk of the lipid (and the incorporated CoQ-10) is present as vesicles of approx. 1000 Å. The NMR spectrum Fig. 7-I shows larger A and C peaks compared to the B and D peaks.

(2) After longer sonication (about 60 min) the average size of liposomes is between 500 and 700

Å and B and D are larger than the A and C peaks (photograph 2 and Fig. 7-II).

(3) After sonication for 150 min peaks A and C disappear and practically all liposomes are 500–700 Å in diameter.

(4) There is no gradual shift of A towards B or C towards D, i.e. only two resonance positions for the methoxyl protons and the 'high-field component' can be observed, with difference of 0.16 = 48 Hz and 0.06 = 18 Hz.

(5) Peaks A and B originate from the methoxyl protons, which was shown when methoxyl-deuterated CoQ-10, prepared by a base-catalysed methoxyl-exchange reaction was incorporated into the phospholipid liposomes. Both peaks disappeared.

The results presented indicate that the two peaks A and B are not resonances from each individual methoxyl group adjacent to the benzene ring (i.e. A from 2-methoxyl and B from 3-methoxyl or vice versa). If this were so one would expect a 1 : 1 or at least a constant ratio if either A or B would contain a contribution from the glycerol protons. As this is, however, not so, the two methoxyl resonances result from two different locations in the membrane.

Clearly membrane curvature affects the location of CoQ-10, as with increasing sonication time the number of small liposomes increases and with that the high-field resonances at 3.82 and 1.52. It is concluded that the 'critical' liposome diameter is 500–700 Å. Above this diameter the low-field resonance position is occupied. CoQ-10 must be moving in the membrane between at least two different locations: the movement being considerably faster than can be detected on the NMR time scale. This implies transverse diffusion of the CoQ-10 either across the lipid bilayer or at least in the monolayer.

The fluid membrane model [22] implies fast lateral and relatively slow transverse diffusion of the phospholipids. CoQ-10 with a comparatively non-polar benzoquinone group should be unrestricted to move in the bilayer. That this is so has been shown [23] for ubiquinol, which can be readily reoxidized totally from the outside with persulfate.

Lanthanide tri-valent cations shift and/or broaden resonances from protons in their immediate vicinity. Long ranging effects have also been reported [24]. Hauser et al. [25] have shown that the glycerol resonances of PC liposomes are affected by lanthanide ions. Even protons adjacent to C-2 of the fatty acyl chains can be influenced [26]. When lanthanide ions (Pr^{3+}, Eu^{3+}) are added in sufficient concentrations to cause peak splitting of PC-proton resonances (such as $-N(CH_3)_3$) no splittings of the CoQ-10 methoxyl or 'high-field component' peaks are observed, regardless of whether CoQ-10 is in the oxidized or reduced form. Consequently these resonances are out of range of lanthanide influence. Then it appears that in any case CoQ-10 is incorporated deep in the membrane core i.e. at least beyond C-2 of the acyl chains.

Acknowledgments

The authors wish to thank Dr. D.F. Ewing at the University of Hull and D. Moorcroft at the University of Manchester for help with the experiments and stimulating discussion about various aspects of NMR spectroscopy.

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