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THE ROLE OF THE SIDE CHAIN IN THE ANTIOXIDANT ACTIVITY OF UBIQUINONES

LAURA LANDI, LUCIANA CABRINI, DIANA FIORENTINI, GIORGIO SARTOR†, PETRONIO PASQUALI† and LANFRANCO MASOTTI

Dipartimento di Biochimica, Universita' di Bologna and †Istituto di Chimica Biologica, Universita' di Parma (Italy)

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The question has been addressed whether the side chain contributes to the antiodixant activity of Ubiquinones. The length, the chemical composition and structure of the chain have been considered.

The effect of the actual concentration of the quinone in egg lecithin vesicles has been investigated by means of both UV spectroscopy and time resolved fluorescence quenching experiments of 12-AS.

The results indicate that the antioxidant properties of the quinone do not seem to depend on the side chain.

KEY WORDS: Ubiquinone, phospholipid vesicles, lipid peroxidation, fluorescence quenching, 12-AS.

ABBREVIATIONS: Q, ubiquinone; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-dibenzoquinone; 12-AS, 12-(9-anthroyloxy) stearic acid; PL, phospholipids.

INTRODUCTION

The antioxidant activity of ubiquinone (Q), discovered over twenty years ago,[1] was not at first considered an important property, and the attention was focused instead on the roles and topology of the quinone in the respiratory chain.[2-7] Only in recent years in many reports concerning the beneficial effects of Q administration in a wide variety of pathological conditions,[8] the role of Q as an antioxidant was again considered and supporting evidence produced. The mechanism by which both the reduced and, with less efficiency, the oxidized form can act as antioxidant is yet unknown. Q could react directly with oxygen radicals and thus interfere with the initiation of lipid peroxidation, or with lipid radicals thus preventing the propagation of peroxidation.

In previous papers we suggested[9,10] that the antioxidant effect of Q mostly resides in its ability to trap peroxyl radicals. This has been recently confirmed by studying the antioxidant activity of Q_3 in the liposomes in which a lipophilic azothermal compound was used as initiator of the autoxidation.[11]

In this study we incorporated short chain Q homologues and a Q analogue into model membranes in order to evaluate whether the side chain were involved in the antioxidant activity of the molecule. We have then tried to determine the amount of

Author to whom all correspondence and reprint requests should be addressed: Dr. Laura Landi, Dipartimento di Biochimica, Universita' di Bologna, Via Irnerio, 48, 40126 Bologna (Italy).

ubiquinones incorporated and the effect of these compounds on the lipoperoxidation induced by iron salts.

MATERIALS AND METHODS

Egg lecithin was obtained from Lipid Products Ltd. (Redhill, U.K.) and used without further purification. Q_1, Q_2, Q_3, and 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone, a Q analogue (DB), were generously supplied by Eisai Co., (Tokyo, Japan). Stock solutions (10–30 mM) of Q_n in absolute ethanol were stored at $-20°C$ and standardized by the change in UV absorption on reduction by $NaBH_4$. The absorption coefficients and wave lengths used were according to Morton.[12] The fluorescent probe 12-(9-anthroyloxy)stearic acid (12-AS) was obtained from Molecular Probes, (Eugene, OR), and stored as a 1 mM solution in tetrahydrofuran at 4°C. All other chemicals of the highest available quality were from Merck (Darmstadt, West Germany) and Sigma Chemicals Co. (St. Louis, MO.).

Vesicles were prepared according to a previously used procedure.[13] When present, Q_n dissolved in CH_3OH was added to lecithin in $CHCl_3-CH_3OH$ (2:1 v/v). The solvent was evaporated to dryness under a stream of nitrogen and the appropriate amounts of 50 mM Hepes buffer, pH 7.2, were added to the lipids (PL) to give a phospholipid concentration of 3 mg/ml and a Q concentration of 70 nmol/ml. The suspensions were then sonified using a Labsonic 2000 sonicator for 20 min at 4°C under nitrogen. Q-containing vesicles were also prepared by adding an ethanolic solution of quinones to the vesicles. The amount of Q incorporated was determined after gel filtration on a Sephadex G-50 column according to a method published elsewhere.[13] Alternatively the different incorporation of ubiquinone homologues was investigated by fluorescence quenching. The fluorescent probe 12-AS in tetrahydrofuran was incubated with an aqueous suspension of vesicles for 40 min at room temperature. The probe: PL molar ratio was 1:500. The measurements were performed using a single photon counter equipped with an Edinburgh F199 nanosecond flash lamp, Philiph XP2020Q fast photomultiplier, Jasco and Farrand monochromators, EG&G Ortec fast NIM electronics and Silena BS27N multichannel analyzer. The half width height of the excitation pulse was 1.2 ns. Excitation and emission wavelengths were set at 350 nm and 430 nm respectively. The channel width was 178 ps. Data were analyzed by global analysis.[14] The adequacy of fits was estimated by the values of χ^2 and from the randomness of the residuals plots. The fluorescence intensity decays were found to be best fitted by a bioexponential function according to the following equation:

$$I_{(t)} = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2} \quad (1)$$

where τ_1 and τ_2 are the two lifetime components of the decay, and α_1 and α_2 represent the relative amounts of the species that emits with τ_1 and τ_2, respectively. The normalized pre exponential factor of the long lived component was calculated as follows:

$$A_1 = \frac{\alpha_1}{\alpha_1 + \alpha_2} \quad (2)$$

A_1, therefore represents the fraction of the species that emits with lifetime τ_1.

Lipid peroxidation in vesicles (0.2–0.3 mg of PL/ml) was initiated by 0.15 mM $FeCl_2$. Samples were incubated for 30 min at 30°C. Lipid peroxide content was measured by the determination of the thiobarbituric acid reactive material (TBAR) acccording to Beuge[15] and chromogenes were measured at 532 nm against an appropriate blank.

Lipid phosphorus was determined after Marinetti.[16]

RESULTS

In order to examine the influence of the length and of the structure of the side chain on the antioxidant properties of ubiquinones, nearly the same amount of Q_0, Q_1, DB, Q_2 and Q_3 was incorporated into different vesicle preparation by ultrasonic irradiation which had been previously shown to allow a comparable pattern of the incorporation of the various Q homologues from Q_2 to Q_{10}.[13] Furthermore, the TBAR formed in these vesicles, after appropriate intervals of time, had been similar for the various Q-containing vesicles.[17] The chosen concentration of about 1 mol % relative to lipid, approached the physiological content in mitochondria and chloroplasts.[18] The vesicles containing the quinone homologues, as well as the control vesicles, were passed through a column of Sephadex G-50, since the antioxidant effect was found to be related to the amount of quinone incorporated into the lipid bilayer,[19,20] and the amount incorporated had usually been determined after gel-filtration.[13] Vesicles were then subjected to peroxidation by using iron salts; the results are reported in Table 1. Q_3, Q_2 and DB showed a very similar inhibitory effect on peroxidation, while Q_0 and Q_1 had none. Furthermore the amounts of Q_0 and Q_1 left after gel filtration were spectrophotometrically undetectable, suggesting a lack of incorporation. Since this phenomenon might depend on the time of incubation, aliquots of control vesicles were incubated either with 60 μM Q_0 or Q_1 for different times. At the end peroxidation was stimulated by 0.15 mM $FeCl_2$. Figure 1 shows that the inhibition of peroxidation

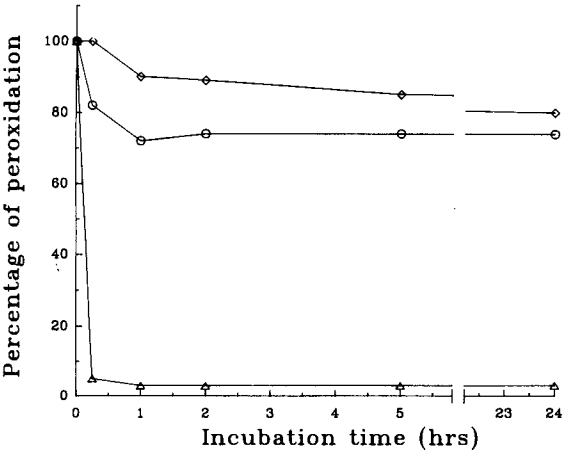

FIGURE 1 Lipid peroxidation in vesicles that were preincubated for different times with Q_0 (O), Q_1 (Δ) and no Q (◇). Percentage of peroxidation was calculated taking the TBAR formation at time 0 as 100.

was virtually completed within the first hour of incubation both in Q_0 and Q_1 containing vesicles. The samples incubated for one hour were gel filtered but, also in this case, the quinone content was undetectable, in spite of the antioxidant properties exhibited by the same vesicles before gel filtration. This finding suggested that the method utilized to evaluate the incorporation of the two quinones into lipid vesicles could be inadequate owing to their different partition equilibrium in comparison with the higher chain homologues.

This possibility was tested by quenching experiments of 12-AS fluorescence. To this purpose Q_0, Q_1 and Q_3 were used as quenchers and they were each separately incorporated into vesicles containing the probe. The two lifetimes of 12-AS recovered from the data analysis were 11.5 $\pm$ 0.2 ns and 7.8 $\pm$ 0.3 ns respectively, and they were found to be constant at each Q concentration tested. The two normalized preexponentials, instead, were dependent on the quencher concentration. A_1, the normalized preexponential factor of the long lifetime, versus $[Q_n]/[PL]$ in gel filtered and not gel filtered vesicles, is reported in Figure 2. $[Q_n]/[PL]$ was calculated according to Ragan[21] (see discussion for details). The fluorescence quenching was dependent on the concentration of the Q_1 and Q_3 added; moreover the longer the chain length, the larger the quenching. Conversely Q_0 did not quench 12-AS fluorescence. Finally Q_3 still acted as a quencher after gel filtration of the vesicles. The insert of Figure 2 shows A_1 versus the concentration of Q_n added. The peroxidizability of the vesicles in the same experimental conditions as those used for the fluorescence experiments, is reported in Figure 3. The inset of Figure 3 shows the percentage of peroxidation versus the concentration of Q_n added.

The maximum protective effect was reached at a ratio of 0.005 $[Q_n]/[PL]$ or 30 μM Q added for all the Q homologues. Q_0 was much less effective in protecting lipids from the radical attack than Q_1 and Q_3, in the order. After gel filtration of the vesicles to which 60 μM Q had been added, Q_0 and Q_1 containing vesicles were peroxidized nearly to the same extent as the controls, while vesicles with Q_3 produced as much TBAR

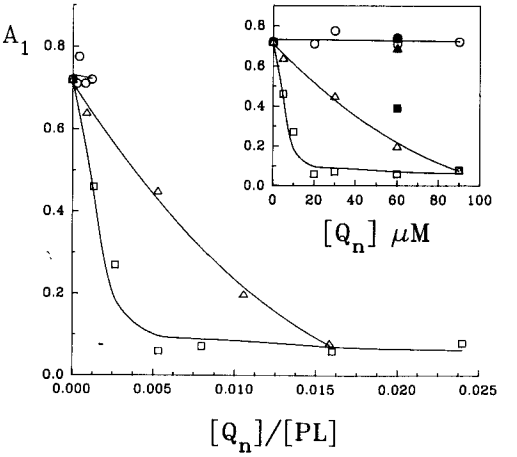

FIGURE 2 Normalized preexponential factor of the long lifetime of 12-AS vs. the ratio $[Q_n]/[PL]$. Q_0 (O), Q_1 (Δ) and Q_3 ($\square$) containing vesicles. Filled symbols refer to gel filtered vesicles. In the insert, A_1 vs. the concentration of Q_n added is reported.

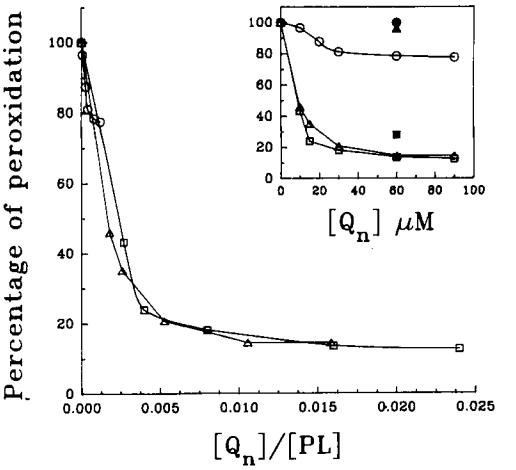

FIGURE 3 Percentage of Fe^{++}-dependent lipid peroxidation vs. the ratio $[Q_n]/[PL]$. Q_0 (O), Q_1 (Δ) and Q_3 (□) containing vesicles. Filled symbols refer to gel filtered vesicles. In the insert, the percentage of peroxidation vs. the concentration of Q_n added is reported.

as the vesicles with Q_1 that were not gel filterted. The Q_3 concentration in the vesicles after gel filtration was determined spectrophotometrically as being 13 nmol/mg PL.

DISCUSSION

The majority of natural and synthetic antioxidants are characterized by the presence of one or more phenolic groups. Oxidized Q_3 protects the phospholipid bilayer from peroxidation[9,10,11,20] even without possessing a phenolic hydrogen. We therefore addressed the question whether the side chain structure and length might confer antioxidation properties to the molecule. We first took into consideration the chemical structure of the side chain. For this purpose we chose DB since it has neither methyl groups nor double bonds. Data in Table 1 show that in our experimental conditions

TABLE 1
Effect of different ubiquinones on Fe^{++} -dependent lipid peroxidation of vesicles

System	TBAR[a] (A_{532nm})	Q incorporated[b] (nmol/mg PL)
PL vesicles	0.225	–
PL vesicles + Q_0	0.220	ND[c]
PL vesicles + Q_1	0.218	ND
PL vesicles + Q_2	0.045	13
PL vesicles + Q_3	0.040	11
PL vesicles + DB	0.035	10

[a] The TBA-reactivity was measured after Beuge (15) at 532 nm
[b] The amount of ubiquinones incorporated by sonication was determined according to a published method (13)
[c] Not detectable

also DB is endowed with antioxidant properties similar to those of two short-chain homologues tested. Since this quinone analogue is provided with a side chain of the same length as Q_2, it can be suggested that the fit of these groups into the pockets created by the *cis* double bonds of arachidonic acid residues postulated by Diplock and Lucy[22] as a mode of action of α-tocopherol, is not a requisite for quinones to exhibit antioxidant activity.

The choice of the length of the side chain is very important because it entails a different solubility of the quinones in the membrane. As a consequence different quinones will have a different actual concentration in model membranes[20] and thus a different antioxidant capability is to be expected. We tried then to incorporate into the vesicles either an ubiquinone lacking completely the side chain, or an ubiquinone having only one isoprenoid unit.

Owing to Q_0 hydrophilicity, it could be expected that its incorporation in the lipid bilayer should be very low: indeed TBAR formation (cfr. Figures 1 and 3) and fluorescence quenching of 12-AS (see Figure 2) confirm such an expectation.

On the basis of Q_0 and Q_1 partition coefficients between phospholipid and water, as determined by Ragan,[21] the aqueous quinone concentrations in the presence of various amounts of PL can be calculated. In our experimental conditions, in which PL concentration varied from 1 to 3 mg/ml and quinone amount from 10 to 90 nmol/ml (see inset of Figures 2 and 3), the percent of total quinone in the aqueous phase was nearly 95% for Q_0 and less than 34% for Q_1. Considering that only 5% of Q_0 is present in the lipid phase, the Q_0 amount incorporated into the lipid can be estimated as ranging from 0.5 to 1.5 nmol/mg of PL (i.e. a ratio $[Q_0]/[PL]$ from 0.0004 to 0.0012). The theoretical amount of incorporated Q_0 can explain the inhibition of peroxidation exhibited by this quinone, which ranged from 20% to 30% (cfr. Figures 1 and 3). These results are in accord with what was reported in a previous paper[20] where the antioxidant effect of Q_3 was compared to the amount of the quinone incorporated into the vesicles. It was shown that maximum protection occurred when the quinone amount was close to physiological content in mitochondria, and that at a concentration of about 3 nmol/mg of PL the lipid peroxidation underwent a 50% inhibition. As far as Q_1 is concerned, its antioxidation effect was very high (cfr. Figures 1 and 3) and so was its percentage in the lipid phase (66% of the quinone added). Nevertheless it was impossible to determine the amount of Q_1 incorporated into the vesicles by our standard procedure. An explanation might be afforded by the consideration of Degli Esposti *et al.*,[23] according to which the partition of the Q homologues in the lipid bilayer reflects the probability of the monomeric form added entering the membrane or forming an aggregate in water:

$$\text{Q int (monomer)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Q ext (monomer)} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Q ext (aggregate)}$$

Only Q_1 would be rather stable in the monomeric form in water so that neither of the two equilibria would be favored ($k_{-1} << k_1$; $k_2 << k_{-2}$). The separation of Q_1 ext by gel filtration from Q_1 int might shift the equilibrium, favoring the continuous loss of the quinone from the membrane. Indeed fluorescence quenching experiments clearly show a different partition of the quinones into the lipid phase, helping, in this way, to explain the different antioxidant activity of the homologues tested.

A few considerations are required for fluorescence measurements. The fluorescence emission decay was best fitted when a three exponential function was used, with the third component representing scattering. The two longer lifetimes, $\tau_1 = 11.5$ ns and

τ_2 = 7.8 ns were virtually unchanged in the presence of the three quinones at different concentrations. Instead large changes in the preexponential factors were derived indicating the quenching to be largely static in nature. The clarifying of the molecular mechanisms of quenching is beyond the scope of the present paper.

In conclusion, the comparison of the antioxidant effects of the Q homologues and a Q analogue supports the view that the side chain is necessary for quinone location and orientation in the bilayer and does not seem to participate in the reaction with free radicals. The same conclusions were also reached by Naumov and Khrapova[24] by a chemiluminescent method in solution.

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