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STUDY OF UBIQUINONE BINDING IN UBIQUINOL-CYTOCHROME c REDUCTASE BY SPIN LABELED UBIQUINONE DERIVATIVE

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SUMMARY-- A functionally active, spin labeled ubiquinone derivative, 2,3-dimethoxy -5-methyl-6-{10-(2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxy)-decyl}-1,4-benzoquinone, has been synthesized for the study of ubiquinone binding in ubiquinol-cytochrome c reductase. When this spin labeled ubiquinone derivative interacted with ubiquinone- and phospholipid-depleted reductase, the spin label was totally immobilized. However, when phospholipids were replenished, the spin label showed mobility behaviour similar to that observed in a hydrophobic environment, indicating that the alkyl side chain of ubiquinone is extended into the hydrophobic region of intact reductase and has some degree of mobility.

Participation of a ubiquinone-binding protein (QPc) in the ubiquinol-cytochrome c region of the mitochondrial electron transfer chain has been established (1-3) through detection of high concentrations of ubisemiquinone radical using controlled reduction of purified ubiquinol-cytochrome c reductase (the cytochrome b-c_1 III complex) with succinate and a catalytic amount of succinate-Q reductase (1, 2), and by redox titration in the presence of redox dyes (3). Using a photoaffinity labeled ubiquinone (Q) derivative (4) coupled with sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis, the protein(s) responsible for ubisemiquinone binding have been identified as two cytochrome b proteins present in the cytochrome b-c_1 III complex. It was not certain whether Q is bound to only one or both cytochrome b proteins, because the photoaffinity labeling group was placed on the end of the alkyl side chain of Q. The length

[1]Abbreviations used: PL, phospholipids; Q, ubiquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_0C_{10}, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; $Q_0C_{10}OH$, 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone; $Q_0C_{10}NAPA$, 2,3-dimethoxy-5-methyl-6-{10-[3-(4-azido-2-nitroanilino)-propionoxy]-decyl}-1,4-benzoquinone; QPc, ubiquinone-binding protein in the cytochrome b-c_1 region; SDS, sodium dodecylsulfate; TMPOC, 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid.

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of this side chain has been shown to affect activity very little beyond a length of 10 carbon atoms (5). If the protein-Q interaction occurs mainly with the benzoquinone ring rather than with whole molecule or the alkyl side chain, then Q is probably bound to only one protein. On the other hand,if the alkyl side chain of Q is the chief factor for binding then it is more likely that both cytochromes b are involved. To establish the binding of Q in the cytochrome b-c_1 III complex we have covalently linked a nitroxide spin label, 2,2,5,5,-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid (TMPOC)[1] (6) to the end of the alkyl side chain of Q and examined the mobility of this spin label after interaction with delipidated, phospholipid (PL) replenished and intact cytochrome b-c_1 III complex preparations. It was observed that in the absence of PL, the spin label was totally immobilized by delipidated cytochrome b-c_1 III complex, whereas the spin label showed a fair degree of mobility when PL were present, indicating that the alkyl side chain of Q is in a hydrophobic environment.

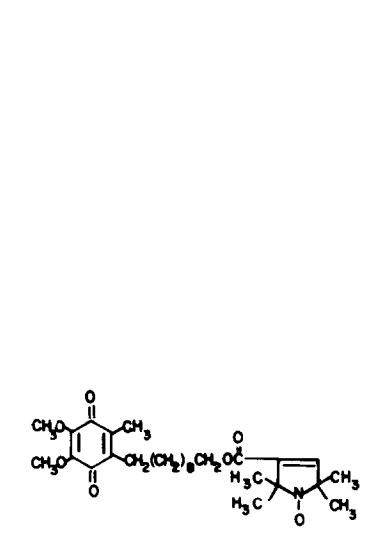
In this communication we report the synthesis and properties of the spin labeled Q derivative and its interaction with the cytochrome b-c_1 III complex.

METHODS AND MATERIALS

Ubiquinol-cytochrome c reductase (the cytochrome b-c_1 III complex) (7) and its PL- and Q-depleted preparations (4, 8) were prepared and assayed according to the reported methods. The PL- and Q-depleted preparation was finally dissolved in 50 mM Na/K phosphate buffer, pH 7.4, containing 20% glycerol to a protein concentration of approximately 10 mg/ml.

The spin labeled Q derivative, 2,3-dimethoxy-5-methyl-6-{10-(2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxy) -decyl}-1,4-benzoquinone (Q_0C_{10}TMPOC), was prepared by the esterification of 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (Q_0C_{10}OH) (9, 10) and TMPOC. Twenty two mg of TMPOC and 25 mg of dicylohexylcarbodiimide were dissolved in 0.25 ml of CH_2Cl_2 which was previously dried over molecular sieves and incubated for 5 min before being admixed with 12 µmoles of Q_0C_{10}OH in 0.1 ml of dry CH_2Cl_2. After addition of 20 µl of pyridine (dried over molecular sieves), the mixture was incubated in a 30° shaker for 12 hours. The mixture was then extracted with one ml acetone, dried with an argon stream, redissolved in 1 ml CH_2Cl_2 and chromatographed on an alumina adsorption column (1 x 4 cm) which was equilibrated and developed with CH_2Cl_2. The Q_0C_{10}TMPOC was recovered in the effluent, while the unreacted Q_0C_{10}OH was eluted with 10% acetone in CH_2Cl_2. The crude Q_0C_{10}TMPOC was applied to a 20 x 20 cm silica gel plate and the plates were developed with 5% acetone in CH_2Cl_2. The Q_0C_{10}TMPOC band was removed and eluted with ether. Upon removal of the solvent, 3.7 µmoles of Q_0C_{10}TMPOC were obtained.

Epr spectra were obtained with a Varian E-4 spectrometer at room temperature. Details of instrument settings are given in the legends of the figures.



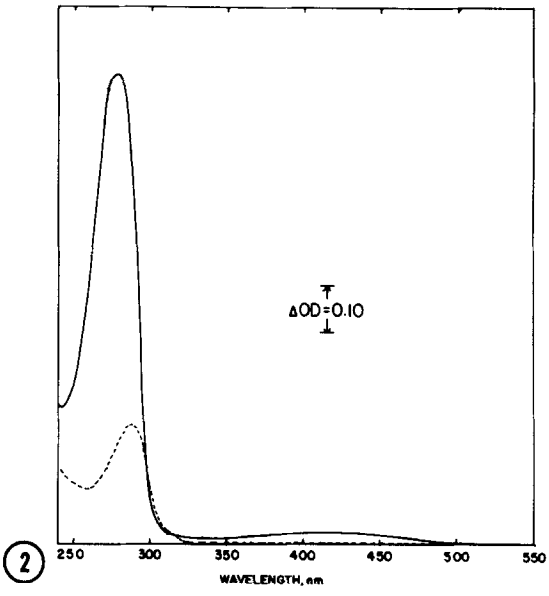


Fig. 1. Structure of Q_0C_{10}TMPOC.

Fig. 2. Absorption spectra of Q_0C_{10}TMPOC. The Q_0C_{10}TMPOC was diluted to 65 μM in 95% ethanol, and the spectra were measured in a Cary spectrophotometer, model 14, at room temperature. One cm light path cuvettes were used. The dotted spectrum was made after the sample was reduced by $NaBH_4$.

Absorption spectra were done with a Cary spectrophotometer, model 14, at room temperature.

TMPOC was obtained from Eastman Organic Company and silica gel thin layer plates were the product of Supelco Inc. Other chemicals were obtained commercially at the highest available purity.

RESULTS AND DISCUSSION

Synthesis and Properties of Spin Labeled Q Derivative (Q_0C_{10}TMPOC)-- The procedure described in the METHODS AND MATERIALS section for the synthesis of spin labeled Q derivative, Q_0C_{10}TMPOC, gave a final yield of about 30%. The unreacted Q_0C_{10}OH was recoverable and can be reused. No special effort was made to perfect the esterification conditons. The chemical structure of Q_0C_{10}TMPOC is given in Fig. 1. Its absorption spectrum is similar to that of 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (Q_0C_{10}) with maximum absorption at 278 nm (see Fig. 2). Since it undergoes hydrolysis easily in alcoholic solvents, Q_0C_{10}TMPOC is usually stored in etheral solution. The spin label of Q_0C_{10}TMPOC was slowly reduced in aqueous solution by succinate when succinate-

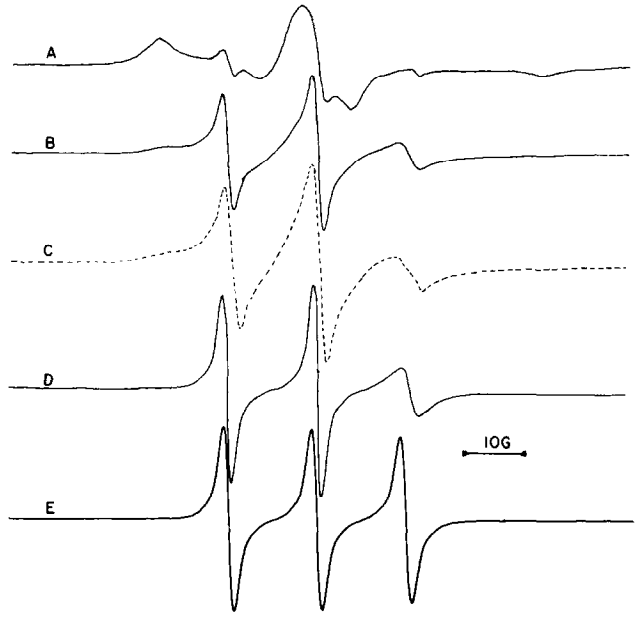


Fig. 3. Epr spectra of spin labeled Q derivative, Q_0C_{10}TMPOC, in different environments. The spectra were taken on a Varian E-4 epr spectrometer, at room temperature, with the instrument settings as follows: field modulation frequency, 100 KHz; microwave power, 10 mW; microwave frequency, 9.5 GHz; modulation amplitude, 1 G; time constant, 0.3 sec; scan rate, 12.5 G/min. Spectrum A represents Q_0C_{10}TMPOC in delipidated cytochrome $\underline{b}$-c_1III complex, 13 mg/ml; B, same as A plus 1% SDS; C, same as A plus PL, asolectin, 0.2 mg/mg protein; D, in 2% sodium cholate. Spectra A to D are in 50 mM Na/K phosphate buffer, pH 7.4, containing 20% glycerol. Spectra E shows Q_0C_{10}TMPOC dissolved in 95% ethanol. The concentrations of Q_0C_{10}TMPOC used and the receiver gain settings are: 140 μM, RG, 2×10^3; 120 μM, RG, 8×10^2; 140 μM, RG, 2×10^3; 93 μM, RG, 8×10^2; 87 μM, RG, 4×10^2 for spectra A to E, respectively.

cytochrome $\underline{c}$ reductase was present, but not when succinate-Q reductase was present. The typical nitroxide spin label epr spectra of Q_0C_{10}TMPOC in ethanol and in 50 mM phosphate buffer containing 20% glycerol and 2% sodium cholate are given in spectra E and D in Fig. 3, respectively.

Comparison of the Effectiveness of Q_0C_{10}TMPOC and Other Synthetic Q Analogues in Succinate-Q and Succinate-Cytochrome $\underline{c}$ Reductases-- Table I compares the electron acceptor and electron mediator activities of synthetic Q analogues. The activity of Q_2 was used as 100%. The saturated straight chain Q analogue, Q_0C_{10}, shows higher activities than does Q_2, both as acceptor for succinate-Q reductase and as mediator in succinate-cytochrome $\underline{c}$ reductase. The latter activity was estimated from the ability of analogues to restore the enzymatic

Table I. Comparison of the Effectiveness of Synthetic Q Analogues as Electron Acceptor for Succinate-Q Reductase and as Electron Mediator in Succinate-Cytochrome c Reductase

Analogues	As Acceptor for Succ.-Q Reductase*		As Mediator in Succ.-cyt. c Reductase**	
	% Activity	Con., μM	% Activity	Con., Q/b
Q_2	100	17	100	5
Q_0C_{10}	111	17	125	5
$Q_0C_{10}OH$	80	20	11	12
Q_0C_{10}TMPOC	91	24	108	7
Q_0C_{10}NAPA	94	17	96	7

*The activity followed was the Q mediated succinate-DCIP reductase activity of Complex II. The concentration refers to the final concentrations of Q analogues in the assay mixture.

**The activity referred to is that restored from the Q- and PL-depleted succinate-cytochrome c reductase upon addition of Q analogues and PL. The concentration represents moles of Q analogues used per mole of cytochrome b in the reconstituted system.

activity to PL- and Q-depleted succinate-cytochrome c reductase upon addition of limiting amounts of Q analogues and sufficient PL. These two activities differ significantly. As an electron acceptor, Q is not re-oxidized after receiving the electron from succinate, while as a mediator the Q analogues must be able not only to accept the electron from its donor but also to be oxidized by the subsequent component. Therefore, a more specific structure is required for mediation. The 6-(10-hydroxydecyl) derivative of Q, $Q_0C_{10}OH$, showed fair activity as the electron acceptor but very poor activity as mediator, indicating that the presence of the hydroxyl group at the end of the side chain of Q may hinder proper binding of ubiquinol-cytochrome c reductase and thus prevent re-oxidation. When this hydroxyl group was esterified with a carboxylic acid such as N-(4-azido-2-nitrophenyl)-β-alanine (NAPA) (4) or TMPOC, it became as effective as Q_2 in mediating the electron transfer reaction in succinate-cytochrome c reductase. Since Q_0C_{10}TMPOC is active both as the electron acceptor of succinate-Q reductase and as the electron mediator in succinate-cytochrome c

reductase, one can use this Q analogue to probe the interaction between Q and the Q-binding protein, QPc.

The Interaction of Spin Labeled Q Derivative (Q_0C_{10}TMPOC) and the PL- and Q-Depleted Cytochrome b-c_1III Complex-- In a previous report, we demonstrated through the use of a photoaffinity labeled Q analogue (Q_0C_{10}NAPA) and SDS polyacrylamide gel electrophoresis, that Q is preferentially bound to cytochrome b proteins. This technique did not indicate where the benzoquinone ring of Q is bound, although it is very likely bound to these same cytochrome b proteins. The formation of ubisemiquinone radical, and the fact that PL have no effect on the labeling pattern produced after photolysis, indicate that binding probably occurs more strongly on the benzoquinone ring than on the alkyl side chain of Q. Spectrum A of Fig. 3 shows the epr spectrum of Q_0C_{10}TMPOC in the PL- and Q depleted cytochrome b-c_1III complex. The broadened spectrum and the appearance of low and high field signals indicate that the spin label is immobilized upon interaction with protein. The immobilization of spin was released, as expected, when sufficient detergent was added to the preparation (spectrum B). A similar release was obtained (spectrum C) when Q_0C_{10}TMPOC was added to the PL- and Q-depleted cytochrome b-c_1III complex followed by the addition of sufficient PL. The similarity of spectra C and B indicates that the spin labels are in similarly hydrophobic environments and are not immobilized. Although this hydrophobic environment could result either directly from the bound PL or from a conformational change of reductase upon PL binding (8), the mobilization of the spin label upon additon of PL is an indication that the alkyl side chain of Q is extended into a hydrophobic environment, and may not be tightly bound to a particular protein subunit. This would explain why the two cytochrome b proteins are equally labeled with photoaffinity labeled Q analogue yet no significant difference in the labeling pattern was observed in the presence and absence of PL. Addition of PL, although increasing the mobility of the alkyl side chain of Q, does not release Q molecule from its binding site, because the benzoquinone ring is anchored at a specific site and therefore no change was observed in the

photoaffinity labeling pattern (4). The possibility exists, although it is unlikely, that a protein which is physically close to both cytochromes b or even sandwitched between them could be the Q-binding protein, because the side chain to which the labeling groups were attached is not very long. Nevertheless, from the results obtained before and described in this communication, it is safe to state that the benzoquinone ring binding site in the cytochrome b-c_1 III complex is either one of the cytochrome b proteins or is located no farther than the length of the side chain of Q_0C_{10}NAPA away from both of the cytochrome b proteins. Further studies with different kinds of photoaffinity and spin labeled Q analogues should give more definite information about the Q binding protein in the cytochrome b-c_1 region at the molecular level.

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