Lateral Diffusion of Ubiquinone during Electron Transfer in Phospholipid- and Ubiquinone-enriched Mitochondrial Membranes*

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After fusion of small unilamellar phospholipid liposomes with mitochondrial inner membranes, the rate of electron transfer between membrane dehydrogenases and cytochrome c decreases as the average distance between integral membrane proteins increases, suggesting that electron transfer is mediated through a diffusional process in the membrane plane (Schneider, H., Lemasters, J. J., Hochli, M., and Hackenbrock, C. R. (1980). J. Biol. Chem. 255, 3748-3756). The role of ubiquinone in this process was evaluated by fusing liposomes containing ubiquinone-10 or ubiquinone-8 with inner membranes. In control membranes enriched with phospholipid only, ubiquinol-cytochrome c reductase and NADH- and succinate-cytochrome c reductase activities decreased proportionally to the increase in bilayer lipid. These decreases were restored substantially in phospholipid plus ubiquinone-supplemented membranes. The degree to which restoration occurred was dependent upon the length of the isoprenoid side chain of the ubiquinone with the shorter chain length ubiquinone-6, always giving greater restoration than ubiquinone-10. It is concluded that electron transfer between flavin-linked dehydrogenases (Complexes I and II) and cytochrome bc₁ (Complex III) occurs by independent, lateral diffusion of ubiquinone as well as the protein complexes within the plane of the membrane.

Ubiquinone is firmly established as an essential component of the electron transfer sequence in mitochondrial respiration (1-8). However, ubiquinone is in large stoichiometric excess over other oxidation-reduction components, and the exact location of this lipid within the energy-transducing membrane as well as its precise role in electron transfer have not been resolved. Three possible modes of function can be considered. Green and Wharton (9) introduced the concept that ubiquinone may function as a mobile electron carrier between the flavin-linked dehydrogenases (Complexes I and II) and cytochrome bc₁ (Complex III). In support, Kroger and Klingenberg (7, 8) demonstrated that 80-90% of ubiquinone present in the mitochondrial membrane functions kinetically as a homogenous pool. The mobile carrier hypothesis implies that ubiquinone shuttles reducing equivalents between spatially separated dehydrogenase complexes and cytochrome bc₁ complexes. It suggests also that such electron transfer involves a diffusional process across or in the plane of the membrane.

Recently, two alternative explanations have been advanced to explain how ubiquinone functions. Yu et al. (10) identified hydrophobic ubiquinone-binding proteins in Complexes I, II, and III and suggested that such binding proteins serve as the actual electron carriers in ubiquinone-dependent electron transfer. The implication is that ubiquinone functions as a prosthetic group for such proteins. In another model advanced by Ragan et al. (11) and Heron, et al. (12), electron transfer between NADH and cytochrome c takes place in physically associated Complex I-Complex III units to which ubiquinone must bind as a coenzyme. Apparent pool behavior of ubiquinone results from dissociation and reassociation of these oxidation-reduction units at rates in excess of overall electron transfer.

In a previous report (13) employing fusion of phospholipid liposomes with mitochondrial inner membranes to increase the bilayer surface area (14), we demonstrated decreases in electron transfer rates which were proportional to the increase in average distance between integral proteins. Although these results indicated that ubiquinone-dependent electron transfer was diffusion limited, we could not determine which of the three possible modes of ubiquinone function described above best described electron transfer in this region of the respiratory sequence. Specifically, the question is whether 1) ubiquinone diffuses independently between widely separated Complexes I, II, and III; 2) ubiquinone diffuses in association with specific membrane proteins (ubiquinone-binding proteins); or 3) ubiquinone diffuses to form short lived associations with Complexes I or II and III which creates a super-complex which is catalytically competent.

In the present study, we fused ubiquinone-enriched phospholipid vesicles with mitochondrial inner membranes in order to examine the diffusional role of ubiquinone in electron transfer. From an assessment of electron transfer rates as a function of membrane ubiquinone concentration, isoprenoid chain length and inner membrane bilayer surface area, we conclude that ubiquinone diffuses independently of other oxidation-reduction components and that this free, lateral diffusion of the ubiquinone molecule is required for distribution of reducing equivalents between independently diffusing membrane dehydrogenases and cytochrome bc₁ complexes.

MATERIALS AND METHODS

Mitochondrial Membranes—Mitochondria from the livers of male Sprague-Dawley rats were isolated by differential centrifugation in a medium containing 70 mM sucrose, 220 mM mannitol, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5 mM/ml of bovine serum albumin, and KOH to pH 7.4. An outer membrane-free, inner membrane-matrix (mitoplast) fraction was prepared by controlled digitonin incubation (15). The mitoplast fraction was washed twice in...
Fusion of Ubiquinone-enriched Liposomes with Inner Membranes—Unilamellar ubiquinone-containing liposomes were prepared as follows: mixed soaybean phospholipid (asolatein, 2.5 g) together with ubiquinone-10 (3.6 mg/g of asolatein) or ubiquinone-6 (3.6 mg/g), was dissolved in 7 ml of chlorofom. The solution was evaporated to dryness under vacuum for 2 h at 30°C using a Buchi rotary evaporator. Of the dry phospholipid-ubiquinone mixture, 1.5 g was suspended in 7 ml of diluted isolation medium. After 1 h of hydration, the lipid mixture was sonicated at 0°C as described previously (13, 14).

Fusion of inner membranes and liposomes was carried out in suspension at 30°C by adjusting the pH to 6.5 as described earlier (13, 14). After an incubation time of 45 min, the inner membrane-liposome mixture was separated on a sucrose density gradient into four buoyant-distinct membrane fractions designated band 1, band 2, band 3, and pellet, from least to most dense.

Enzyme Measurements and Analytical Methods—Enzyme activities were measured at room temperature as previously described (13) and expressed as turnover numbers in units of electrons/s/heme a, the equivalent of pairs of electrons/s/molecule of cytochrome c oxidase. All succinate-linked activities were measured after a 18-h preincubation in the absence of electron acceptor.

Heme a concentration was determined by dual wavelength spectrophotometry in an Amino DW-2a spectrophotometer from the oxidized minus dithionite reduced absorbance change at the wavelength pair, 600-630 nm, using an extinction coefficient of 13.1 mM-1 cm-1 (16). Ubiquinone was extracted from membrane samples with a methanol/chloroform mixture essentially as described by Kräger and Klingenberg (6). Ubiquinone concentration was determined from the oxidized minus borohydride reduced absorbance change at 280-289 nm using an extinction coefficient of 8.8 mM-1 cm-1. Lipid phosphorus was measured by the method of Bartlett (17). Freeze-fracture electron microscopy was performed as described previously (13, 14).

Materials—Horse heart cytochrome c (type VI), ubiquinone-10, and ubiquinone-6 were obtained from Sigma. Soybean phospholipid (asolatein) was obtained from Associated Concentrates (New York). Ubiquinone-1 was the generous gift of Hoffmann-LaRoche, Basel, Switzerland, and DBH was kindly donated by Dr. B. L. Trumpower, Dartmouth Medical School, Hanover, NH. Other chemicals were of the highest purity available commercially.

RESULTS

Fusion of Ubiquinone-enriched Liposomes with Inner Membranes—Fusion of inner membranes with liposomes containing phospholipid plus ubiquinone proceeded to roughly the same extent as fusion with liposomes containing phospholipid alone. Density gradient centrifugation of the fusion mixture yielded four fractions designated bands 1, 2, 3, and pellet, from least to most dense. These fractions were examined by freeze-fracture electron microscopy (results not shown). As the buoyant density of the membrane fractions decreased, there was an increase in the average distance between integral proteins (intramembrane particles) and an increase in the overall size of the inner membranes. The distribution of membrane integral proteins was random in all membrane fractions and at all ubiquinone concentrations. The increases in protein spacing and membrane surface area were proportional to the amount of lipid incorporated. The membranes fused with phospholipid plus ubiquinone were structurally indistinguishable from membranes fused with phospholipid alone. Thus, as with phospholipid incorporation alone (13, 14), the electron microscopic observations showed that the native membranes became highly enriched with exogenous phospholipid plus ubiquinone, that the native membrane proteins remained laterally in the expanded bilayer, and that there was essentially complete mixing of endogenous and exogenous lipid.

The membrane fractions were analyzed for heme a of cytochrome oxidase, lipid phosphorus, and ubiquinone (Table I). Lipid phosphorus to heme a increased progressively from pellet to band 1, the most buoyant fraction, and showed a 6-fold increase in phospholipid over pellet, the densest fraction. Ubiquinone incorporation did not alter the molar ratios of phospholipid to heme a in any membrane fraction.

Electron microscopic analysis of phospholipid and phospholipid plus ubiquinone-enriched mitochondrial inner membranes

| Lipid phosphorus to heme a | Molar ratio of lipid phosphorus to heme a | Molar ratio of ubiquinone to heme a |
|---------------------------|-----------------------------------------|-----------------------------------|
|                           | control                                | Q-10                             |
| Pellet                    | 1.1                                    | 7.5                              |
| Band 3                    | 1.6                                    | 7.6                              |
| Band 2                    | 3.3                                    | 8.1                              |
| Band 1                    | 7.5                                    | 8.6                              |

Table I

Molar ratios of ubiquinone to lipid phosphorus in phospholipid and phospholipid plus ubiquinone-enriched mitochondrial inner membranes

| Lipid phosphorus to heme a | Phospholipid only | Phospholipid + Q-10 | Phospholipid + Q-6 |
|----------------------------|-------------------|---------------------|--------------------|
| Pellet                     | 6.3               | 11.1                | 10.6               |
| Band 3                     | 4.7               | 11.5                | 11.3               |
| Band 2                     | 2.6               | 10.6                | 12.6               |
| Band 1                     | 1.2               | 11.9                | 9.6                |

Table II

1 The abbreviations used are: DBH, ubiquinone having a decyl side chain; Q-10, ubiquinone-10; Q-6, ubiquinone-6; Q-1, ubiquinone-1.
relationship between enzyme activity and membranous ubiquinone concentration. In membranes enriched in phospholipid plus ubiquinone, there was substantial recovery of NADH-cytochrome c reductase activity. Incorporation of ubiquinone-6 or ubiquinone-10 affected different activities in different ways. NADH dehydrogenase activity increased with incorporation of ubiquinone, especially with ubiquinone-6. This effect was unspecific (all fractions were affected nearly equally) and there was no consistent relationship between enzyme activity and membranous ubiquinone concentration. In membranes enriched in phospholipid plus ubiquinone, there was substantial recovery of NADH-ubiquinone and NADH-cytochrome c reductase activities, especially the latter. Recovery of NADH-cytochrome c reductase activity was nearly complete in bands 3 and 2. In band 1, although recovery was not complete, activity was still 4-fold greater after ubiquinone incorporation than control membranes enriched only with phospholipid. The activity of NADH-ubiquinone reductase showed relatively smaller decreases after phospholipid incorporation and less obvious restorations after ubiquinone incorporation. Thus, it is evident that the activities of NADH-cytochrome c reductase and, to a lesser extent, NADH-ubiquinone reductase were dependent upon the concentration of ubiquinone in the membrane. At constant membranous ubiquinone, these activities were much less dependent on the phospholipid to protein ratio.

Effects of Ubiquinone Incorporation on Succinate-linked Electron Transfer Activities—In control membranes enriched in phospholipid alone, the activities of succinate dehydrogenase, succinate-ubiquinone reductase, and succinate-cytochrome c reductase all decreased with increasing lipid incorporation (Fig. 3). As observed with NADH-linked activities, the greatest decreases occurred in cytochrome c reduction, and band 1 activity again decreased by 94%. Incorporation of ubiquinone had variable effects on succinate dehydrogenase and succinate-ubiquinone reductase activities. Ubiquinone-6 increased these activities while ubiquinone-10 decreased them. These effects appear unrelated to membranous ubiquinone concentration or to the total amount of exogenous ubiquinone incorporated. Although all succinate-linked activity measurements were made after preincubation with succinate to achieve maximal activation (20), the observed changes may represent differential activation of succinate dehydrogenase or some other type of ubiquinone chain length-dependent modulation of activity. In contrast, there was substantial
recovery of succinate-cytochrome c reductase activity in membranes enriched additionally with ubiquinone.

In order to distinguish modulatory effects of the two ubiquinones from restorative effects, the percent activity of succinate-cytochrome c reductase was compared in ubiquinone-enriched and control, phospholipid-enriched membranes relative to the pellet membrane fraction (Fig. 4). Both ubiquinones provided substantial restoration of activity, especially in the denser membrane fractions. Restoration of activity was chain length–dependent with ubiquinone-6 being more effective than ubiquinone-10. These experiments show that the activities of NADH-succinate and ubiquinol-cytochrome c reductase are dependent upon membranous ubiquinone concentration and, at equal concentration, the length of the ubiquinone side chain.

**DISCUSSION**

Our previous studies (13, 14) showed that incorporation of phospholipid into mitochondrial inner membranes by fusion with unilamellar liposomes caused an increase in the average distance between integral proteins. Electron transfer activity in these fused membranes decreased in proportion to the increase in bilayer surface area. This decrease was especially obvious between the flavin-linked dehydrogenases (Complexes I and II) and cytochrome bc₆ (Complex III). Since the decreased electron transfer rates correlated with the increased lateral distances between integral proteins, we concluded that there was a diffusion-limited step in the transfer of reducing equivalents between the hydrogenases and cytochrome bc₆.

In the present study, we have examined more closely the role of ubiquinone in diffusion-mediated electron transfer. As compared to membranes enriched in phospholipid alone, membranes enriched in both phospholipid and ubiquinone showed substantial restoration of NADH, succinate-, and ubiquinol-cytochrome c reductase activities. This ability of ubiquinone to restore electron transfer activity lost after phospholipid incorporation strongly supports the concept that during its catalytic cycle, ubiquinone diffuses independently and individually in the lateral plane of the membrane. If ubiquinone were required to codiffuse with a protein such as an ubiquinone-binding protein (10), or if ubiquinone-mediated electron transfer required collisional interaction between integral protein complexes (11, 12), then there should have been little or no recovery of activity after ubiquinone incorporation.

Since ubiquinol-cytochrome c reductase activity decreased with phospholipid incorporation and this effect was reversed by ubiquinone incorporation, it is likely that DBH, the electron donor for this assay, donates into the membranous pool of ubiquinone rather than into Complex III directly (22). This would account for the dependence of ubiquinone-cytochrome c reductase activity on membranous ubiquinone concentration.

The extent of recovery of NADH-, succinate-, and ubiquinol-cytochrome c reductase activity was related to the chain length of the newly incorporated ubiquinones with the shorter chain length, Q-6, giving greater recoveries than the longer chain length, Q-10. Since lateral mobility of a lipid molecule increases with decreasing chain length (21), the relationship between chain length and activity also supports the view that ubiquinone mediates electron transfer by diffusion.

Not all activity was restored after ubiquinone incorporation, especially in band 1, the most lipid-enriched membrane fraction. Relatively unspecific effects caused by incorporation of nonmitochondri al lipid may, in part, account for the lack of full restoration. For example, succinate dehydrogenase, a single enzyme activity, decreased slightly but progressively with increasing phospholipid incorporation, an effect not reversed by ubiquinone incorporation. Other single enzyme activities, such as NADH dehydrogenase and cytochrome c oxidase (13, 14), have been found to increase with incorporation of nonmitochondrial phospholipid. These unspecific effects are not reversed by ubiquinone incorporation.

There also appeared to be some direct modulatory effects of the ubiquinones on some individual enzymes. NADH dehydrogenase, succinate dehydrogenase, and thenoyltrifluoroacetone-sensitive succinate-ubiquinone reductase (probably also a single enzyme activity) all showed changes in activity dependent on the type of exogenous ubiquinone incorporated. We cannot explain why these modulatory effects were not dependent on the total amount of exogenous ubiquinone incorporated, but they may be related to differential activation of these enzymes. In this regard, we should point out that in the most dense membrane fractions the absolute rate of succinate dehydrogenase was consistently lower than the absolute rate of succinate-cytochrome c reductase. This lower activity may reflect a kinetic hindrance in transferring electrons to an artificial electron acceptor, phenazine methosulfate, that may not exist for the endogenous acceptor.

Our findings provide definitive experimental support of the early proposals by Green and Wharton (9) and Kröger and Klingenberg (7, 8) that ubiquinone is a mobile electron carrier which functions in a homogenous pool during the transfer of electrons between the dehydrogenases and the cytochrome bc₆ complex. Our results do not support proposals in which ubiquinone functions as a protein-bound electron carrier. In view of our observation that newly incorporated ubiquinone readily participates in electron transfer despite its great molar excess over other electron carriers, we expect that the majority of the ubiquinone is unbound and freely mobile. Of course, we do not exclude the possibility that there are some forms of protein-bound ubiquinone, since there is good evidence that Complexes I, II, and III all contain subunits which are able to bind ubiquinone specifically, the so-called ubiquinone-binding proteins (10, 23–28). In addition, the stable semiubiquinone forms observed by EPR in mitochondrial membranes during redox titrations (9, 29–33) can be accounted for by the existence of a protein-bound form of ubiquinone. Such protein-bound ubiquinones would be consistent with the observation by Kröger and Klingenberg (7) of two ubiquinone populations, a large pool (80–90%) that reacts in a kinetically homogenous fashion, and a small pool (10–20%) that is nonreducible by NADH or succinate. These two populations may represent free ubiquinone and protein-bound ubiquinone, respectively.

Our model of electron transfer from NADH and succinate
to cytochrome $c$ in the intact mitochondrial membrane is that individual electron transfer components, i.e. the membrane dehydrogenase complexes, the cytochrome $bc_{1}$ complex, and ubiquinone, diffuse randomly and independently of one another in the membrane plane. In addition, hydrophobic subunits of the dehydrogenase and cytochrome $bc_{1}$ complexes provide specific binding sites for stabilization of ubiquinone during electron transfer (ubiquinone-binding proteins). Further protein-protein interactions in the membrane plane between the dehydrogenase complexes and the cytochrome $bc_{1}$ complex are not required for electron transfer.

Our model accounts for the nonstoichiometric ratios of membrane dehydrogenases to $bc$, cytochromes (34), the abundance of ubiquinone relative to other electron carriers (9, 34), and the experimental observation that ubiquinone functions as a kinetically homogenous pool during electron transfer (7, 8). This model is consistent with the observation by Weiss and Wingfield (35) that direct protein-protein interactions are not necessary for electron transfer between isolated complexes of succinate-ubiquinone reductase (Complex II) and ubiquinol-cytochrome $c$ reductase (Complex III) in nonionic detergent.

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