The role of DT-diaphorase in the maintenance of the reduced antioxidant form of coenzyme Q in membrane systems

[NAD(P)H:quinone reductase/ubiquinone/liposomes/lipid peroxidation/free radicals]

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ABSTRACT The experiments reported here were designed to test the hypothesis that the two-electron quinone reductase DT-diaphorase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] functions to maintain membrane-bound coenzyme Q (CoQ) in its reduced antioxidant state, thereby providing protection from free radical damage. DT-diaphorase was isolated and purified from rat liver cytosol, and its ability to reduce several CoQ homologs incorporated into large unilamellar vesicles was demonstrated. Addition of NADH and DT-diaphorase to either large unilamellar or multilamellar vesicles containing homologs of CoQ, including CoQ9 and CoQ10, resulted in the essentially complete reduction of the CoQ. The ability of DT-diaphorase to maintain the reduced state of CoQ and protect membrane components from free radical damage as lipid peroxidation was tested by incorporating either reduced CoQ9 or CoQ10 and the lipophilic azoinititator 2,2'-azobis(2,4-dimethylvaleronitrile) into multilamellar vesicles in the presence of NADH and DT-diaphorase. The presence of DT-diaphorase prevented the oxidation of reduced CoQ and inhibited lipid peroxidation. The interaction between DT-diaphorase and CoQ was also demonstrated in an isolated rat liver hepatocyte system. Incubation with adriamycin resulted in mitochondrial membrane damage as measured by membrane potential and the release of hydrogen peroxide. Incorporation of CoQ9 provided protection from adriamycin-induced mitochondrial membrane damage. The incorporation of dicoumarol, a potent inhibitor of DT-diaphorase, interfered with the protection provided by CoQ. The results of these experiments provide support for the hypothesis that DT-diaphorase functions as an antioxidant in both artificial membrane and natural membrane systems by acting as a two-electron CoQ reductant that forms and maintains the antioxidant form of CoQ. The suggestion is offered that DT-diaphorase was selected during evolution to perform this role and that its conversion of xenobiotics and other synthetic molecules is secondary and coincidental.

Coenzyme Q (CoQ) was originally discovered as an essential member of the mitochondrial electron transfer chain (1) and later hypothesized and shown to function in the energy-conserving protonmotive Q cycle (2). Its antioxidative capacity was demonstrated in vitro (3, 4) and, subsequently, a large number of studies have been published demonstrating the antioxidative role of CoQ at various levels of biological organization (for reviews, see refs. 5–8). In addition, its efficacy in the treatment of several clinical entities has received attention (8–10). Since its primary function has been associated with its role in energy metabolism, the role of CoQ as an antioxidant was considered to be secondary or coincidental (5, 11). The demonstration that high levels of CoQ exist in the majority of cellular membranes (12) and that it is found largely in the reduced form (13) prompted the question of the function of CoQ in these membranes and, in addition, the means by which the reduced state is maintained, especially in those membranes containing no obvious means of catalyzing its reduction.

The enzyme DT-diaphorase, discovered shortly after CoQ (14), is a two-electron quinone reductase [NAD(P)H:(quinone-acceptor) oxide reductase, EC 1.6.99.2] (15), the majority of which resides in the cytosolic fraction of the cell, with minor activity in mitochondria and endoplasmic reticulum (14). DT-diaphorase is an inducible enzyme (16) that may protect the cell from oxidative damage by preventing oxyradical formation from one-electron reduction of quinones and the subsequent formation of superoxide radicals via dismutation of the semiquinone (17, 18). Its protective capacity with respect to quinone carcinogenesis has been demonstrated (19, 20), and it has been labeled an “anticancer enzyme” (21).

A role for DT-diaphorase in the regeneration and maintenance of the antioxidative form of CoQ has been hypothesized (22), and data from experiments performed in situ and in vivo, which support the concept of such a vital role for DT-diaphorase, have been reported (23). We report here the results of experiments on the kinetic interaction of DT-diaphorase and CoQ homologs of various side-chain lengths, the reduction and maintenance of the reduced form of CoQ, including CoQ9 and CoQ10, by DT-diaphorase in large unilamellar vesicles (LUVET) and multilamellar vesicles (MLVs), and the direct relationship between the reduced state of CoQ and the prevention of free radical damage to liposomal membrane components. In addition, results of experiments with intact hepatocytes, consistent with a role for DT-diaphorase–CoQ interaction in the prevention of adriamycin (adr)-generated free radical damage, are presented.

Such data provide a supportive basis for the hypothesis that DT-diaphorase was selected during evolution for the purpose of providing antioxidant protection to cellular membranes by means of its ability to generate and maintain the reduced, antioxidant state of CoQ in such membrane systems.

MATERIALS AND METHODS

Chemicals. Sources of chemicals were as follows: rhodamine 123 (RH-123) and propidium iodide (PI) (Lambda Fluoro...
Preparation of DT-Diaphorase. Twenty liters of liver cytosol from 3-methylcholanthrene-treated Sprague–Dawley rats was used for the purification of DT-diaphorase (24). The cytosolic fraction was prepared according to Ernst et al. (25) and was purified as described (26), with modifications (27). An azodicoumarol Sepharose-6B affinity column was used and the enzyme eluted with NADH, resulting in an essentially pure preparation of DT-diaphorase. The concentrated sample was passed through a CM-Sepharose column equilibrated with a solution of 50 mM Tris-HCl, pH 7.0/0.25 M sucrose in order to remove contaminants by two proteins with high isoelectric points (28). DT-diaphorase activity was assayed with NADH and NADPH as electron donors and menadione as acceptor by following the absorbancy change of cytochrome c as secondary acceptor at 550–540 nm (29). Essentially equal activities of the purified enzyme with NADH and NADPH, together with high sensitivity to inhibition by dic, ensured its identity as DT-diaphorase (29). When CoQ homologs were tested as electron acceptors, the time course of the reaction was followed by monitoring the absorbancy decrease of NADH at 340 nm, using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹ in a Tasco 7850 double-beam spectrophotometer. The extent of nonenzymatic CoQ reduction by NADH was determined in all cases either in the absence of enzyme or in the presence of 20 μM dic.

Preparation of CoQ-Containing MLVs and LUVETs. MLVs were prepared by adding PC and CoQ homologs to a round-bottomed tube, removing each solvent with a stream of nitrogen to obtain a thin film, followed by the addition of 1–4 ml of 0.14 M NaCl/50 mM Tris buffer, pH 7.5/NADH (9 μg/ml)/DT-diaphorase (54 μg/ml). Final PC, CoQ, and NADH concentrations were 15 mM, 10–30 μM, and 3–6 mM respectively. The suspension was vortex-stirred for 12 min. To prepare LUVETs, MLVs void of NADH and DT-diaphorase were prepared using an extruder as described (30). The final volume of LUVETs was adjusted to final PC and CoQ concentrations of approximately 15 mM and 10–30 μM, respectively. NADH and DT-diaphorase were then added to achieve the concentrations noted above. Both types of vesicles were maintained under air and continuous stirring at 37°C. At time intervals, aliquots were transferred to an equal volume of isopropanol, and CoQ and CoQH2 contents were determined by HPLC.

Auctionization Experiments. MLVs used in these experiments contained 36 μM CoQ2H2 or CoQ10H2 and the lipophilic initiator AMVN (4 mM) added at the beginning of the preparation. Reduction of CoQ analogs was according to Rieske (31). DT-diaphorase was added to 54 μg/ml. Control samples, devoid of either NADH or DT-diaphorase, were prepared under the same experimental conditions. Autoxidation was initiated by increasing the temperature to 37°C under air and continuous stirring. Aliquots were removed at time intervals to determine the redox state of the CoQ homologs by HPLC and the formation of thiobarbituric acid reactive material (TBARS) (32).

Determination of CoQ Redox States. Analysis was by HPLC using a Spherisorb ODS2 column (15 mm × 4.6 mm). Elution was isocratic at a flow rate of 1.5 ml/min with methanol/isopropanol (75:25, vol/vol) for CoQ9 and CoQ8H2 and methanol/isopropanol (65:35, vol/vol) for CoQ10 and CoQ9H2. Detection was at 275 and 291 nm for the oxidized and reduced forms, respectively.

Hepatocyte Isolation and Incubation. Male Wistar rats of 200–300 g body weight were used for isolation of hepatocytes and for standard laboratory diet and libitum. Hepatocytes were isolated by a modification (33) of the two-step collagenase liver-perfusion technique of Seglen (34). Cell viability was determined by trypan blue exclusion or PI staining (5 mg/ml) for 5 min. Hepatocytes (1 × 10⁶ cells/ml) were suspended in Krebs-Henseleit medium (pH 7.4) supplemented with 5 mM glucose under an atmosphere of 95% O2/5% CO2 in a shaking bath at 37°C and incubated with 50 μM NADH for 2 hr. The incorporation of CoQ10 was achieved by coinoculation of isolated hepatocytes and CoQ9H2-enriched liposomes, prepared according to Fleischer and Fleischer (35). When indicated, dic was added to hepatocytes at 20 μM to inhibit endogenous DT-diaphorase.

Flow Cytometry. Flow cytometry analyses were performed with an EPICS C cell sorter (Coulter). Fluorochrome were excited at 488 nm with an argon laser at 200 mW. Forward-angle light scatter (FALS) and right-angle light scatter (90LS) were used to select live and dead hepatocytes (36). Fluorescence was collected through a 488-nm blocking filter, a 560-nm long-pass dichroic mirror, and a 520-nm short-pass filter (RH-123, DCHF-DA) or a 570-nm long-pass filter (PI). Correlated histograms and listmode data were collected for linearity (FALS, 90LS) or logarithmically (3 decades, 256 channels) amplified signals (RH-123, DCHF-DA). Mitochondrial membrane potential was estimated by incubating hepatocytes (3 × 10⁶ cells/ml) with RH-123 (50 ng/ml) for 10 min (37). Hydrogen peroxide, released during treatment with ad, was assayed by incubation at 37°C with DCHF-DA (2 μM) for 15 min (38) and fluorescence units were converted to concentration units using a calibration curve.
lecithin liposomes, the rates of reduction catalyzed by DT-diaphorase were higher for LUVET-incorporated CoQ10. The rate of NADH oxidation, monitored spectrophotometrically, matched the rate of CoQ reduction monitored by HPLC analysis (data not shown).

Reduction of CoQ6 and CoQ10 by DT-Diaphorase in Liposomal Systems. Two types of vesicles, MLVs (Fig. 1A) and LUVETs (Fig. 1B), were prepared with CoQ6 or CoQ10 (15–20 μM) incorporated, and the time course of CoQ decrease and CoQH2 increase, in the presence of NADH and DT-diaphorase, was monitored by HPLC analyses (Fig. 1). Once again, DT-diaphorase reduced the two long-chain CoQ homologs, and LUVETs were the preferred preparation. Fig. 2 shows the rates of decrease of oxidized CoQ10 and increase of CoQ10H2 in LUVETs with two concentrations of DT-diaphorase and in the absence of DT-diaphorase. These results show a rate dependence on DT-diaphorase concentration and a lack of CoQ10 reductase activity in the control void of DT-diaphorase.

Maintenance of CoQ6H2 and CoQ10H2 and Prevention of Lipid Peroxidation by DT-Diaphorase in MLVs. As shown in Fig. 3, incorporation of reduced CoQ6 or CoQ10 and the azoinitiator AMVN in the lipid bilayer in the presence of DT-diaphorase and NADH resulted in the maintenance of the reduced state of the quinone and inhibition of TBARS formation (lipid peroxidation). Omission of NADH as a source of reducing equivalents for DT-diaphorase resulted in oxidation of the reduced CoQ molecules and, upon depletion of CoQH2, commencement of lipid peroxidation.

Evidence That Endogenous CoQ Protection Against Oxidative Stress Requires Its Reduction by DT-Diaphorase. Oxidative stress by adr in perfused rat liver (39) and isolated hepatocytes (40) has been shown to induce loss of mitochondrial membrane potential concomitant with increases of cell superoxide and hydrogen peroxide levels. Exogenous CoQ10 administration provided protection of mitochondrial activities and membrane potential by virtue of its antioxidant effect. We have utilized the ability of adr to induce oxidative stress in isolated hepatocytes in order to examine the involvement of DT-diaphorase in production of the reduced form of CoQ by treating isolated hepatocytes, incubated with adr and CoQ10h2, with dic, a potent inhibitor of DT-diaphorase. As shown in Table 2, dic prevented the protective effect of CoQ both against adr-induced hydrogen peroxide generation and loss of mitochondrial membrane potential. Determination of DT-diaphorase activity of hepatocyte cytosols (100,000 X g supernatants) indicated a direct relation between protection by CoQ incubation and DT-diaphorase activity (Table 2). Levels of CoQH2 determined by HPLC analysis, were significantly lower in the dicitreated samples (Table 2).

**DISCUSSION**

In addition to its function in mitochondrial electron transport and oxidative phosphorylation, a primary role for CoQH2 as a vital hydrophobic phase antioxidant has been well established (see refs. 23 and 42 for reviews). In fact, CoQ10H2 has been reported to act more efficiently than α-tocopherol in the antioxidative protection of human low density lipoprotein (43). The discovery (44, 45) and confirmation (12, 46) that CoQ exists at relatively high concentrations in all subcellular membranes and that significantly >50% is in the reduced state (13) prompted the question of its role in these extramitochondrial membranes and the means by which CoQ in these membranes is maintained largely in the hydroquinone state. As early as 1986, it was suggested (22) that the two-electron quinone reductase DT-diaphorase functioned to maintain CoQ in its reduced, antioxidant state.

Although much is known about the prosthetic group, structure, induction, mechanism, gene, pharmacological and toxicological functions, and other properties of DT-diaphorase (47, 48), the physiological role for which it was selected during evolution has not been elucidated. Results of *in vivo* and *in situ* experiments designed to test the DT-diaphorase-CoQ reduc-
tase hypothesis have provided indirect supportive evidence (23). The experiments reported here were designed to test this hypothesis more directly by (i) determining the ability of highly purified DT-diaphorase to catalyze the two-electron reduction of various CoQ homologs, (ii) testing the interaction of DT-diaphorase and long-chain CoQ homologs incorporated into phospholipid vesicles, and (iii) studying the role of hepatocyte DT-diaphorase in the protection, provided by incorporation of CoQ\textsubscript{10}, from free radical damage.

The results of all three experimental approaches provide strong support for a CoQ reductase role for DT-diaphorase. In the presence of phospholipid vesicles, DT-diaphorase catalyzed the dicoumarol-sensitive reduction of CoQ homologs of various isoprenoid chain lengths, including CoQ\textsubscript{10}. Incorporation of long-chain homologs of CoQ in both LUVETs and MLVs in the presence of DT-diaphorase resulted in NADH-supported, enzyme concentration-related reduction of CoQ. Incorporation of long-chain CoQ\textsubscript{H2} and the free radical generator AMVN into MLVs resulted in NADH and DT-diaphorase-dependent maintenance of CoQH\textsubscript{2} and prevention of free radical damage to liposomal membrane components. These results thus provide evidence supporting the ability of DT-diaphorase to reduce CoQ in membrane models. The results of experiments with isolated hepatocytes in which inhibition of DT-diaphorase by dic prevented the protective effect of incorporated CoQ\textsubscript{10} against free radical mitochondrial membrane damage induced by adr, provide strong support for the DT-diaphorase-CoQ reductase hypothesis in a natural cellular system. Results of HPLC analyses of CoQ\textsubscript{10} and CoQ\textsubscript{10H2} in these experiments are also consistent with the DT-diaphorase-CoQ reductase hypothesis.

The results of this multiple experimental approach, together with previous findings (23), provide significant support for the hypothesis that DT-diaphorase functions in antioxidation by interacting with hydrophobic phase CoQ to act as a two-electron quinone reductase to form and maintain the antioxidative form of CoQ—i.e., CoQH\textsubscript{2}. Although the majority of DT-diaphorase activity resides in the cytosolic compartment, its assay in vitro requires the presence of nonionic detergent for maximal activity (29). This is consistent with the probability that cytosolic DT-diaphorase may interact with membrane components located near the membrane—cytosolic interface, similar to a recently discovered (49) liver cytosolic factor involved in the regeneration, by cytosolic ascorbate, of membrane-bound vitamin E from the \( \alpha \)-tocopheroxy radical (7) and a NADH–CoQ reductase recently identified in liver plasma membrane (50). The results reported here also support the suggestion (23) that DT-diaphorase was selected during evolution to act as a CoQ reductase to protect cellular membrane components from free radical damage.

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### Table 2. Effect of dic on protection of hepatocytes by coenzyme Q\textsubscript{10} against adr-induced oxidative damage

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Adr</th>
<th>Adr + CoQ\textsubscript{10} + Dic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane permeability*</td>
<td>233.1 ± 25.2</td>
<td>55.8 ± 6.0</td>
<td>163.0 ± 18.7</td>
</tr>
<tr>
<td>Peroxide production(a)</td>
<td>17.8 ± 3.2</td>
<td>207.9 ± 5.8</td>
<td>28.2 ± 6.1</td>
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<tr>
<td>Cytosolic DT-diaphorase*</td>
<td>648 ± 116</td>
<td>525 ± 141</td>
<td>475 ± 116</td>
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<tr>
<td>CoQ\textsubscript{H2}</td>
<td>181.9 ± 22.9</td>
<td>460.2 ± 37.8</td>
<td>281.9 ± 22.6</td>
</tr>
<tr>
<td>CoQ\textsubscript{H2}</td>
<td>545.7 ± 32.2</td>
<td>621.8 ± 25.7</td>
<td>445.7 ± 28.2</td>
</tr>
<tr>
<td>CoQ\textsubscript{H2}</td>
<td>30.6 ± 11.7</td>
<td>145.6 ± 32.1</td>
<td>750.4 ± 72.2</td>
</tr>
<tr>
<td>CoQ\textsubscript{H2}</td>
<td>94.6 ± 21.3</td>
<td>ND</td>
<td>403.6 ± 34.3</td>
</tr>
</tbody>
</table>

*Mitochondrial membrane potential assayed by RH-123 uptake in arbitrary fluorescence units.
\(a\)nmol per 10\(^6\) cells determined according to ref. 41.
\(b\)Activity as nmol per min per mg of cytosolic protein.

ND, not detectable.

Fig. 3. CoQ\textsubscript{H2} content (---) and TBARS formation (····) in MLVs subjected to lipid peroxidation induced by AMVN at 37°C in the presence of NADH and DT-diaphorase (●, ●) and in the absence of NADH (○, ○). (A) CoQ\textsubscript{H2}-containing MLVs. (B) CoQ\textsubscript{10H2}-containing MLVs. NADH, lecithin, and DT-diaphorase concentrations are as in Fig. 1.


