Autoxidation of Ubiquinol-6 Is Independent of Superoxide Dismutase†

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Received January 31, 1996; Revised Manuscript Received March 27, 1996⊗

ABSTRACT: Ubiquinone (Q) is an essential, lipid soluble, redox component of the mitochondrial respiratory chain. Much evidence suggests that ubiquinol (QH_2) functions as an effective antioxidant in a number of membrane and biological systems by preventing peroxidative damage to lipids. It has been proposed that superoxide dismutase (SOD) may protect QH_2 from autoxidation by acting either directly as a superoxide−semiquinone oxidoreductase or indirectly by scavenging superoxide. In this study, such an interaction between QH_2 and SOD was tested by monitoring the fluorescence of *cis*-parinaric acid (cPN) incorporated phosphatidylcholine (PC) liposomes. Q_6H_2 was found to prevent both fluorescence decay and generation of lipid peroxides (LOOH) when peroxidation was initiated by the lipid-soluble azo initiator DAMP, dimethyl 2,2′-azobis (2-methylpropionate), while Q_6 or SOD alone had no inhibitory effect. Addition of either SOD or catalase to Q_6H_2-containing liposomes had little effect on the rate of peroxidation even when incubated in 100% O_2. Hence, the autoxidation of QH_2 is a competing reaction that reduces the effectiveness of QH_2 as an antioxidant and was not slowed by either SOD or catalase. The *in vivo* interaction of SOD and QH_2 was also tested by employing yeast mutant strains harboring deletions in either CuZnSOD and/or MnSOD. The *sod* mutant yeast strains contained the same percent Q_6H_2 per cell as wild-type cells. These results indicate that the autoxidation of QH_2 is independent of SOD.

Coenzyme Q[1] or ubiquinone (Q) is an essential lipid-soluble electron transport component of the respiratory chain located in the inner mitochondrial membrane (Brandt & Trumpower, 1994). Much *in vitro* evidence supports the idea that reduced coenzyme Q (QH_2) acts as an effective antioxidant in membrane systems by scavenging lipid peroxyl radicals (Mellors & Tappel, 1966; Stocker et al., 1991; Frei et al., 1990; Ernster & Forsmark-Andree, 1993; Ernster & Dallner, 1995). QH_2 performs this function through the redox transitions of the benzoquinone ring. In this capacity, one molecule of QH_2 may reduce two lipid peroxyl radicals, and it either may act independently of α-tocopherol (Forsmark et al., 1991; Forsmark-Andree et al. 1995) or may act to regenerate α-tocopherol by reducing the α-tocopheroxyl radical (Kagan et al., 1990; Stoyanovsky et al., 1995; Cabrini et al,. 1991; Ernster & Dallner, 1995).

$$QH_2 + 2LOO^{\bullet} \rightarrow Q + 2LOOH \quad (1)$$

$$QH_2 + 2\alpha\text{-}TO^{\bullet} \rightarrow Q + 2\alpha\text{-}TOH \quad (2)$$

Subcellular fractionation studies show that QH_2 is present in other intracellular locations such as microsomes (Takada et al., 1982), Golgi, lysosomes, peroxisomes, and plasma membranes (Kalen et al., 1987). QH_2 may function primarily as an antioxidant in these membrane regions by inhibiting lipid peroxidation or protein carboxylation (Aberg et al., 1992; Forsmark-Andree et al., 1995). Alternatively, there is evidence of participation in the plasma membrane electron transport chain, growth control, and regulation of membrane secretion (Crane et al., 1993, Sun et al., 1992).

In order to understand how QH_2 acts as an antioxidant, it is important to determine how QH_2 is maintained in the reduced form in the cell. It is clear that Q is reduced to QH_2 in the mitochondria by several membrane bound enzymes including various electron transport chain dehydrogenases, and also other distinct systems including pyrimidine dihydroorotate dehydrogenase [EC 1.99.11] (Nagy et al., 1992; Jones, 1980), the fatty acid β oxidation enzyme electron-transferring flavoprotein:ubiquinone reductase [EC 1.5.5.1] (Frerman, 1988), and perhaps also the NADPH dehydrogenase (quinone) [EC 1.6.99.6] (Frei et al., 1986). In addition, there are other systems that act to maintain QH_2 in the reduced form outside of the mitochondria. These systems either operate as quinone reductases (Villalba et al., 1995; Navarro et al., 1995; Takahashi et al., 1995; Stocker & Suarna, 1993) or are thought to slow the autoxidation of QH_2 (Beyer, 1992).

QH_2 autoxidation proceeds through an initial generation of the semiquinone and superoxide (eq 3) which is a slow reaction at physiological pH (Sugioka et al., 1988). Once the semiquinone is present, it can react rapidly with dioxygen, due to favorable redox potential, and generate more superoxide (eq 4) (Ingold et al., 1993). If the superoxide reacts rapidly with the hydroquinone (eq 5), then a chain reaction ensues, autoxidizing QH_2 to yield Q plus H_2O_2, with superoxide as the chain carrier (Frei et al., 1990; Ernster &

† This work was first presented at the American Society for Biochemistry and Molecular Biology Annual Meeting (Schultz et al., 1995). This study was supported by NIH Grants GM45952 (C.F.C.) and GM46828 and DK46828 (J.S.V.).

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⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations: cPN, *cis*-parinaric acid; CumOOH, cumene hydroperoxide; ECD, electrochemical detection; DAMP, dimethyl 2,2′-azobis(2-methylpropionate); HPLC, high performance liquid chromatography; LOOH, lipid peroxides; Q, oxidized ubiquinone; Q_6, ubiquinone-6; QH_2, ubiquinol/reduced ubiquinone; Q_6H_2, ubiquinol-6; PC, phosphatidylcholine; SOD, superoxide dismutase.

S0006-2960(96)00245-0 CCC: $12.00

Beyer, 1991). Equation 6 results from the sum of eqns 4 and 5.

$$QH_2 + O_2 \rightarrow QH^{\bullet} + O_2^{\bullet -} + H^+ \quad (3)$$

$$QH^{\bullet} + O_2 \rightarrow Q + O_2^{\bullet -} + H^+ \quad (4)$$

$$QH_2 + O_2^{\bullet -} + H^+ \rightarrow QH^{\bullet} + H_2O_2 \quad (5)$$

$$QH_2 + O_2 \rightarrow Q + H_2O_2 \quad (6)$$

$$O_2^- + O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (7)$$

This type of mechanism is well-documented for several hydroquinones and is frequently used as the basis for superoxide dismutase assays including the 6-hydroxydopamine assay (Heikkila & Cabbat, 1976). In such assays, the introduction of low levels of SOD to solution containing hydroquinone is found to inhibit the autoxidation of hydroquinone by removing the superoxide (eq 7) and hence preventing the chain carried autoxidation reactions (4) and (5) (Fridovich, 1982; Heikkila & Cohen, 1973; Bandy et al., 1990). Alternatively, it has been postulated that superoxide dismutase [EC 1.15.1.1] (SOD) may protect quinols from autoxidation by functioning as a superoxide−semiquinone oxidoreductase (eq 8) (Cadenas et al., 1988, 1992). However, the direct reduction of $QH^{\bullet}$ by SOD seems unlikely, since the crystal structure of SOD indicates the presence of a narrow cleft in the active site channel which restricts access to superoxide and other ions and competitive inhibitors smaller than 4 Å (Tainer et al., 1983; Getzoff et al., 1983).

$$QH^{\bullet} + O_2^{\bullet -} + H^+ \rightarrow QH_2 + O_2 \quad (8)$$

In either case, the proposed ability of SOD to protect QH_2 from autoxidation would result in the maintenance of QH_2 levels and may offer an explanation for the chemical basis of superoxide toxicity. In this study, the interaction of SOD with QH_2 was tested both *in vitro* and *in vivo*. Phosphatidylcholine (PC) liposomes containing *cis*-parinaric acid (cPN) were employed to test the ability of SOD to enhance Q_6H_2 protection of lipid peroxidation. cPN fluoresces when incorporated into membranes, and the loss in fluorescence is used as an indirect indicator of lipid peroxidation to study the properties of a number of antioxidants, including QH_2 (van den Berg et al., 1990, 1994; Tsuchiya et al., 1994; Kagan et al., 1994). In such systems, the onset of lipid peroxidation is not observed until QH_2 is oxidized (Tribble et al., 1994). In addition, the possible interaction of QH_2 and SOD was studied *in vivo* by making use of yeast mutant strains lacking SOD (Gralla & Valentine, 1991; Lui et al., 1992). These mutant strains were studied to determine whether the relative percentage of QH_2 was maintained in *sod* deletion mutants.

MATERIALS AND METHODS

Chemicals. Ubiquinone-6 (Q_6), bovine CuZnSOD (SOD), bovine liver catalase, *Escherichia coli* manganese superoxide dismutase, and soybean phosphatidylcholine (PC) were purchased from Sigma (St. Louis, MO). DAMP was a generous gift of Wako Chemicals USA, Inc. (Richmond, VA). cPN was from Molecular Probes, Inc. (Eugene, OR). All other chemicals used were of the highest grade available.

Liposome Preparation and Fluorescence Assay. Liposomes were prepared by an adaptation of previously reported protocols (Cipollone et al., 1994; Frei et al., 1990). An aliquot of a PC stock solution (125 mM in chloroform) was mixed with 2 mL of methanol and Q_6H_2 (0.3−144 μM), when present, and dried by rotary evaporation. The thin film was resuspended in 1.25 mL of phosphate buffered saline (PBS: 1.407 M NaCl/12 mM NaH_2PO_4/81 mM Na_2HPO_4, pH 7.4) by vortexing for 3 min, and unilammelar vesicles were formed by extrusion using the LiposoFast of Avestin, Inc. (Ottawa, Canada) (MacDonald et al., 1991). This unilamellar liposome preparation was diluted with PBS to a final concentration of 600 μM. To remove trace metal ions, PBS was treated with Chelex 100 (100−200 mesh, sodium form) from Bio-Rad. cPN was added in ethanol to the liposomes to a final concentration of 1 μM, and liposomes were kept on ice. Fluorescence of cPN added to the 2 mL liposome aliquots containing 500 μM PC was optimal at 1 μM and was linear to 2 μM cPN (data not shown). The PC concentration provides maximal fluorescence in the assay at 600 μM as determined by titrating various concentrations of PC and measuring the level of fluorescence generated with 1 μM cPN (data not shown). PC aliquots (3 mL) were warmed to 37 °C for 6 min, and lipid peroxidation was initiated by addition of either 25 μM $CuSO_4$, 1.93 mM cumene hydroperoxide (CumOOH), or both $CuSO_4$ and CumOOH. Fluorescence was measured at regular intervals in a Kontron Instruments SFM25 spectrofluorometer equipped with a thermostated multiple cuvette holder and a magnetic stirring device, with excitation and emission wavelengths set at 324 nm (slit width 10 nm) and 413 nm (slit width 10 nm) as previously described for PC and cPN analysis (Tribble et al., 1994). The percent relative fluorescence was determined after subtraction of background levels of lipid fluorescence without cPN. Results represent at least three separate experiments.

Q_6 was reduced for the liposome experiments as described by Cipollone et al. (1994). A 5 mg sample of Q_6 in 2.5 mL of ethanol was combined with 15 mL of a solution of 0.1 M phosphate, pH 7.4, 0.2 M sucrose, and 0.2 g of sodium dithionite, and the mixture was vortexed for 1 min. Q_6H_2 was extracted by addition of 5 mL of cyclohexane to the solution, followed by vortexing for 1 min and centrifugation for 5 min. The upper cyclohexane layer was dried down by rotary evaporation, resuspended in acidic ethanol, and stored at 4 °C. This Q_6H_2 preparation was stable up to 1 month.

To test for a correlation between fluorescence and lipid peroxidation, PC liposomes containing cPN and either 140, 5, or 0.3 μM Q_6 or Q_6H_2 were prepared as previously described. Liposome aliquots (3 mL) were warmed to 45 °C for 6 min, peroxidation was initiated by addition of the azo initiator DAMP in ethanol, and fluorescence was measured at regular intervals. Time point 0 was taken prior to DAMP addition. Sample aliquots (330 μL) were taken at 0, 30, 60, and 90 min to determine lipid peroxides (LOOH) by the FOX assay (Jiang et al., 1992). A 100 μL sample aliquot was combined with 900 μL of 90% methanol, containing 125 μM xylenol orange, 250 μM ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)\cdot 6H_2O$), 25 mM H_2SO_4, and 4 mM butylated hydroxytoluene, and incubated at room temperature in the dark for 30 min, and absorbance at 562

Table 1: Genotype and Sources of *Saccharomyces cerevisiae* Strains Used in Antioxidant Assays

strain	genotype	source
EG103	α *leu2-3,112 his3D1 trp1-289a ura3-52*	Gralla & Valentine, 1991
EG118	EG103-*sod1*Δ*::URA3*	Liu et al., 1992
EG119	EG103-*sod1*Δ*::URA3*	this study
CC118	EG103-*sod1*Δ*::URA*3	this study
EG110	EG103-*sod2*Δ*::TRP1*	Liu et al., 1992
EG133	EG103-*sod1*Δ*::URA3*, *sod2*Δ*::TRP1*	Liu et al., 1992

nm was measured with a Kontron Instruments DB-3500 UV/vis spectrophotometer. The amount of LOOH present in PC samples was determined from a hydrogen peroxide standard curve (hydroperoxide $\Sigma = 2.8 \times 10^4\ M^{-1}\ cm^{-1}$ in methanol at 233 nm; Jiang et al., 1992). LOOH values represent an average of three measurements per time point in each experiment, and all results are an average of three separate experiments.

The effect of SOD on Q_6H_2 was tested by addition of SOD to liposomes containing Q_6H_2 prior to DAMP peroxidation initiation, under normoxia and hyperoxia conditions. PC liposomes plus or minus 0.3 μM Q_6 or 0.3 μM Q_6H_2 were prepared, and 3 mL aliquots of liposomes were combined with either PBS buffer, CuZnSOD, MnSOD, or catalase [EC 1.11.1.6] in 10 mL glass tubes and vortexed, and 100% O_2 was bubbled through the samples for 1 min, followed by incubation at 37 °C in a shaking water bath. The samples were tested in the cPN/DAMP assay to assess the ability of SOD to slow Q_6H_2 autoxidation at time 0, 1, 2, or 4 h. The samples were transferred to cuvettes, cPN was added, and aliquots were warmed to 45 °C in the fluorometer for 3 min. DAMP was then added to initiate peroxidation, and 330 μL aliquots were taken from the samples before DAMP initiation to determine whether lipid peroxidation was initiated and chain-carried by O_2 during incubation. Another 330 μL aliquot was taken at 90 min to measure LOOH generated during the cPN/DAMP assay. LOOH were determined using the FOX assay (Jiang et al., 1992), and values represent an average of 3 measurements per time point.

Yeast Ubiquinol Determination. Mutant strains of *Saccharomyces cerevisiae* are described in Table 1. The *sod1* null mutation (*sod1*Δ::URA3) was introduced into EG103 to generate EG119 and CC118 as previously described (Liu et al., 1992). To determine the relative percentage of Q_6H_2 present in *sod* mutant strains, yeast were grown in 100 mL of liquid cultures of 1% bacto yeast extract, 2% bacto peptone, and either 2% dextrose, glycerol, ethanol, or lactate at 30 °C overnight (YPD) to an OD_{600} 12−18 or for 2 days (YPG, YPE, YPL) to an OD_{600} 1.0−1.5. Cultures were harvested and an equal number of cells per strain lysed by vortexing for 2 min with 4 volumes of glass beads (425−600 μm) per yeast cell pellet in 5 mL of buffer (0.24 M sorbitol, 0.30 M mannitol, and 12 mM Tris-HCl, pH 7.4). Lipids were extracted into 18 mL of methanol and 12 mL of petroleum ether (Aberg et al., 1992) by vortexing the glass bead pellet for 30 s in 50 mL borosilicate glass tubes with PTFE lined caps, and the organic and aqueous layers were separated by a 5 min spin at 2000 rpm with an IEC Centra-7R tabletop centrifuge. A 9 mL aliquot of the upper petroleum ether layer was concentrated under N_2 and analyzed by reversed-phase high performance liquid chromatography (HPLC) and electrochemical detection (ECD). Chromatography was performed with a silica C18 column (Econosphere 5-μm, 4.6 × 250 mm, Alltech, Deerfield, IL) and a Waters HPLC/ECD system composed of two M-45 pumps, a 441 absorbance detector, a 464 pulsed electrochemical detector with a glassy carbon working electrode at 0.5 V, and a 720 system controller. Data were collected using a Shimadzu C-R3A chromatopac. The mobile phase contained methanol/reagent alcohol (95:5) and 20 mM lithium perchlorate at a flow rate of 1 mL/min (Lang et al., 1986). Q_6H_2 was quantitated directly from the ECD results, using an external standard of Q_6H_2 generated by reduction with $^1/_{10}$ volume 2.5% $NaBH_4$ and incubated on ice for 35 min in the dark (Motchnik et al., 1994). Q_6 was quantitated as the increase of the Q_6H_2 peak after sample treatment with $NaBH_4$. Statistical significance was determined using Student's *t* test, and paired differences were considered significant if $p < 0.05$.

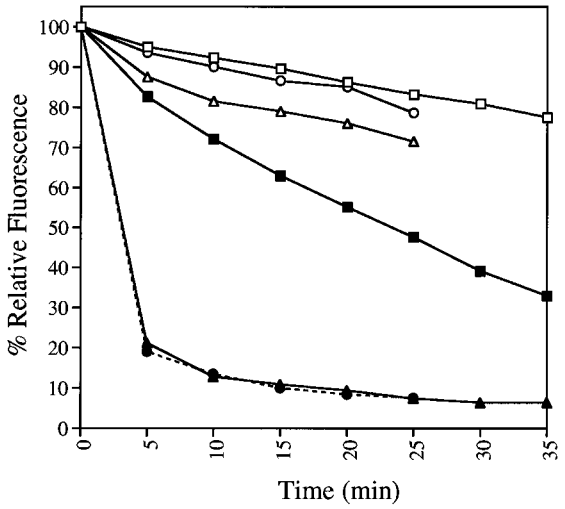

FIGURE 1: $CuSO_4$ catalyzes Q_6H_2 autoxidation in cPN-containing phosphatidylcholine liposomes. Unilamellar PC (600 μM) liposomes containing PBS, pH 7.3, with (●, ▲, ○, △) or without (□, ■) 144 μM Q_6H_2 were prepared. cPN was added to a final concentration of 1 μM, and lipid peroxidation was either not initiated (□) or initiated by addition of 25 μM $CuSO_4$ (●), 1.93 mM cumene hydroperoxide (CumOOH) (△), or both CuSO4 and CumOOH (■, ▲). Fluorescence was measured at regular intervals; % relative fluorescence was determined after subtraction of background fluorescence. Results are representative of at least three separate experiments.

RESULTS

Q_6H_2 Functions as a Pro-oxidant with Cu^{2+}. The ability of Q_6H_2 to function as an antioxidant was originally tested by initiating lipid peroxidation with Cu^{2+}/CumOOH. As shown in Figure 1, addition of both Cu^{2+} and CumOOH to PC liposomes is required for peroxidation and is observed as a decrease in cPN fluorescence. When Cu^{2+} or Cu^{2+} and CumOOH were added to liposomes containing Q_6H_2, a greater than 80% decay in fluorescence was observed within the first 5 min, and is due to the addition of Cu^{2+} to the system. This loss of fluorescence correlates to an increase in lipid peroxidation products (data not shown). These results suggest that $CuSO_4$ catalyzes Q_6H_2 autoxidation in cPN-containing phosphatidylcholine liposomes since Q_6H_2 does not protect against peroxidation as expected. Fluorescence decay of PC alone is not significantly enhanced by the sole addition of either $CuSO_4$ or CumOOH (data not

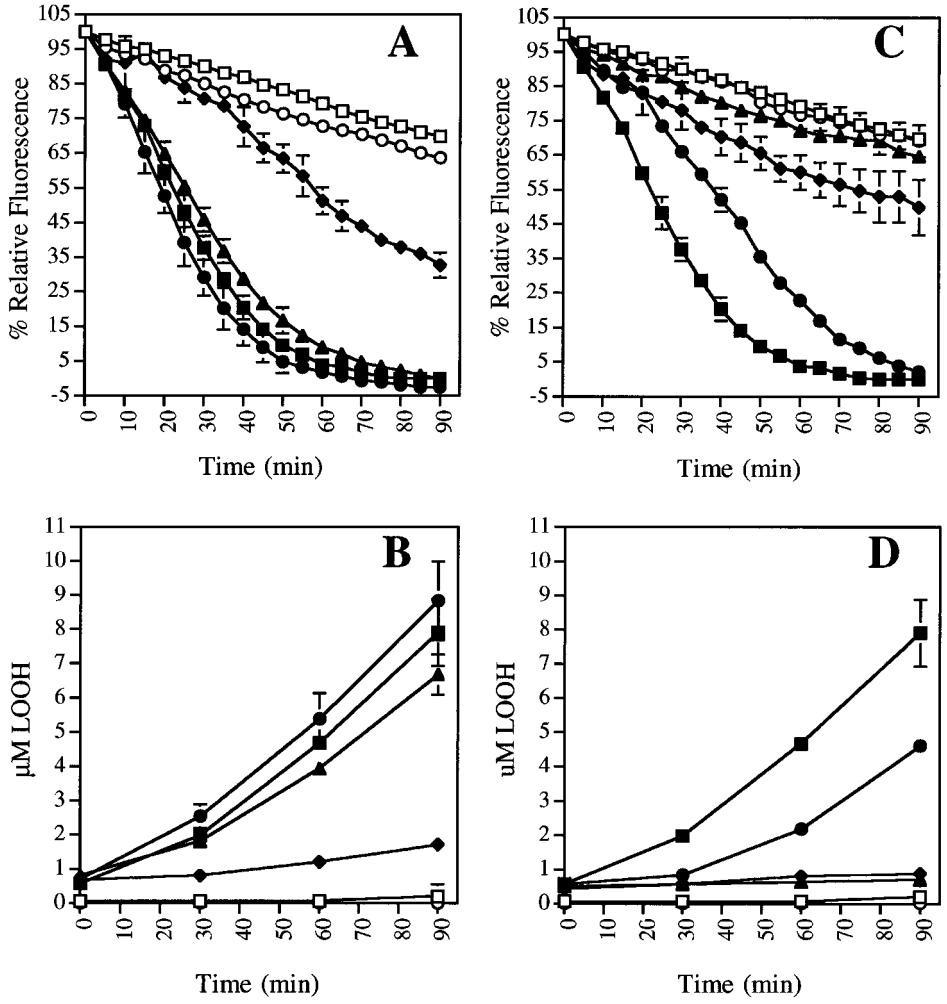

FIGURE 2: The cPN fluorescence decay correlates with a concomitant increase in DAMP-generated lipid peroxides and can be slowed by Q_6H_2. Liposomes were prepared as in Figure 1 and contained either Q_6 (panels A and B) or Q_6H_2 (panels C and D) at the designated concentrations: 140 μM (♦), 5 μM (▲), 0.3 μM (○, ●), and control liposomes without Q (□, ■). Peroxidation was initiated by addition of 1 mM DAMP (closed symbols) and compared to controls (open symbols). Fluorescence was measured at regular intervals (panels A and C). Aliquots (330 μL) were taken at 0, 30, 60, and 90 min and used to measure lipid peroxides by the FOX assay (panels B and D). LOOH values represent an average of three measurements per time point in each experiment, and all results are an average of three separate experiments.

shown). In this system it is evident that Q_6H_2 in the presence of Cu^{2+} acts as a pro-oxidant, generating more peroxidation than CumOOH and Cu^{2+}, due to Fenton or Habor−Weiss reactions with a reducing agent and free metal (Beyer, 1994; Halliwell & Gutteridge, 1989).

In Vitro/DAMP Initiated Peroxidation Assays. Lipid peroxidation is also induced by azo initiators which decay upon heating to generate N_2 and a carbon centered radical at a known and consistent rate. Azo initiators have been used to study both hydrophilic and lipophilic antioxidants, including QH_2 (Ingold et al., 1993; Niki, 1990; Stocker et al., 1991; Frei et al., 1990). Upon addition of DAMP to PC liposomes, a continual decrease in fluorescence is observed due to oxidation of cPN (Figure 2, panels A and C), and this decay in fluorescence corresponds to a concomitant generation of LOOH (Figure 2, panels B and D). When 0.3 or 5 μM Q_6 was added to the liposomes, the rate of fluorescence decay was similar to the oxidative control, indicating that Q_6 does not act as an antioxidant at these concentrations (Figure 2A). Liposomes containing 0.3 μM Q_6H_2 showed a significant decrease in the rate of peroxidation, compared to control and Q_6 liposomes, reflecting the ability of Q_6H_2 to act as an antioxidant and inhibit lipid peroxidation (Figure 2, panels C and D). The observed protection was transient, indicating that Q_6H_2 is oxidized over the course of the experiment. A higher concentration of Q_6H_2 (5 μM) completely inhibits peroxidation since the fluorescence decay follows that of PC without DAMP, further supporting the potency of Q_6H_2 as an antioxidant. Curiously, although addition of 144 μM Q_6H_2 slows peroxidation, the level of protection is less than that observed at 5 μM Q_6H_2, and 144 μM Q_6 protects against peroxidation with a similar rate of fluorescence decay as observed for the same concentration of Q_6H_2. Together, these results indicate that cPN fluorescence decay correlates with a concomitant increase in DAMP-generated lipid peroxides and can be slowed by Q_6H_2 and that Q_6H_2 functions as a potent antioxidant and protects against DAMP-initiated lipid peroxidation in this system. Since 0.3 μM represents the physiological ratio of 1/2000 oxidized and reduced Q to phospholipids in human tissues (Frei et al., 1990) and demonstrates a transient protection over time, this concentration was used to test whether SOD protects QH_2 from autoxidation.

To determine the ability of SOD to inhibit the autoxidation of Q_6H_2, SOD was added to Q_6H_2-containing liposomes and

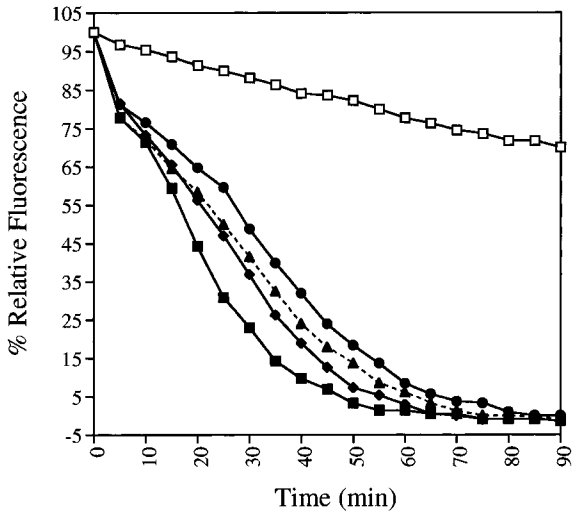

FIGURE 3: Addition of SOD does not slow the rate of cPN fluorescence decay in liposomes containing Q_6H_2. The experimental design is the same as described in Figure 2. PC liposomes containing 0.30 μM Q_6H_2 (●, ▲, ♦) were prepared; 150 μg/mL (▲) or 0.15 μg/mL (♦) SOD was added; and peroxidation was initiated by addition of 1 mM DAMP. Levels of fluorescence were compared to control liposomes with (■) and without (□) DAMP. Addition of either 15 or 1.5 μg/mL SOD to Q_6H_2 liposomes showed similar rates of fluorescence decay as addition of 150 or 0.15 μg/mL SOD (data not shown).

rate of fluorescence decay was followed over time (Figure 3). If SOD slows Q_6H_2 autoxidation, then the rate of fluorescence decay and lipid peroxidation should be slower than the decay rate for liposomes containing only Q_6H_2. Liposomes with added SOD showed the same rate of decay as liposomes alone, indicating that SOD has no adverse effects on the assay (data not shown). When SOD was added to Q_6H_2 liposomes, SOD consistently caused a slight increase in the fluorescence decay of the samples independent of concentration and enhanced the rate of cPN peroxidation in the presence of Q_6H_2 without slowing Q_6H_2 autoxidation, suggesting that SOD is unable to protect Q_6H_2 from autoxidation.

Peroxidation in phospholipid membranes will increase the hydrophilicity of the membrane and generate conditions favoring quinol autoxidation through superoxide (Nohl, 1986). It is possible, however, in the experimental protocol presented in Figure 3, that Q_6H_2 could be oxidized by DAMP without generation of superoxide. Bowry and colleagues recently concluded that, in QH_2-depleted LDL, peroxidation initiated by the lipid-soluble azo initiator AMVN did not proceed through superoxide since addition of SOD did not influence the rate of LOOH generation (Bowry et al., 1995). Azo initiators react to generate lipid peroxyl radicals upon heating which initiate lipid peroxidation (eqs 9−11) and chain carry peroxidation to cPN (Ingold et al., 1993), resulting in a continued propagation of peroxyl radicals through lipid radicals and a loss in fluorescence (eqs 9−14).

$$\mathrm{RCN{=}NCR} \rightarrow 2\mathrm{RC}^{\bullet} + \mathrm{N_2} \quad (9)$$

$$\mathrm{RC}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{RCOO}^{\bullet} \quad (10)$$

$$\mathrm{RCOO}^{\bullet} + \mathrm{LH} \rightarrow \mathrm{RCOOH} + \mathrm{L}^{\bullet} \quad (11)$$

Since QH_2 can chain terminate peroxidation reactions as described in reactions 1 and 2, it could quench cPN peroxidation in a similar manner, allowing the reaction sequence to proceed without superoxide production (eqs 15−17).

$$\mathrm{L}^{\bullet} + \mathrm{O_2} \rightarrow \mathrm{LOO}^{\bullet} \quad (12)$$

$$\mathrm{LOO}^{\bullet} + \mathrm{LH} + \rightarrow \mathrm{LOOH} + \mathrm{L}^{\bullet} \quad (13)$$

$$\mathrm{LOO}^{\bullet} + \mathrm{cPN} \rightarrow \mathrm{cPN\text{-}OO}^{\bullet} + \mathrm{L}^{\bullet} \quad (14)$$

$$\mathrm{cPN\text{-}OO}^{\bullet} + \mathrm{QH_2} \rightarrow \mathrm{cPN\text{-}OOH} + \mathrm{QH}^{\bullet} \quad (15)$$

$$\mathrm{cPN\text{-}OO}^{\bullet} + \mathrm{QH}^{\bullet} \rightarrow \mathrm{cPN\text{-}OOH} + \mathrm{Q} \quad (16)$$

$$\mathrm{QH}^{\bullet} + \mathrm{QH}^{\bullet} \rightarrow \mathrm{QH_2} + \mathrm{Q} \quad (17)$$

To ensure that superoxide is generated in the reaction system, liposomes containing Q_6H_2, Q_6, or PBS were incubated, with either SOD or catalase, in 100% O_2 at 37 °C for 4 h, and the ability of Q_6H_2 to protect cPN against peroxidation was assessed at 0, 1, 2, and 4 h. Hyperoxia conditions are known to induce formation of reactive oxygen species, including superoxide (Gardner et al., 1994). LOOH were measured at each time point before the cPN protection assay to determine whether lipid peroxidation was initiated and chain-carried during O_2 incubation, and again at 90 min to measure the extent of peroxidation incurred during the cPN assay. Figure 4A indicates that, at 0 h incubation, liposomes containing Q_6H_2 or Q_6H_2 in the presence of SOD show similar levels of fluorescence during the cPN assay, as was observed in Figure 3. The addition of catalase to the liposomes did not affect lipid peroxidation, since the rate of fluorescence decay was the same as that observed upon addition of SOD (data not shown). Control liposomes, or liposomes containing Q_6, SOD, or catalase, exhibit fluorescence decay, and this rate is increased compared to samples containing Q_6H_2. After 1 h O_2 incubation (Figure 4B), the rate of fluorescence decay for the samples containing Q_6H_2 is markedly less than at the 0 h time point due to a reduced ability of Q_6H_2 to protect cPN against peroxidation, indicating the autoxidation of Q_6H_2 in the presence of O_2. Q_6H_2 is fully oxidized within 2 h (Figure 4C), as indicated by the same rate of fluorescence decay for samples containing either Q_6H_2, Q_6, or no Q; however, increased levels of LOOH were detected at 4 h even though Q_6H_2 was unable to protect cPN fluorescence after 2 h in 100% O_2 (Figure 4C and Table 2). SOD was unable to slow Q_6H_2 oxidation as there is no observable difference in peroxidation over time in the presence or absence of SOD. Similar results were obtained when *E. coli* MnSOD was tested under the same conditions (data not shown).

Lipid peroxidation products associated with the liposomes studied in Figure 4 were quantified by the FOX assay. As indicated in Table 2, levels of LOOH increased concomitant to the length of exposure to 100% O_2. It is likely that the peroxidation reactions are initiated and chain carried by superoxide since various reactive oxygen species are generated under hyperoxia conditions (Gardner et al., 1994). LOOH detected were the same for both control and Q_6 liposomes over the time course of the experiment, while Q_6H_2 samples showed consistently lower levels of LOOH. After a 22 h incubation, the rate of fluorescence decay for all samples tested was the same as shown in Figure 4C, and

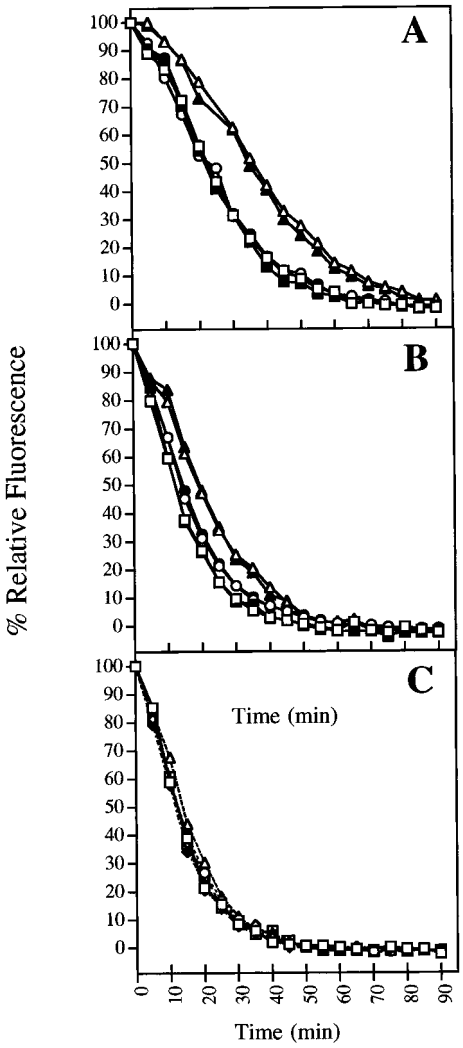

FIGURE 4: SOD is unable to prevent Q_6H_2 autoxidation when liposomes are incubated in 100% O_2 at 37 °C. Liposomes were prepared with (closed symbols) or without (open symbols) SOD and contained either 0.3 μM Q_6H_2 (△, ▲); 0.3 μM Q_6 (○, ●); or no Q (□, ■). Samples were treated with 100% O_2 as described under Materials and Methods, and following incubation for 0 h (panel A), 1 h (panel B), or 2 h (panel C), samples were tested in the cPN/DAMP assay as described in Figure 2. Aliquots (330 μL) were taken at 0 and 90 min to measure LOOH generated during the cPN/DAMP assay (Table 2).

LOOH levels of Q_6H_2 liposomes were equal to the control liposomes, indicating that Q_6H_2 can be fully oxidized (data not shown). When SOD and catalase were both added to liposomes, no protection of Q_6H_2 was observed in either assay (data not shown). Q_6H_2 most likely delays the onset of LOOH formation, and once Q_6H_2 is depleted, the rate of LOOH formation is the same. As Table 2 indicates, the LOOH levels in liposomes with or without Q_6H_2 increase by approximately 2-fold between 2 and 4 h of oxygen exposure. Together, these results suggest that, during this incubation period, neither SOD nor catalase acts to "protect" Q_6H_2 under high oxidizing conditions and *in vitro* there is no apparent interaction between Q_6H_2 and SOD.

In Vivo Assays. The ability of SOD to inhibit the autoxidation of Q_6H_2 *in vivo* was tested in yeast mutant strains lacking SOD and compared with the isogenic wild-type parental strain. If Q_6H_2 autoxidation is inhibited by SOD, then *sod* deletion mutants might be expected to contain a decreased amount of Q_6H_2. Yeast strains containing

Table 2: Lipid Peroxides Generated from cPN/DAMP Time Course in 100% O_2 Determined by the FOX LOOH Assay[a]

	preincubation (h)							
	μM LOOH (0 min)				μ LOOH (90 min)			
sample	0	1	2	4	0	1	2	4
PC	2.8	6.3	12.3	19.2	10.8	16.6	21.1	21.4
PC + SOD	3.0	6.7	14.8	21.2	12.2	17.5	22.1	23.0
PC + catalase	2.4	5.4	12.9	20.5	10.5	16.1	20.1	22.0
PC + Q_6	1.1	6.9	10.4	18.4	9.1	15.5	19.1	22.7
PC + Q_6 + SOD	1.1	7.2	11.4	19.9	9.9	15.3	19.8	23.9
PC + Q_6 + catalase	0.4	5.6	9.7	18.2	9.9	13.9	17.3	23.0
PC + Q_6H_2	1.4	3.2	6.4	11.7	7.3	12.1	14.1	17.8
PC + Q_6H_2 + SOD	1.4	3.3	6.1	14.1	8.0	13.0	14.3	18.6
PC + Q_6H_2 + catalase	0.7	2.3	6.4	13.2	7.0	12.0	13.9	18.2

[a] PC liposomes containing 1 μM cPN, 1 mM DAMP, either 0.30 μM Q_6 or Q_6H_2, and 1.5 μg/mL SOD or catalase were prepared, treated with 100% O_2 for 1 min, and subjected to preincubation, and LOOH were determined at various time points as described in Materials and Methods. Values indicate the amount of LOOH present in 100 μL of sample tested, at either 0 min or 90 min following addition of DAMP, and are an average of three separate measurements made from the corresponding time points in Figure 4.

deletions in either *SOD1* (encoding Cu,ZnSOD) or *SOD2* (encoding MnSOD) or deletions in both genes were grown, lipids were extracted, and the relative percent Q_6H_2 was determined with HPLC-coupled electrochemical detection. When strains were grown in the fermentable carbon source dextrose, wild-type yeast (EG103) maintained 80% of total Q (Q and QH_2) as QH_2 (Figure 5A). The *sod* mutant strains contained approximately the same relative percent Q_6H_2 compared to the wild-type strain, 68% to 82%, respectively. Thus, Q_6H_2 levels are maintained near normal levels even in the complete absence of SOD1 and/or SOD2. However, when *sod* mutant strains were grown in glycerol, a nonfermentable carbon source, *sod1* strains were found to contain a small, but significantly lower percentage Q_6H_2 (58% in *sod1* versus 85% wild-type; Figure 5B). Similar results were also observed from strains grown in either ethanol or lactate (data not shown). The decrease in percent Q_6H_2 in *sod1* mutant yeast grown under oxidative conditions implies that diminished Q_6H_2 levels may result from the imposed oxidative stress and that inhibiting Q_6H_2 autoxidation is not a major role of SOD *in vivo*.

DISCUSSION

Many *in vitro* studies show QH_2 to act as a very effective lipid-soluble antioxidant, although the effectiveness of QH_2 may be compromised by its tendency to autoxidize rapidly. The autoxidation of QH_2 is considered to proceed through chain-carried reactions with superoxide (Frei et al., 1990; Ernster & Beyer, 1991; Stoyanovsky et al., 1995). The superoxide, chain-carried autoxidation of aqueous-soluble hydroquinones (such as 6-hydroxydopamine) forms the basis of an extremely sensitive assay for SOD activity, in which the addition of a small amount of SOD may be observed to completely inhibit hydroquinone autoxidation. It has been proposed that SOD may function to inhibit QH_2 autoxidation. While the inhibition of small aqueous-soluble hydroquinone autoxidation by SOD is well documented, it is not clear whether SOD could protect a lipid-soluble hydroquinone such as QH_2 from similar autoxidation; and the autoxidation rate per se may depend on the orientation of Q in the membrane.

In the current study, the ability of SOD to inhibit autoxidation and maintain high levels of ubiquinol-6 was

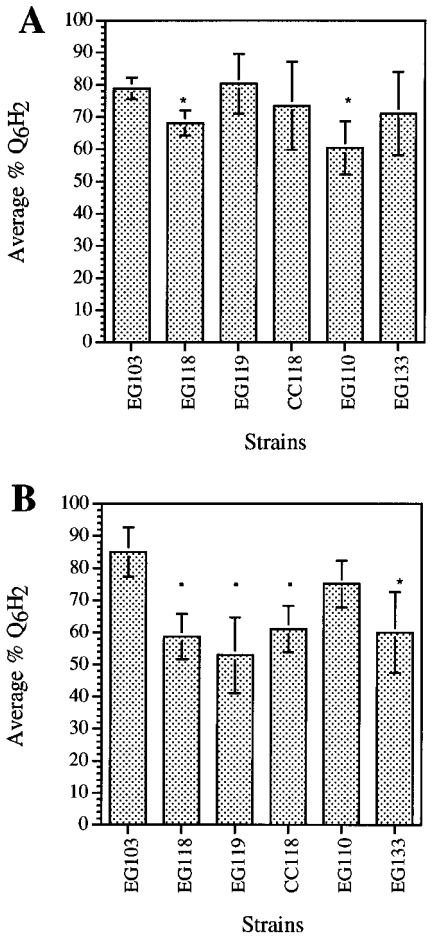

FIGURE 5: Sod-delete yeast strains maintain near normal levels of Q_6H_2. Yeast were grown at 30 °C overnight in YPD (panel A, $n = 3$) or for 2 days in YPG (panel B, $n = 5$). Cells were harvested, lysed, and extracted for total lipids. Lipid extracts were analyzed by C18 HPLC-coupled electr Chemical detection, and the % Q_6H_2 was determined as described under Materials and Methods. (•, $p < 0.01$; *, $p < 0.05$).

tested *in vitro* with liposomes containing cPN as a fluorescent indicator of lipid peroxidation. In this system, Q_6H_2 protects against azo-initiated lipid peroxidation as measured by a decreased rate of cPN fluorescence decay and the inhibition of LOOH products (Figure 2). Thus, a protection of Q_6H_2 autoxidation by SOD should be manifested by a further decrease in both cPN fluorescence decay and LOOH formation. In fact, when SOD is added to Q_6H_2-containing liposomes, a slightly increased rate of fluorescence decay is observed compared to PC plus Q_6H_2 (Figure 3). A similar increase in the rate of decay of cPN was observed when catalase was added, suggesting that this effect is not specific to SOD (data not shown). Since azo initiators generate peroxyl radicals, it is possible that Q_6H_2 may be oxidized by reactions independent of superoxide. In this case SOD would be unable to protect Q_6H_2. However, SOD is also unable to prevent Q_6H_2 autoxidation when incubated over time in the presence of 100% oxygen (Figure 4 and Table 2). Under such hyperoxia conditions, superoxide and various peroxides are known to be generated (Gardner et al., 1994), and liposome peroxidation was initiated and chain-carried in 100% O_2 as determined by assays of LOOH (Table 2). Thus, the addition of SOD to this liposome system does not slow Q_6H_2 oxidation, even under conditions which promote the formation of superoxide. Although SOD has been observed to slow the oxidation of a variety of water-soluble hydroquinones (Ishii & Fridovich, 1990; Linderson et al., 1994; Heikkila & Cabbat, 1976), SOD may be unable to protect QH_2 when it is incorporated into a membrane system because the enzyme and lipid are in separate environments and SOD cannot diffuse into the lipid bilayer to scavenge superoxide.

The *in vitro* liposome studies reported here also indicate that the ability of QH_2 to act as an antioxidant is influenced greatly by the method used to initiate oxidation. Addition of $CuSO_4$ to liposomes containing QH_2 generated more peroxidation, as measured by an increased rate of cPN fluorescence decay, than Cu^{2+} and CumOOH. It is well documented that any reductant which can reduce O_2, such as ascorbic acid or α-tocopherol, can act as a pro-oxidant in the presence of Fe^{3+} or Cu^{2+} and promote lipid peroxidation (Niki, 1991; Yoshida et al., 1994), and Q_1H_2 oxidation was shown to be catalyzed by either Fe^{3+} or Cu^{2+} (Mordente et al, 1992). Li and Trush (1993) reported the oxidation of hydroquinone to benzoquinone by Cu^{2+}, and Bandy et al. (1990) and Eyer (1991) have independently found that Cu,-ZnSOD could catalyze the autoxidation of hydroquinone in solution, due to Cu^{2+} in the enzyme. It has also been reported that soybean PC liposomes are oxidized by either copper or iron ions, in the absence of added hydroperoxides or preformed lipid peroxides (Yoshida & Niki, 1992). However, the rate of copper-induced PC oxidation is greatly decreased in our system since the rate of fluorescence decay for Q_6H_2-containing liposomes plus Cu^{2+} is significantly faster than for PC liposomes with Cu^{2+}. The decay in fluorescence observed by addition of Cu^{2+} to liposomes containing Q_6H_2 is significantly more pronounced at higher concentrations (144 μM), while lower concentrations (5 and 0.3 μM) show little, if any, fluorescence loss and generate the same level of peroxides as oxidized control liposomes (data not shown). Our results indicate that high concentrations of Q_6H_2 in the presence of Cu^{2+} can act as a pro-oxidant and greatly enhance the rate of lipid peroxidation.

Also of interest is the observation that at high concentrations oxidized Q (Q_6) appears to protect against lipid peroxidation (Figure 2). Stoyanovsky et al. (1995) have recently shown that Q can react with superoxide, generating oxygen and the semiquinone radical, which can concomitantly reduce the α-tocopheroxyl radical, forming reduced α-tocopherol and Q. This study indicates that oxidized Q in the presence of superoxide may function as a potential antioxidant. This type of mechanism may also account for the finding that high concentrations of Q_3 can inhibit azo-initiated peroxidation in both liposomes and LDL (Landi et al., 1990, 1992; Merati et al., 1992). In these studies concentrations of Q ranged from 72 to 288 μM. Despite the observation that high concentrations of oxidized Q show a protective effect, we found that high concentrations of Q_6H_2 (144 μM) actually provided less protection than 5 μM Q_6H_2 (Figure 2). We speculate that high concentrations of Q/QH_2 may result in formation of altered liposomes. In the presence of detergents, rates of lipid peroxidation are significantly increased due to a change in liposomes to micelles (Maiorino et al., 1995). This may explain the apparent paradox in our system; i.e., high concentrations of oxidized Q_6 provide protection (perhaps via semiquinone formation), yet high concentrations of Q/Q_6H_2 may disrupt the liposome structure and increase susceptibility of lipids and QH_2 to oxidation

(Nohl et al., 1996). Additionally, our experiments indicate that the autoxidation of Q_6H_2 in the absence of added oxidant, either Cu^{2+} or azo initiator, is very minimal since the rate of fluorescence decay is considerably slower in liposomes containing Q_6H_2 than the rate of oxidation in similar QH_2/PC liposome systems described by other investigators (Frei et al., 1990), indicating that Q_6H_2 is more stable in our liposome preparation.

The possible interaction of SOD and QH_2 was studied *in vivo* in yeast lacking SOD. Strains containing deletions in either *SOD1* or *SOD2* showed similar levels of percent Q_6H_2 as the wild-type strain when grown in fermentable conditions. Strains harboring a deletion in *SOD1* contained slightly lower levels of % Q_6H_2 in nonfermentable/respiratory conditions. Since this slight decrease in the redox level of Q_6H_2 occurs under conditions favoring generation of reactive oxygen species (Gralla & Kosman, 1992), this loss of Q_6H_2 may arise as a net result of increased oxidative stress and not as a direct effect of the loss of SOD. Thus, SOD does not play a significant role *in vivo* in maintaining high levels of Q_6H_2 and in preventing or slowing its autoxidation. Similarly, Western blot analysis on wild-type and *sod* mutant strains grown to stationary phase did not show a change in the levels of dihydroxyhexaprenyl benzoate methyltransferase, an enzyme required for Q biosynthesis, suggesting that levels of this Q biosynthetic enzyme are not increased to compensate for the lack of SOD in the mutant cells (data not shown).

These findings suggest that any interaction between QH_2 and SOD *in vivo* is probably a secondary effect resulting from altered metabolism. When Shindo et al. (1993) measured the *in vivo* levels of antioxidant enzymes, and hydrophilic and lipophilic antioxidants in murine epidermis and dermis exposed to chronic ultraviolet light, they found a 50% decrease in SOD activity, a 50% decrease in both reduced glutathione and total glutathione levels, an 80% decrease in catalase activity, and a greater than 90% decrease in ubiquinol-9, while α-tocopherol levels, oxidized glutathione, glutathione peroxidase, and glutathione reductase activities remained the same. They interpreted these changes as a general loss of antioxidants resulting from a global response to increased oxidation and lipid peroxides. This interpretation adds further support to the secondary effect of loss of Q_6H_2 under stressful or oxidizing conditions.

Together, these results indicate that there is no direct functional interaction between Q and SOD, that the autoxidation of Q_6H_2 is independent of SOD, and that Q_6H_2 is primarily maintained in its reduced state in the cell by other enzymatic systems. One seldom-mentioned system for generating QH_2 is *de novo* biosynthesis (Stocker & Suarna, 1993). Although the Q biosynthetic pathway generally depicts oxidized Q as the product, the last *O*-methylation step requires demethyl-QH_2 as a hydroquinone substrate (Houser & Olson, 1977). In addition to mitochondrial reactions that generate reduced Q, other systems that operate either in the cytosol or extracellularly may be required to generate QH_2 in these locations. Cytosolic enzyme systems that may function to reduce Q to QH_2 include DT-diaphorase [EC 1.6.99.2], an NAD(P)H:(quinone-acceptor) oxidoreductase (Cadenas, 1995; Cadenas et al., 1992; Lind et al., 1982), and a recently identified NADPH-Q reductase activity in rat liver cytosol that is separate from DT-diaphorase (Takahashi et al., 1995, 1996). Stocker and Suarna (1993) have shown that human blood cells and a human hepatoma cell line can act to reduce extracellular Q. Such extracellular Q reduction may be mediated by transplasma membrane redox systems, which utilize intracellular NADH to reduce Q in the plasma membrane (Sun et al., 1992; Crane et al., 1993; Villalba et al., 1995; Navarro et al., 1995). Similar systems that utilize NADH to reduce Q have been detected in the Golgi (Crane et al., 1984) and microsomes (Takada et al., 1982; Kagan et al., 1990). Sugioka et al. (1988) have also observed that NADPH−cytochrome P-450 reductase [EC 1.6.2.4] or hypoxanthine/xanthine oxidase [EC 1.2.3.2] generated superoxide ions can reduce ubiquinone-1. Further characterization of these distinct Q reductase systems will be very important, since the maintenance of QH_2 *in vivo* is vital for its function as an antioxidant.

ACKNOWLEDGMENT

We thank Tracy Fang for providing technical assistance.

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BI960245H