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NADH and NADPH-Dependent Reduction of Coenzyme Q at the Plasma Membrane

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ABSTRACT

High affinity for NADH, and low affinity for NADPH, for reduction of endogenous coenzyme Q_{10} (CoQ_{10}) by pig liver plasma membrane is reported in the present work. CoQ reduction in plasma membrane is carried out, in addition to other mechanisms, by plasma membrane coenzyme Q reductase (PMQR). We show that PMQR-catalyzed reduction of CoQ_0 by both NADH and NADPH is accompanied by generation of CoQ_0 semiquinone radicals in a superoxide-dependent reaction. In the presence of a water-soluble vitamin E homologue, Trolox, this reduction leads to quenching of the Trolox phenoxyl radicals. The involvement of PMQR versus DT-diaphorase under the conditions of vitamin E and selenium sufficiency and deficiency was evaluated for CoQ reduction by plasma membranes. The data presented here suggest that both nucleotides (NADH and NADPH) can be accountable for CoQ reduction by PMQR on the basis of their physiological concentrations within the cell. The enzyme is primarily responsible for CoQ reduction in plasma membrane under normal (nonoxidative stress-associated) conditions. Antiox. Redox Signal. 2, 251–262.

INTRODUCTION

COENZYME Q (CoQ, UBIQUINONE) functions as an ubiquitous antioxidant in biological membranes as well as an integral component of the mitochondrial electron transport coupled to ATP biosynthesis (Sastry *et al.*, 1961; Crane and Morré, 1977). CoQ is the only lipid-soluble antioxidant synthesized *de novo* by all organisms, and its antioxidant function has been associated with its reduced form, the ubiquinol ($CoQH_2$). Several mechanisms, both enzymatic and nonenzymatic, have been reported for CoQ reduction in those compartments in which it resides (for review, see Villalba *et al.* 1999).

In plasma membrane, this reduction can be achieved through the participation of several CoQ-reductases that can be either integral membrane proteins or cytosolic enzymes. Among these CoQ-reductases, DT-diaphorase, mostly located in the cytosol, catalyzes the two-electron reduction of the quinone using both NADH and NADPH with almost equal affinities (Ernster *et al.*, 1962). Takahashi and coworkers have reported another cytosolic NADPH-CoQ reductase, different from DT-di-

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aphorase, that also catalyzes the reduction of CoQ through a two-electron reaction mechanism (Takahashi *et al.*, 1992). On the other hand, an NADH-CoQ reductase consisting of a cytochrome b_5 reductase has been isolated and purified from pig liver plasma membrane (Navarro *et al.*, 1995; Villalba *et al.*, 1995). This integral plasma membrane CoQ reductase (PMQR) catalyzes the one-electron reduction of CoQ with the generation of semiquinone radical (Kagan *et al.*, 1998; Quinn *et al.*, 1999) and recycles the water-soluble vitamin E homologue, Trolox, in a superoxide-dependent reaction (Kagan *et al.*, 1998). This PMQR has been reported to be highly specific for NADH as an electron donor and no significant activities have been measured when NADPH was used as electron donor (Navarro *et al.*, 1995; Villalba *et al.*, 1995).

Deficiency of vitamin E and selenium has been reported to increase the activity and the protein level of PMQR present in rat liver plasma membrane (Navarro *et al.*, 1998). While only minimal amounts of DT-diaphorase activity can be measured in liver plasma membranes from rats fed vitamin E and selenium-sufficient diet, association of this enzyme with plasma membrane is dramatically increased during vitamin E and Se deficiency (Navarro *et al.*, 1998). Thus, by using liver plasma membranes obtained from rats fed diets either sufficient or deficient in vitamin and Se, we can easily manipulate the levels of one-electron and two-electron CoQ-reductases in the membrane to estimate the relative contribution of each enzyme activity to antioxidant regeneration.

In the present work, we have systematically studied NADH- and NADPH-dependent coenzyme Q_{10} reductase activities in liver plasma membranes and report the kinetic parameters for both nucleotides. In our assays, we have used the natural CoQ homologue CoQ_{10}, and the reduction kinetics for both endogenous and exogenous CoQ_{10} are presented. Furthermore, we have evaluated the relative contributions of NADH and NADPH to support the antioxidant regeneration capacity of plasma membranes, and their specificities in supplying electrons for PMQR and DT-diaphorase under conditions of both adequate and deficient vitamin E and selenium.

MATERIALS AND METHODS

Ethylenediaminetetraacetic acid (EDTA), phenyl-methylsulfonyl fluoride (PMSF), dithiothreitol (DTT), 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), egg yolk-phosphatidylcholine, L-alpha-phosphatidylcholine, dioleoyl (C18:1, [cis]-9) synthetic (DOPC), 2,3-dimethoxy-5-methyl-1,4 benzoquinone (CoQ_0), CoQ_{10}, deferroxamine mesylate (DFO), 2-mercaptoethanol, dicumarol, rotenone, partially acetylated cytochrome *c*, NADH, NADPH, and superoxide dismutase (SOD, EC 1.15.1.1) from bovine erythrocytes were obtained from Sigma (St. Louis, MO). Trolox was purchased from Aldrich (Milwaukee, WI).

Plasma membrane preparation

Male Long-Evans hooded rats (Harlan, Indianapolis, IN) were fed vitamin E and selenium-sufficient and -deficient diets following a protocol approved by the Animal Care and Use Committee at Purdue University as described by Navarro *et al.* (1998). Pig livers were obtained from a slaughterhouse. Unless otherwise stated, all procedures for plasma membrane isolation were performed at 4°C. Crude membrane fractions were prepared from pig and rat liver homogenates by differential centrifugation, and plasma membranes were then purified from crude fractions by the two-phase partition method. Isolated plasma membranes were resuspended in 25 m*M* Tris-HCl, pH 7.6, containing 10% glycerol, 1 m*M* EDTA, and 1 m*M* PMSF, and stored at −70°C until use. Purity was assessed by marker enzyme analysis as described previously (Alcaín *et al.*, 1992). In some preparations, extrinsic and adsorbed soluble proteins were removed by treating membranes with 0.5 *M* KCl and then separating the membrane residue by ultracentrifugation for 1 hr at 105,000 × *g*.

Protein purification

All procedures for protein purification were carried out at 4°C. PMQR was solubilized from salt-extracted pig liver plasma membranes with 2.5% CHAPS in 25 m*M* Tris-HCl, pH 7.6, containing 1 m*M* EDTA, 1 m*M* PMSF, and 0.1 m*M* DTT. The protein extract was separated

from the insoluble residue by centrifugation at 105,000 × *g* for 1 hr. PMQR was then purified by size-exclusion chromatography on Sephacryl S-300 HR, ion-exchange chromatography on DEAE-Sepharasose 6B CL, and affinity chromatography on 5'-ADP agarose followed by biospecific elution with NADH, as described in detail elsewhere (Villalba *et al.*, 1995). DTT was omitted from the last purification step to avoid nonenzymatic reduction of ubiquinone in the reductase activity assay (Takahashi *et al.*, 1996). The enzyme activity assayed by NADH oxidation in the presence of ferricyanide or CoQ_0 was 53.8 μmol/mg protein per min and 5.5 μmol/mg protein per min, respectively. One enzyme unit was defined as the amount of protein required to oxidize 1 μmol NADH/min in the ferricyanide reductase assay.

CoQ_{10} reductase determination

NAD(P)H-CoQ_{10} reductase activity was measured by following the accumulation of endogenous $CoQ_{10}H_2$ in liver plasma membranes upon incubation with NAD(P)H. Stock solutions of NAD(P)H were prepared daily in 50 m*M* phosphate buffer, pH 7.4, and concentrations of the reduced nucleotides were calculated based on spectrophotometric determinations at 340 nm. Purity of commercial NAD(P)H was checked by HPLC as described by Noack *et al.* (1992).

Membranes (10 mg/ml) were incubated with NAD(P)H (up to 1 m*M*) in 25 m*M* Tris-HCl, pH 7.6, for 30 min at 37°C. 2-Mercaptoethanol was added to a final concentration of 0.5 m*M* to avoid reoxidation of $CoQ_{10}H_2$. Levels of endogenous CoQ_{10} and $CoQ_{10}H_2$ were then determined by HPLC with electrochemical detection as described previously (Lagendijk *et al.*, 1996; Arroyo *et al.*, 1998). Briefly, samples were vigorously vortexed after addition of 1 ml of *n*-propanol and then left resting for 3 min. After a second vortexing, samples were centrifuged for 5 min at 2,500 × *g* and a 100-μl sample from the supernatant was used immediately for reversed-phase HPLC separation with a C18 column (5-μm particle, 5 × 0.45 cm). The flow rate was 1 ml/min and the mobile phase was a mixture of methanol and *n*-propanol (4:1) containing 89.5 m*M* $HClO_4$ and 57 m*M* NaOH. The quinone and hydroquinone forms of CoQ were monitored with an Coulochem II electrochemical detector (ESA, Chelmsford, MA) fitted with a Model 5010 analytical cell with the electrodes set at potentials of −500 mV and +300 mV. Both CoQ_{10} and $CoQ_{10}H_2$ were detected as the oxidized quinone at the second electrode. Concentrations were calculated by integration of peak areas and comparison with external standards.

To measure the reduction of exogenous CoQ_{10} by plasma membranes, a concentrated stock solution of CoQ_{10} (4.5 m*M*) was prepared in heated ethanol to increase its solubility and then added to egg yolk phosphatidylcholine liposomes to a final concentration of 50 μ*M*. This liposomal-CoQ_{10} solution was used to add exogenous CoQ_{10} to the plasma membrane, and the final amount of liposomes (5 mg/ml) was kept constant by adjusting with CoQ_{10}-free liposomes. Plasma membranes (2.5 mg/ml) were incubated under N_2 atmosphere with liposomal-CoQ_{10} (0–35 μ*M*) for 24 hr at 37°C in 25 m*M* Tris-HCl, pH 7.6, containing 0.5 m*M* 2-mercaptotethanol, 0.2 m*M* DFO, 5 μ*M* dicumarol, 5 μ*M* rotenone, and 1 m*M* NAD(P)H. After the incubation period, lipids were extracted as described above and concentrations of $CoQ_{10}H_2$ determined by HPLC using UV detection at 290 nm, instead of electrochemical detection. The same conditions were used to measure reduction of exogenous CoQ_{10} at increasing NAD(P)H concentrations (0–1 m*M*), keeping CoQ_{10} constant at 25 μ*M*.

EPR measurements

For EPR detection of free radicals, reactions were carried out at 25°C in 50 m*M* phosphate buffer, pH 7.4, containing 1.5 mg/ml of DOPC liposomes, 0.2 m*M* DFO, 100 mU/ml PMQR, 0.2 m*M* NAD(P)H, and 1 m*M* CoQ_0. Trolox phenoxyl radicals were generated by UV-light irradiation of 3.5 m*M* Trolox by using an Oriel UV-source (output power 200 W) and interference fiter λ_{max} 290.6 nm, bandwidth 10 nm (Oriel Instruments, Stratford, CT). EPR spectrometer (JEOL JES-RE1X) settings were center field 335.5 mT, scan range 10 mT, scan sweep 2.5 mT/min, time constant 0.1 sec, magnetic field 100 kHz,

modulation amplitude 0.2 mT, microwave frequency 9.44 gHz, microwave power 30 mW, receiver gain 1×10^3, and at 25°C.

Assays for acetylated cytochrome c reduction

The CoQ-dependent reduction of acetylated cytochrome *c* was recorded to estimate superoxide generation by CoQ-reductase. Assays were carried out at 37°C with continuous gentle stirring in 50 m*M* phosphate buffer, pH 7.4, containing 0.2 m*M* DFO, 0.2 m*M* NAD(P)H, 0.125 mg/ml acetylated cytochrome *c*, 25 μg/ml rat liver plasma membrane, and 20 μM CoQ_0. The reaction was monitored at 550 nm in a DU-640 Beckman spectrophotometer. An extinction coefficient of 29.5 mM^{-1} cm^{-1} was used to calculate rates of cytochrome *c* reduction.

RESULTS

Pig liver plasma membrane contains both CoQ_{10} and $CoQ_{10}H_2$. Total (oxidized plus reduced) CoQ_{10} concentration is 116.3 ± 2.7 pmoles/mg protein, and about 38% is found in its reduced form (Arroyo *et al.*, 1998). These membranes also contain NADH-cytochrome b_5 reductase as intrinsic quinone reductase (PMQR) that catalyzes the CoQ reduction in the membrane (Villalba *et al.*, 1995; Navarro *et al.*, 1995; Kagan *et al.*, 1998; Arroyo *et al.*, 1998). Several other enzymes have been reported to catalyze the reduction involving NADH and/or NADPH as electron donor (Ernster *et al.*, 1962; Takahashi *et al.*, 1992). PMQR has been reported to use only NADH as electron donor and no activity has been observed with NADPH (Navarro *et al.*, 1995; Villalba *et al.*, 1995). However, this observation may have been inaccurate because the assays with PMQR were made using a liposome-reconstituted system rather than the natural membranes. Also, CoQ reduction was mainly determined by using the artificial coenzyme Q homologue CoQ_0 and quantified by measuring the increase in absorbance at 410 nm, which is not very sensitive (Navarro *et al.*, 1995; Villalba *et al.*, 1995). By using more sensitive techniques (EPR and HPLC with electrochemical and UV detection) and endogenous CoQ_{10}, we have studied the ability of plasma membranes to utilize both NADH and NADPH to catalyze CoQ_{10} reduction.

Reduction of endogenous CoQ_{10} in plasma membranes was carried out in the presence of different amounts of either NADH or NADPH, and quantified by HPLC analysis with electrochemical detection (Fig. 1). Kinetics were very similar at concentrations of NAD(P)H higher than 0.2 m*M*, but at lower concentrations the activity with NADH was much greater than that with NADPH (Fig. 1). For example, 10 μM NADH yielded about 60% of the maximum activity, whereas 10 μM NADPH yielded no activity. Maximal amounts of reduced CoQ_{10} was about 57% of total CoQ_{10} after the 30-min incubation period with 1 m*M* NADH or NADPH. Reciprocal plots showed biphasic kinetics of CoQ_{10} reduction at increasing concentrations of NADH, where a high- and a low-affinity range could be recognized. Data from these plots were used to calculate K_m and V_{max} values corresponding to both ranges of NADH concentration. High-affinity K_m for NADH was about 10–11 μM, whereas the low-affinity K_m was one order of magnitude higher (Table 1). In contrast to the results obtained with NADH, kinetic pa-

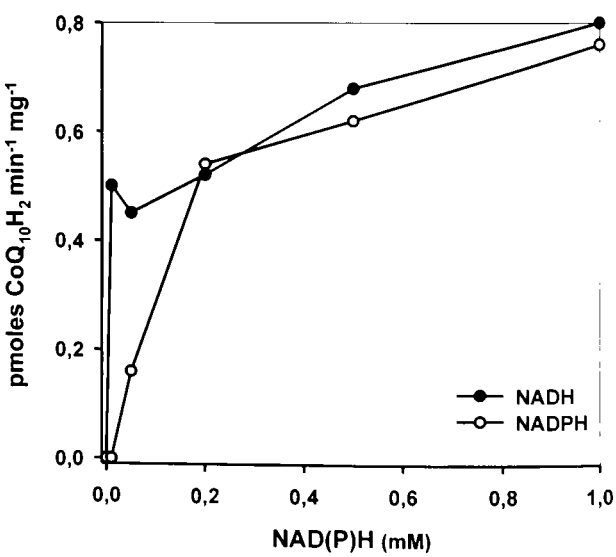

FIG. 1. NAD(P)H dose–response of endogenous CoQ_{10} reduction by plasma membranes. Assays were carried out at 37°C in 25 m*M* Tris-HCl, pH 7.6, containing 0.5 m*M* 2-mercaptoethanol, 10 mg/ml pig liver plasma membranes, and 0–1 m*M* NAD(P)H. After 30 min of incubation, CoQ_{10} and $CoQ_{10}H_2$ were extracted and measured by HPLC and electrochemical detection.

rameters obtained for NADPH were of the same order, irrespective of the nucleotide concentration (Table 1).

To assess the kinetic parameters at varying amounts of CoQ_{10} keeping NAD(P)H constant, pig liver plasma membranes were incubated in the presence of 1 m*M* NAD(P)H and different amounts of CoQ_{10} that had been added exogenously from a liposomal stock solution. The $CoQ_{10}H_2$ generated was quantified by HPLC analysis using UV determination at 290 nm (Fig. 2). Because of the lower sensitivity of the UV detector and the low of plasma membrane NAD(P)H-CoQ_{10} reductase activity, a longer incubation period (24 hr) was needed to detect $CoQ_{10}H_2$ accumulation accurately. As shown in Fig. 2, no significant differences in the rate of CoQ_{10} reduction were obtained with either nucleotide, which was corroborated by calculation of K_m and V_{max} values from reciprocal plots (K_m = 24.33 and 22.44 μM, V_{max} = 0.468 and 0.449 pmoles $CoQ_{10}H_2$/min per mg of protein using NADH and NADPH, respectively).

To compare data obtained with endogenous CoQ_{10} with those obtained with exogenous CoQ_{10} in terms of CoQ_{10} reduction at varying concentrations of NAD(P)H, we measured $CoQ_{10}H_2$ accumulation in plasma membranes with constant exogenous CoQ_{10} (25 μM) and increasing amounts of NAD(P)H (0–1 m*M*) (Fig. 3). If we compare results depicted in Figs. 1 and 3, which represent NAD(P)H dose–response reduction curves for endogenous and exogenous CoQ_{10}, respectively, we can see a similar behavior, *i.e.*, similar CoQ_{10} reduction at high NAD(P)H concentrations, and a much higher CoQ_{10} reduction with NADH than with NAD(P)H when low concentrations of these nucleotides were used. Although kinetic analysis of these results also demonstrated a higher affinity for NADH than for NADPH (Table 1), some quantitative differences between reduction of endogenous and exogenous CoQ_{10} should however be noted. These include higher K_m values for pyridine nucleotides and no biphasic kinetics when exogenous CoQ_{10} was used. Also, while NADH and NADPH at 0.2 m*M* gave very similar rates of endogenous CoQ_{10} reduction, a significant difference in the reduction rates was observed for NADH and NADPH at 25 μM of exogenous CoQ_{10}.

To study the mechanism reaction of the PMQR with NADH and NADPH, we measured semiquinone radical generation by EPR using the purified enzyme. As shown in Fig. 4, PMQR purified from pig liver plasma mem-

TABLE 1. KINETIC PARAMETERS FOR NADH- AND NADPH-DRIVEN CoQ_{10} REDUCTION BY PIG LIVER PLASMA MEMBRANE DETERMINED IN THE PRESENCE OF ENDOGENOUS OR EXOGENOUS CoQ_{10}.

	Endogenous CoQ_{10}				*Exogenous CoQ_{10}*	
	K_m[a]		V_{max}[b]		K_m[a]	V_{max}[b]
NADH	10.9	148	0.55	0.91	125	0.331
NADPH	150	95.7	0.64	0.79	400	0.333
	K_{m1}	K_{m2}	V_{max1}	V_{max2}	—	—

Endogenous CoQ_{10} reduction was determined by HPLC analysis with electrochemical detection. Plasma membranes (10 mg/ml) were incubated at 37°C for 30 min in 25 m*M* Tris-HCl, pH 7.6, containing 0.5 m*M* 2-mercaptoethanol and NAD(P)H (0–1 m*M*). Exogenous CoQ_{10} reduction was determined by HPLC analysis and UV detection. Plasma membranes (2.5 mg/ml) were incubated at 37°C under N_2 atmosphere for 24 hr in 25 m*M* Tris-HCl, pH 7.6, containing 0.5 m*M* 2-mercaptoethanol, 0.2 m*M* DFO, 5 μM dicumarol, 5 μM rotenone, 25 μM CoQ_{10} (added from a liposomal stock solution, the final concentration of egg yolk-phosphatidylcholine was 5 mg/ml), and NAD(P)H (0–1 m*M*). After incubations, CoQ_{10} and $CoQ_{10}H_2$ were extracted and measured as described (Arroyo *et al.*, 1998).

[a] μM.

[b] pmoles $CoQ_{10}H_2$/min per mg protein.

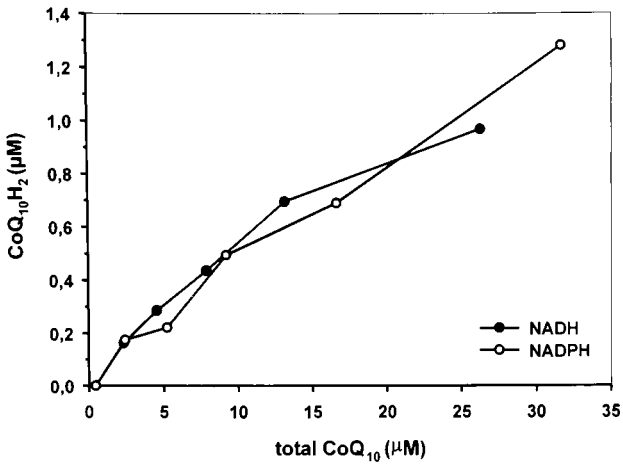

FIG. 2. CoQ_{10} dose-response of NAD(P)H-dependent CoQ_{10} reduction by plasma membranes. Assays were carried out at 37°C and under N_2 atmosphere in 25 m*M* Tris-HCl, pH 7.6, containing 0.5 m*M* 2-mercaptoethanol, 0.2 m*M* DFO, 5 μ*M* dicumarol, 5 μ*M* rotenone, 2.5 mg/ml pig liver plasma membrane, 1 m*M* NAD(P)H. Different amounts of CoQ_{10} from a liposomal stock solution were added to the reaction mixture. After 24 hr, $CoQ_{10}H_2$ was extracted and measured by HPLC with UV detection at 290 nm.

brane catalyzed the generation of CoQ_0 semiquinone radical in the presence of either NADH or NADPH, although a weaker semiquinone radical signal was observed with the latter electron donor. In both cases, the semiquinone signal was decreased upon addition of SOD to the reaction mixture, indicating the involvement of superoxide.

NADH-CoQ_0 reductase activity of PMQR

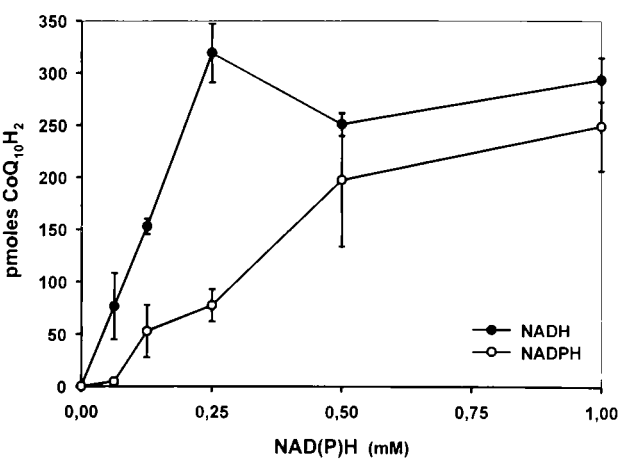

FIG. 3. NAD(P)H-dose response of exogenous CoQ_{10} reduction by plasma membranes. Assays were carried out as described in Fig. 2, except that the added CoQ_{10} was kept constant at 25 μ*M* and NAD(P)H was varied in the range 0–1 m*M*.

mediates the recycling of a water-soluble vitamin E homologue, Trolox, in a superoxide-dependent reaction (Kagan *et al.*, 1998). We induced Trolox phenoxyl radical by UV irradiation (Kagan and Packer, 1994), and tested the ability of PMQR to quench the radical signal. As previously reported (Kagan *et al.*, 1998), NADH was very effective in CoQ_0/PMQR-dependent quenching of Trolox phenoxyl radical, with the concomitant appearance of the CoQ_0 semiquinone radical (Fig. 5). When NADPH was present in the reaction mixture, the CoQ_0/PMQR-dependent quenching of Trolox phenoxyl radical was also observed, but in this case no signal of the CoQ_0 semiquinone radical was detected (Fig. 5). Quenching of Trolox phenoxyl radicals was also sensitive to SOD, suggesting the involvement of superoxide in the reaction.

In addition to PMQR, DT-diaphorase can also mediate $CoQ_{10}H_2$ regeneration in the plasma membrane, mainly under oxidative stress conditions (Beyer *et al.*, 1996; Navarro *et al.*, 1998). According to the different reaction mechanisms for PMQR and DT-diaphorase in CoQ reduction, and the different affinities of both enzymes for NADH and NADPH, we quantified superoxide generation by measuring CoQ_0-mediated reduction of acetylated cytochrome *c* in vitamin E and selenium-sufficient and -deficient rat liver plasma membranes (the latter membranes containing dramatically increased levels of DT-diaphorase; Navarro *et al.*, 1998). As shown in Table 2, reduction of acetylated cytochrome *c* was higher with NADH than with NADPH (both at 0.2 m*M*), and vitamin E and Se deficiency induced an increase in NADH-driven reduction but not in the reaction mediated by NADPH. Salt-extraction of control plasma membranes produced a significant increase of the activity measured with NADH but not with NADPH. However, salt-extraction of deficient membranes significantly increased superoxide generation with both nucleotides (Table 2).

DISCUSSION

CoQ represents a unique lipophilic antioxidant as it is the only lipid-soluble antioxi-

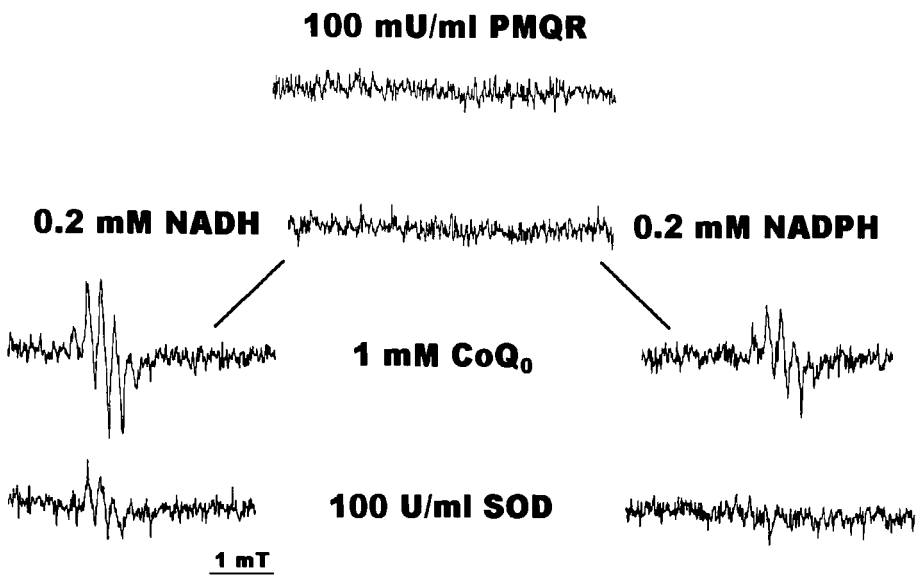

FIG. 4. EPR spectra of CoQ_0 semiquinone radicals generated by PMQR in the presence of NAD(P)H. Reactions were carried out at 25°C in 50 m*M* phosphate buffer, pH 7.4, containing 1.5 mg/ml DOPC liposomes, 0.2 m*M* DFO, and 100 mU/ml PMQR. NAD(P)H (0.2 m*M*), CoQ_0 (1 m*M*), and SOD (100 U/ml) were added as indicated. EPR spectrometer (JEOL JES-RE1X) settings were center field 335.5 mT, scan range 10 mT, scan sweep 2.5 mT/min, time constant 0.1 sec, magnetic field 100 kHz, modulation amplitude 0.2 mT, microwave frequency 9.44 gHz, microwave power 30 mW, receiver gain 1×10^3 and at 25°C.

dant so far known that is synthesized by all organisms (Ernster and Dallner, 1995). Its reduction through different enzymatic processes involving reducing agents, such as NADH and NADPH, allows cells to connect its antioxidant capacity with metabolism, which has been also proposed for vitamin E (Liebler, 1993; Kagan and Tyurina, 1998). Different cytosolic enzymes such as DT-diaphorase or NADPH-CoQ reductase can act as CoQ reductases, as has been described for DT-diaphorase under oxidative stress conditions induced by vitamin E and selenium deficiency (Navarro *et al.*, 1998). However, in the absence of oxidative stress, or basal conditions, these enzymes may not play a substantial role in CoQ reduction in plasma membrane. Under these conditions PMQR, an intrinsic plasma membrane enzyme, can fulfil this function and modulate the antioxidant capacity of the membrane in both oxidative stress and basal conditions.

Coenzyme Q was reduced in plasma membrane in the presence of NADH as well as NADPH (Figs. 1 and 3). These nucleotides are the major sources of reducing equivalents in cells. The concentrations of their oxidized and reduced forms in rat liver have been determined to be approximately 335, 94, 89, and 178 μM for NAD^+, NADH, $NADP^+$, and NADPH, respectively (Pasos and Telepneva, 1985). Our data on K_m values for NADH and NADPH (10.9 and 150 μM, respectively) for endogenous CoQ_{10} reduction are in agreement with the content of nucleotides reported by these authors. The K_m value obtained here for NADH is also in the same range as the values of 6–8 μM calculated previously from assays with purified PMQR using the artificial homologue CoQ_0 as electron acceptor (Navarro *et al.*, 1995; Villalba *et al.*, 1995). These data also indicate that CoQ_{10} reduction in plasma membrane could take place at physiological concentrations of NADH and NADPH *in vivo* (in the latter case at a rate about one-half of the V_{max}).

CoQ_{10} added exogenously from a liposomal stock suspension was also reduced by plasma membranes by both NADH and NADPH. Although reduction kinetics were qualitatively quite similar to those obtained with endogenous CoQ_{10}, the K_m values calculated for both nucleotides were significantly higher than those calculated from the reduction assay using endogenous quinone. This difference could be explained by the different assay conditions

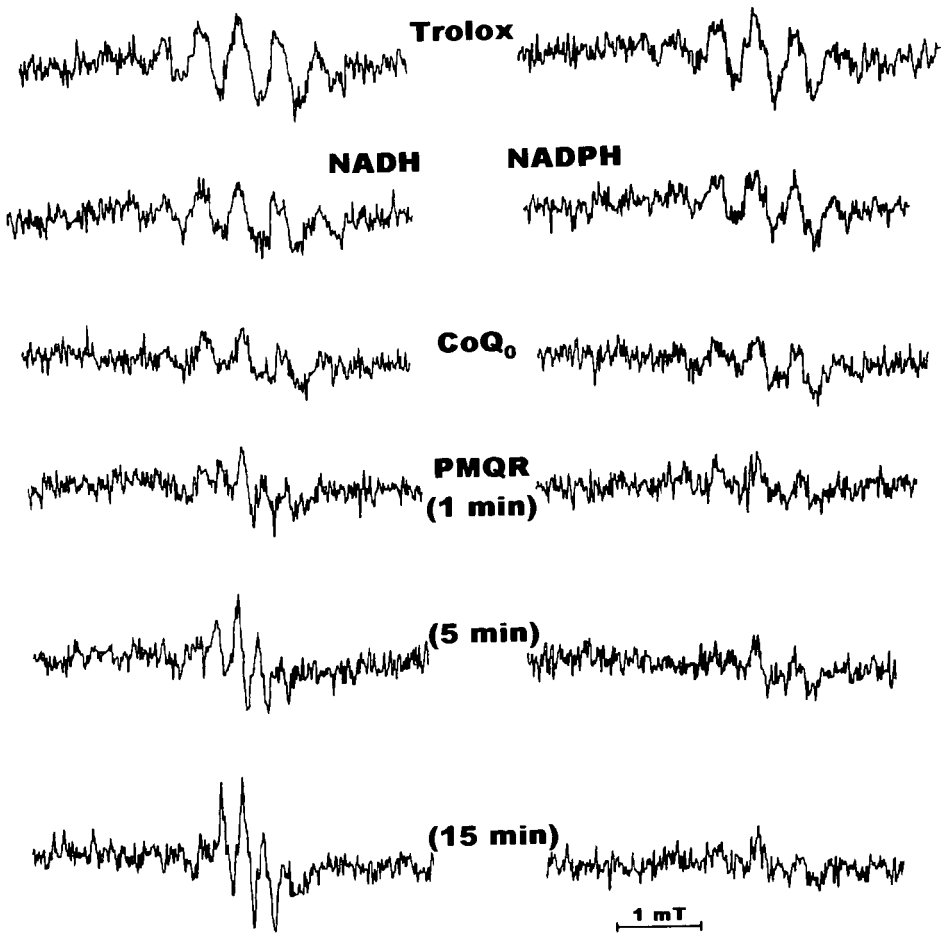

FIG. 5. EPR spectra of Trolox phenoxyl radicals and their quenching by PMQR/CoQ_0 system in the presence of NADH or NADPH. Trolox phenoxyl radicals were generated by irradiation at 290 nm and 25°C of 3.5 m*M* Trolox in 50 m*M* phosphate buffer, pH 7.4, containing 1.5 mg/ml DOPC liposomes and 0.2 m*M* DFO. NAD(P)H (0.2 m*M*), CoQ_0 (1 m*M*), and PMQR (100 mU/ml) were added as indicated. EPR spectrometer settings were the same as described in Fig. 4.

used to measure reduction of endogenous and exogenous CoQ_{10}. Also, it could represent reduction at nonphysiological sites of plasma membrane when an excess of exogenous CoQ_{10} is used. Further, V_{max} values for exogenous CoQ_{10} reduction were lower (about one-half) than those with endogenous CoQ_{10}. This could be explained if reoxidation of semiquinones or hydroquinones was taking place during the 24 hr incubation period used to detect $CoQ_{10}H_2$ accumulation. This seems to be unlikely given that these assays were carried under N_2 atmosphere and in the presence of 0.5 m*M* 2-mercaptoethanol. These conditions completely prevent reoxidation of $CoQ_{10}H_2$ without reducing chemically the quinone (Takahashi *et al.*, 1996; Arroyo *et al.*, 1998). We can also discard denaturation and/or inactivation of PMQR during the 24-hr incubation at 37°C because accumulation of $CoQ_{10}H_2$ was fully linear over time during the whole incubation period (data not shown).

The one-electron reduction of relatively stable radical intermediates, such as ubisemiquinone, has been proposed as a potential role for one-electron quinone reductases (such as PMQR) (Goldman *et al.*, 1997). This mechanism may be important for $CoQH_2$ recycling *in vivo*, particularly if the semiquinone is stabilized (Stocker and Suarna, 1993). Thus, an interesting hypothesis can be put forward that some ubiquinone-binding proteins, such as those associated with mitochondrial complexes (Yu *et al.*, 1995), may be required for efficient CoQ_{10} reduction and/or stabilization.

According to the K_m values calculated for the

TABLE 2. SUPEROXIDE GENERATION BY NAD(P)H-DEPENDENT ELECTRON TRANSPORT IN LIVER PLASMA MEMBRANE OBTAINED FROM VITAMIN E AND SELENIUM-SUFFICIENT AND -DEFICIENT RATS

		Type of membrane		
	Treatment	*+ Vit. E + Se*	*− Vit. E − Se*	*Increment*
NADH	None	22.04 ± 0.20	31.56 ± 1.48	43 %
	+ 0.5 M KCl	37.00 ± 0.12	67.64 ± 3.80	83 %
NADPH	None	14.04 ± 1.52	14.88 ± 5.75	6 %
	+ 0.5 M KCl	13.12 ± 1.40	21.56 ± 1.40	64 %

Acetylated cytochrome *c* reduction was monitored spectrophotometrically at 550 nm in 50 m*M* phosphate buffer, pH 7.4, containing 0.2 m*M* DFO, 0.2 m*M* NAD(P)H, 0.125 mg/ml acetylated cytochrome *c*, 25 μg/ml plasma membrane, and 20 μM CoQ_0 at 37°C and gentle stirring. No cytocrome *c* reduction was observed when 150 U/ml SOD was present in the reaction mixture or when heated-inactivated plasma membranes were used (not shown). Data are expressed in nmoles of acetylated cytochrome *c* reduced min^{-1} mg^{-1} protein (mean ± SD, $n = 2$). The "*Increment*" column represents the activities measured in deficient-membranes in relation to their respective control (in %).

pyridine nucleotides, reduction rates of exogenous CoQ_{10} with 1 m*M* NADH or NADPH were almost identical. Exogenous CoQ_{10} reduction also followed a saturation kinetics with respect to the concentration of the quinone, and a K_m of about 23–24 μM was calculated. This value is much lower than K_m for the homologue CoQ_0, reported to be about 625 μM (Navarro *et al.*, 1995; Villalba *et al.*, 1995), and this fact agrees with CoQ_{10} as a natural substrate. However, it is very likely that this value underestimates the affinity of PMQR for CoQ_{10} because the reduction is probably limited by the effective incorporation of the quinone into plasma membranes in a monomeric and redox-active form (Lenaz *et al.*, 1994).

The generation of CoQ_0 semiquinone radical by the PMQR purified from pig liver plasma membrane clearly demonstrated that this enzyme can use NADH as well as NADPH as electron donors. However, and in accordance with the previous results, the CoQ_0 semiquinone radical signal intensity obtained with NADPH was lower than that obtained with NADH. As previously described for NADH (Kagan *et al.*, 1998), this NADPH-dependent reduction of CoQ_0 was also sensitive to SOD, indicating the involvement of superoxide. Superoxide could be generated during the catalytic reduction of CoQ_0 and then react with the quinone to contribute, at least partially, to the overall reduction of CoQ_0 observed (Kagan *et al.*, 1998).

Regeneration of the reduced coenzyme Q is an essential step for ubiquinol-mediated recycling vitamin E in membranes, which increases the antioxidant capacity of both compounds (Beyer, 1994; Ernster and Dallner, 1995; Kagan *et al.*, 1996). The ubisemiquinone radical can also contribute to regenerate α-tocopherol from the corresponding radical (Landi *et al.*, 1992). As previosly reported using NADH/PMQR/CoQ_0 and the lipoxygenase-linoleic acid method to generate Trolox phenoxyl radical (Kagan *et al.*, 1998), the steady-state concentrations of semiquinone radicals generated by NADH or NADPH were sufficient to quench the Trolox phenoxyl radicals generated by UV irradiation. Quenching of Trolox phenoxyl radicals also involved superoxide because it was inhibited by SOD. In the absence of oxidative stress, *i.e.*, basal conditions, the amount of the vitamin E tocopheroxyl radical generated is expected to be minimal, and thus NADPH could also be an adequate electron donor for CoQ_{10} reduction and vitamin E recycling in plasma membrane. According to Stoyanovsky *et al.* (1995), no net reduction of α-tocopherol phenoxyl radical is carried out directly by superoxide. Thus, as proposed earlier by these authors, a role for superoxide/CoQ as a mechanism to increase antioxidant protection

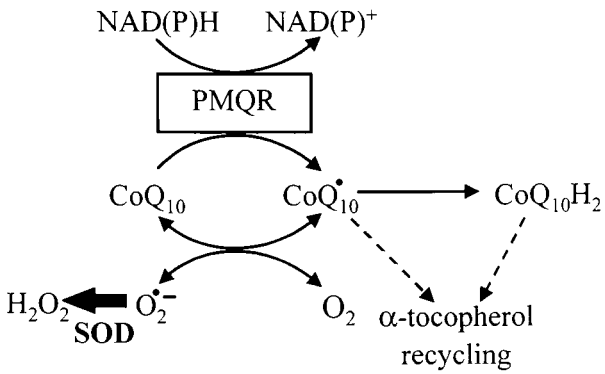

FIG. 6. Antioxidants regeneration by PMQR. Under physiological concentrations, both NADH and NADPH can be electron donors for PMQR to reduce CoQ_{10} to CoQ_{10} semiquinone. This radical can dismutate or accept a second electron to generate the fully reduced hydroquinone. Both the semiquinone and the hydroquinone can regenerate α-tocopherol by reducing its phenoxyl radical. CoQ_{10} semiquinone can also react with oxygen to produce superoxide. This reaction is reversible in such a way that CoQ_{10} can also accept electrons from superoxide to increase regeneration of α-tocopherol. By eliminating superoxide and thus displacing the equilibrium, SOD diminishes the amount of semiquinone radicals and decreases regeneration of α-tocopherol.

through the regeneration of α-tocopherol from the α-tocopheroxyl radical can be considered. A scheme showing the interactions between PMQR, CoQ, superoxide, and α-tocopherol is depicted in Fig. 6.

CoQ reduction is increased in the membranes lacking vitamin E by increasing both CoQ_{10} content and CoQ_{10} reductase activities to overcome this deficiency (Navarro *et al.*, 1998). Among these CoQ_{10} reductase activities, DT-diaphorase associated with plasma membrane is highly increased as no activity is found in vitamin E-adequate plasma membranes (Navarro *et al.*, 1998). To evaluate the role of both NADH and NADPH in the antioxidant capacity of plasma membrane and their use by PMQR and DT-diaphorase, we took advantage of some known properties of these two enzymes: (1) PMQR catalyzes one-electron CoQ reduction with the generation of superoxide, whereas DT-diaphorase catalyzes this reduction by a two-electron mechanism, and no superoxide is produced in this reaction (Li *et al.*, 1995); (2) K_m values for PMQR were about 11 and 150 μ*M*, for NADH and NADPH, respectively (see above), whereas the values reported for DT-diaphorase are 85 and 45 μ*M* (Lind *et al.*, 1990); (3) plasma membrane-associated DT-diaphorase activity is increased under conditions of vitamin E and selenium deficiency (Navarro *et al.*, 1998), and (4) DT-diaphorase (as an extrinsic membrane protein) can be extracted from plasma membrane by treatment with 0.5 *M* KCl (Navarro *et al.*, 1998).

We have used the reduction of acetylated cytochrome *c* as an indicator of superoxide generation, in vitamin E and selenium-deficient and -sufficient rat liver plasma membranes. Reduction of acetylated cytochrome *c* was higher with NADH than with NADPH in control membranes, which corroborates that NADPH is a poor electron donor for PMQR at this concentration (0.2 m*M*). Accordingly, CoQ_0 reductase measured with NADPH is much more lower than the activity measured with NADH in these membranes (Navarro *et al.*, 1998). Vitamin E and Se deficiency induced an increase in NADH-driven generation of superoxide, which is consistent with a similar increase in PMQR level in plasma membrane (Navarro *et al.*, 1998), but no such increase was observed with NADPH. Since at 0.2 m*M* NAD(P)H, CoQ reduction by PMQR is much lower with NADPH than with NADH, the enrichment of PMQR would make minimal contribution to CoQ reduction because, under these conditions, DT-diaphorase would be the major CoQ reductase (Navarro *et al.*, 1998). When control and deficient membranes were extracted with high salt to remove adsorbed and peripheral membrane proteins, the activity measured with NADH was increased about 1.6- and 2-fold respectively. Because specific activity is referred to protein, a part of the increase can be attributed to the enrichment in intrinsic proteins (*i.e.*, PMQR) in plasma membrane after salt-extraction (Arroyo *et al.*, 1998). No such increase in reduction of acetylated cytochrome *c* was observed with NADPH in control membranes. However, salt extraction of deficient membranes significantly increased superoxide generation. In this case, removal of DT-diaphorase (that might compete with PMQR for NADPH at 0.2 m*M*) from deficient membranes might have contributed to activation of superoxide generation, an effect mainly observed in the absence of vitamin E. Although NADPH-dependent dehydrogenase activity of salt-extracted

membranes was decreased due to removal of DT-diaphorase, $CoQ_{10}H_2$ regeneration was even increased about 1.4-fold in these membranes with NADH at 1 m*M* (Arroyo *et al.*, 1998), which agrees with the increase in superoxide generation shown here.

In view of our results, we conclude that NADH as well NADPH can be used for CoQ reduction in plasma membrane under the physiological concentrations of both nucleotides. Under basal conditions of low oxidative stress, PMQR may be the predominant enzyme responsible for the reduction. Under oxidative stress conditions caused by vitamin E and selenium deficiency, PMQR levels are elevated and DT-diaphorase associates with the plasma membrane, and both enzymes contribute to CoQ reduction.

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ABBREVIATIONS

CHAPS, 3-[3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CoQ_{10}, coenzyme Q_{10}; CoQn, coenzyme Qn (oxidized); $CoQnH_2$, ubiquinol, coenzyme Qn (reduced); DFO, deferroxamine mesylate; DOPC, L-alpha-phosphatidylcholine, dioleoyl C18:1, [*cis*]-9) synthetic; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMQR, plasma membrane coenzyme Q reductase; PMSF, phenyl-methylsulfonyl fluoride; SOD, superoxide dismutase.

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