

Plasma membrane NADH-coenzyme Q₀ reductase generates semiquinone radicals and recycles vitamin E homologue in a superoxide-dependent reaction

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Abstract We investigated the ability of plasma membrane CoQ reductase (PMQR) purified from pig liver to reduce phenoxyl radicals of a vitamin E homologue, Trolox. We report that NADH-driven one-electron reduction of CoQ₀ catalyzed by PMQR produced CoQ₀ semiquinone radical and CoQ₀H₂. These in turn, recycle vitamin E homologue, Trolox, via reducing its phenoxyl radical. A significant part of NADH/PMQR-catalyzed reduction of CoQ₀ (and Trolox recycling) was superoxide-dependent. Overall, our results demonstrate that PMQR in the model system used can act as an antioxidant enzyme that recycles water-soluble homologues of coenzyme Q and vitamin E.

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Key words: NADH-coenzyme Q₀ reductase; Semiquinone radical; Vitamin E recycling; Superoxide; Coenzyme Q₀; Trolox

1. Introduction

Coenzyme Q (CoQ, or ubiquinone) is an indispensable participant of mitochondrial electron transport and proton-motive cycle. Recent evidence suggests that the reduced form of ubiquinone (CoQH₂) may play another important role as a potent lipid-soluble antioxidant, (reviewed in [1]). There are at least two different mechanisms through which coenzyme Q is implicated in antioxidant roles: a direct scavenging of oxygen and organic radicals [1] and enhancement of vitamin E via its recycling from the phenoxyl radical [2]. The emergence of coenzyme Q as an antioxidant has special significance as it is the only lipid-soluble antioxidant so far known that is manufactured in the body [3]. It is easily converted from its ox-

dized form back to its reduced, antioxidant form, by well-established electron transport pathways.

Recently, additional enzymatic mechanisms have been described that catalyze NADPH- or NADH-dependent reduction of CoQ in the cytosolic environments and in plasma membrane, respectively [4,5]. In both cases, protective antioxidant effects of NAD(P)H-driven regeneration mechanisms for CoQ against oxidative stress have been established. Interaction of these enzymatic pathways with vitamin E, however, has not been reported so far. In the present work, we investigated the ability of plasma membrane CoQ reductase (PMQR) from pig liver to reduce phenoxyl radicals of a vitamin E homologue, Trolox. We report that PMQR-catalyzed NADH-driven one-electron reduction of ubiquinone Q₀ to its semiquinone radical via a superoxide-dependent process caused reduction of phenoxyl radicals of Trolox, i.e. regenerated Trolox.

2. Materials and methods

CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); potassium ferrocyanide; L-α-phosphatidylcholine, dioleoyl (C18:1,[cis]-9) synthetic (DOPC); 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ₀); deferoxamine mesylate (DFO); NADH; linoleic acid; lipoxigenase (EC 1.13.11.12) type I-B from soybean; superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes (SOD); acetonitrile and ammonium acetate were obtained from Sigma (St. Louis, MO). Trolox and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were obtained from Aldrich (Milwaukee, WI).

2.1. Isolation and purification of plasma membrane NADH-CoQ reductase (PMQR)

PMQR was extracted from purified pig liver plasma membranes [6] with the non-denaturing zwitterionic detergent, CHAPS (2.5%). After removal of peripheral proteins and solubilization, PMQR was purified by size exclusion chromatography on Sephacryl S-300 HR, ion exchange chromatography on DEAE-Sepharose 6B CL and affinity chromatography on 5'-ADP agarose followed by elution with NADH as described in [5]. The enzyme was homogenous as judged by SDS-PAGE assay (34 kDa). The enzyme activity assayed by NADH oxidation in the presence of ferrocyanide or CoQ₀ was 53.8 μmoles/mg protein/min and 5.5 μmol/mg protein/min, respectively.

2.2. EPR measurements

For EPR measurements, reactions were carried out at 25°C in 50 mM phosphate buffer, pH 7.4 containing DOPC liposomes (1.5 mg/ml), DFO (0.2 mM), PMQR (100 mU/ml), NADH (0.2 mM) and CoQ₀ (1 mM), lipoxigenase (150 U/μl), linoleic acid (1.5 mM) and Trolox (3.5 mM). 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 s, magnetic field 100 kHz, modulation amplitude 0.2 mT, microwave frequency 9.44 GHz, microwave power 30 mW, receiver gain 1 × 10³ and at 25°C.

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Abbreviations: PMQR, plasma membrane NADH-CoQ reductase; CoQ₀, 2,3-dimethoxy-5-methyl-1,4-benzoquinone, ubiquinone Q₀; CoQ₀H₂, ubiquinol Q₀; DFO, deferoxamine mesylate; DOPC, L-α-phosphatidylcholine, dioleoyl (C18:1,[cis]-9) synthetic; SOD, superoxide dismutase; EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate)

2.3. HPLC measurements

For HPLC measurements, the reaction mixture contained DOPC liposomes (1.5 mg/ml), Trolox (0.125 mM), coenzyme Q₀ (0.032 mM), DFO (0.2 mM), lipoxygenase (9.0 U/μl) and linoleic acid (0.72 mM) in 50 mM phosphate buffer, pH 7.4 at 25°C. PMQR (88 mU/ml) and NADH (0.05 mM) were added when necessary. A C-18 reverse-phase column (Ultrasphere ODS, 5 μm, 4.6 mm×25 cm, Beckman) was used for HPLC determinations (Shimadzu LC-600 pump and diode-array detector (SPD-M10A). Detector was set at a primary monitoring wavelength of 292 nm (to detect reduced Trolox and CoQ₀) and a second monitoring wavelength of 266 nm (to detect CoQ₀-oxidized). The mobile phase was CH₃CN:H₂O, 30:70 v/v containing 2 mM ammonium acetate adjusted to pH 3.0 with CH₃COOH. The flow rate was 1.2 ml/min. Under these conditions the retention times for Trolox, CoQ₀-reduced and CoQ₀-oxidized were 12.5, 4.2 and 6.8 min, respectively. The data acquired were exported from the detector using Shimadzu Class VP software.

2.4. PMQR-catalyzed NADH oxidation

The time course of PMQR-catalyzed NADH oxidation under the conditions of EPR and HPLC measurements was determined spectrofluorimetrically (λ_{excit} 340 nm, λ_{emis} 460 nm) (spectrofluorofotometer RF-5301PC, Shimadzu, Kyoto, Japan) using RFPC software.

3. Results and discussion

3.1. PMQR-catalyzed formation of CoQ₀ semiquinone radicals

To assess the ability of PMQR to catalyze one-electron reduction of CoQ₀, we used EPR to directly assay CoQ₀ semiquinone radicals (Fig. 1). Incubating PMQR with NADH and CoQ₀ produced a partially resolved (five lines) characteristic signal of CoQ₀ semiquinone radical with hyperfine structure due to splittings from methyl protons (2.37 G) [7]. An identical signal was observed when CoQ₀ was exposed to superoxide produced by xanthine oxidase (10 mU/ml)/xanthine (2.5 mM) (data not shown). Combinations of PMQR with either CoQ₀ alone or NADH alone did not result in any discernible signals of CoQ₀ radicals. NADH did not directly reduce CoQ₀ as no detectable EPR signals were discernable during incubation of NADH with CoQ₀ (data not shown). In separate experiments, we also demonstrated that NADH did not directly react with Trolox phenoxyl radicals. We gener-

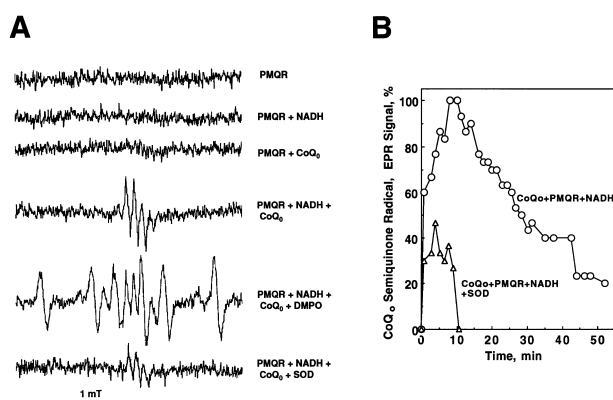


Fig. 1. EPR spectra (A) and time course (B) of NADH-induced CoQ₀ semiquinone radicals generated in the presence of PMQR. A: Reactions were carried out at 25°C in 50 mM phosphate buffer, pH 7.4 containing DOPC liposomes (1.5 mg/ml), DFO (0.2 mM) and PMQR (100 mU/ml). NADH (0.2 mM) and/or CoQ₀ (1 mM) were added as indicated. B: Incubation conditions were the same as in A. Both NADH and CoQ₀ were added. 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 s, 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.

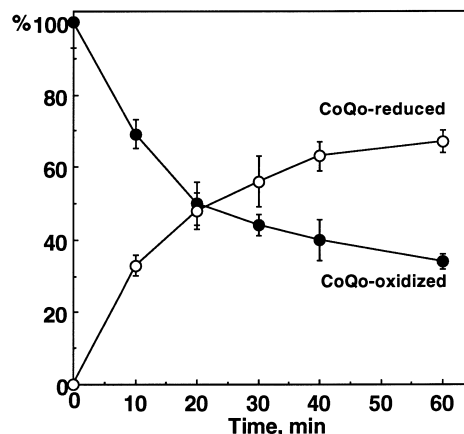


Fig. 2. NADH-dependent PMQR-catalyzed reduction of CoQ₀ in DOPC liposomes as measured by HPLC. Incubation conditions: DOPC (1.5 mg/ml) in 50 mM phosphate buffer, pH 7.4 containing (at 25°C) PMQR (88 mU/ml) and NADH (0.05 mM). The results of one (out of three) prototypical experiment are shown.

ated Trolox phenoxyl radicals by UV-irradiation (using Oriel UV-source and interference filter λ_{max} 290.6 nm, bandwidth 10 nm, Oriel Instrument, Stratford, CT, USA). The EPR signal of Trolox phenoxyl radical was not affected by addition of NADH (data not shown). The CoQ₀ semiquinone radical signal intensity increased to maximum during 10 min of incubation after which it decayed (Fig. 1B) (the signal was no longer detectable after 50–60 min). The disappearance of the signal coincided in time with complete oxidation of NADH (data not shown). HPLC measurements demonstrated a continuous decrease of CoQ₀ concentration in the course of its incubation with PMQR and NADH accompanied by accumulation of CoQ₀H₂ (Fig. 2). Quantitatively, formation of CoQ₀H₂ com-

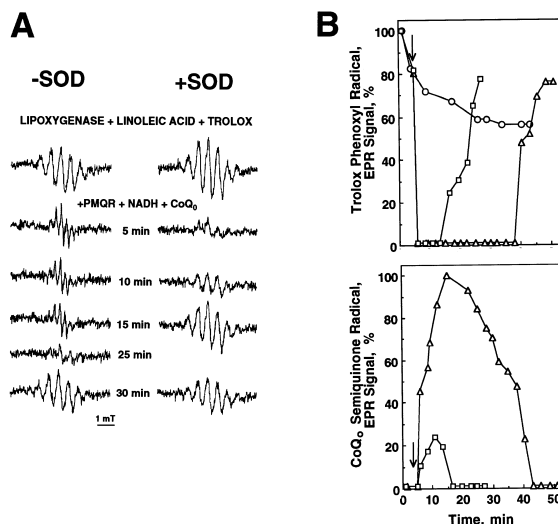


Fig. 3. EPR spectra (A) and time course (B) of Trolox phenoxyl radicals and CoQ₀ semiquinone radicals generated in the reaction system containing lipoxygenase/linoleic acid/Trolox and PMQR/NADH/CoQ₀ (added as indicated by the arrow) in the presence (opened squares) and in the absence (opened triangles) of SOD. (Circles show phenoxyl radicals of Trolox generated in the absence of PMQR/NADH/CoQ₀). Reaction conditions and EPR spectrometer settings were the same as in the legend of Fig. 1A. Phenoxyl radicals of Trolox (3.5 mM) were generated by lipoxygenase (150 U/μl), linoleic acid (1.5 mM). SOD was 100 U/ml.

pletely accounted for the consumption of CoQ₀. SOD significantly inhibited formation of CoQ₀ semiquinone radicals by both decreasing their magnitude and shortening their duration (Fig. 1). CoQ₀ semiquinone radical is known to be able to reduce molecular oxygen to produce superoxide [8,9]. In line with this, when a spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was added to the incubation system the resulting EPR signal represented superposition of the characteristic signal of DMPO-OOH adduct and the signal of CoQ₀ semiquinone radical (Fig. 1).

3.2. Reduction of Trolox phenoxyl radicals by PMQR/NADH/CoQ₀

Lipoxygenase-catalyzed oxidation of linoleic acid via intermediate formation of peroxy radicals can be successfully used to generate phenoxyl radicals of vitamin E and its homologues [10]. We utilized this system in order to measure the reduction of the phenoxyl radicals by PMQR/NADH/CoQ₀ (Fig. 3). A characteristic EPR signal of Trolox phenoxyl radicals was observed in the presence of lipoxygenase/linoleic acid. Under the conditions used, the signal was observable for at least 40 min. This signal immediately disappeared and was substituted by the CoQ₀ semiquinone radical signal upon addition of PMQR/NADH/CoQ₀. The signal of Trolox phe-

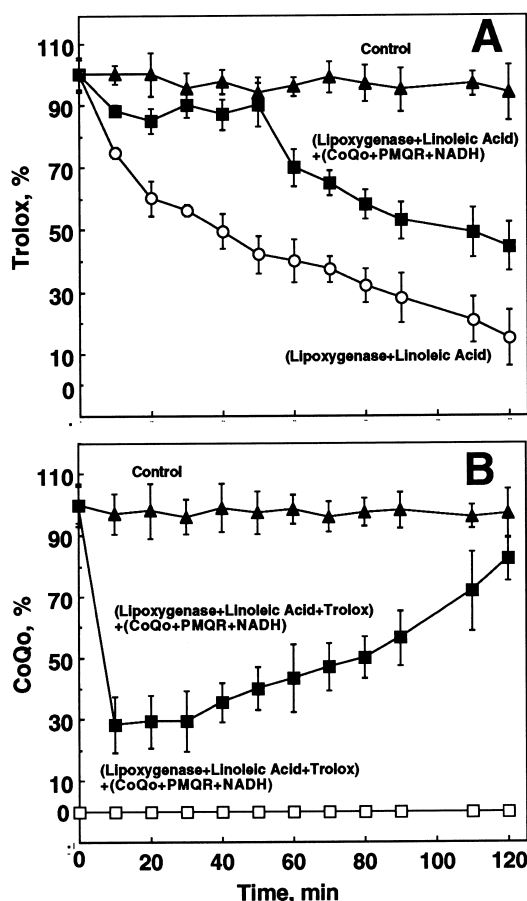


Fig. 4. Time course of Trolox (A) and coenzyme Q₀ (B) in the reaction system containing lipoxygenase/linoleic acid/Trolox and PMQR/NADH as measured by HPLC. Reaction conditions were the same as in the legend of Fig. 2. Lipoxygenase (9.0 U/μl) and linoleic acid (0.72 mM) were present in the incubation medium. B: Closed symbols, CoQ₀-oxidized; opened squares, CoQ₀H₂-reduced. Results are means ± S.E.M. (*n* = 3).

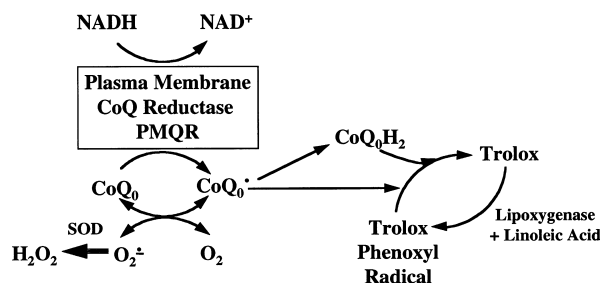


Fig. 5. A diagram of the postulated reaction scheme for NADH-dependent reduction of CoQ₀ and recycling of Trolox by PMQR.

noxyl radical reappeared at about 30 min (after NADH was consumed) and its intensity was only slightly lower than that before addition of PMQR/NADH/CoQ₀ (Fig. 3). This suggests that the PMQR/NADH/CoQ₀ reaction mixture did not inhibit lipoxygenase-catalyzed oxidation of linoleic acid and generation of Trolox phenoxyl radicals. Neither PMQR, CoQ₀ and NADH alone nor combinations PMQR/NADH and PMQR/CoQ₀ affected the typical EPR signal of Trolox phenoxyl radical. SOD significantly weakened PMQR/NADH/CoQ₀-induced effects of Trolox phenoxyl radical signals. Mn²⁺ known to inhibit PMQR [11] prevented PMQR/NADH/CoQ₀-induced effect on Trolox phenoxyl radicals (data not shown). Zn²⁺ which blocks ferrocyanide reductase activity of the enzyme but does not inhibit PMQR-catalyzed reduction of CoQ₀ [11] did not change the ability of the enzyme to eliminate Trolox radicals in the presence of CoQ₀ and NADH (data not shown). These results suggest that NADH-supported PMQR-catalyzed reduction of CoQ₀ caused reduction of Trolox phenoxyl radicals.

3.3. Protective effect of PMQR/NADH/CoQ₀ against lipoxygenase/linoleic acid-induced oxidation of Trolox

To directly prove that PMQR/NADH/CoQ₀ regenerates Trolox from its phenoxyl radicals we performed HPLC measurements of Trolox, CoQ₀ and CoQ₀H₂. Incubation of Trolox with lipoxygenase/linoleic acid caused rapid consumption of Trolox (Fig. 4A). In the presence of PMQR/NADH/CoQ₀, oxidation of Trolox was significantly inhibited. CoQ₀ concentration in the system decreased within 10 min and then returned to its initial level. In contrast to continuous accumulation of CoQ₀H₂ by PMQR/NADH/CoQ₀ (Fig. 2), no CoQ₀H₂ was detectable in the course of incubation of PMQR/NADH/CoQ₀ with Trolox/lipoxygenase/linoleic acid (Fig. 4B). These data suggest that CoQ₀H₂ generated from CoQ₀ by PMQR/NADH was consumed during the reduction of Trolox phenoxyl radicals.

Overall our results demonstrate that PMQR can act as an important antioxidant enzyme in the model system used by catalyzing NADH-dependent one-electron reduction of CoQ₀ to produce CoQ₀ semiquinone radical and CoQ₀H₂ that, in turn, recycle the water-soluble vitamin E homologue, Trolox, via reducing its phenoxyl radical (Fig. 5).

Results of our experiments with SOD indicate that superoxide may be involved in PMQR-associated antioxidant mechanisms. Since SOD was able to significantly inhibit both PMQR-catalyzed reduction of CoQ₀ and reduction of Trolox phenoxyl radical it is feasible that the enzyme's catalytic cycle involves one-electron reduction of oxygen along

with the reduction of CoQ₀. Subsequent reaction of superoxide with CoQ₀ to produce the semiquinone radical might be responsible, at least in part, for the enzymatic reduction of CoQ₀. This type of superoxide-driven ubiquinone-mediated reduction of vitamin E phenoxyl radicals as a potentially important antioxidant mechanism has been described earlier [12].

Lipoxygenase-catalyzed monooxygenation of polyunsaturated fatty acids has been reported to be accompanied by production of superoxide [13] that might contribute to the reduction of phenoxyl radicals by PMQR/NADH/CoQ₀. This was not likely to be the case in our experiments with Trolox because release of superoxide by lipoxygenase is known to be fully inhibitable by different phenolic compounds [13]. To completely eliminate lipoxygenase-produced superoxide as a potential source of CoQ₀ reduction in separate experiments we repeated the measurements in which we generated Trolox phenoxyl radicals by UV-irradiation [14]. We found that phenoxyl radicals of Trolox were reduced by PMQR/NADH/CoQ₀ similar to our results with lipoxygenase/linoleic acid (vide supra).

In conclusion, we demonstrated that PMQR can act as an antioxidant enzyme in the model system used and recycle water-soluble homologues of coenzyme Q and vitamin E.

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