Plasma membrane NADH-coenzyme Q_0 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 CoQ_0 catalyzed by PMQR produced CoQ_0 semiquinone radical and CoQ_0H_2. These in turn, recycle vitamin E homologue, Trolox, via reducing its phenoxyl radical. A significant part of NADH/PMQR-catalyzed reduction of CoQ_0 (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.

Key words: NADH-coenzyme Q_0 reductase; Semiquinone radical; Vitamin E recycling; Superoxide; Coenzyme Q_0; 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 ($CoQH_2$) 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 oxidized 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 Q_0 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-alpha-phosphatidylcholine, dioleoyl (C18:1,[cis]-9) synthetic (DOPC); 2,3-dimethoxy-5-methyl-1,4 benzoquinone (CoQ_0); deferoxamine mesylate (DFO); NADH; linoleic acid; lipoxygenase (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 CoQ_0 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 CoQ_0 (1 mM), lipoxygenase (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 1×10^3 and at 25°C.

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Abbreviations: PMQR, plasma membrane NADH-CoQ reductase; CoQ_0, 2,3-dimethoxy-5-methyl-1,4 benzoquinone, ubiquinone Q_0; CoQ_0H_2, ubiquinol Q_0; DFO, deferoxamine mesylate; DOPC, L-alpha-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)

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2.3. HPLC measurements

For HPLC measurements, the reaction mixture contained DOPC liposomes (1.5 mg/ml), Trolox (0.125 mM), coenzyme Q_0 (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 CoQ_0) and a second monitoring wavelength of 266 nm (to detect CoQ_0-oxidized). The mobile phase was CH_3CN:H_2O, 30:70 v/v containing 2 mM ammonium acetate adjusted to pH 3.0 with CH_3COOH. The flow rate was 1.2 ml/min. Under these conditions the retention times for Trolox, CoQ_0-reduced and CoQ_0-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 CoQ_0 semiquinone radicals

To assess the ability of PMQR to catalyze one-electron reduction of CoQ_0, we used EPR to directly assay CoQ_0 semiquinone radicals (Fig. 1). Incubating PMQR with NADH and CoQ_0 produced a partially resolved (five lines) characteristic signal of CoQ_0 semiquinone radical with hyperfine structure due to splittings from methyl protons (2.37 G) [7]. An identical signal was observed when CoQ_0 was exposed to superoxide produced by xanthine oxidase (10 mU/ml)/xanthine (2.5 mM) (data not shown). Combinations of PMQR with either CoQ_0 alone or NADH alone did not result in any discernible signals of CoQ_0 radicals. NADH did not directly reduce CoQ_0 as no detectable EPR signals were discernable during incubation of NADH with CoQ_0 (data not shown). In separate experiments, we also demonstrated that NADH did not directly react with Trolox phenoxyl radicals. We generated 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 CoQ_0 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 CoQ_0 concentration in the course of its incubation with PMQR and NADH accompanied by accumulation of CoQ_0H_2 (Fig. 2). Quantitatively, formation of CoQ_0H_2 com-

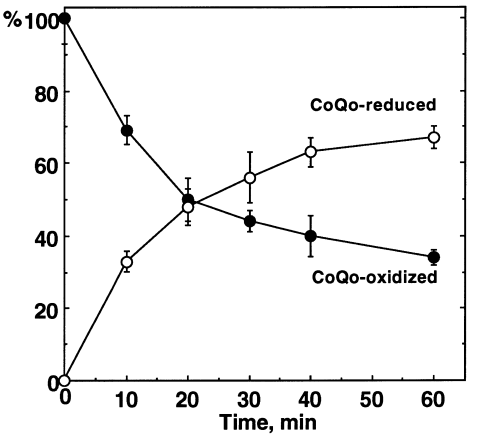

Fig. 2. NADH-dependent PMQR-catalyzed reduction of CoQ_0 in DOPC liposomes as measured by HPLC. Incubation conditions: DOPC (1.5 mg/ml) in 50 mM phosphate buffer, pH 7.4 containing (at 25°C) PMQR (88 mU/ml) and NADH (0.05 mM). The results of one (out of three) prototypical experiment are shown.

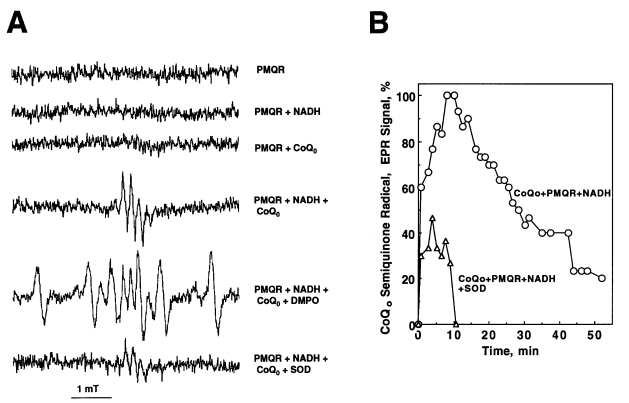

Fig. 1. EPR spectra (A) and time course (B) of NADH-induced CoQ_0 semiquinone radicals generated in the presence of PMQR. A: Reactions were carried out at 25°C in 50 mM phosphate buffer, pH 7.4 containing DOPC liposomes (1.5 mg/ml), DFO (0.2 mM) and PMQR (100 mU/ml). NADH (0.2 mM) and/or CoQ_0 (1 mM) were added as indicated. B: Incubation conditions were the same as in A. Both NADH and CoQ_0 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.

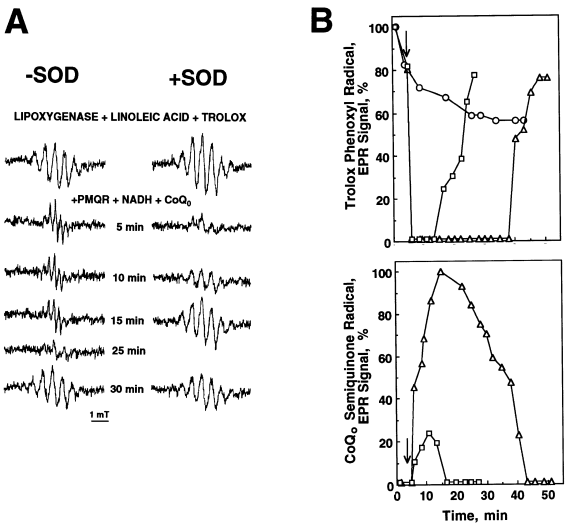

Fig. 3. EPR spectra (A) and time course (B) of Trolox phenoxyl radicals and CoQ_0 semiquinone radicals generated in the reaction system containing lipoxygenase/linoleic acid/Trolox and PMQR/NADH/CoQ_0 (added as indicated by the arrow) in the presence (opened squares) and in the absence (opened triangles) of SOD. (Circles show phenoxyl radicals of Trolox generated in the absence of PMQR/NADH/CoQ_0). 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 CoQ_0. SOD significantly inhibited formation of CoQ_0 semiquinone radicals by both decreasing their magnitude and shortening their duration (Fig. 1). CoQ_0 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 CoQ_0 semiquinone radical (Fig. 1).

3.2. Reduction of Trolox phenoxyl radicals by PMQR/NADH/CoQ_0

Lipoxygenase-catalyzed oxidation of linoleic acid via intermediate formation of peroxyl 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/CoQ_0 (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 CoQ_0 semiquinone radical signal upon addition of PMQR/NADH/CoQ_0. The signal of Trolox phenoxyl radical reappeared at about 30 min (after NADH was consumed) and its intensity was only slightly lower than that before addition of PMQR/NADH/CoQ_0 (Fig. 3). This suggests that the PMQR/NADH/CoQ_0 reaction mixture did not inhibit lipoxygenase-catalyzed oxidation of linoleic acid and generation of Trolox phenoxyl radicals. Neither PMQR, CoQ_0 and NADH alone nor combinations PMQR/NADH and PMQR/CoQ_0 affected the typical EPR signal of Trolox phenoxyl radical. SOD significantly weakened PMQR/NADH/CoQ_0-induced effects of Trolox phenoxyl radical signals. Mn^{2+} known to inhibit PMQR [11] prevented PMQR/NADH/CoQ_0-induced effect on Trolox phenoxyl radicals (data not shown). Zn^{2+} which blocks ferrocyanide reductase activity of the enzyme but does not inhibit PMQR-catalyzed reduction of CoQ_0 [11] did not change the ability of the enzyme to eliminate Trolox radicals in the presence of CoQ_0 and NADH (data not shown). These results suggest that NADH-supported PMQR-catalyzed reduction of CoQ_0 caused reduction of Trolox phenoxyl radicals.

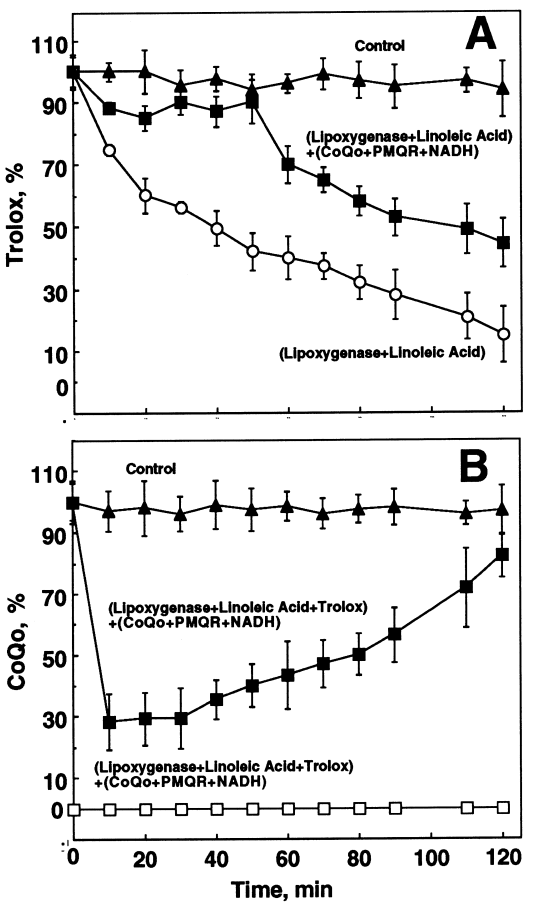

Fig. 4. Time course of Trolox (A) and coenzyme Q_0 (B) in the reaction system containing lipoxygenase/linoleic acid/Trolox and PMQR/NADH as measured by HPLC. Reaction conditions were the same as in the legend of Fig. 2. Lipoxygenase (9.0 U/μl) and linoleic acid (0.72 mM) were present in the incubation medium. B: Closed symbols, CoQ_0-oxidized; opened squares, CoQ_0H_2-reduced. Results are means ± S.E.M. ($n = 3$).

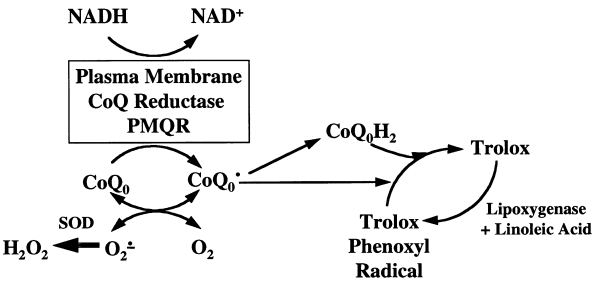

Fig. 5. A diagram of the postulated reaction scheme for NADH-dependent reduction of CoQ_0 and recycling of Trolox by PMQR.

3.3. Protective effect of PMQR/NADH/CoQ_0 against lipoxygenase/linoleic acid-induced oxidation of Trolox

To directly prove that PMQR/NADH/CoQ_0 regenerates Trolox from its phenoxyl radicals we performed HPLC measurements of Trolox, CoQ_0 and CoQ_0H_2. Incubation of Trolox with lipoxygenase/linoleic acid caused rapid consumption of Trolox (Fig. 4A). In the presence of PMQR/NADH/CoQ_0, oxidation of Trolox was significantly inhibited. CoQ_0 concentration in the system decreased within 10 min and then returned to its initial level. In contrast to continuous accumulation of CoQ_0H_2 by PMQR/NADH/CoQ_0 (Fig. 2), no CoQ_0H_2 was detectable in the course of incubation of PMQR/NADH/CoQ_0 with Trolox/lipoxygenase/linoleic acid (Fig. 4B). These data suggest that CoQ_0H_2 generated from CoQ_0 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 CoQ_0 to produce CoQ_0 semiquinone radical and CoQ_0H_2 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 CoQ_0 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 CoQ_0. Subsequent reaction of superoxide with CoQ_0 to produce the semiquinone radical might be responsible, at least in part, for the enzymatic reduction of CoQ_0. 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/CoQ_0. 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 CoQ_0 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/CoQ_0 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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References

[1] Beyer, R.E. (1990) Free Radic. Biol. Med. 8, 545–565.
[2] Kagan, V.E., Nohl, H. and Quinn, P.J. (1996) in: E. Cadenas and L. Packer (Eds.), Handbook of Antioxidants, Marcel Dekker, New York, pp. 157–201.
[3] Ernster, L. and Dallner, G. (1995) Biochim. Biophys. Acta 1271, 195–204.
[4] Takahashi, T., Okamoto, T. and Kishi, T. (1996) J. Biochem. 119, 256–263.
[5] Villalba, J.M., Navarro, F., Cordoba, F., Serrano, A., Arroyo, A., Crane, F.L. and Navas, P. (1995) Proc. Natl. Acad. Sci. USA 92, 4887–4891.
[6] Alcain, F.J., Villalba, J.M., Low, H., Crane, F.L. and Navas, P. (1992) Biochem. Biophys. Res. Commun. 186, 951–955.
[7] Das, M.R., Connor, H.D., Leniart, D.S. and Freed, J.H. (1970) J. Am. Chem. Soc. 92, 2258–2269.
[8] Boveris, A., Cadenas, E. and Stoppani, A.O.M. (1976) Biochem. J. 156, 435–444.
[9] Sugioka, K., Nakano, M., Totsune-Nakano, H., Minakami, H., Tero-Kubota, S. and Ikegami, Y. (1988) Biochim. Biophys. Acta 936, 377–385.
[10] Packer, L., Maguire, J.J., Melhorn, R.J., Serbinova, E.A. and Kagan, V.E. (1989) Biochem. Biophys. Res. Commun. 159, 229–235.
[11] Navarro, F., Villalba, J.M., Crane, F.L., Mackellar, W.C. and Navas, P. (1995) Biochem. Biophys. Res. Commun. 212, 138–143.
[12] Stoyanovsky, D.A., Osipov, A.N., Quinn, P.J. and Kagan, V.E. (1995) Arch. Biochem. Biophys. 323, 343–351.
[13] Chamulitrat, W., Hughes, M.F., Eling, T.E. and Mason, R.P. (1991) Arch. Biochem. Biophys. 290, 153–159.
[14] Kagan, V.E. and Packer, L. (1994) Methods Enzymol. 234, 316–320.