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Research article

ESR studies of ascorbic acid-dependent recycling of the vitamin E homologue Trolox by coenzyme Q_0 in murine skin homogenates

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The recycling of Trolox, a water-soluble vitamin E homologue, by coenzyme Q_0 (CoQ_0) during Cu^{2+}-initiated oxidation of ascorbate in mouse skin homogenates was investigated using electron spin resonance (ESR) spectroscopy. In a mixture containing CoQ_0, Cu^{2+} and mouse skin homogenates, the ESR signal of CoQ_0 semiquinone radical ($CoQ_0^{\bullet-}$) appeared and declined with time; addition of Trolox accelerated the $CoQ_0^{\bullet-}$ signal decay. Only after the disappearance of the $CoQ_0^{\bullet-}$ signal was the appearance of the Trolox phenoxyl radical signal observed. In addition, the lifetime of the $CoQ_0^{\bullet-}$ signal and the length of the lag period during which the Trolox radical ESR signal could not be detected were dependent on the presence of Trolox, CoQ_0 or Cu^{2+}. The results suggest that $CoQ_0^{\bullet-}$, formed by the interaction between CoQ_0 and endogenous ascorbic acid ($AscH^-$) in skin homogenates, regenerates Trolox from its phenoxyl radical.

INTRODUCTION

Free radicals are now known to play an important role in many areas of biology and are, therefore, being actively investigated in connection with various human health problems.[1,2] Reactive oxygen species are also thought to play an important role in cutaneous pathology, such as skin carcinogenesis and skin aging.[3–5] Skin has developed several antioxidant defense mechanisms to protect itself from free radical processes.[6,7] Water-soluble antioxidants include ascorbic acid, glutathione, and enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase. Lipid soluble antioxidants include α-tocopherol (TOH) and ubiquinones. Much attention has been paid to the biological properties of vitamin E because it is the major lipid-soluble chain-breaking antioxidant of membranes. The biological antioxidant activity of vitamin E is believed to be due to its ability to inhibit lipid peroxidation in biological membranes by scavenging the peroxyl radical ($LOO^{\bullet}$):

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \qquad \text{Eq. 1}$$

To be an effective antioxidant, oxidized tocopherol must be recycled. Although enzyme systems capable of reducing oxidized tocopherol have not been described at present, the synergistic antioxidant effects of vitamin E and vitamin C upon lipid peroxidation have been observed in model systems,[8–12] suggesting that vitamin C can regenerate vitamin E from its oxidized form.

Ubiquinone (CoQ, oxidized coenzyme Q) is well-known as a biological quinone-compound. The main function of ubiquinone in biology is to act as redox component of transmembrane electron transport systems, such as the respiratory chain of mitochondria. Ubiquinol ($CoQH_2$, reduced coenzyme Q) is the two-electron reduction product of ubiquinone. Ubiquinol has been found to have a strong activity in inhibiting lipid peroxidation in model systems, such as liposomal systems,[13] and in biological systems such as low-density lipoproteins.[14] Moreover, $CoQH_2$ has been shown to be an efficient regenerator of α-tocopherol from the tocopheroxyl radical.[15–19]

It has been proposed that superoxide radical may serve to reduce CoQ to the semiquinone radical ($CoQ^{\bullet-}$) which can, in turn, regenerate α-tocopherol from its phenoxyl radical.[19] It is also possible that ascorbic acid could serve

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this function. The purpose of the present study was to investigate the interactions of these antioxidants. However, vitamin E and ubiquinone-10 are insoluble and are poorly incorporated into membranes and within membranes are often associated in clusters. For this reason, research has been conducted with water-soluble vitamin E and ubiquinone-10 analogues.[15,20–22] In order to examine directly their regeneration reactions using electron spin resonance (ESR), we investigated the water-soluble homologues Trolox and CoQ_0 in a mouse skin homogenate system in which production of Trolox radicals was initiated by Cu^{2+}.

Materials and Methods

Reagents

$CuSO_4 \cdot 5H_2O$, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ_0), superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes (SOD), ascorbate oxidase (EC 1.10.3.3), dioleoylphosphatidylcholine (DOPC) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were obtained from Sigma (St Louis, MO, USA). Trolox was from Aldrich (Milwaukee, WI, USA).

Preparation of mouse skin homogenates

Skin was obtained from female 8–10-week-old hairless mice (Charles River Laboratories, Wilmington, MA, USA). After cervical dislocation of the mice, the skin samples were dissected immediately from the side and back. Adherent subcutis was gently removed by scraping the dermal side. Skin homogenates were prepared as reported previously with slight modifications.[23] Briefly, by using an Ultra-Turrax homogenizer (Ika-Works, Inc., Cincinnati, OH, USA), skin homogenates were prepared in 100 mM phosphate buffer (pH 7.4) which had been treated with Chelex-100 to remove transition metals. Protein concentration was determined by Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA).

ESR study of the recycling of Trolox by CoQ_0 in mouse skin homogenates

Reactions were carried out in 50 mM phosphate buffer, pH 7.4 containing skin homogenates (9.7 mg protein/ml), CoQ_0 (3.5–7.0 mM), Trolox (0.9–1.8 mM) and Cu^{2+} (0.35–7.0 mM). The reaction mixtures were immediately introduced into a quartz capillary and fitted into a flat cell and ESR spectra were recorded over time using an IBM ER 200D-SRC ESR spectroscopy. ESR spectroscopy settings: central field 3475 G; modulation frequency 100 kHz; modulation amplitude 2.5 G; microwave power 20 mW; scan width 100 G; gain 8.0 x 10^5; temperature 298ºK.

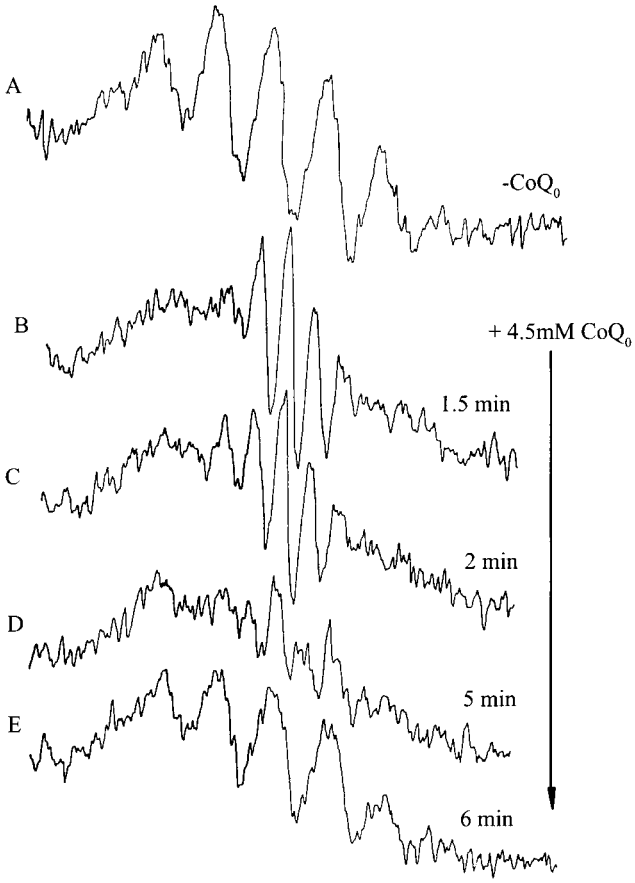

Fig. 1. Time-dependent changes in ESR spectra of CoQ_0 semiquinone radicals and Trolox phenoxyl radicals generated by Cu^{2+}-mediated oxidation of mouse skin homogenates. Reaction medium contained skin homogenates (9.7 mg protein/ml) in 50 mM phosphate buffer (pH 7.4), 0.9 mM Trolox and 0.7 mM Cu^{2+} without (A) or with 4.5 mM CoQ_0 (B–E).

ESR detection of the interaction between CoQ_0 and ascorbic acid

ESR spectra were recorded after the mixtures containing 0.7 mM ascorbic acid and 2.2 mM CoQ_0 in phosphate buffer (pH 7.4) in the presence or absence of Cu^{2+} (0.7 mM) were transferred into a quartz capillary and fitted into a flat cell. ESR spectroscopy settings were the same as above except modulation amplitude 2 G and gain 5.0 x 10^5.

Results

Trolox recycling by CoQ_0 in mouse skin homogenates

Incubating skin homogenates with 0.7 mM Cu^{2+} and 0.9 mM Trolox resulted in the appearance of a typical

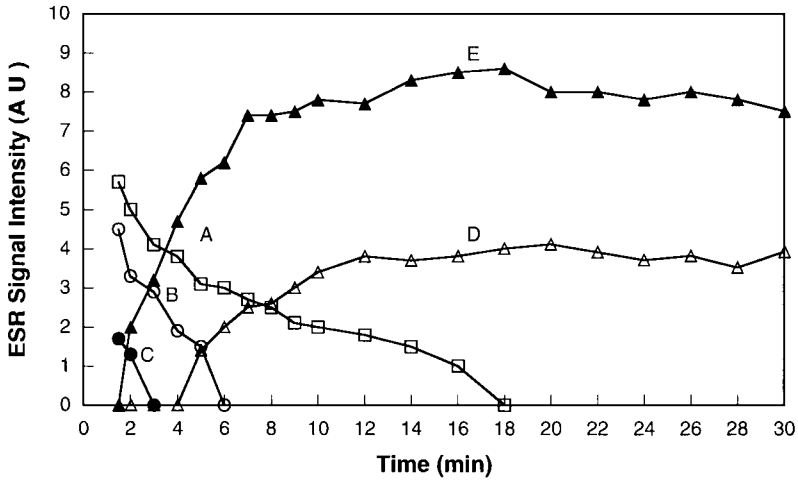

Fig. 2. Effect of Trolox concentration on the time course of the appearance and disappearance of $CoQ_0^{\bullet-}$ and Trolox radical signals in mouse skin homogenates. The reaction mixture contained skin homogenates (9.7 mg protein/ml), 4.5 mM CoQ_0 and the indicated concentrations of Trolox in the presence of 0.7 mM Cu^{2+}. (A) No Trolox, $CoQ_0^{\bullet-}$; (B) 0.9 mM Trolox, $CoQ_0^{\bullet-}$; (C) 1.8 mM Trolox, $CoQ_0^{\bullet-}$; (D) 0.9 mM Trolox, Trolox radical; (E) 1.8 mM Trolox, Trolox radical.

Trolox phenoxyl radical ESR signal (Fig. 1A). The signal was observable for at least 60 min under the conditions used. Upon addition of 4.5 mM CoQ_0, this radical signal immediately disappeared and was replaced by a partially resolved (five lines) signal characteristic of $CoQ_0^{\bullet-}$ (Fig. 1B). Its hyperfine splitting constant (a^H = 2.39 G) was the same as that reported in the literature.[21] After the disappearance of the $CoQ_0^{\bullet-}$ signal, the ESR signal of the Trolox radical reappeared at about 5 min and increased with time (Fig. 1D,E). The results indicate that Trolox radicals can be recycled to Trolox by CoQ_0 in skin homogenates.

Effects of Trolox and CoQ_0 concentrations on the recycling of Trolox

The effect of the Trolox concentration on the time course of $CoQ_0^{\bullet-}$ and of Trolox radical signals in the skin homogenate containing 4.5 mM CoQ_0 in the presence of 0.7 mM Cu^{2+} is shown in Figure 2. In the absence of Trolox, the lifetime of the $CoQ_0^{\bullet-}$ signal was 18 min (Fig. 2A). However, the presence of 0.9 mM Trolox decreased the lifetime of the $CoQ_0^{\bullet-}$ signal to 6 min (Fig. 2B). When the concentration of added Trolox was increased to 1.8 mM, the life-time of the $CoQ_0^{\bullet-}$ signal further declined to

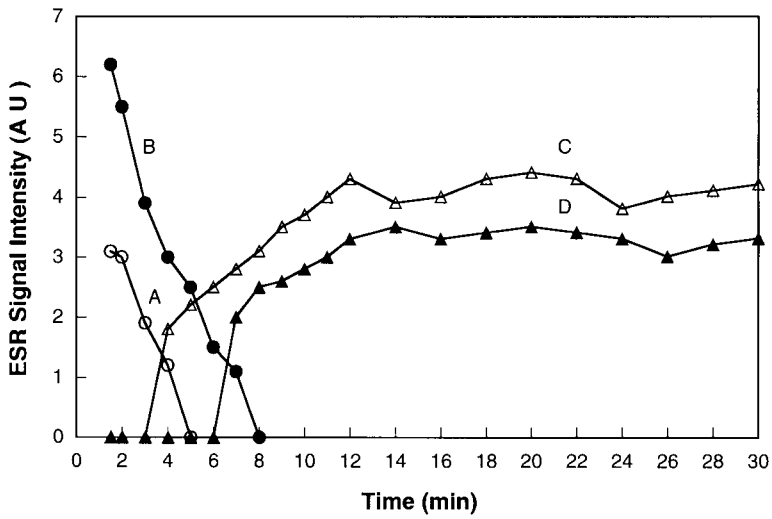

Fig. 3. Effect of CoQ_0 concentration on the time course of the appearance and disappearance of $CoQ_0^{\bullet-}$ and Trolox radical signals in mouse skin homogenates. The reaction mixture contained skin homogenates (9.7 mg protein/ml), 0.9 mM Trolox and the indicated concentrations of CoQ_0 in the presence of 0.7 mM Cu^{2+}. (A) 3.5 mM CoQ_0; $CoQ_0^{\bullet-}$; (B) 7.0 mM CoQ_0, $CoQ_0^{\bullet-}$; (C) 3.5 mM CoQ_0, Trolox radical; (D) 7.0 mM CoQ_0, Trolox radical.

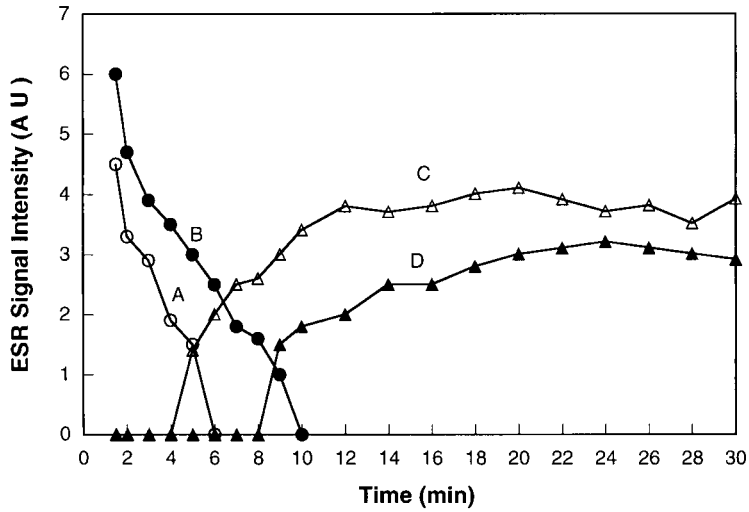

Fig. 4. Effect of Cu^{2+} concentration on the time course of the appearance and disappearance of $CoQ_0^{\bullet-}$ and Trolox radical signals in mouse skin homogenates. Reactions were carried out at room temperature in 50 mM phosphate buffer, pH 7.4 containing skin homogenates (9.7 mg protein/ml), 4.5 mM CoQ_0, 0.9 mM Trolox and the indicated concentrations of Cu^{2+}. (A) 0.70 mM Cu^{2+}, $CoQ_0^{\bullet-}$; (B) 0.35 mM Cu^{2+}, $CoQ_0^{\bullet-}$, (C) 0.70 mM Cu^{2+}, Trolox radical; (D) 0.35 mM Cu^{2+}, Trolox radical.

3 min (Fig. 2C), consistent with the idea that CoQ_0 regenerated Trolox from Trolox radical: Trolox in skin homogenates was preserved through recycling of Cu^{2+}-induced Trolox radical back to Trolox at the expense of CoQ_0. So, addition of Trolox accelerated CoQ_0 consumption in a concentration-dependent manner. At the same time, the delay in the re-appearance of the Trolox radical signal decreased and the ESR signal intensity of Trolox radical greatly increased (Fig. 2D,E).

The effect of CoQ_0 concentration on this recycling system was also tested in skin homogenates containing fixed concentrations of Trolox (0.9 mM) and Cu^{2+} (0.7 mM). As shown in Figure 3, there was a concentration-dependent prolongation of the lifetime of the $CoQ_0^{\bullet-}$ signal (Fig. 3A,B), and the $CoQ_0^{\bullet-}$-induced delay in the re-appearance of the Trolox radical signal was also concentration-dependent (Fig. 3C,D).

Effects of Cu^{2+} concentration and EDTA on the recycling of Trolox

The results shown in Figure 4 indicate that when the Cu^{2+} concentration was increased from 0.35 mM to 0.7

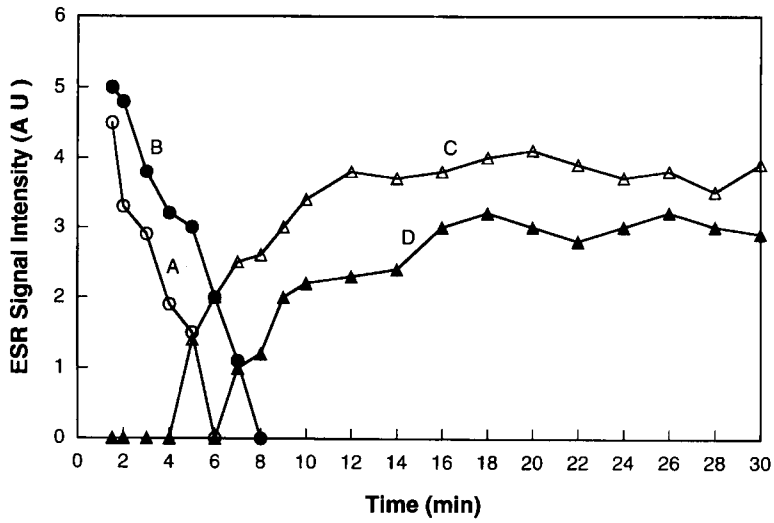

Fig. 5. Time course of the appearance and disappearance of $CoQ_0^{\bullet-}$ and Trolox radical signals in mouse skin homogenates with or without 0.3 mM EDTA (control). Reactions were carried out at room temperature in skin homogenates (9.7 mg protein/ml) containing 4.5 mM CoQ_0, 0.9 mM Trolox and 0.7 mM Cu^{2+}. (A) Control, $CoQ_0^{\bullet-}$; (B) Control + 0.3 mM EDTA, $CoQ_0^{\bullet-}$; (C) Control, Trolox radical; (D) Control + 0.3 mM EDTA, Trolox radical.

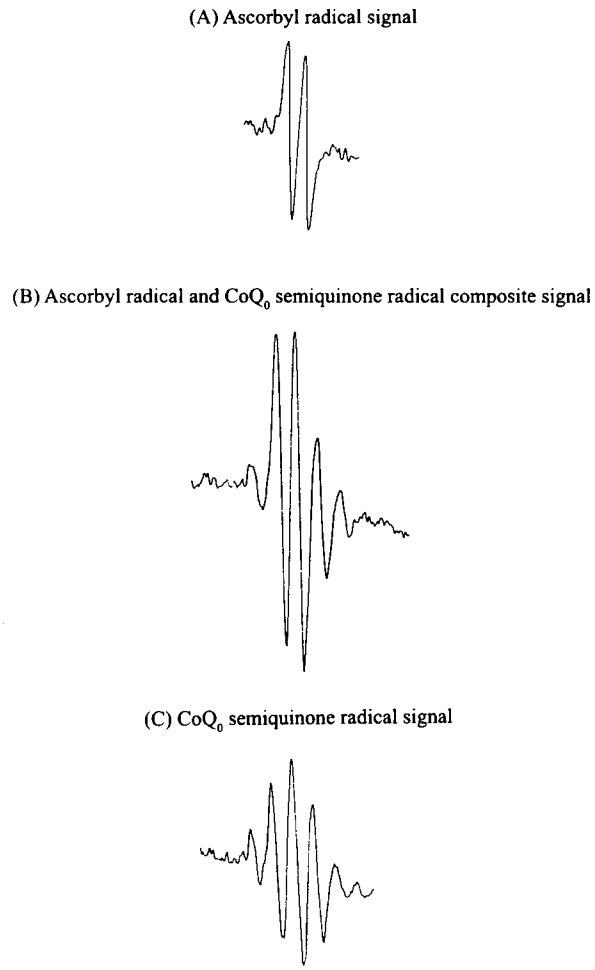

Fig. 6. ESR spectra of radicals generated in pH 7.4 phosphate buffer in the presence of 0.7 mM Cu^{2+}. (A) Ascorbyl radical signal in the presence of 0.7 mM ascorbic acid; (B) ascorbyl radical and CoQ_0 semiquinone radical composite signal in the presence of 0.7 mM ascorbic acid and 2.2 mM CoQ_0; (C) CoQ_0 semiquinone radical signal alone after the disappearance of ascorbyl radical.

mM, the lifetime of the $CoQ_0^{\bullet-}$ signal in this system was shortened from 10 min to 6 min, indicating that increasing Cu^{2+} concentration accelerated the consumption of CoQ_0. The delay in the reappearance of Trolox radical was also shortened.

The effect of EDTA, a chelator of Cu^{2+}, on this recycling system was investigated (Fig. 5). When 0.3 mM EDTA was added to the skin homogenate containing 4.5 mM CoQ_0, 0.9 mM Trolox and 0.7 mM Cu^{2+}, the decay rate of the CoQ_0 semiquinone radical was decreased, suggesting that the presence of EDTA could inhibit the consumption of CoQ_0 by chelating Cu^{2+}. In addition, the lag period during which the Trolox radical ESR signal could not be detected was longer than that in the control (without EDTA). The Trolox radical ESR signal could not be detected even after 24 min of incubation when the EDTA concentration was increased up to 0.7 mM (data not shown).

These results suggest that the extent of lipid peroxidation induced by Cu^{2+} significantly influenced the recycling of Trolox by CoQ_0 in skin homogenates. The lower the extent of lipid peroxidation, the lower the $CoQ_0^{\bullet-}$ decay rate and the weaker the signal intensity of Trolox radical.

Interaction between CoQ_0 and ascorbate

Addition of 0.7 mM ascorbate to phosphate buffer (pH 7.4) containing 0.7 mM Cu^{2+}-generated ascorbyl radical ($Asc^{\bullet-}$; Fig. 6A). The $Asc^{\bullet-}$ signal and $CoQ_0^{\bullet-}$ composite signal appeared upon addition of 2.2 mM CoQ_0 to this mixture (Fig. 6B). The same ESR signal also appeared in the absence of Cu^{2+}, but the presence of Cu^{2+} accelerated the decay of this signal. Only the $CoQ_0^{\bullet-}$ signal was observed in this system after the disappearance of the $Asc^{\bullet-}$ signal (Fig. 6C). No signals were detected in a solution containing 2.2 mM CoQ_0 and 0.7 mM Cu^{2+}. Also, addition of CoQ_0 alone to skin homogenates produced both $Asc^{\bullet-}$ and $CoQ_0^{\bullet-}$ signals.

DISCUSSION

Ascorbic acid, α-tocopherol and coenzyme Q are important antioxidants in skin. Ascorbic acid may perform its antioxidant function either by quenching various free radical species directly or by reducing membrane-bound oxidized vitamin E.[8–12] The ability of reduced coenzyme Q to regenerate the antioxidant form of vitamin E has also been demonstrated.[15–19] Trolox and CoQ_0, the water-soluble homologues of vitamin E and CoQ_{10}, respectively, are effective antioxidants.[24] It has been reported that diets supplemented with multiple antioxidants containing Trolox, ascorbic acid and CoQ_0 provided protection against oxidative damage to rat blood and tissues *in vivo*.[25] This work provides evidence that there is an interaction between ascorbate, CoQ_0 and Trolox; namely, $CoQ_0^{\bullet-}$, formed by direct or indirect interaction of ascorbate and CoQ_0, can reduce the Trolox radical to Trolox (Fig. 7).

In this study, Cu^{2+} was used to initiate lipid peroxidation of mouse skin homogenates. This oxidation system is a simple and convenient system for investigating, by ESR spectroscopy, the recycling of Trolox by CoQ_0, and indirectly by ascorbic acid present in mouse skin homogenates. Phenoxyl radicals of exogenously added Trolox can be directly generated in skin homogenates either by interaction with peroxyl radicals produced by Cu^{2+}-induced lipid peroxidation, or by direct interaction with Cu^{2+}.

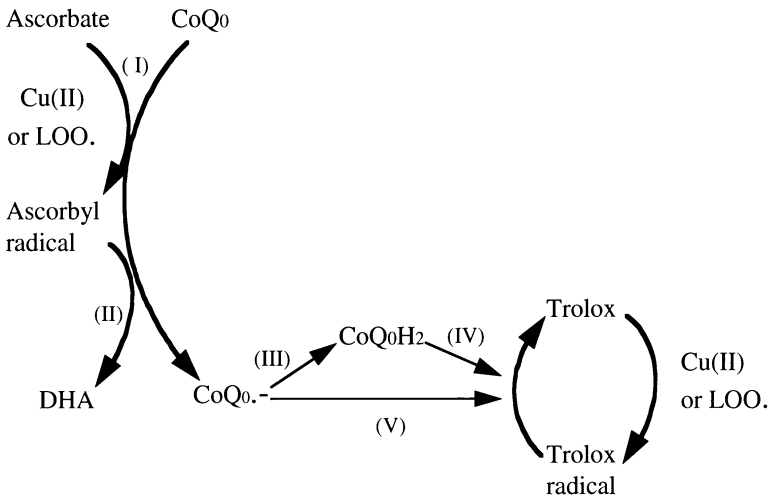

Fig. 7. Scheme of the proposed reactions for the reduction of CoQ_0 and recycling of Trolox indirectly by CoQ_0 in skin homogenates.

The reduced form of CoQ, ubiquinol ($CoQH_2$), is able to recycle α-tocopherol. Thermodynamically, CoQ_0 itself can not reduce Trolox radical to Trolox, as seen from the redox potentials for Trolox radical, H^+/Trolox (0.48 V) and $CoQ/CoQ_0^{\bullet-}$ (–0.11 to –0.15 V).[26] But, based on our results, CoQ_0, the oxidized form of ubiquinol, did appear to recycle Trolox from its corresponding radical form, because the Trolox radical signal re-appeared after the disappearance of the $CoQ_0^{\bullet-}$ signal (Fig. 1) and the lifetime of the $CoQ_0^{\bullet-}$ signal and the delay in the re-appearance of the Trolox radical signal were dependent on the concentration of Trolox or CoQ_0 (Figs 2 & 3).

Why and how did CoQ_0 apparently recycle Trolox in mouse skin homogenates? This question was further investigated. Addition of Cu^{2+} did not result in the formation of $CoQ_0^{\bullet-}$ or the recycling of Trolox by CoQ_0 in phosphate buffer (pH 7.4) or in DOPC liposomes (data not shown). But, using mouse skin homogenates instead of phosphate buffer or DOPC, we observed that the $CoQ_0^{\bullet-}$ signal appeared and Trolox was recycled. Why? Roginsky *et al.*[27] have demonstrated that ascorbic acid can reduce CoQ to $CoQ_0^{\bullet-}$ and/or $CoQH_2$. The concentration of ascorbic acid is estimated to be 0.2–0.5 mM in skin homogenates.[28] Thus, it is possible that the presence of endogenous ascorbic acid may be necessary for CoQ_0 to recycle Trolox in skin homogenates.

To test this hypothesis, we studied the interaction of CoQ_0 and ascorbate. Both the $CoQ_0^{\bullet-}$ signal and the $Asc^{\bullet-}$ signal appeared simultaneously after mixing ascorbate with CoQ_0 in phosphate buffer in the presence or absence of Cu^{2+} (Fig. 6B). In addition, incubation of skin homogenates and CoQ_0 also generated the same signal. Thus, as shown in Figure 7, the following reactions exist in this system:

$$CoQ_0 + AscH^- \rightarrow CoQ_0^{\bullet-} + Asc^{\bullet-} + H^+ \quad \text{Eq. 2: Fig. 7(I)}$$

$$Cu^{2+} + AscH^- \rightarrow Cu^+ + Asc^{\bullet-} + H^+ \quad \text{Eq. 3}$$

$$CoQ_0 + Asc^{\bullet-} \rightarrow DHA + CoQ_0^{\bullet-} \quad \text{Eq. 4: Fig. 7(II)}$$

$$CoQ_0^{\bullet-} + CoQ_0^{\bullet-} \rightarrow CoQ_0 + CoQ_0H_2 \quad \text{Eq. 5: Fig. 7(III)}$$

Stoyanovsky *et al.* have reported that one-electron reduction of ubiquinone-10 by superoxide anion results in the formation of ubiquinone-10 radicals causing redox-recycling of α-tocopherol from its phenoxyl radical.[19] However, in this investigation, it was found that superoxide dismutase (SOD) had no effects on Trolox recycling by CoQ_0 and no ESR signals of DMPO-OOH or DMPO-OH adducts were detected in the presence of 0.25 M DMPO in the incubation system (data not shown), suggesting that superoxide anion was not involved in Trolox recycling by CoQ_0. Thus, ascorbic acid may provide another mechanism for reducing CoQ_0 to $CoQ_0^{\bullet-}$ and/or CoQ_0H_2, and may indirectly regenerate Trolox from its phenoxyl radical (Fig. 7(IV),(V)). It was also found that addition of exogenous ascorbic acid to DOPC liposomes containing CoQ_0 and Trolox, in the presence of Cu^{2+}, resulted in the recycling of Trolox by CoQ_0, similar to the results seen in skin homogenates. In contrast, addition of CoQ_0 to skin homogenates where endogenous ascorbate was oxidized by ascorbate oxidase did not result in the appearance of CoQ_0 semiquinone radical signals in the presence of Cu^{2+} (data not shown).

Thus, our results demonstrate that, in skin homogenates, endogenous ascorbic acid may mediate, directly or indirectly, the formation of CoQ_0 semiquinone radical and/or CoQ_0H_2 (directly or via disproportionation of CoQ_0 semiquinone radicals), which, in turn, can recycle Trolox (Fig. 7). This mechanism is in agreement with the redox potentials for $CoQ_0^{\bullet-}$, $2H^+/CoQH_2$ (0.15 to 0.20 V), $CoQ/CoQ_0^{\bullet-}$ (–0.11 to –0.15 V), $ascorbate^{\bullet-}$,

H^+/ascorbate (0.282 V) and dehydroascorbic/ascorbate$^{\bullet-}$ (-0.174 V).[26] In addition to superoxide anion-mediated recycling of α-tocopherol by CoQ_{10}, its homologue, CoQ_0, can participate in ascorbic acid-dependent recycling of the vitamin E homologue Trolox. Usually both lipophilic and aqueous oxygen reactive species are responsible for oxidative damage to tissues *in vivo*. Thus, synergistic interactions among these antioxidants – CoQ or $CoQH_2$, ascorbic acid and α-tocopherol or Trolox – may strengthen their ability to protect tissues from damage induced by reactive oxygen species.

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