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Redox-interaction of α-tocopheryl quinone with isolated mitochondrial cytochrome bc_1 complex

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Abstract

The homogenous distribution of vitamin E in lipid membranes is a prerequisite for its universal function as lipophilic antioxidant. Its antioxidant activity leads to the irreversible formation of α-tocopheryl quinone (TQ) in those membranes. Very little is known about the interference of TQ with redox-cycling enzymes normally interacting with ubiquinone (UQ), which exerts important bioenergetic functions in the mitochondrial respiratory chain. One of the most complex redox reactions of the respiratory chain is the interaction of reduced UQ (UQH_2) with the cytochrome bc_1 complex (ubiquinol:cytochrome *c* reductase, EC 1.10.2.2). The aim of this study was to elucidate the influence of TQ on the electron transfer from UQH_2 to cytochrome *c* via the isolated mitochondrial cytochrome bc_1 complex. Although TQ is present in substoichiometric amounts with respect to UQ in mitochondria and in our experiments with isolated bc_1 complex, we observed a decrease of the total electron transfer rate via the bc_1 complex with increasing amounts of TQ. Both reduced TQ (TQH_2) and UQH_2 are able to reduce *b*-cytochromes in the bc_1 complex, however, they act in a completely different way. While reduction of *b*-cytochromes by UQH_2 can occur both via the Q_o and the Q_i pocket of the cytochrome bc_1 complex, TQH_2 can preferably reduce *b*-cytochromes via the Q_i pocket. These differences are also reflected by the extremely low turnover numbers of the bc_1 activity for TQ/TQH_2 compared to UQ/UQH_2 suggesting that TQ/TQH_2 acts as a weak competitive inhibitor for binding sites of UQ/UQH_2. In contrast, the oxidation properties of TQ and UQ are similar. Furthermore, oxidized TQ was observed to decrease the $O_2^{\bullet -}$ release rate of UQH_2-consuming cytochrome bc_1 complex. These findings suggest that the irreversible oxidation of vitamin E to TQ in mitochondrial membranes causes a downregulation of respiratory activities as well as a lower $O_2^{\bullet -}$ formation rate by the cytochrome bc_1 complex.

Keywords: α-Tocopheryl quinone; Ubiquinone; Cytochrome bc_1 complex; Mitochondria; Superoxide radicals; Vitamin E

Vitamin E (predominantly natural α-tocopherol) is the most important lipophilic antioxidant protecting biomembranes from oxidative damage. While the hepatic metabolism of this compound was shown to lead to oxidation of the phytyl side chain [1], during the radical-scavenging activity vitamin E is irreversibly degraded to α-tocopheryl quinone (TQ) [2]. Confirmation comes from in vivo studies which showed increased amounts of TQ following oxidative stress occurring in the pathophysiology of arteriosclerosis [3], reperfusion of ischemic hearts [4] or chronic ethanol intoxication in liver [5]. The transport of this metabolite was recently recognized to be of (patho-)physiological significance since a TQ-selective carrier protein was described [6].

The existence of TQ resulting from oxidation of vitamin E in peroxidizing mitochondrial membranes in vivo is likely because of the following findings: (i) a 10 times higher rate constant for the reaction of peroxyl radicals with vitamin E ($3.3 \times 10^6\ M^{-1}\ s^{-1}$) than with ubiquinol-9 ($3.4 \times 10^5\ M^{-1}\ s^{-1}$) [7]; (ii) the demonstration that

Abbreviations: AA, antimycin A; BHT, 2,6-di-tert-butyl-4-methylphenol; cyt b_{562}, high potential cytochrome *b* (b_H) in the bc_1 complex (Q_i pocket); cyt b_{566}, low potential cytochrome *b* (b_L) in the bc_1 complex (Q_o pocket); cyt *c*, cytochrome *c* (oxidized); DTPA, diethylenetriaminepentaacetic acid; dUQ, decyl ubiquinone (2-decyl-5,6-dimethoxy-3-methyl-[1,4]benzoquinone); $dUQH_2$, reduced dUQ; FeS_R, Rieske iron-sulfur protein; MOPS, 3-morpholino-1-propanesulfonic acid; Myx, myxothiazol; P_i, inorganic phosphate; SOD, superoxide dismutase; TQ, α-tocopheryl quinone, 2-(3-hydroxy-3,7,11,15-tetramethyl-hexadecyl)-3,5,6-trimethyl-[1,4]benzoquinone; TQ_0, 2-(3-hydroxy-3-methyl-butyl)-3,5,6-trimethyl-[1,4]benzoquinone; TQ_0H_2, reduced TQ_0; TQH_2, reduced TQ; UQ_n, ubiquinone (*n* indicates the number of isoprene units in the side chain); UQ_nH_2, reduced UQ_n

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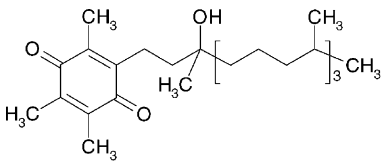
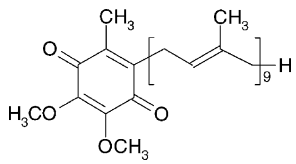
vitamin E is the preferable radical scavenger in mitochondrial membranes while ubiquinol partially recycles chromanoxyl radicals [8]; and (iii) the identification of TQ as oxidation product of vitamin E in liver mitochondrial membranes in vitro [9].

Although conformity exists about the existence of TQ in mitochondrial membranes, the amount reported in the literature is still controversial. For liver mitochondria values of up to 2 nmol/mg protein were published while newer analytical procedures yielded much lower values [10–12]. In a recent study, we detected in heart mitochondrial membranes (submitochondrial particles) amounts of 0.1–0.3 nmol/mg protein [13]. In experiments with isolated perfused rat hearts, it was demonstrated that the tissue concentration can be increased by perfusion of TQ-containing liposomes by a factor of 10 [13]. This suggests that the high content of TQ in certain vegetables [14,15] could contribute to the enrichment of TQ in subcellular membranes. Enrichment of TQ could be of particular significance if it interacts with mitochondrial redox couples which account for oxygen radical release of the respiratory chain under certain conditions [13].

The TQ molecule is highly lipophilic and its quinone moiety exhibits a structural analogy to ubiquinone (UQ). On the other hand, the lipophilic side chains of both molecules display structural differences. While natural α-tocopheryl quinone has a phytyl side chain with three chiral centers in the *RRR*-configuration, natural ubiquinone has a polyisoprenic chain in the all-*E*-configuration.



α-tocopheryl quinone (TQ)



ubiquinone-9 (UQ_9)

Due to the analogy of the head groups and the fact that UQ is a mobile electron carrier in the mitochondrial respiratory chain [16], the question arises whether TQ can interfere with mitochondrial functions of UQ. One of the most susceptible redox couples of the respiratory chain with respect to oxygen radical release is the interaction of UQ with the cytochrome bc_1 complex (ubiquinol:cytochrome *c* reductase, EC 1.10.2.2) located in the inner mitochondrial membrane [17]. This complex possesses two quinone-binding sites, which can harbor up to three UQ molecules in total [18].

The aim of the present study was to clarify whether or not the different redox states of TQ and UQ can coexist in mitochondrial membranes and whether TQH_2 can replace UQH_2 as a reductant of the cytochrome bc_1 complex, which is supposed to be a major source of oxygen radicals during certain conditions of respiration [19].

1. Materials and methods

1.1. Materials and animals

Ubiquinones (dUQ, UQ_6, UQ_9), SOD, MOPS, Triton X-100, Sepharose CL-6B, horse heart cytochrome *c*, antimycin A, myxothiazol and BSA were purchased from Sigma. Vitamin E, pentamethylchromanol, substances for the preparation of buffers, HPLC eluents and all other compounds (analytical grade) were obtained from Merck. Beef hearts were from the local slaughterhouse. Male Sprague–Dawley rats were provided by the Research Institute for Laboratory Breeding in Himberg (Austria).

1.2. Isolation of the mitochondrial cytochrome bc_1 complex

One hundred milliliter beef heart mitochondrial suspension was prepared from fresh beef heart by differential centrifugation according to Smith [20]. The preparation method of the bc_1 complex was adopted from Schägger et al. [21]. Briefly, after a centrifugation of the mitochondrial suspension at 27,000 × *g* for 15 min the pellet was resuspended in MOPS buffer (20 mM, pH 7.2) to give a protein concentration of 35 mg/ml. Mitochondria were partially solubilized by the addition of Triton X-100 (1.75%) and NaCl (600 mM) giving a protein concentration of 26 mg/ml. The pellet obtained by ultracentrifugation at 100,000 × *g* for 45 min was resuspended in a buffer containing 300 mM sucrose, 20 mM MOPS (pH 7.2) giving a protein concentration of 35 mg/ml. The resulting suspension was mixed with an equal volume of extraction buffer (4% Triton X-100, 1.2 M NaCl, 20 mM MOPS, 300 mM sucrose, 2 mM NaN_3, pH 7.2) and stirred for 5 min. The supernatant obtained by ultracentrifugation (45 min, 100,000 × *g*) was mixed with an equal volume of hydroxyapatite (prepared according to Tiselius et al. [22]) equilibrated with 0.5% Triton X-100, 250 mM NaCl and 80 mM NaP_i to bind cytochrome bc_1 complex. After a slow centrifugation (1 min, 430 × *g*) the bc_1/hydroxyapatite sediment was washed with five volumes of buffer (0.05% Triton X-100, 250 mM NaCl, 110 mM NaP_i, 2 mM NaN_3, pH 7.2). The washed sediment was filled into a preparative column and the crude bc_1 complex was eluted with buffer (0.25% Triton X-100, 0.2 M KP_i, 2 mM NaN_3, pH 7.2). The crude detergent-solubilized bc_1 complex was concentrated to 15 mg protein/ml by pressure filtration using Amicon YM100 membranes (Millipore). Finally, the enzyme preparation was purified by gel chromatography using a Sepharose CL-6B column eluted with

0.05% Triton X-100, 100 mM NaCl, 20 mM MOPS, 2 mM NaN_3, pH 7.2. A typical purified bc_1 preparation contained about 13 nmol cytochrome *b* per mg protein (corresponding to 6.5 nmol bc_1 complex/mg protein) and was stored at 77 K after addition of 50% glycerol. The maximal turnover number of the bc_1 complex without further reconstitution with phospholipids was about 200 nmol reduced cyt *c*/(nmol bc_1 complex × second). HPLC analysis confirmed that the isolated bc_1 complex exhibited a residual UQ_{10} content of 0.039 ± 0.007 nmol UQ_{10} per nmol bc_1 complex. A residual TQ content could not be detected in the isolated bc_1 complex by HPLC analysis above the detection limit of about 0.001 nmol TQ per nmol bc_1 complex.

1.3. Preparation of TQ and TQ_0

TQ and TQ_0 (2-(3-hydroxy-3-methyl-butyl)-3,5,6-trimethyl-[1,4]benzoquinone) were prepared by $FeCl_3$ oxidation of α-tocopherol and 2,2,5,7,8-pentamethyl-chroman-6-ol and chromatographic purification as described previously [13].

1.4. Preparation of TQ_0H_2 and $dUQH_2$

Reduced TQ_0 (TQ_0H_2) and reduced dUQ ($dUQH_2$) were prepared from oxidized quinones by reduction with KBH_4 in ethanolic solution as described previously [13].

1.5. Redox-interaction of TQ_0/TQ_0H_2 and $dUQ/dUQH_2$ in a model system

TQ_0, TQ_0H_2, dUQ and $dUQH_2$ (8 μM each) were separately dissolved in deoxygenated buffer (50% ethanol/50% H_2O, 20 mM KP_i, pH 6 or 11.4) and UV spectra were recorded. From these data the difference spectrum of a reaction of equimolar amounts of TQ_0H_2 and dUQ to TQ_0 and $dUQH_2$ was calculated. Experimentally TQ_0H_2 and dUQ were dissolved in two different cuvettes with 0.5 cm path length each and a reference spectrum of both cuvettes together (in tandem position) was recorded with a DW-2000 (SLM AMINCO) spectrophotometer. Afterwards the contents of both cuvettes were mixed in the absence of oxygen, aliquots were filled into the same cuvettes and the difference spectrum was obtained.

1.6. HPLC analysis of quinones and vitamin E in subfractions of rat liver

Liver mitochondria from male Sprague–Dawley rats with an age of 3 month ($n = 6$) were obtained by differential centrifugation as described in [23]. For each sample 2 mg of mitochondrial protein in 1 ml H_2O were admixed with BHT (0.2 mM) and SDS (5 mM) prior to extraction with 3 ml ethanol/hexane (2:5) under an argon atmosphere. After phase separation the organic phase was removed and evaporated to dryness. The residue was dissolved in ethanol and analyzed by HPLC on a Waters LC1 module equipped with an UV detector for measurements of oxidized quinones at 268 nm (TQ) and 275 nm (UQ). Reduced quinones and vitamin E were determined by an electrochemical detector (Shimadzu L-ECD-6A), which was set to a potential of +0.6 V. The column (Merck, Nova-Pak C18 3.9 mm × 150 mm) was eluted by 1 ml/min mobile phase consisting of $NaClO_4$ (50 mM) dissolved in a mixture of ethanol, methanol, acetonitrile and $HClO_4$ (400:300:300:1). Concentrations of TQ, TQH_2, UQ_9 UQ_9H_2, and vitamin E were calculated using an internal standard of UQ_6. Protein concentrations were determined according to the method of Biuret [24].

1.7. Measurement of cytochrome b reduction kinetics in the cytochrome bc_1 complex by TQ_0H_2 and $dUQH_2$

Air–oxidized cytochrome bc_1 complex (0.61 μM) was diluted in 1 ml buffer (250 mM sucrose, 0.2 mM EDTA, 50 mM KP_i, pH 7.2, 0.1% BSA, 2 mM KCN, 1 mM NaN_3) and absorptions at 562 and 575 nm (reference wavelength) [25] were recorded simultaneously with a diode-array photometer (Milton Roy MR3000). After 100 s the reduction was initiated by the addition either of $dUQH_2$ or TQ_0H_2 (25 μM each) using a Hamilton syringe. Experiments were repeated in the presence of inhibitors of the bc_1 complex antimycin A (36 μM) and/or myxothiazol (41 μM). The cytochrome *b* reduction kinetics were calculated from the absorption changes at 562 nm minus 575 nm.

1.8. Measurement of cytochrome b oxidation in the cytochrome bc_1 complex by TQ_0 and dUQ

Detergent-solubilized cytochrome bc_1 complex (3.4 μM) was diluted in 1 ml buffer (250 mM sucrose, 0.2 mM EDTA, 50 mM KP_i, pH 7.2, 0.1% BSA, 2 mM KCN, 1 mM NaN_3) and reduced by dithionite (9.9 mM) in a cuvette of a DW-2000 (SLM AMINCO) spectrometer. The complete reduction of the bc_1 complex was verified photometrically and further additions of buffer did not reoxidize the bc_1 complex. Afterwards oxidized quinones, either dUQ (100 μM) or TQ_0 (100 μM), were added to non-inhibited and inhibited bc_1 complex (10 μM antimycin A or 10 μM myxothiazol) and the oxidation and re-reduction kinetics (by excessive dithionite) of the *b*-cytochromes were obtained from absorption changes at 566 nm minus 575 nm.

1.9. Measurement of the quinol:cytochrome c oxidoreductase activity of the bc_1 complex

The activity was measured in a buffer containing 250 mM sucrose, 0.2 mM EDTA, 0.1% BSA, 50 mM KP_i, pH 7.2, 2 mM KCN, 1 mM NaN_3, 100 μM cyt *c*. First the non-enzymatic reduction of cytochrome *c* by

$dUQH_2$ was followed for 1 min at 550 nm minus 540 nm in a DW-2000 (SLM AMINCO) dual-wavelength photometer. One microliter bc_1 complex was added and the cytochrome *c* reduction was recorded for another minute. If required purified bc_1 complex was diluted with buffer (200 mM KCl, 10 mM MOPS, pH 7.2) prior to activity measurements. The enzyme activity was calculated from the difference of the two rates using an extinction coefficient of 19 mM^{-1} cm^{-1} [26] and was expressed as turnover numbers, i.e. nmol reduced cyt *c*/(nmol bc_1 complex × second). Alternatively TQ_0H_2 was used instead of $dUQH_2$. For experiments with different ratios of the quinones 30 μM cyt *c*, 16 μM $dUQH_2$ and a constant sum of 16 μM oxidized quinones (0–16 μM TQ_0, 0–16 μM dUQ) was used.

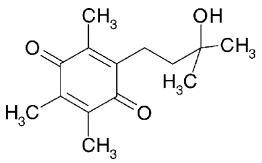
1.10. Measurement of $O_2^{\bullet -}$ production of the isolated bc_1 complex

The release of $O_2^{\bullet -}$ from isolated bc_1 complex was assessed from the difference of the cytochrome *c* reduction in the presence and in the absence of SOD (“SOD-sensitive cytochrome *c* reduction”). In 3 ml buffer (150 mM KCl, 1 mM EDTA, 20 mM triethanolamine (pH 7.5), 1 mM DTPA, 1 mM KCN) 18 nM bc_1 complex, 30 μM cyt *c* were premixed. Two cuvettes were placed in the sample and reference beam of a DW-2000 (SLM AMINCO) spectrometer containing 200 μl buffer with 15 μM (final concentration) $dUQH_2 \pm 15$ μM TQ_0. Additionally, SOD (7 μg/ml final concentration) was added to the sample cuvette. Photometric measurements at 550 nm (double beam mode) were started and the premixed solution (3 ml) containing bc_1 complex and cytochrome *c* was filled from a syringe via a Y-joint into the two cuvettes. $O_2^{\bullet -}$ formation was determined from the rates of SOD-sensitive cytochrome *c* reduction (1 mol $O_2^{\bullet -}$ reduces 1 mol cytochrome *c*) using an extinction coefficient of 21 mM^{-1} cm^{-1} [27] and expressed as nmol $O_2^{\bullet -}$/(nmol bc_1 complex × second) [17,19].

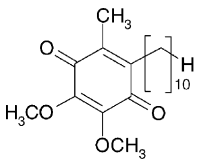
2. Results

Various studies demonstrated the accumulation of TQ in different tissues after acute or chronical periods of oxidative stress [3–5]. Since the amount of TQ in mitochondrial membranes is still a matter of debate we studied the content of TQ in mitochondrial membranes from livers of male Sprague–Dawley rats ($n = 6$). HPLC analysis of TQ in liver mitochondria revealed amounts of 0.033 ± 0.006 nmol/mg protein. The analysis of the vitamin E content in those membranes gave values of 1.2 ± 0.1 nmol/mg protein establishing a reservoir for an increased TQ formation under conditions of oxidative stress in mitochondria. In comparison with the total content of ubiquinones (1.3 ± 0.1 nmol/mg protein) TQ amounts to 2.5% of the total quinone pool, ranging between 1 and 5% for the individual animals.

In context with the observation that TQ levels in membranes were increased under certain pathophysiological conditions these findings prompted us to study the influence of substoichiometric amounts of TQ (with respect to UQ) on the transfer of electrons from reduced ubiquinone (UQH_2) to cytochrome *c* via the isolated mitochondrial bc_1 complex. Due to the poor solubility of natural TQ and UQ these quinones cannot be used directly as substrates for kinetic measurements [28]. However, the facts that binding pockets at the bc_1 complex specifically interact with the quinone moiety of substrates [29] while binding of the hydrophobic tail seems to have only a modulating effect [30] render the use of TQ and UQ with modified side chains possible. For these reasons a low molecular weight analogue of natural UQ, the 2-decyl-5,6-dimethoxy-3-methyl-[1,4]benzoquinone (dUQ) was used in this study, which was also applied as artificial mitochondrial substrate by other groups [31]. In order to compare this substrate with a TQ-derivative of about the same lipophilicity, 2-(3-hydroxy-3-methyl-butyl)-3,5,6-trimethyl-[1,4]benzoquinone (TQ_0) was synthesized from pentamethylchromanol. Thus, the relative decrease of the lipophilicity of both artificial substrates with respect to their natural counterparts is comparable. Due to the dominating role of the quinone moiety binding at the bc_1 complex, these low molecular weight compounds should be suitable to predict the binding behaviour of their natural analogues.



α-tocopheryl quinone-0 (TQ_0)



decyl-ubiquinone (dUQ)

Since UQ is permanently cycled in the mitochondrial membrane between the oxidized and the reduced state, the question arises whether TQ and UQ in different redox states can exchange reducing equivalents with each other.

$$E_{m7}\left(\frac{UQ}{UQH_2}\right) = +90\,mV$$

$$E_{m7}\left(\frac{TQ}{TQH_2}\right) = +50\,mV^{1}$$

The redox potentials, which were calculated from the half sum of the one-electron potentials at pH 7 $E(Q/Q^{\bullet -})$

[1] This redox potential was adopted from duroquinone which possesses the same quinone moiety as TQ.

and $E(Q^{\bullet -}/QH_2)$ [32,33], predict that for equimolar concentrations reduced TQ should not coexist with oxidized ubiquinone.

$$TQH_2 + UQ \rightleftharpoons TQ + UQH_2 \quad (1)$$

$$K = \frac{[TQ] \times [UQH_2]}{[TQH_2] \times [UQ]} \approx 22.5 \quad (2)$$

Calculation of the equilibrium constant at pH 7 yielded a value of 22.5 which is in favor of reduced UQ and oxidized TQ. However, extraction of quinones from mitochondrial membranes often yields TQH_2 besides oxidized UQ. In order to clarify this contradiction, model experiments were performed in a solvent which mimics the situation in mitochondrial membranes (Fig. 1). Due to spectral similarity of TQ ($\lambda_{max} = 268$ nm, $\varepsilon = 18.2\ mM^{-1}\ cm^{-1}$) [34] and UQ ($\lambda_{max} = 275$ nm, $\varepsilon = 14.2\ mM^{-1}\ cm^{-1}$) [35] and their corresponding reduced species the photometric analysis required a simulation of the expected spectral changes during equilibration. Fig. 1A shows UV-spectra of the two opposite redox states, i.e. equimolar amounts of TQ_0H_2 and dUQ or TQ_0 and $dUQH_2$, which were obtained by placing two cuvettes with the individual quinones in series in the light path of the UV-spectrometer. By spectral subtraction it was shown that reaction (1) using equimolar amounts of reactants should result in the difference spectrum indicated by the continuous line in Fig. 1A. Fig. 1B displays difference spectra which were obtained after the reaction between TQ_0H_2 and dUQ in buffered anaerobic ethanol solutions. The shape of the difference spectrum and the isosbestic point at 277 nm for pH 11.4 clearly indicate that a rapid reaction takes places only at very high pH values implicating the requirement of deprotonated quinone species for this reaction. The small shift of the maximum for the positive slope is due to the slightly different absorption of half-reduced species [28], which were not considered in the simulation. In pure lipid membranes, however, charged quinone species are unlikely to exist, excluding the equilibration by this chemical reaction. Therefore, in lipid membranes of respiring mitochondria a coexistence of TQH_2 and UQ appears to be likely and prompted us to compare the activity of TQ_0H_2 and $dUQH_2$ as substrates for the mitochondrial bc_1 complex, the native quinol-oxidizing enzyme in the inner mitochondrial membrane.

The measurement of the quinol:cytochrome *c* oxidoreductase activity for the two hydroquinones revealed that $dUQH_2$ (Fig. 2A) is a significantly more efficient substrate for the bc_1 complex than TQ_0H_2 (Fig. 2B). Maximal turnover numbers (v_{max}) for $dUQH_2$ of $286 \pm 35\ s^{-1}$ and for TQ_0H_2 of $0.5 \pm 0.04\ s^{-1}$ were obtained by fitting the experimental data with the Michaelis–Menten equation. This suggests that tocopheryl quinone cannot replace ubiquinone as mobile electron carrier in the regular respiratory chain. However, the K_m values of $dUQH_2$ and TQ_0H_2 were of the same order of magnitude (398 ± 91 and 85 ± 17 μM, respectively) and imply a stronger binding of TQ_0/TQ_0H_2 to the cytochrome bc_1 complex.

In order to elucidate the mechanistic details behind this difference we studied the reduction/oxidation kinetics of the bc_1 complex by the respective quinols/quinones. The kinetic traces in Fig. 3 demonstrate the ability of the two hydroquinones to reduce *b*-cytochromes in the bc_1 complex via the ubiquinone-binding sites associated with cytochromes b_{562} (b_H) and b_{566} (b_L)/FeS_R, named Q_i and Q_o, respectively. Both hydroquinones reduce the two *b*-cytochromes (b_{562} and b_{566}) in the absence of

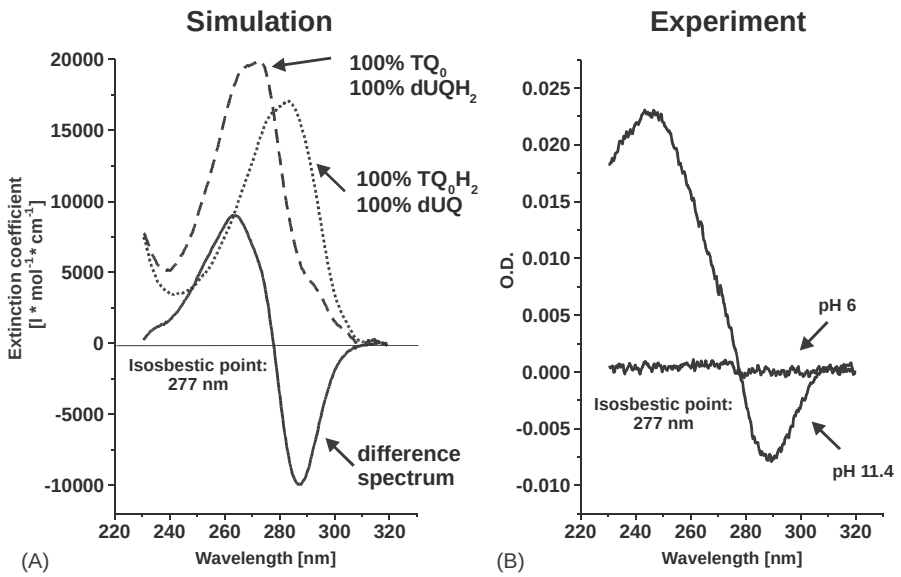


Fig. 1. Equilibration of TQ_0H_2 and dUQ to TQ_0 and $dUQH_2$. (A) UV-spectra of a simulated mixture of TQ_0H_2 and dUQ (dotted line) and TQ_0 and $dUQH_2$ (dashed line) obtained from two staggered cuvettes containing the individual quinones. From these spectra the difference spectrum simulating the reduction of dUQ by TQ_0H_2 was calculated (solid line). (B) UV-spectra obtained after mixing of equimolar amounts (8 μM each) of TQ_0H_2 and dUQ in a buffered ethanol solution at pH 6 and 11.4 under anaerobic conditions.

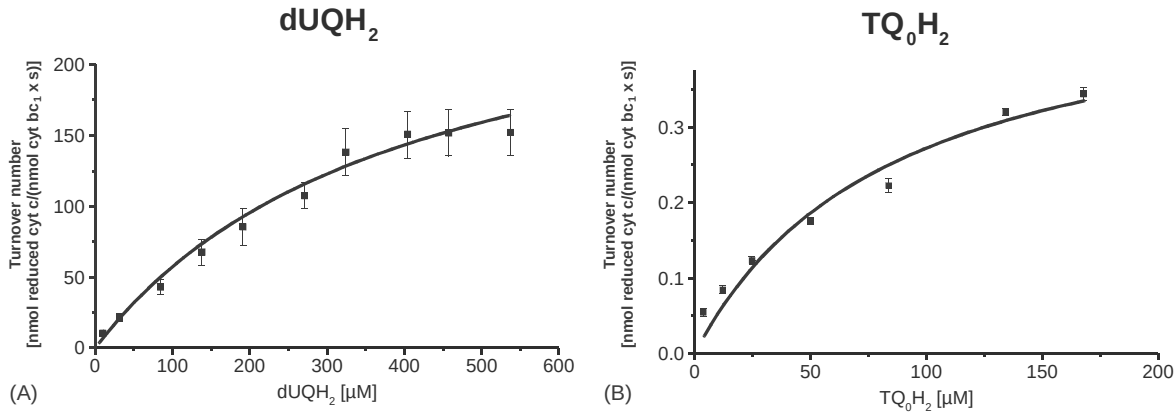


Fig. 2. Quinol:cytochrome *c* oxidoreductase activity of the isolated bc_1 complex ((A) 8.4 nM, (B) 6.8 µM) for (A) $dUQH_2$ and (B) TQ_0H_2 as substrate. The activity was expressed as nmol reduced cyt *c*/(nmol bc_1 complex × second). Each data point represents the mean of three measurements. The data were fitted by a non-linear regression according to the Michaelis–Menten equation. The buffer contained 100 µM cyt *c* as acceptor.

inhibitors to the same reduction level (about 40–50% of the dithionite-reducible amount). In the chosen timescale the rates of cytochrome *b* reduction were similar for $dUQH_2$ (Fig. 3A) and TQ_0H_2 (Fig. 3B). Repeating the experiments in the presence of inhibitors for the Q_i site (antimycin A) and the Q_o site (myxothiazol) did not significantly change the cytochrome *b* reduction rates and levels achieved by $dUQH_2$ as substrate. Only under double block conditions [36] with both inhibitors present, the reduction was slowed down and a lower final reduction state was reached. However, in the case of TQ_0H_2 already the presence of antimycin A significantly hindered cytochrome *b* reduction by this substrate. In addition, under double block conditions a lower reduction state resulted as well. The sensitivity of the reduction by TQ_0H_2 to antimycin A suggests a preferred binding of this hydroquinone to the Q_i pocket of the protein. In contrast, $dUQH_2$ as substrate obviously can equilibrate with *b*-cytochromes both via the Q_i and the Q_o site.

In analogous experiments we studied the response of fully reduced bc_1 complex to oxidized dUQ and TQ_0 (Fig. 4). In the non-inhibited bc_1 complex equal amounts of quinones caused a temporary reoxidation of *b*-cytochromes with a major absorbance change at 566 nm. This was followed in both cases by a slow re-reduction driven by an excessive amount of dithionite in our system. Therefore, the resulting oxidation/reduction kinetics of *b*-cytochromes were followed as absorption differences between 566 and 575 nm. In the absence of inhibitors equal amounts of quinone (100 µM each) caused a rapid oxidation of cyt b_{566} of about 18% (dUQ) and 9% (TQ_0). After addition of myxothiazol (Q_o inhibitor) the oxidation/reduction kinetics for both quinones were similar to control conditions. In contrast, the Q_i site inhibitor antimycin A completely prevented an oxidation of *b*-cytochromes by both TQ_0 and dUQ, suggesting similar substrate properties of oxidized quinones at the Q_i site. In contrast to the reduction of *b*-cytochromes, which can occur both via the Q_i and Q_o site, the oxidation of the *b*-cytochromes by either quinone takes place exclusively via the Q_i site.

The latter findings, however, suggest that TQ can modulate the electron transfer at the bc_1 complex. Therefore,

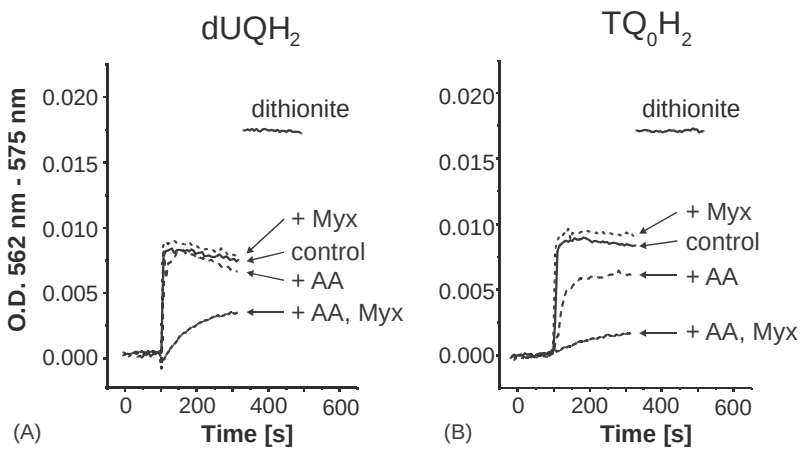


Fig. 3. Kinetics of cytochrome *b* reduction in the isolated bc_1 complex (0.61 µM) by: (A) $dUQH_2$ and (B) TQ_0H_2 (25 µM each) in the absence (''control'') and presence of inhibitors: 41 µM myxothiazol (Myx), 36 µM antimycin A (AA). The total amount of reducible *b*-cytochromes was obtained by the reduction with dithionite.

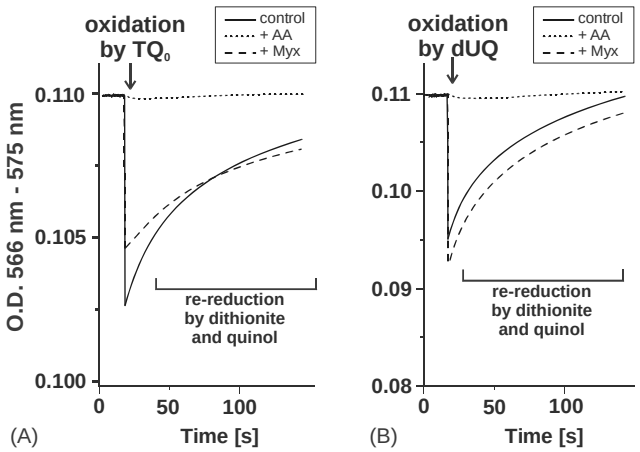


Fig. 4. Oxidation and subsequent re-reduction of *b*-cytochromes in dithionite-reduced isolated bc_1 complex (3.4 μM) by (A) TQ_0, (B) dUQ (100 μM each) in the absence (“control”) or presence of inhibitors: 10 μM myxothiazol (Myx) and 10 μM antimycin A (AA).

we studied the electron flow rates in the non-inhibited bc_1 complex using $dUQH_2$ as electron donor and cytochrome *c* as acceptor in the presence of different ratios of dUQ and TQ_0. For the establishment of the same redox state of the Q-pool, the molar amounts of oxidized quinones (TQ_0 plus dUQ) were kept constant. As shown in Fig. 5, increasing portions of TQ_0 decreased overall electron transfer rates already at physiologically relevant ratios of UQ to TQ of 20:1 (corresponding to 5% TQ of total quinone in Fig. 5). Furthermore, a linear relationship between increasing amounts of TQ_0 and the initial rates of cytochrome *c* reduction was observed.

All results presented so far demonstrate the unique selectivity of the bc_1 complex for UQ as substrate but

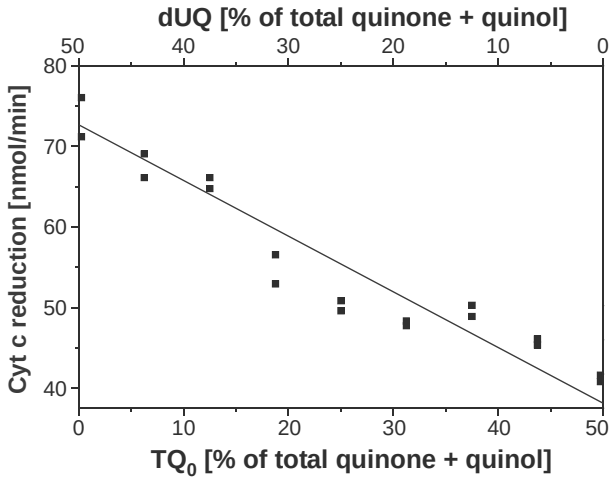


Fig. 5. Modulation of the quinol:cytochrome *c* oxidoreductase activity by different ratios of TQ_0 and dUQ. The bc_1 complex was preincubated with 16 μM (TQ_0 + dUQ) in different ratios and subsequently the reaction was initiated by the addition of 16 μM $dUQH_2$. The amount of 5% TQ_0 corresponds to the natural TQ/UQ ratio in liver mitochondria of 1:20. The buffered solution (1 ml) contained 0.091 μM bc_1complex, 30 μM cyt *c*, 20 μg/ml SOD and 16 μM (TQ_0 + dUQ) plus 16 μM $dUQH_2$ as substrates.

show the possible interference of substoichiometric amounts of TQ in its catalytic activities as well. Due to this observation the question arises whether the accumulation of TQ in mitochondrial membranes could be critical for the univalent reduction of oxygen by the mitochondrial electron transfer system. Therefore, we compared the ability of $dUQH_2$ to produce superoxide radicals during consumption via the bc_1 complex in the absence and in the presence of TQ_0. The rate of $O_2^{\bullet -}$ formation was assessed from the difference of the quinol-dependent cytochrome *c* reduction by the bc_1 complex with and without SOD. After supplementation with $dUQH_2$ (15 μM) as substrate a release rate of 0.149 ± 0.007 nmol $O_2^{\bullet -}$ per nmol bc_1 per second ($n = 9$) during consumption via the bc_1 complex was detected. However, if additionally oxidized TQ_0 (15 μM) was present, a statistically significant ($P < 0.05$) decrease of the $O_2^{\bullet -}$ release rate to 0.091 ± 0.006 nmol $O_2^{\bullet -}$ per nmol bc_1 per second ($n = 9$) was observed. This supports the concept of a competition of TQ with UQ-binding to the cytochrome bc_1 complex.

3. Discussion

Vitamin E exerts a variety of physiological effects. Among those the antioxidant activity is of major interest. In contrast, one of the stable degradation products emerging from its antioxidant function, α-tocopheryl quinone (TQ), was not considered so far as a metabolite of biological interest.

While steady-state levels of TQ in mitochondrial membranes are rather low due to an efficient recycling of the chromanoxyl radical back to α-tocopherol, it was demonstrated that under pathophysiological conditions such as arteriosclerosis, ischemia/reperfusion, aging and high intake of TQ-containing food, TQ concentrations in biomembranes increase [3,4,14,15]. This is also valid for mitochondrial membranes as demonstrated for ethanol-intoxicated rats [37]. The recently discovered TQ-specific lipid transporter [6] rises the question whether TQ can be removed from or transported to mitochondria by this protein factor maintaining a steady state concentration in mitochondrial membranes. Therefore, also the hypothesis that TQ exerts certain physiological functions in mitochondrial membranes has to be considered [38].

In addition, in a previous study [13] it was suggested that reduced tocopheryl quinone can supply electrons to the mitochondrial bc_1 complex in submitochondrial particles.

However, it remains unclear whether TQH_2 itself can act as a substrate for the bc_1 complex or whether TQH_2 effects are indirectly mediated by reduction of the ubiquinone pool which in turn can reduce the bc_1 complex. This open question prompted us to investigate the interaction of tocopheryl quinone with the isolated bc_1 complex. The extremely low residual amount of 0.039 nmol UQ_{10} per nmol bc_1 complex and the virtual absence of TQ in the bc_1

preparations allowed to study the substrate properties of TQ and UQ independently from each other.

The physiological ratio of UQ to TQ is about 20:1 in liver mitochondria from young healthy rats. Our data did not confirm the 1:1 ratio reported by Hughes and Tove [10]. The high stoichiometric excess of UQ with regard to TQ is in contrast to the quasi one-to-one ratio of vitamin E and UQ in mitochondrial membranes which can give rise to much higher TQ concentrations under conditions of oxidative stress, e.g. ethanol intoxication [37].

Physico-chemical experiments have shown (Fig. 1) that TQH_2 and UQ may coexist in lipid membranes. This is due to the fact that equilibration preferably occurs via charged, i.e. deprotonated, quinol species as well as deprotonated semiquinones, which are not stabilized in pure lipid membranes. Furthermore, this observation corresponds to the general rule that the rate of electron transfer is decreased by participation of protonated species [33].

The K_m value of $dUQH_2$–binding to the cytochrome bc_1 complex (398 μM) (Fig. 2) corresponds to findings of Lenaz et al. [39]. They found K_m values of about 560 μM (0.28 nmol UQ/mg mitochondrial protein) for UQ binding to succinate oxidase proteins (including the bc_1 complex) in native mitochondrial membranes.

The lower K_m value for TQ_0H_2 (85 μM) as compared with $dUQH_2$ indicates a stronger binding of TQ_0H_2 to the cytochrome bc_1 complex. However, as demonstrated by the different maximal turnover numbers of quinol:cytochrome *c* oxidoreductase activity it is unlikely that TQ can replace UQ as an electron carrier.

Thus, an interference of TQ with the regular electron transfer can be expected by competing with UQ for binding sites of the bc_1 complex.

As demonstrated in this study, TQ_0 could partially replace dUQ at the Q_i site of the bc_1 complex. This was concluded from the same inhibition pattern of cytochrome *b* oxidation by TQ_0 and dUQ at the Q_i site (Fig. 4). Support for this finding comes from the fact that TQ can provide at least two groups (2 C═O) for H-bond formation at the Q_i site. For ubiquinone in the bc_1 complex, three groups (2 C═O, OCH_3) have been detected to bind to the amino acid residues Lys^{227}/Asp^{228}, His^{201} and Ser^{205} in the Q_i pocket [29]. However, H-bonding via a methoxy group to Ser^{205} as for ubiquinone is not possible for the methyl groups of TQ.

In contrast, electron bifurcation at the Q_o site seems to be more specific for UQ than for TQ. This was demonstrated by the low quinol:cytochrome *c* oxidoreductase activity of TQH_2 (Fig. 2B) and the slow and incomplete electron transfer from TQH_2 to *b*-cytochromes in the presence of antimycin A (Fig. 3B). Recently, Brandt and coworkers demonstrated that two UQ molecules occupy the Q_o pocket [18] which suggests a cooperative action of both molecules during electron bifurcation. Our data and structural differences between both quinones corroborate the concept that TQH_2 is not able to mimic UQH_2-guided electron bifurcation.

The $dUQH_2$:cytochrome *c* oxidoreductase activity in the presence of substoichiometric amounts of TQ_0 reveals that TQ_0 is able to slow down the electron transfer via the bc_1 complex (Fig. 5). A similar effect was observed by Yu and Yu [40], who, however, attributed this effect to vitamin E itself. Under pathophysiological conditions which cause lipid peroxidation of biomembranes, accumulation of TQ arising from vitamin E [9] or TQH_2 [41–43] can be expected to decrease mitochondrial energy-linked respiration. The presence of increasing amounts of oxidized TQ_0 not only inhibited the electron transfer from $dUQH_2$ to cytochrome *c* (Fig. 5), it diminished the quinol-driven $O_2^{\bullet -}$ formation of the isolated bc_1 complex as well. Our findings support the concept that TQ/TQH_2 could have regulatory functions in the mitochondrial membrane under certain metabolic conditions.

Acknowledgments

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