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A New Ubiquinone Metabolite and Its Activity at the Mitochondrial bc_1 Complex

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Ubichromanol, a reductive cyclization product of ubiquinone, acts as radical scavenging antioxidant and is similarly effective as α-tocopherol. However, nothing is known so far on the two-electron oxidation product of this antioxidant and its bioactivity. This study demonstrates that ubichromanol yields a ubiquinone-like compound with a hydroxyl-substituted side chain (UQOH) on oxidation. HPLC/MS and HPLC/ECD measurements revealed its natural presence in bovine liver mitochondria. The bioactivity of this formerly unknown compound as substrate for mitochondrial complex III was tested by measurements of the quinol:cytochrome c oxidoreductase activity in bovine submitochondrial particles and isolated mitochondrial bc_1 complex. Consistently in both model systems, reduced UQOH exhibited substrate efficiencies below that of native ubiquinone but a significantly higher efficiency than α-tocopheryl quinone. Model calculations revealed that on binding of reduced UQOH to the bc_1 complex the polar hydroxyl group was located close to hydrophobic amino acid residues. This fact could in part explain the lower efficiency of reduced UQOH in comparison to ubiquinone as a substrate for the mitochondrial bc_1 complex. Therefore, the hydroxylation of the aliphatic or isoprenoid side chains of bioquinones, which is typical for quinoid oxidation products of chromanols, such as α-tocopherol and ubichromanol, disturbs substrate binding at the mitochondrial electron-transfer complexes, which usually interact with ubiquinone.

Introduction

Ubiquinones (UQ)[1] and α-tocopherol (Toc) are compounds occurring in mammalian cells, which play important roles in bioenergetics (*1*) and antioxidative defense (*2*) as well as modulation of signal transduction (*3*). While these functions are considered as clearly physiologically distinct activities, there is a close chemical relation between bioquinones and biochromanols: Reductive cyclization and oxidative ring opening can interconvert both compounds into each other.

For the biologically most important component of vitamin E, Toc, the relation between the chromanol state and its two-electron oxidation product α-tocopheryl quinone (TQ) is well-known (*4*−*6*). While all organisms able to perform photosynthesis can cyclize the vitamin E precursors to chromanol compounds, mammalian species have lost this biosynthetic ability during evolution (*7*). Therefore, mammals have to supply their biomembranes with vitamin E through the diet (*8*−*10*).

Mammalian organisms metabolize tocopherols through two distinct pathways (*8*). Especially, the liver degrades excessive dietary tocopherols, which are not consumed in an antioxidant reaction, by side chain oxidation (*11*). Toc, which is distributed in the lipid membranes of various organs, acts there as a radical-scavenging antioxidant forming the chromanoxyl radical on one-electron oxidation. This radical, which can be recycled by other antioxidants, can also be further oxidized to the two electron-oxidation product TQ (*12*−*14*).

Because this quinoid oxidation product has a structure similar to UQ, it has been suggested that TQ interferes with certain mitochondrial UQ functions (*15*). This was confirmed by the finding that TQ acts as a weak competitive inhibitor at the mitochondrial complex II and complex III (*16*).

The presence of a cyclase activity leading from quinones to chromanols in mammalian tissues was never confirmed for UQ and TQ. Therefore, it would be of interest whether chromanol analogues of UQ, such as ubichromanol (UCa) and ubichromenol (UCe), are natively present in mammalian cells and whether,

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[1] Abbreviations: BLM, bovine liver mitochondria; BSA, bovine serum albumin; cyt, cytochrome; MOPS, 3-morpholino-1-propane sulfonic acid; RLM, rat liver mitochondria; RT_{rel}, relative retention time; SDS, sodium dodecyl sulfate; SMA, stigmatellin; SMP, submitochondrial particles; OPLS, optimized potentials for liquid simulations; Toc, α-tocopherol; TQ, α-tocopheryl quinone; TQ-H_2, α-tocopheryl hydroquinone; UCa, ubichromanol; UCe, ubichromenol; UQ, ubiquinone; UQ-H_2, ubihydroquinone; UQOH, hydroxylated ubiquinone; UQOH-H_2, hydroxylated ubihydroquinone.

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in principle, chromanol analogues of UQ oxidize to UQ-like products. In the past, there have been only a few reports on the presence of UCe in liver homogenates (*17*−*19*). Links questioned some of these reports by proving that chromatographic materials, such as aluminum oxide (*20*), can transform UQ to UCe. As long as the mechanism of the mitochondrial energy transduction was still unclear (*21*), it was speculated that cyclized (ubi-)quinones (UCe and UCa) could be energy-transducing intermediates during oxidative phosphorylation (*22*, *23*). On the basis of this theory, several phosphorylated ubichroma(e)nols were synthesized in the past (*24*, *25*). However, the nature and the properties of UCa/UCe oxidation products were never clarified.

Therefore, we designed a study to explore the presence of bicyclic UQ analogues in mammalian liver mitochondria as well as the nature and properties of oxidation products as substrates for the mitochondrial bc_1 complex in comparison to UQ and TQ.

Materials and Methods

Chemicals. UQ_{10} was obtained from Kanegafuchi. UQ_9, $dUQH_2$, horse heart cytochrome (cyt) c, Triton X-100, Sepharose CL6B, and glycerol were purchased from Sigma, and organic solvents for synthesis and HPLC, as well as NaCl, Na_2SO_4, NaH_2PO_4, KH_2PO_4, 3-morpholino-1-propane sulfonic acid (MOPS), $CaCl_2$, $HClO_4$, $NaClO_4$, KBH_4, NaN_3, KCN, and $Ce^{IV}(NH_4)_2(NO_3)_6$ were from Merck. All chemicals were at least of analytical grade purity.

Synthesis of UCe. UCe_9[2] was synthesized by cyclization of 250 mg of UQ_{10} in 1 mL of deoxygenated triethylamine for 2 h at 95 °C according to Imada and Morimoto (*26*). After removal of the solvent, the product was purified by column chromatography (Florisil, Merck), using a mixture of *n*-hexane:$CHCl_3$ (v/v = 8:2) as the eluent. The UV spectrum (peak maxima at 275, 283, and 332 nm) of the slightly yellow fraction (ca. 20−45% yield) was identical to the published one (*27*). NMR data of the compound were published previously (*28*). An UCe_8 standard for HPLC measurements was prepared as described for UCe_9 except that the chromatography step was omitted. The identity was verified by its UV absorptions at 275 and 332 nm, its coulometric oxidation at +450 mV, and an HPLC retention time different from UQ_9.

Synthesis of UCa. UCa_9 was obtained by selective reduction of UCe (ca. 45−65% yield) (*29*). Purified UCe_9 (500 mg) was dissolved in 50 mL of EtOH, and the solution was heated. To the boiling solution, 1 g of Na in small pieces was added rapidly and the solution was further refluxed for 2 h until all Na was dissolved. The cooled solution was mixed with a saturated NaCl solution in a ratio of 1:1 and extracted with ether. The ether was evaporated from the organic extract, and the residue was dissolved in *n*-hexane/$CHCl_3$ (5:2) and dried over Na_2SO_4. This crude product was purified by column chromatography on Florisil (Merck) eluting with *n*-hexane/$CHCl_3$ (v/v = 8:2). The purity assessed by NMR and HPLC was >97%. NMR data were reported in a previous work (*28*).

Preparation of Liver Mitochondria and Submitochondrial Particles (SMPs). Liver mitochondria were isolated from male Sprague−Dawley rats (Him:OFA/SPF) as described previously (*30*) in a buffer containing 0.25 M sucrose, 20 mM triethanolamine, 1 mM EDTA, and 0.1 mg/mL bovine serum albumin (BSA) (pH 7.4, 4 °C). Analogously, liver mitochondria were prepared from fresh bovine liver, which was obtained from a local slaughterhouse. The protein content of the protein suspension was measured by the Biuret method using BSA as a standard (*31*). SMPs were obtained from beef heart mitochondria by sonication as described in ref *32*.

[2] For ubiquinones (UQ_n), ubichromenols (UCe_n), and ubichromanols (UCa_n), the index *n* indicates the number of isoprenic units in the side chain. The abbreviations UQ, UCe, and UCa without index refer to those compounds in general.

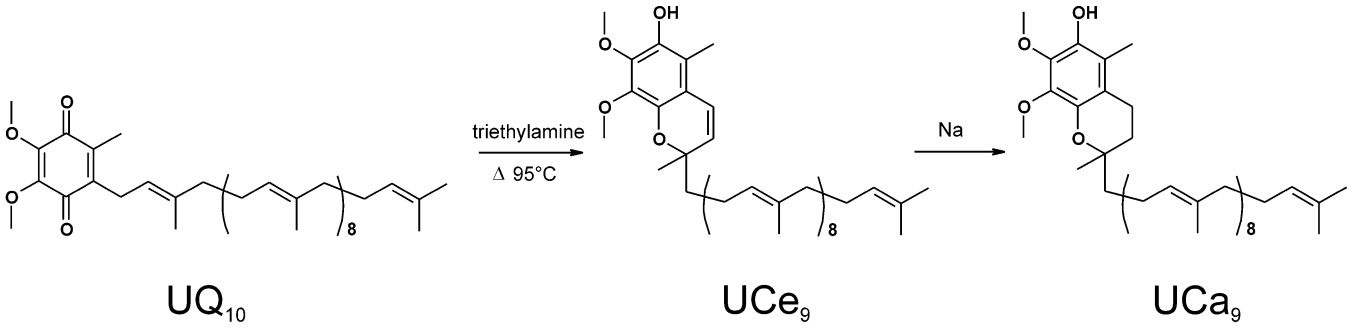
Preparation of the Mitochondrial cyt bc_1 Complex. One hundred milliliters of a beef heart mitochondrial suspension was prepared from fresh beef heart by differential centrifugation according to Smith et al. (*33*). The preparation method of the bc_1 complex was adopted from Schägger et al. (*34*). Briefly, after a centrifugation of the mitochondrial suspension at 27000*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 adding Triton X-100 (1.75%) and NaCl (600 mM), giving a protein concentration of 26 mg/mL. The pellet obtained by ultracentrifugation at 100000*g* for 45 min was resuspended in a buffer containing 300 mM sucrose and 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, and 2 mM NaN_3, pH 7.2) and stirred for 5 min. The supernatant obtained by ultracentrifugation (45 min, 100000*g*) was mixed with an equal volume of hydroxyapatite [prepared according to Tiselius et al. (*35*)] equilibrated with 0.5% Triton X-100, 250 mM NaCl, and 80 mM NaH_2PO_4 to bind cyt bc_1 complex. After a slow centrifugation (1 min, 430*g*), the bc_1/hydroxyapatite sediment was washed with 5 volumes of buffer (0.05% Triton X-100, 250 mM NaCl, 110 mM NaH_2PO_4, and 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 KH_2PO_4, and 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, and 2 mM NaN_3, pH 7.2. A typical purified bc_1 preparation contained about 13 nmol cyt b per mg protein (6.5 nmol bc_1 complex/mg protein) and was stored at 77 K after addition of 25% glycerol.

Detection of UQ-Related Compounds in Rat and Bovine Liver Mitochondria (BLM) by HPLC. Electrochemical detection was used in our study because of its superior selectivity and sensitivity for the detection of lipophilic antioxidants such as tocopherols and UQ derivatives (*36*). Samples of 2−5 mg of protein in 1 mL of H_2O were mixed with 5 mM sodium dodecyl sulfate (SDS) and extracted with 3 mL of anaerobic ethanol/hexane (2:5). HPLC analysis was performed on a Waters LC1 module equipped with an internal UV detector and a coulometric detector Coulochem III (ESA) equipped with a conditioning cell (5021A) and an analytical cell (5010A). UQ-related compounds were eluted from a reversed phase column [Hibar RT 125-4, LiChrospher 100 RP-18 (5 μm)] equipped with a precolumn [LiChroCART 4-4, LiChrospher 100 RP-18 (5 μm)] with 50 mM $NaClO_4$ in ethanol/methanol/acetonitrile/$HClO_4$ (400:300:300:1) at 1 mL min^{-1} and detected optically at 275 nm or coulometrically in the oxidative mode using the analytical cell of the ESA detector (E_2 = +450 mV). To detect oxidized quinones electrochemically, the conditioning cell between column and detector was set to −500 mV and E_1 of the analytical cell was set to −450 mV for postcolumn reduction [modified from Tang et al. (*37*, *38*)].

HPLC/MS of UCa Oxidation Product and Liver Mitochondrial Extracts. To identify the chemical structures of metabolites, liquid chromatography−mass spectrometry (HPLC/MS) measurements were performed using an HPLC system fitted with a PE series 200 pump, a PE 235C DAD detector, and a PE series 200 autosampler (Perkin-Elmer Instruments, Wellesley, MA). The system was coupled in line to an API 150EX mass spectrometer (Perkin-Elmer Sciex) fitted with an atmospheric pressure ionization source for APCI ionization in the negative mode. The operating conditions were as follows: capillary voltage, −4.2 kV; orifice voltage, −40 V; and temperature, 400 °C. The column, mobile phase ($HClO_4$ and $NaClO_4$ were omitted), flow rate, and injection volume (10 μL) were identical to those conditions used in the analytical HPLC assay (see above).

Oxidation of UCa_9. Oxidation of the chromanol was performed according to a modified method of Jacob et al. (*39*). The mild

Scheme 1. Stepwise Synthesis of UCa_9



UQ_{10} UCe_9 UCa_9

oxidizing agent $Ce^{IV}(NH_4)_2(NO_3)_6$ (200 μmol) was dissolved in 10 mL of H_2O/acetonitrile (1:9). This solution was added dropwise to a solution of 113 μmol of UCa_{10} in 12.5 mL of acetonitrile. This mixture was diluted by 60 mL of H_2O and subsequently extracted three times with 30 mL of ether. The pooled extracts were dried over $CaCl_2$. After evaporation of the ether, the residue was dissolved in hexane and purified by column chromatography on Kieselgel 60 (Merck) eluted by hexane/$CHCl_3$.

NMR of $UQ_{10}OH$. ^{1}H NMR spectra were recorded at 300.13 MHz, and ^{13}C NMR spectra were recorded at 75.47 MHz with $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shift data are given in ppm units, and coupling constants are given in Hz. ^{1}H peaks were assigned by means of ^{1}H and H−H COSY spectra, ^{13}C peaks were assigned by means of APT (attached proton test), HMQC (heteronuclear multiquantum coherence), and HMBC (^{1}H-detected multiple bond connectivity) spectra. Multiplets that were not resolved are indicated by "m", and resonances of double intensity originating from two magnetically equivalent carbons are indicated by "d.i.". ^{1}H NMR: δ 1.27 (s, 3H, $^{3a'}CH_3$), 1.50 (m, 2H, $^{2'}CH_2$), 1.60 [s, 24H, C═C(*CH*$_3$)CH$_2$], 1.63 [s, 3H, C═C(^{cis}*CH*$_3$)(^{trans}-CH$_3$)], 1.68 [s, 3H, C═C(^{cis}CH$_3$)(^{trans}*CH*$_3$)], 1.97 (m, 18H, *CH*$_2$−CH$_2$−CH═C), 2.02 (s, 3H, ^{3a}CH$_3$), 2.08 (m, 18H, CH$_2$−*CH*$_2$−CH═C), 2.54 (m, 2H, $^{1'}CH_2$), 3.99 (s, 6H, OMe), 5.11 (m, 9H, CH$_2$−CH$_2$−*CH*═C), 5.80 (1H, s, br, OH). ^{13}C NMR: δ 11.8 ($^{3a}CH_3$), 15.5 [C═C(^{cis}*CH*$_3$)(^{trans}CH$_3$)], 16.0 [m, CH═C(*CH*$_3$)], 21.1 ($^{1'}CH_2$), 22.7 ($^{5'}CH_2$), 25.7 [C═C(^{cis}CH$_3$)(^{trans}*CH*$_3$)], 26.5 ($^{3a'}CH_3$), 26.7−26.8 (m, CH$_2$−*CH*$_2$−CH═C), 39.7 (m, *CH*$_2$−CH$_2$−CH═C), 40.2 ($^{4'}CH_2$), 41.6 ($^{2'}CH_2$), 61.0 (d.i., 2 × OMe), 72.6 ($^{3'}C$), 124.0−124.4 [m, *CH*═C(CH$_3$)], 131.2 [C═*C*(^{cis}CH$_3$)(^{trans}CH$_3$)], 134.9−135.1 [m, CH═*C*(CH$_3$)], 135.7 ($^{5'}$CH$_2$−CH═*C*), 138.4 (^{3}C), 142.9 (^{2}C), 144.3 (d.i., ^{5}C, ^{6}C), 184.8 (^{1}C), 185.1 (^{4}C).

Reduction of TQ, UQ_{10}, and $UQ_{10}OH$. The strongly lipophilic quinols [α-tocopheryl hydroquinone (TQ-H_2), UQ_{10}-H_2, and $UQ_{10}OH$-H_2] were prepared by reduction of the respective quinones with sodium dithionite/KBH_4 in ethanol/water (1:1) and subsequent extraction with anaerobic diethyl ether/cyclohexane (2:1).

Measurement of the Quinol:Cyt c Oxidoreductase Activity. SMPs (0.105 mg of protein) were suspended in 1 mL of buffer (pH 7.2, 25 °C) containing 250 mM sucrose, 50 mM KH_2PO_4, 0.2 mM EDTA, 2 mM KCN, 1 mM NaN_3, 2.5 mg/mL BSA, 5 mM $MgCl_2$, and 50 μM cyt c^{3+}. The reduction of cyt c^{3+} was measured photometrically at 550 minus 540 nm after addition of 0−150 μM hydroquinones (TQ-H_2, UQ_{10}-H_2, and $UQ_{10}OH$-H_2) over a time period of 3 min using a Shimadzu Multispec 1501 diode array photometer (*40*). Experiments were carried out in the presence of KCN and NaN_3 in order to block reoxidation of cyt c^{2+} by traces of cyt oxidase. For the concentration determination of TQ-H_2, UQ_{10}-H_2, and $UQ_{10}OH$-H_2, the extinction coefficients 5000, 4003, and 4003 M^{-1} cm^{-1} at 290 nm were used, respectively. The obtained slopes were corrected for the chemical reduction of cyt c^{3+} by hydroquinones obtained in the absence of SMP. The enzymatic reduction rates were calculated using an extinction coefficient of $\epsilon_{550-540\ nm} = 19\ mM^{-1}\ cm^{-1}$ (*41*). The activities of the respective hydroquinones at the isolated bc_1 complex were measured under identical conditions using 0.121 mg/mL bc_1 protein.

Position of Hydroxylated Ubihydroquinone ($UQOH$-H_2) in the Q_{out} Pocket of the bc_1 Complex. So far, no crystal structure of the cyt bc_1 complex with bound ubiquinol exists. Therefore, a structure containing the inhibitor stigmatellin (SMA) was used as a model. From the crystal structure 2A06 of the bovine bc_1 complex, a sphere of about 24 Å around the SMA molecule bound in the Q_{out} pocket of the protein was extracted using HyperChem 7.5 (*42*). A geometry-optimized UQ_2OH-H_2 structure was superimposed to the SMA molecule using a root-mean-square fit for selected atoms 8C, 7aO, and 5C in SMA and 1C, 6aO, and 4C in UQ_2OH-H_2. Afterward, SMA was removed from the protein sphere and the geometry of the UQ_2OH-H_2 molecule was optimized using the force field method OPLS (optimized potentials for liquid simulations) (*43*). To demonstrate the hypothetic position of the side chain OH group of UQ_2OH-H_2 within the protein, amino acid residues within a distance of 4 Å were extracted together with UQ_2OH-H_2 from the optimized structure. By analogy, SMA together with the same amino acid residues was extracted from the 2A06 structure. From these data, Figure 5 was prepared using MOLEKEL (*44*, *45*).

Results

A basic prerequisite for evaluating the bioactivity of bicyclic UQ derivatives is the availability of reference compounds and the identification of their oxidation products.

Synthesis of Chroma(e)nol Analogues of UQ. UQs can be considered as analogues of TQ. Likewise, there exist chromanol and chromenol analogues of UQs. During formation of the bicyclic chroma(e)nols, the proximal isoprene group of the side chain of UQ is involved in pyran ring formation.

We synthesized the corresponding chromenol by cyclization of UQ_{10} by heating in triethylamine at 95 °C (Scheme 1). According to general nomenclature with the index indicating the number of isoprene units in the molecule, this compound is referred to as UCe_9, also in accordance with other publications (*29*) (see footnote 2). In a second step, UCa_9 was synthesized from UCe by reduction with sodium metal. Because of its structure, UCa_9 can be considered as an UQ analogue of Toc.

Two-Electron Oxidation Products of UCa. By analogy to typical oxidation procedures in the chemistry of Toc, we performed several oxidation reactions of UCa_9 with different oxidants, such as PbO_2 and $FeCl_3$. The crude oxidation products were analyzed by HPLC/MS with electrospray ionization. The mass spectrum of the major oxidation product, which was eluted in our RP-HPLC system before UCa_9, UCe_9, and UQ_{10}, is shown in Figure 1A.

The observed molecular peak at *m*/*z* 880.9 was evidently different from that of UCa_9 (*m*/*z* = 865.39) and UCe_9 (*m*/*z* = 863.37), suggesting a cleavage of the oxa-ring of the chromanol with a concomitant mass gain. The fragmentation peak at 863.0 corresponded to the loss of H_2O (*m*/*z* = 18) and suggests a labile hydroxy-alkyl moiety in the molecule. To gain more structural information, UCa_9 was reacted with the milder oxidant $Ce^{IV}(NH_4)_2(NO_3)_6$. This procedure neatly produced the same compound as the major oxidation product in purities between

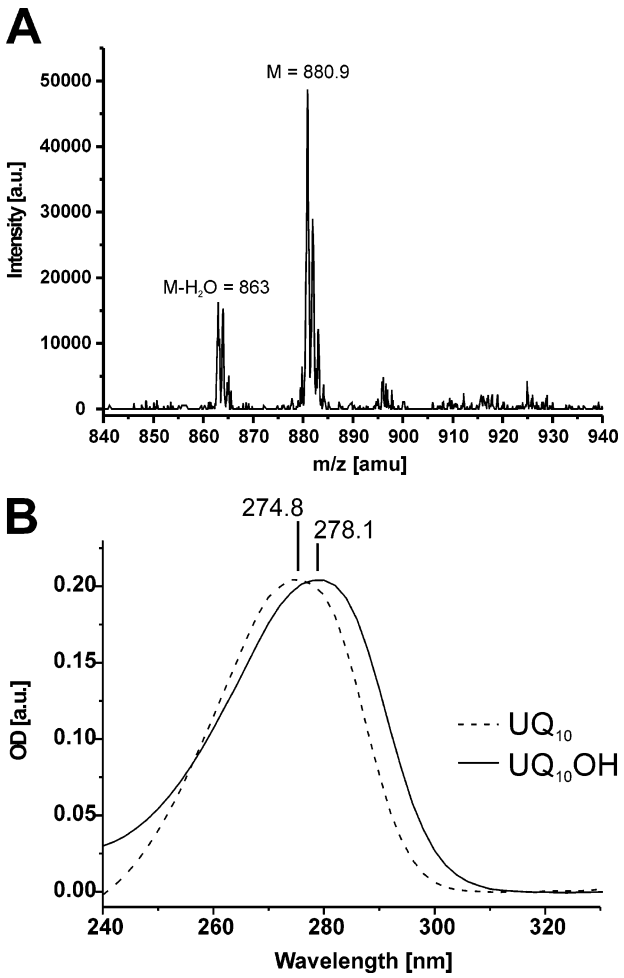
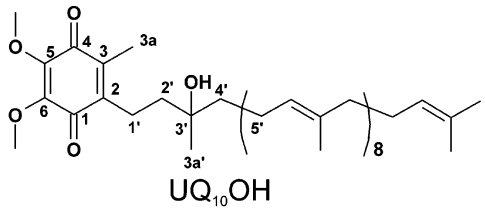


Figure 1. (A) Mass spectrum of the major oxidation product of UCa_9 after oxidation with $FeCl_3$ obtained by HPLC/MS. (B) UV spectrum of the oxidation product of UCa_9 in ethanol.

Scheme 2. Oxidation Product of UCa_9: $UQ_{10}OH$



90 and 95% assessed by HPLC. The UV spectrum (Figure 1B) of the compound exhibited an absorption maximum at 278.1 nm in ethanol, which is similar to that of UQ_{10} (λ_{max} = 274.8 nm). Nuclear magnetic resonance spectra (^1H and ^{13}C; see the Materials and Methods) showed signals at 184.8 and 185.1 (^{13}C) characteristic of keto carbon atoms in a benzoquinone ring system. The resonance at 72.6 ppm is typical of a tertiary alcohol carbon, while a signal at 5.80 (^1H) corresponds well to an OH group attached to an aliphatic carbon atom. Considering these plain data and the additional connectivity information obtained from specialized NMR techniques (H−H COSY, APT, HMQC, and HMBC), the structure in Scheme 2 was assigned to the new UQ derivative $UQ_{10}OH$ {2-[(6*E*,10*E*,14*E*,18*E*,22*E*,26*E*,-30*E*,34*E*)-3-hydroxy-3,7,11,15,19,23,27,31,35,39-decamethyltetraconta-6,10,14,18,22,26,30,34,38-nonaenyl]-5,6-dimethoxy-3-methyl-[1,4]benzoquinone}. The ^1H spectrum of $UQ_{10}OH$ is in good agreement with previously published data on a UQ_7OH derivative, although the respective report provided only data of low resolution (*46*). The first exocyclic methyl group

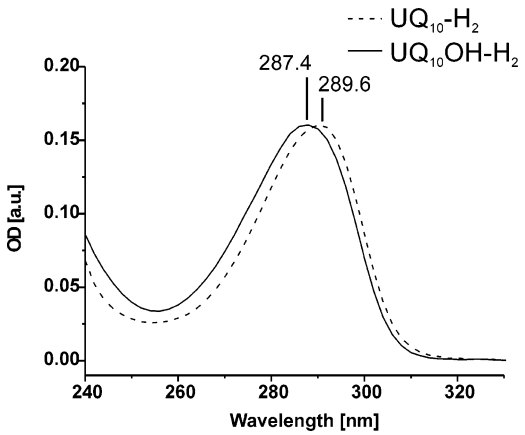


Figure 2. UV spectra of reduced hydroxylated UQ ($UQ_{10}OH$-H_2) and reduced UQ (UQ_{10}-H_2) in ethanol.

(CH_3-3a in $UQ_{10}OH$ and CH_3-2a in UCa and UCe) resonated at around 1.28 ppm for $UQ_{10}OH$ and UCa but was shifted downfield to 1.38 ppm by the double bond of the chromenol in UCe. Another feature to readily distinguish the quinone from the chromanol/chromenol counterparts is the shift of the methoxyl groups, being around 3.88/3.94 ppm for the heterocyclic derivatives and 3.99 ppm for both methoxyls in the quinone $UQ_{10}OH$. The NMR resonances of the CH_2-CH_2 structural motif adjacent to the quinoid ring are found at 2.54 and 1.50 ppm, respectively, for ^1H and at 21.1 and 41.6 ppm, respectively, for ^{13}C, which is an unambiguous proof for the presence of a chroman-derived compound rather than a chromene-derived structure. Structures like hydroxy-iso-UQs, as mentioned in ref *46*, can therefore be ruled out for compound $UQ_{10}OH$.

$UQ_{10}OH$-H_2 as Substrate for the Cyt bc_1 Complex. Previous studies have shown that mitochondrial respiratory complexes have a variable substrate specificity largely favoring UQ/ubiquinol (hydroquinones were designated with the -H_2 suffix) as the native substrate (*16*). Both bc_1 complex constitutively present in SMP and isolated bc_1 complex demonstrated a substrate preference for ubihydroquinone (UQ-H_2) over TQ-H_2. To study the bc_1 activity of $UQ_{10}OH$, it was chemically reduced to the quinol ($UQ_{10}OH$-H_2). $UQ_{10}OH$-H_2 exhibited an absorption spectrum (λ_{max} = 287.4 nm) almost identical with ubiquinol (λ_{max} = 289.6 nm) (Figure 2). It was used as a substrate for measuring the quinol:cyt c oxidoreductase activity of the bc_1 complex in SMP (Figure 3A) and isolated bc_1 complex (Figure 3B) in the presence of cyt c^{3+} as an electron acceptor.

The native substrate UQ_{10}-H_2 demonstrated a continuously growing reaction rate, which was not saturable under our experimental conditions, preventing the deviation of kinetic parameters as for short-chain homologues in our previous works (*15*, *16*). This is due to the slow interchange of these lipophilic substrate molecules from UQ_{10}-H_2 micelles with the mitochondrial membrane (*47*). TQ-H_2 once more showed its poor substrate properties as an electron donor for the bc_1 complex. The newly synthesized UQ_{10} derivative ($UQ_{10}OH$-H_2), which exhibited the same quinone moiety as UQ_{10}-H_2, but an additional hydroxylation site of the side chain like TQ-H_2, shows intermediate substrate properties. Its activity is about one-third of the UQ_{10}-H_2 activity but significantly higher than that of TQ-H_2.

Occurrence of UQ Analogues in Liver Mitochondria. In contrast to plants, mammalian cells apparently do not possess a cyclase able to convert the quinone form of these antioxidants back to the chroma(e)nol species. Nevertheless, there have been sporadic reports on the occurrence of such compounds in

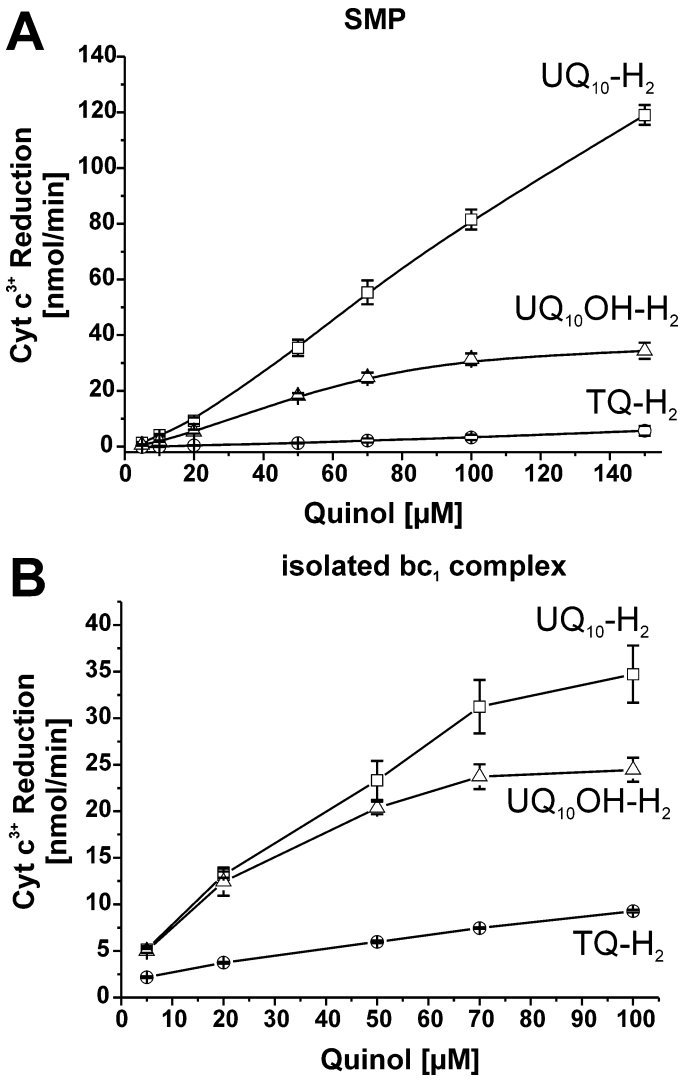


Figure 3. Reduction of cyt c^{3+} by the mitochondrial bc_1 complex in SMP (A) using reduced UQ_{10} (UQ_{10}-H_2), TQ-H_2, and reduced side chain-hydroxylated UQ_{10} ($UQ_{10}OH$-H_2) as substrates. Each data point is the mean ± SE of seven measurements.

mammals. Therefore, we tried to detect such analogues by different analytical methods and used the synthesized compounds as reference standards. Because of the complex mixture of lipophilic extracts from liver mitochondria, an HPLC separation in combination with UV, coulometric, and alternatively MS detection was required for qualitative identification. To make the obtained retention time data comparable between the different instruments and the slightly different solvent systems, we calculated a relative retention time (RT_{rel}) scale (Table 1) using UQ_9 as an internal reference ($RT_{rel} = 1$). All quinones are typically detectable by the coulometric detector after postcolumn reduction and show strong UV absorption at 275 nm in contrast to matrix compounds such as cholesteryl esters. In addition, chromenols and chromanols are detected by the electrochemical detector (ECD), while only chromenols exhibit a significant absorption at 275 nm. Considering these properties, the chromatographic separation was repeated using a mass spectrometer as the detector. Combining information obtained from UV/ECD detection and RT_{rel} data, the molecular mass peaks were assigned to the respective structures. For extracts of liver mitochondria from rats (RLM) and bovines (BLM), the data and assignments are listed in Tables 2 and 3, respectively. Besides the predominant UQ_9/UQ_9-H_2, traces of UCe_8 and UQ_8 were detected in rat liver mitochondrial (RLM) extracts. In addition, mass spectrometry suggested the presence of certain cholesteryl esters, which, however, are neither detected by UV (275 nm) nor by ECD under our experimental conditions. In contrast, in BLM, UQ_{10}/UQ_{10}-H_2 dominated besides traces

Table 1. Chromatographic Retention and Concentration of UQ Derivatives in Mitochondria[a]

compound	RT_{rel}	nmol/mg protein	
		RLM	BLM
UQ_9-H_2	0.71	0.8891 ± 0.0914	0.0011 ± 0.0004
$UQ_{10}OH$	0.83	0.0327 ± 0.0003	0.1297 ± 0.0371
UCe_8	0.86	0.2799 ± 0.0209	ND
UQ_9	1.00	1.0302 ± 0.2879	0.0271 ± 0.0276
UQ_{10}-H_2	1.02	0.0563 ± 0.0357	0.0436 ± 0.0112
UCe_9	1.28	0.0340 ± 0.0049	0.0032 ± 0.0019
UCa_9	1.37	ND	ND
UQ_{10}	1.48	0.0741 ± 0.0225	0.3531 ± 0.1328
sum UQ_9 + UQ_9-H_2		1.9193 ± 0.1965	0.0282 ± 0.0278
sum UQ_{10} + UQ_{10}-H_2		0.1303 ± 0.0132	0.3967 ± 0.1282

[a] Retention times of lipophilic UQ standards relative to UQ_9 ($RT_{rel} = 1$) measured using a mobile phase with 50 mM $NaClO_4$ in ethanol/methanol/acetonitrile/$HClO_4$ (400:300:300:1) (for further details, see the Materials and Methods). The day-to-day variation of those RT_{rel} values is for all components about ±0.02 arbitrary units, and the agreement between retention times on the HPLC/UV/ECD and the HPLC/MS system is about ±0.05 arbitrary units. The concentration of UQ derivatives in RLM (n = 2) and BLM (n = 4). The sum of quinones and hydroquinones (UQ_n + UQ_n-H_2) was calculated from the individual samples and does, therefore, not exactly match the sum of the means of UQ_n plus UQ_n-H_2. UQ_8, UCa_8, and UQ_9OH could not be quantified due to the lack of appropriate standards. Data are given as means ± SD.

Table 2. UQ-Related and Coeluting Components of RLM Extracts Detected by HPLC/MS Using a Mobile Phase Consisting of Ethanol/Methanol/Acetonitrile (400:300:300) (For Further Details, See the Materials and Methods)[a]

RT_{rel}	molecular ion	formula weight	assignment
0.67	726.5 (strong)	727.13	UQ_8[c]
	795.5 (weak)	797.27	UQ_9-H_2[b]
0.82	646.5	647.09	C 18:3 (linolenic acid) cholesteryl ester[c]
0.86	793.5	795.25	UCe_8[b]
1.00	794.5	795.25	UQ_9[b]
1.23	648.5	649.11	C 18:2 (linoleic acid) cholesteryl ester[c]
1.49	863.0	863.37	UQ_{10}[b]

[a] The RT_{rel} values were calculated from the UV peak maximum of UQ_9 (11.7 min). [b] Assignments based on mass numbers and retention time data of standards (see Table 1). [c] Assignments based on mass numbers and literature data for cholesterol esters (*61*) and UQ_8 (*62*).

Table 3. UQ-Related and Coeluting Components of BLM Extracts Detected by HPLC/MS Using a Mobile Phase Consisting of Ethanol/Methanol/Acetonitrile (400:300:300) (For Further Details, See the Materials and Methods)[a]

RT_{rel}	molecular ion	formula weight	assignment
0.86	880.5 (weak)	881.39	$UQ_{10}OH$[b]
	646.5 (strong)	647.09	C 18:3 (linolenic acid) cholesteryl ester[c]
1.00	794.5	795.25	UQ_9[b]
1.16	648.5	649.11	C 18:2 (linoleic acid) cholesteryl ester[c]
1.23	861.5	863.37	UCe_9[b]
1.45	862.5	863.37	UQ_{10}[b]

[a] The RT_{rel} values were calculated from the UV peak maximum of UQ_9 (11.7 min). [b] Assignments based on mass numbers and retention time data of standards (see Table 1). [c] Assignments based on mass numbers and literature data for cholesterol esters (*61*).

of UQ_9, UCe_9, and cholesteryl esters. In addition, traces of $UQ_{10}OH$ were detected, which eluted before UQ_9, in the RP-HPLC system.

To corroborate the presence of $UQ_{10}OH$ in BLM, the organic extract was subjected to HPLC/MS measurements, which were specifically adjusted to detect fragmentations between 850 and 900 amu. For identifying the component in the BLM extract, a mass spectrum was recorded (Figure 4A) in which the molecular ion peak at 880.5 and the fragmentation peak at 862.5 matched the spectrum of pure $UQ_{10}OH$ shown in Figure 1A. Furthermore, an HPLC method with electrochemical detection was applied to verify this finding. In Figure 4B, an overlay of chromatographic traces recorded by coulometric detection is shown. The original BLM extract showed a not completely resolved peak at $RT_{rel} = 0.84$ (other HPLC and eluent system than in HPLC/MS). On spiking with $UQ_{10}OH$, this peak is increased in a dose-dependent fashion. These combined findings strongly suggest the natural presence of $UQ_{10}OH$ in BLM extracts. Interestingly, MS measurements to detect its chemical precursor UCa_9 failed to confirm its presence in these BLM extracts.

Using this analytical information, the concentrations of UQ_9-H_2, $UQ_{10}OH$, UCe_8, UQ_9, UQ_{10}-H_2, UCe_9, UCa_9, and UQ_{10} in BLM and RLM extracts were determined and listed in Table 1. The UQ_9OH expected in rat mitochondria could no be identified so far due to the lack of an appropriate standard, but its presence is very likely.

Discussion

This work was aimed at clarifying (i) whether UCa or its metabolites are naturally occurring compounds, (ii) whether UCa is a potential precursor of UQ-like molecules, and (iii) whether these have a bioactivity at the mitochondrial bc_1 complex.

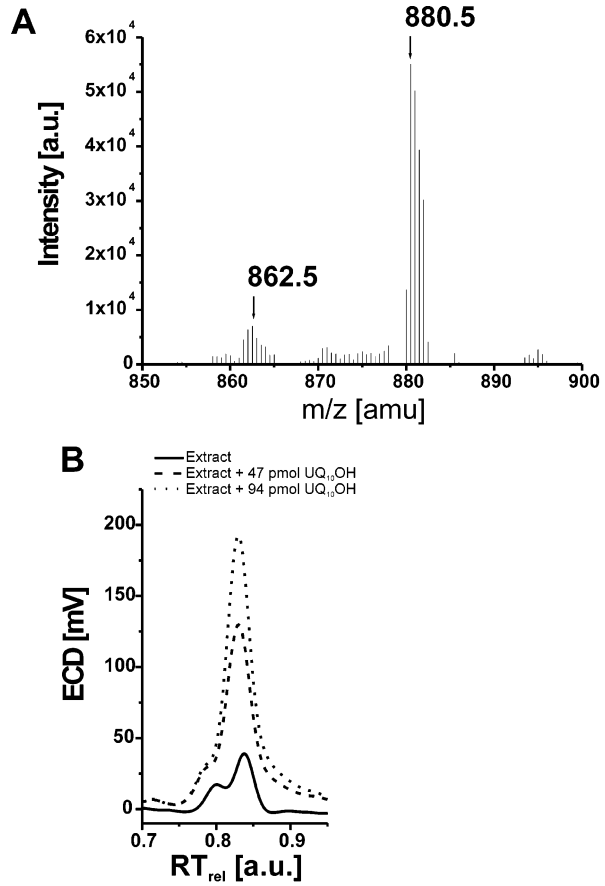


Figure 4. Detection of $UQ_{10}OH$ in BLM. (A) Mass spectrum of the BLM extract recorded at $RT_{rel} = 0.83$ of the chromatogram, and (B) overlay of chromatographic traces from coulometric detection (for settings, see the Material and Methods) of a BLM extract (solid line), spiked with 47 pmol of $UQ_{10}OH$ (dashed line) and 94 pmol of $UQ_{10}OH$ (dotted line).

It is well-known that the chain length of UQ derivatives in organisms is species specific. However, the fact that UQ_8 and UQ_{10} were detected besides UQ_9 (major rat UQ homologue) in RLM (Tables 1 and 2) and UQ_9 besides UQ_{10} (major bovine or human UQ homologue) in BLM (Tables 1 and 3) suggests that biosynthetic enzymes for UQ in mammalian tissues have only a low specificity forming both UQ species with shortened and prolonged isoprenic side chains. Therefore, other UQ derivatives (UCe_{n-1} and UQ_nOH) detected in our study were always derived from the respective UQ_n species and can amount up to 30% of the UQ_n present (Table 1). The natural presence of UCe in rat tissues was—and still is—controversially discussed because of biological and analytical uncertainties. It was shown that saponification of tissue homogenates (*48*) and aluminum oxide used as chromatographic material (*20*) could lead to an artificial conversion of UQ to UCe. It has been described that the formation of UCe from UQ during chromatographic procedures can be accelerated by acidic eluents (*49*) and by photochemical processes (*26*). Already, Green and co-workers described that some tissues contain both UQ and UCe and some others only UQ (*49*). Joshi and Ramasarma demonstrated that UCe is accumulated in livers of vitamin A-deficient rats in comparison to control animals due to a diminished catabolism (*18*). Mechanistic studies of Raman and Rudney have shown UCe not to be a precursor of UQ and, conversely, UQ to be only slowly converted by a nonenzymatic reaction to UCe in tissues (*50*). More detailed studies by Olson and Rudney reported that in liver slices incubated with side chain-labeled UQ_9, the tissue

UQ_9 became labeled obviously by exchange. In addition, UCe_8 was partially labeled, suggesting a transformation of UQ_9 to UCe_8 (*51*). However, the reverse experiment with labeled UCe_8 yielded nearly no labeled UQ_9 in this study. Therefore, if there is any enzymatic formation of UCe, most likely, UQ is the precursor. Our measurements confirmed the presence of UCe in liver mitochondria of rats and bovines (Tables 1−3). Nevertheless, the detailed mechanism of an enzymatic UCe synthesis is still unknown (*50*).

In contrast to UCe, a natural presence of UCa was never reported. In addition, also, our measurements provided no evidence for a natural occurrence so far. However, because UCa is an analogue of Toc and could be of interest as antioxidant supplement, a more detailed study to clarify biochemical and potentially pharmacological properties of UCa is required. Although the chemical structure of UCa has already been known for several decades, only a few attempts have been made to test the benefit of UCa as an antioxidant. The reasons for this failure are the lack of natural sources of UCa and of biochemical and clinical data for this group of compounds. The data of Mukai and our own measurements showed that UCa and UCe have between 10 and 50% of the radical scavenging activity of Toc (*28*, *52*). However, there have been only sporadic reports on the benefit of UCa as an antioxidant in living organisms (*53*) and as an ingredient of topically applied cosmetics (*54*, *55*). On the basis of the photochemical conversion of UQ to UCe (*26*), a pharmacological impact of such bicyclic UQ derivatives in topical applications is likely but has never been studied so far. A single publication reported that UCa_9 cannot restore the fertility in vitamin E-deficient rats (*56*). Besides the radical-scavenging properties of UCa, its oxidation product (an UQ-like compound), which was characterized in this study, could be potentially important to support mitochondrial functions in a similar manner as UQ.

Our previous work on the influence of TQ, which is a natural oxidation product of Toc, on the respiratory chain has shown that UQ-binding complexes exhibit a different specificity for UQ in comparison to TQ (*16*). While mitochondrial complex II does not accept TQ at all, complex I is most flexible in its substrate requirements. The complex III activity is ruled by binding properties of both oxidized and reduced (ubi-)quinone. While oxidized TQ acts as a mildly competitive inhibitor, TQ-H_2 is ineffective as an electron donor at this complex. These findings have been verified with both native TQ and a short-chain analogue. Because of these observations, it was of major interest to identify the two-electron oxidation product of UCa_9 (an UQ analogue of α-Toc). The one-electron oxidation product was characterized by ESR previously (*28*, *57*). The molecular peak obtained in MS measurements of the UCa_9 oxidation product (Figure 1A) is not identical with UQ_9 (m/z = 795.25), UQ_9-H_2 (m/z = 797.25), UQ_{10} (m/z = 863.37), UQ_{10}-H_2 (m/z = 865.39), UCa_9 (m/z = 865.39), and UCe_9 (m/z = 863.37). Its fragmentation (loss of H_2O) indicates a hydroxylation of the molecule. This assignment was corroborated by NMR data, which confirmed a hydroxylation in the side chain. The UV spectrum of this compound ($UQ_{10}OH$) resembles that of UQ_{10} (Figure 1B), and the spectrum of the reduced species ($UQ_{10}OH$-H_2) matches that of UQ_{10}-H_2 (Figure 2). In contrast to these similar UV spectroscopic properties, $UQ_{10}OH$ is eluted much faster than UQ_{10} in the reversed phase chromatographic system, suggesting a higher polarity because of hydroxylation. Morimoto et al. described the oxidative formation of UQ_7OH from UCa_6 (*46*). These multiple evidence support our conclusion that we detected a formerly unknown UQ_{10} species, which is formed in analogy to TQ from its corresponding chromanol. Because UCa_9 was not detected in BLM in contrast to UCe_9, a metabolic conversion between $UQ_{10}OH$ and UCe_9 cannot be excluded. Because UCe was suggested to be an intermediate in UQ catabolism, this could be true for $UQ_{10}OH$ as well. The concentration ratios of UQ_{10} + $UQ_{10}H_2$:UCe_9:$UQ_{10}OH$ were 1:0.26:0.43 and 1:0.008:0.11 for RLM and BLM, respectively. The variable ratio between the concentrations in different mammals suggests that the quantitative relationship between UQ and its metabolites strongly depends on the species and possibly its nutrition. This, however, is very likely since also the major UQ derivatives for rats (UQ_9) and bovines (UQ_{10}) are different. However, so far, no information on putative functions of $UQ_{10}OH$ was obtained in current experiments.

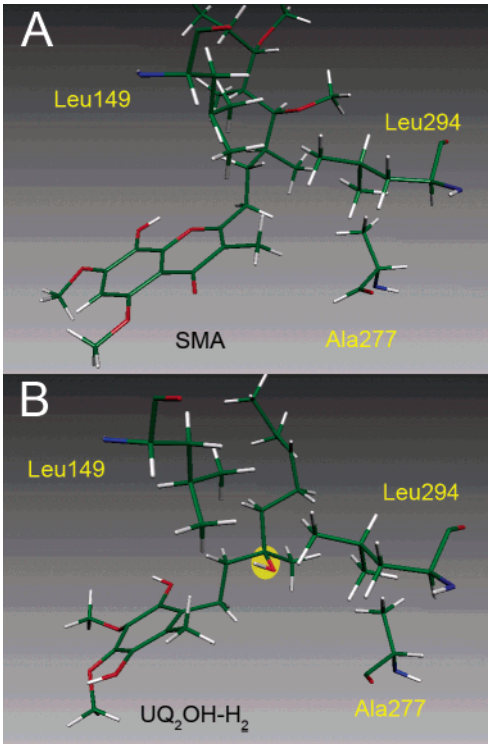


Figure 5. Approximation of the OH group position of UQ_2OH-H_2 in the Q_{out} pocket of the bc_1 complex. (A) Position of the SMA molecule in the crystal structure 2A06 of the bovine bc_1 complex in relation to three surrounding amino acid residues. (B) Hypothetic position of UQ_2-OH-H_2 obtained by an OPLS force field optimization using the aromatic ring of SMA as a starting position for the quinone ring of UQ_2OH-H_2. The yellow highlighted area shows the position of the OH group in the side chain and the position of the residues Leu149, Leu294, and Ala277 of the cyt b subunit.

Because of the finding that TQ interferes with the binding of UQ to mitochondrial complexes, the substrate properties of $UQ_{10}OH$ were of interest. Our data in two model systems show that reduced $UQ_{10}OH$-H_2 is much more effective than TQ-H_2 but still not as effective as UQ_{10}-H_2, which is present in the lipid phase of the inner mitochondrial membrane in concentrations of up to 5 mM (*58*) (Figure 3). The slightly different relative activities of quinols between SMP and isolated bc_1 complex may arise from their different lipophilicities in combination with the larger lipophilic bulk phase in SMP as compared with the detergent-solubilized bc_1 complex. The higher activity of $UQ_{10}OH$-H_2 as compared with TQ-H_2 suggests that methoxy groups attached to the quinone ring could be important for H-bond formation to amino acid residues in the Q_{out} pocket of the bc_1 complex. Usually, only the H-bond formation of the quinol hydroxyl groups to the amino acid residues Glu272 of the cyt b subunit and His161 of the Rieske

ISP (2BCC crystal structure) were considered as important for substrate binding (*59*). Unfortunately, there exists no crystal structure of the bc_1 complex with UQ-H_2 bound in the Q_{out} pocket. SMA is a strong inhibitor of the mitochondrial cyt bc_1 complex blocking the quinol oxidation at the Q_{out} pocket of this protein. Because of its strong binding, several crystal structures of the bc_1 complex including this inhibitor exist. The lower effectiveness of $UQ_{10}OH$-H_2 in comparison to UQ_{10}-H_2 suggests that hydroxylation of the side chain disturbs substrate binding at the Q_{out} pocket of the bc_1 complex as well. The position of the OH group can roughly be estimated from bound SMA in the crystal structure 2A06 of bovine bc_1 complex (*60*).

Figure 5A shows the original position of SMA surrounded by three amino acid residues in the 2A06 structure. In comparison, the hypothetic position of a UQ_2OH-H_2 molecule, which was obtained by force field optimization within this subunit, is displayed (Figure 5B). The OH group of UQ_2OH-H_2 is surrounded by the protein structure of the Q_{out} pocket. Within a distance of 4 Å to the O atom of the OH group, there are found residues of Leu149, Leu294, and Ala277. These rather apolar amino acids could be responsible for the retarded binding of $UQ_{10}OH$-H_2 bearing a polar OH group in the side chain in comparison to the native substrate UQ_{10}-H_2. The detection of $UQ_{10}OH$ in BLM and in RLM in context with redox properties of $UQ_{10}OH$ similar to UQ_{10} suggest the natural presence of $UQ_{10}OH$-H_2 in these organelles as well, which, however, was not explicitly studied in this work. Furthermore, because of its structure, $UQ_{10}OH$-H_2 is expected to act as an antioxidant in analogy to UQ_{10}-H_2. Because UQ_{10} is the major UQ species in bovines and humans, our findings suggest the presence of $UQ_{10}OH$ in certain human tissues, too.

Further work is needed to clarify the origin of $UQ_{10}OH$ and the modulation of its quantity in certain mammalian tissues as well as its influence on other mitochondrial and cellular functions. Only this information would allow predicting the potential benefit of the precursor UCa_9 as a supplement.

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