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Are Reduced Ubiquinones Oxygen Radical Generators?

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Ubiquinone (UQ) is the only natural compound which was reported both to generate and to scavenge oxygen-derived radicals. Redox-cycling of ubiquinone may yield six different species of the parent compound: UQH_2, UQH^-, UQ^{2-}, $UQH^\bullet$, $UQ^{\bullet-}$, and UQ. Ubiquinol (UQH_2) is unequivocally considered to be the ubiquinone species capable of scavenging oxygen-derived radicals. In contrast, the ubiquinone species responsible for one e^- reduction of dioxygen (O_2) thereby initiating the cascade of oxidative stress is still a matter of controversial debate. In the present study this question was approached by following the effect of O_2 on the stability of the various reduced forms of UQ. For this purpose conditions were designed allowing the selective accumulation of the two protonated and of the two deprotonated forms of reduced ubiquinones. Our results exclude both protonated (ubiquinol, UQH_2) and anionic (ubiquinol anion, UQH^-, and ubiquinol dianion, UQ^{2-}) fully reduced ubiquinones as the source exerting one e^- reduction of O_2. Ubisemiquinone (semiquinone radical, $UQH^\bullet$), when protonated, underwent rapid disproportionation, while transition to the semireduced anionic form (semiquinone radical anion, $UQ^{\bullet-}$) was found to favor autoxidation. The results obtained in this study provide a chemical base for the assessment of one e^- transfer from redox-cycling UQ to O_2 in the respiratory chain and in biomembranes where ubihydroquinol is suggested to exert antioxidant activities.

INTRODUCTION

The role of ubiquinone (UQ)[1] as an e^- carrier and H^+ translocator in mitochondria has been extensively studied for many years. Apart from this bioenergetic activities which contribute to mitochondrial ATP-synthesis redox-cycling UQ was also suggested to be involved in $O_2^{\bullet-}$ release into the cell (*1–7*). This assumption was based on extraction/reincorporation experiments of mitochondrial UQ more than 20 years ago (*2*) and is still considered as an unequivocal evidence on the *in vivo* generation of $O_2^{\bullet-}$ radicals associated with regular mitochondrial respiration. However, one e^- donors such as redox-cycling ubisemiquinones of the Q-cycle are stabilized against autoxidation through binding to the respective redox partners. This was supported by the demonstration that ubisemiquinones of intact mitochondria were insensitive to O_2. The significance of the suggested side effect of mitochondrial respiration is

[1] Abbreviations used: UQ, general formula for ubiquinones; UQ_0, 2,3 dimethoxy-5-methyl-1,4 benzoquinone (605-94-9); UQ_{10}, ubiquinone Q_{10} (1339-63-5); UQH_2, ubiquinol; UQH^-, ubiquinol anion; UQ^{2-}, ubiquinol dianion; $UQH^\bullet$, semiquinone; $UQ^{\bullet-}$, semiquinone anion; $O_2^{\bullet-}$, superoxide radical; SOD, superoxide dismutase (EC 1.15.1.1); O_2, dioxygen; EPR, electron paramagnetic (spin) resonance; e^-, electron.

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becoming clear if one considers that $O_2^{\bullet-}$ triggers a cascade of subsequent radical reactions (oxidative stress) which may be deleterious to all biomolecules. An evaluation on the suggested role of redox-cycling UQ in the development of oxidative stress requires both the demonstration of the particular UQ species involved and the analysis of the conditions under which this natural e^- carrier will shuttle single electrons to O_2 out of sequence. Candidates to be considered as potential one e^- donors are the various forms of semi- and fully reduced UQ. Both fully and semireduced UQ are expected to exist in the anionic and protonated form so that five different species, namely UQH_2, UQH^-; UQ^{2-}, $UQH^\bullet$, and $UQ^{\bullet-}$, come into question as one e^- reductant of O_2. All of these five species occur in mitochondria during e^- transfer (*8–10*). They are also expected to exist in any other biomembrane undergoing lipid peroxidation in the presence of UQH_2. However, the literature does not address the form of UQ capable of one e^- reduction of O_2. Since oxidation and reduction of ubiquinones are performed by the alternative addition or release of single e^- and protons it was of interest to systematically study whether any/all of these intermediates can become a direct reductant(s) of O_2. The answer to this question is significant for the evaluation of a possible prooxidant role of UQ in biomembranes.

MATERIALS AND METHODS

Chemicals

KBH_4, KH_2PO_4, and ethanol were obtained from Merck, UQ (coenzyme Q) was obtained from Kanegafuchi Chemical Industry Co., Ltd. All the chemicals were of analytical grade purity.

Experimental Design

Experiments with the water soluble homologue ubiquinone UQ_0. O_2 consumption was measured simultaneously with the formation of UQ (from UQH_2) or with the appearance of $UQ^{\bullet-}$ radicals by inserting an O_2-sensitive Clark electrode into the cuvette of the spectrophotometer or into the flat cell of the EPR spectrometer. UQ_0 concentrations were followed photometrically at 275 nm (ε = 14,900 M^{-1} cm^{-1}). Initial concentrations used: UQ_0H_2 0.09 mM, UQ_0 0.01 mM, superoxide dismutase (SOD) 0.02 mg/ml, O_2 0.24 mM, in phosphate buffer 50 mM adjusted to pH 7.4.

Experiments with native UQ_{10}. The experiments were carried out in ethanol because O_2 contents both in ethanol and in aqueous solutions were almost identical (*11*). For testing the effect of O_2 $UQ_{10}H_2$ solution was saturated with air O_2 immediately before starting the reaction upon the addition of buffered UQ_{10} solution adjusted to the desired pH values (from pH 5 to 13.5). Ethanol solution, containing 2.5% water, was buffered with TRIS buffer (final concentration of TRIS buffer was 2.5 mM) adjusted to pH values required (in the range of 5 to 13.5). When the experiments were carried out under anaerobic conditions, O_2 was removed from the corresponding solutions by bubbling argon for 10 min prior to starting the

reaction. Initial concentrations used: $UQ_{10}H_2$ 0.15 mM, UQ_{10} 0.15 mM, oxygen 0.24 mM.

Reduction of ubiquinones. UQ_{10} (UQ_0) was reduced to $UQ_{10}H_2$ (UQ_0H_2) according to Maguire *et al.* (*12*).

EPR Spectroscopy

EPR spectra were recorded with a Bruker ER-200 SRP in a quartz flat cell. General EPR settings: microwave frequency 9.6–9.7 GHz, modulation frequency 100 kHz, microwave power 10 mW, modulation amplitude 0.28 G, sweep 30 G. Specific EPR settings for measuring $UQ_0^{\bullet-}$ in water phase: microwave power 20 mW, modulation amplitude 0.5 G, sweep 30 G, center field 3492 G, scan rate 5.4 G/min, time constant 0.6 s, receiver gain 10^6.

Optical Spectroscopy

Optical spectra were recorded with a uv–vis diode array spectrometer (Milton Roy, MR 3000) in a quartz cell (1 = 1 cm) made for anaerobic experiments (Aminco SLM Instruments, Inc.). UQ_0 (UQ_{10}) concentrations were followed photometrically at 275 nm (ε = 14,900 M^{-1} cm^{-1}).

O_2 Consumption

O_2 consumption was measured by an OM-4 oxygen meter (Microelectrodes, Inc.). The O_2 sensitive electrode was inserted in a round glass cell provided with an access to insert reagents.

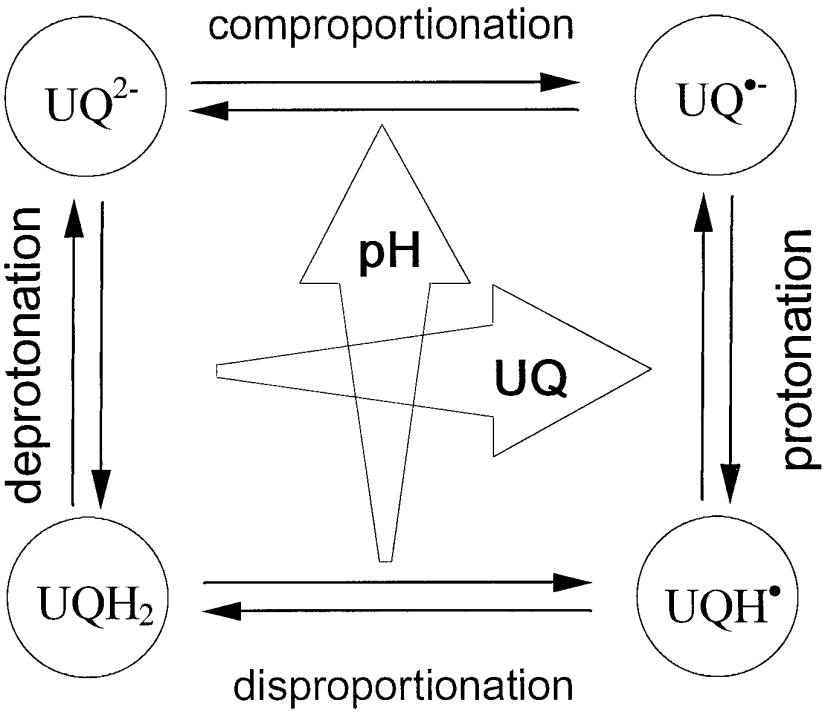
RESULTS

Depending on the pH of a solution, reduced UQ_0 can exist in the protonated and in the deprotonated form. If UQ_0 is present in addition to ubiquinols (UQ_0H_2, UQ_0H^-, UQ_0^{2-}), ubisemiquinones ($UQ_0H^{\bullet}$, $UQ_0^{\bullet-}$) can also be expected to be formed as shown in Scheme 1. The possible autoxidation of reduced UQ species was first investigated with UQ_0, which can be dissolved in water. The aqueous reaction medium was adjusted to the physiological pH level (7.4), allowing the use of SOD for checking the presence or absence of the expected autoxidation product $O_2^{\bullet-}$. According to Scheme 1 the addition of UQ_0 to UQ_0H_2 dissolved in the aqueous reaction system resulted in the formation of $UQ^{\bullet-}$ radicals.

The existence of $UQ_0^{\bullet-}$ was made safe by means of EPR spectroscopy which demonstrated the existence of a typical $UQ_0^{\bullet-}$ related spectrum (see Fig. 1, inset) with characteristic splitting constants (3H (5CH3) = 2.4 G; 1H(6H) = 2.1 G) previously described (*13*).

In the presence of SOD, ubisemiquinones decline under the detection limit, indicating the existence and involvement of $Q_2^{\bullet-}$. This proves that autoxidation of a reduced UQ_0 species present in the reaction system can be expected.

All further experiments were performed with the natural ubiquinone species



SCHEME 1. Schematic presentation of the procedure for equilibrium shifts between protonated and deprotonated uni- and divalently reduced UQ.

UQ_{10} in an ethanol/buffer mixture which in contrast to UQ_0 in water is closer to the biological conditions of ubiquinone activities. In this model system SOD, which is water soluble, cannot be applied to $O_2^{\bullet-}$ detection. Autoxidation of $UQ_{10}H_2$ was therefore estimated by oxygen consumption and the formation of UQ_{10}. The existence of the predominant species of reduced UQ_{10} can be addressed in the following way. Addition of UQ_{10} to a solution containing fully reduced UQ_{10} gives rise to the establishment of an equilibrium between all reduction states of UQ_{10} according to comproportionation reaction. According to the mass law a maximum of semiquinones are formed when UQ_{10} concentration equals $UQ_{10}H_2$ concentration. Based on pK values of semiquinones (pK($UQ_{10}^{\bullet-}$/$UQ_{10}H^{\bullet}$) = 5.9) and hydroquinones (pK($UQ_{10}H^{-}$/$UQ_{10}H_2$) = 11.3) (pK(UQ_{10}^{2-}/$UQ_{10}H^{-}$) = 13.2) (*9*, *10*) it is possible to shift the predominant species of the two reduction states of UQ_{10} as a function of the pH. Thus, depending on the respective conditions chosen, different patterns of reduced UQ_{10} species can be adjusted (see Scheme 1). When applying these conditions for the variation of the predominant UQ_{10} species, it turned out that reduced UQ_{10} started to oxidize only at pH values higher than 10 (Fig. 2). Formation of (oxidized) UQ_{10} from the reduced forms was found to increase with increasing pH values (Fig. 2A). O_2 disappeared from the reaction system accordingly (Fig.

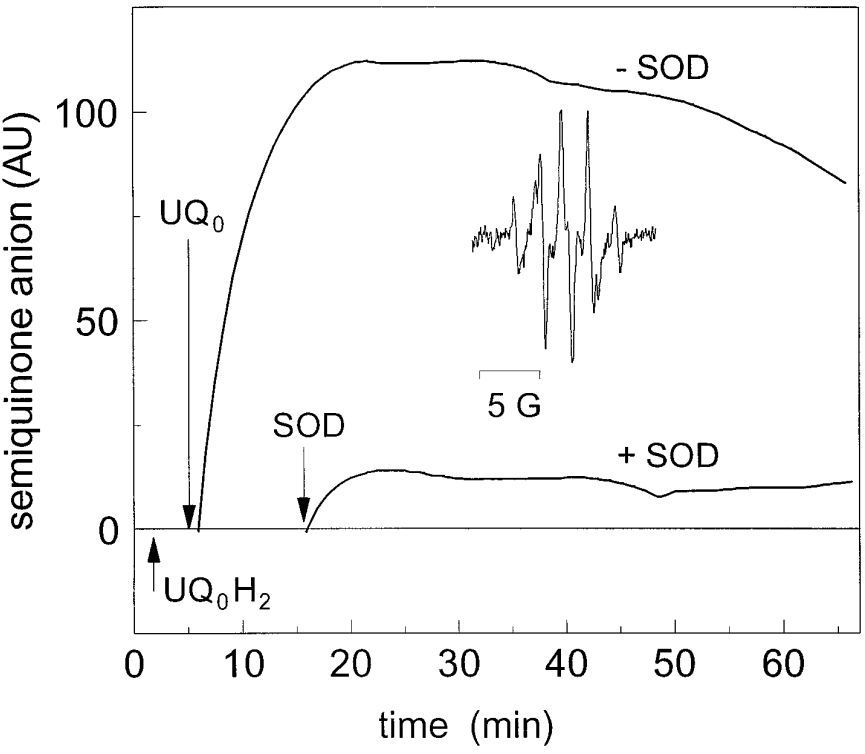


FIG. 1. Time course of $UQ_0^{\bullet-}$ following initiation of comproportionation in an aqueous phosphate buffer (50 mM, pH 7.4) equilibrated with air O_2. The identity of the deprotonated $UQ_0^{\bullet-}$ species was made safe by comparing splitting constants (3H α_{H1} = 2.4 G; 1H α_{H2} = 2.1 G) obtained with those of the literature (*13*). Experimental conditions: UQ_0H_2 0.09 mM, UQ_0 0.01 mM, superoxide dismutase (SOD) 0.02 mg/ml, O_2 0.24 mM, in phosphate buffer 50 mM adjusted to pH 7.4.

2B). The presence of an ubisemiquinone related EPR signal was detected in the alkaline range as long as O_2 was absent (Fig. 4A, inset). The analysis of the splitting constants (3H α_{H1} = 2.08 G; 2H α_{H2} = 1.04 G) elicits the presence of the ubisemiquinone anion ($UQ_{10}^{\bullet-}$) (*13*). In the lower pH range the expected protonated $UQ_{10}H^{\bullet}$ species was not detectable, irrespective of whether or not UQ_{10} was added to initiate $UQ_{10}H^{\bullet}$ formation from the comproportionation reaction. Since O_2 was also not removed from the reaction protonated $UQ_{10}H^{\bullet}$ formed most likely disappeared from the reaction system by spontaneous disproportionation. The lack of O_2 consumption in combination with the lack of detectable amounts of UQ_{10} expected from the oxidation of reduced UQ_{10} rule out protonated forms of UQ_{10} species as a source of e^- for O_2. Oxygen consumption, which was found to increase with increasing pH values (Fig. 2) reveals the presence of an autoxidizable UQ_{10} species in the reaction system. Since $UQ_{10}^{\bullet-}$ and fully reduced UQ_{10} anions ($UQ_{10}H^-$, UQ_{10}^{2-}) become the dominant forms in the alkaline pH range the UQ_{10} form ultimately interacting with O_2 cannot be identified from experiments shown in Fig. 2.