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NADPH-CYTOCHROME REDUCTASE CATALYSED REDOX CYCLING OF 1,4-BENZOQUINONE; HAMPERED AT PHYSIOLOGICAL CONDITIONS, INITIATED AT INCREASED pH VALUES

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Abstract—In the present study the inability of 1,4-benzoquinone to support NADPH-cytochrome reductase catalysed redox cycling was investigated. The results obtained demonstrate that NADPH-cytochrome reductase is able to initiate a rapid two-electron reduction of 1,4-benzoquinone resulting in formation of the hydroquinone. The intermediate one-electron reduced semiquinone form does not pass its electron on to molecular oxygen, i.e. giving rise to redox cycling, but is reduced by a second electron, either by NADPH-cytochrome reductase upon protonation of the semiquinone or through disproportionation, both giving rise to the two-electron reduced hydroquinone. At pH values below the pK_a of the hydroquinone, the electrons of the hydroquinone are also not passed on to molecular oxygen due to efficient protonation. However, at pH values around or above the pK_a (9.85) of the two-electron reduced hydroquinone form, significant redox cycling activity is observed in a 1,4-benzoquinone containing incubation. Further experiments demonstrate a similarity in both the concentration and pH dependence of 1,4-benzoquinone or 1,4-hydroquinone supported NADPH-cytochrome reductase catalysed redox cycling. From these observations it is concluded that 1,4-benzoquinone is able to redox cycle from its deprotonated two-electron reduced hydroquinone form, but only at relatively high pH values. Together the data provide an insight into why the NADPH-cytochrome reductase catalysed redox cycling of 1,4-benzoquinone is inhibited at physiological conditions, but initiated at increased pH values.

Key words: 1,4-benzoquinone; 1,4-hydroquinone; redox cycling; NADPH-cytochrome reductase; pH effect

The toxicity of quinones is generally ascribed to their capacity to bind covalently to cellular nucleophilic macromolecules, and/or to their capacity to support a process called redox cycling [1–3]. The redox cycling of quinones is initiated by their one-electron reduction catalysed by flavin-containing enzymes like NADPH-cytochrome reductase (EC 1.6.2.4), NADH-cytochrome reductase (EC 1.6.2.2) or xanthine oxidase (EC 1.2.3.2) [4], resulting in formation of a reactive semiquinone radical. The semiquinone radical may pass its unpaired electron on to molecular oxygen, giving rise to the formation of reactive oxygen species and regeneration of the quinone which can participate in a new redox cycle. In addition, upon protonation and a second single-electron reduction of the semiquinone radical the two-electron reduced deprotonated hydroquinone form arises. This form might also react with molecular oxygen passing on one or both of its electrons, resulting in formation of respectively superoxide anion radicals or hydrogen peroxide.

The extent to which redox cycling may add to the toxicity of the benzoquinones varies with the substituent pattern of the quinone [3, 5]. BQ† as well as monosubstituted BQ derivatives have been reported to redox cycle very poorly, or not at all [6]. The poor redox cycling of BQ has been ascribed to its very positive one-electron reduction potential in aqueous solutions, although the reason for poor redox cycling due to a relatively positive single-electron reduction potential remained unclear [2, 6, 7]. Other investigators ascribed the lack of redox cycling ability of BQ to the slow reaction of its semiquinone radical with molecular oxygen (k = 0.5–4.5 × 10^4/M/sec) [3, 8–10].

Generally, factors not taken into consideration when studying the redox cycling capacities of the quinones in biological systems, are the protonation–deprotonation equilibria of their semiquinone and of their two-electron reduced hydroquinone form, which are, however, well documented in the literature [11, 12]. This, in spite of the fact that protonation can be expected to stabilise the negative charge resulting from the one- or two-electron reduction, thereby decreasing the possibility of a reaction of the one- or two-electron reduced form with molecular oxygen, i.e. redox cycling. That deprotonation is a requisite for electron transfer is a well established concept [13].

The objective of the present study was to

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† Abbreviations: BQ, 1,4-benzoquinone; HQ, 1,4-hydroquinone; LUMO, lowest unoccupied molecular orbital.

investigate the lack of redox cycling of 1,4-benzoquinone and the importance of protonation equilibria of its one- and two-electron reduced forms for the inability of BQ to redox cycle in a NADPH-cytochrome reductase catalysed reaction.

MATERIALS AND METHODS

Purification of NADPH-cytochrome reductase. NADPH-cytochrome reductase was purified from liver microsomes of female Wistar rats pretreated with phenobarbital (Brocacef b.v., Maarssen, The Netherlands) (0.1% (w/v) in drinking water for 7 days), essentially as described by Yasukochi and Masters [14]. Emulgen 911 (Kao Corporation, Tokyo, Japan) was used instead of Renex 690, and all buffers contained 100 μM phenylmethanesulfonyl fluoride (Merck, Darmstadt, Germany) to inhibit protease activities. The preparation was made detergent-free as described before [15].

NADPH-cytochrome reductase activity was determined as described by Phillips and Langdon [16], using cytochrome *c* (Boehringer, Mannheim, Germany) as the final electron acceptor. One μmol cytochrome *c* reducing activity per min was taken as the unit to quantify the NADPH-cytochrome reductase activity. Protein concentrations were measured using the method of Lowry *et al.* [17] using bovine serum albumin (Sigma, St Louis, MO, U.S.A.) as the standard. The final detergent-free preparation had a specific activity of at least 35 units/mg protein and was homogeneous as judged by SDS–PAGE, carried out as described by Laemmli [18].

Redox cycling of benzoquinones. BQ, HQ and tetramethyl-1,4-benzoquinone (duroquinone) were purchased from Aldrich (Steinheim, Germany). The redox cycling of benzoquinones was measured at room temperature in 0.1 M potassium phosphate buffer pH 7.5 (unless indicated otherwise) containing 0.15 mM NADPH (final concentration). The desired concentrations of quinones were added as 1% (v/v) of a 100 times concentrated, freshly prepared solution in dimethyl sulfoxide. Reactions were started by addition of 0.13 units of NADPH-cytochrome reductase. NADPH oxidation was detected by measuring the absorption at 340 nm. Activities were corrected for NADPH-cytochrome reductase-independent NADPH oxidation which was generally between 0 and 30% of the reductase-dependent activity.

Production of H_2O_2 was measured as described by Werringloer [19]. Oxygen consumption was measured using a Clark oxygen electrode at room temperature.

Molecular orbital computer calculations. Molecular orbital calculations were performed as described before [15].

Statistical analysis of data. The results represent the mean ± standard error of the mean.

RESULTS

Redox cycling of the BQs

The redox cycling of BQ and tetramethyl-1,4-benzoquinone (used as a well-known redox active control) was investigated as a function of the benzoquinone concentration. From the results obtained (Fig. 1) it follows that there is a significant difference in the extent to which the BQ and tetramethyl-1,4-benzoquinone redox cycle. Whereas redox cycling of BQ itself was hardly detectable at pH 7.5, substitution with four-electron donating substituents (tetramethyl-1,4-benzoquinone) resulted in increased rates of redox cycling. The apparent K_m and V_{max} for the NADPH-cytochrome reductase catalysed redox cycling of tetramethyl-1,4-benzoquinone derived from the data presented in Fig. 1 were respectively 19 ± 1 μM and 367 ± 22 nmol NADPH oxidized/min/U_{red}.

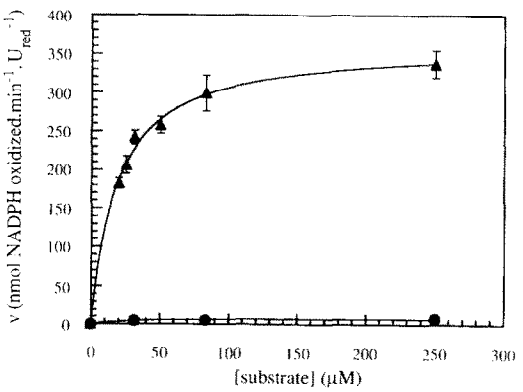

Fig. 1. NADPH-cytochrome reductase catalysed NADPH oxidation in the presence of tetramethyl-1,4-benzoquinone (-▲-) or BQ (-●-) at pH 7.5 and increasing concentration of the benzoquinone. NADPH-oxidation was measured as described in Materials and Methods. The results represent the mean ± standard error of the mean of three experiments.

In the presence of superoxide dismutase (0.1 mg/mL) at physiological pH values redox cycling of BQ was still not observed, indicating that elimination of superoxide anions from the incubation medium does not result in a shift of the equilibrium semiquinone + oxygen ⇄ quinone + superoxide anion to the right, initiating possibilities for redox cycling.

Single electron reduction potentials of the BQs

Previous studies reported in the literature suggest a relationship between electronic affinities, polarographic single-electron reduction potentials and the energy (E) of the LUMO of a molecule [11, 20–22]. The LUMO is the molecular orbital into which the electron will be positioned upon one-electron reduction. To investigate whether the non-redox cycling of BQ could be due to a deviating single-electron reduction potential compared to the single-electron reduction potential of tetramethyl-1,4-benzoquinone, the E_{LUMO} values of these and additional BQs were calculated and compared to data available in the literature [11, 22] for their one-electron reduction potentials. The results obtained (Fig. 2) demonstrate that the calculated E_{LUMO} values correlate with the single-electron reduction potential of the benzoquinones (correlation coefficient = 0.909). The data in Fig. 2 also demonstrate that the one-electron reduction potential of BQ is higher than that of methyl substituted

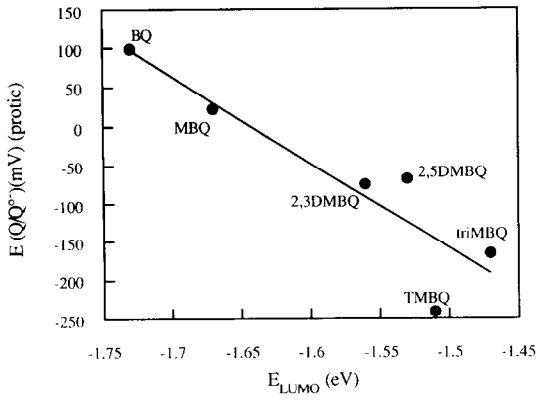

Fig. 2. Relation between the single-electron reduction potential ($E(Q/Q^{\cdot-})$ for a series of BQs in aqueous solution and their calculated E_{LUMO}. The correlation coefficient was 0.909. The one-electron reduction potentials in aqueous solution of the $Q/Q^{\cdot-}$ couples, indicated here as $E(Q/Q^{\cdot-})$, were taken from Chambers [11]. The E_{LUMO} values were calculated as described in Materials and Methods. BQ = 1,4-benzoquinone; MBQ = methyl-1,4-benzoquinone; 2,3DMBQ = 2,3-dimethyl-1,4-benzoquinone; 2,5DMBQ = 2,5-dimethyl-1,4-benzoquinone; triMBQ = trimethyl-1,4-benzoquinone; TMBQ = tetramethyl-1,4-benzoquinone

benzoquinones, suggesting that the reduction of BQ (higher $E_{1/2}$, lower E_{LUMO}) is energetically more favourable than that of tetramethyl-1,4-benzoquinone. From this it is concluded that the absence of redox cycling of BQ is unlikely to be due to an inability to accept electrons.

NADPH-cytochrome reductase catalysed reduction of BQ

In Fig. 3 data are presented that confirm that the poor redox cycling of BQ is not due to its inability to accept electrons. Immediately following the addition of NADPH-cytochrome reductase to a BQ containing incubation a rapid decrease in NADPH is observed, which levels off after a short period of time (Fig. 3a). During this rapid decrease in NADPH no oxygen consumption is observed (data not shown). The amount of NADPH oxidized varies stoichiometrically with the amount of BQ added (Fig. 3b). From this observation it follows that two electrons are consumed per molecule of BQ. This implies that the BQ can be one-electron reduced by NADPH-cytochrome reductase, but instead of passing its electron on to molecular oxygen, the semiquinone radical either disproportionates or becomes quickly reduced by a second electron, both processes resulting in formation of the two-electron reduced hydroquinone.

Redox cycling of BQ at varying pH

The lack of redox cycling of BQ might be ascribed to efficient protonation of either its one- or its two-electron reduced form. Thus, a shift in the pH of the incubation to a value around or above the pK_a of the one-electron reduced semiquinone or the two-electron reduced hydroquinone might result in increased possibilities for redox cycling of BQ. Therefore, the effect of varying pH of the incubation mixture on the NADPH-cytochrome reductase catalysed redox cycling of 1,4-benzoquinone was investigated. Tetramethyl-1,4-benzoquinone was included in this experiment as a positive control, i.e. a compound capable of redox cycling at physiological pH values. From the data, presented in Fig. 4, it

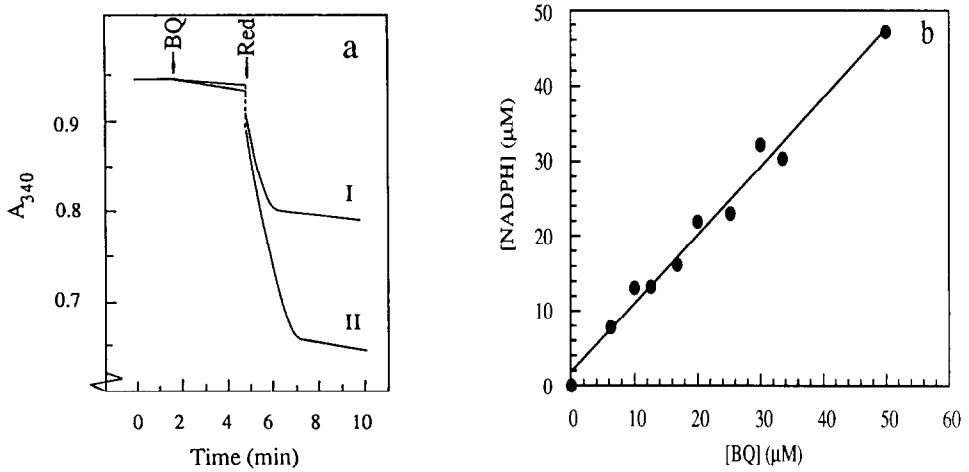

Fig. 3. Time- (a) and BQ concentration- (b) dependent NADPH-cytochrome reductase catalysed NADPH oxidation. Incubation conditions were as described in Materials and Methods. NADPH oxidation was determined by monitoring the absorbance at 340 nm. At the arrows in (a) respectively BQ (in two concentrations) I and II and NADPH-cytochrome reductase were added to the incubation. In (b) the relationship between the amount of BQ added and the amount of NADPH oxidized is depicted. The curve obtained fits the equation [NADPH] = 1.87 + 0.91.*[BQ], correlation coefficient = 0.991.

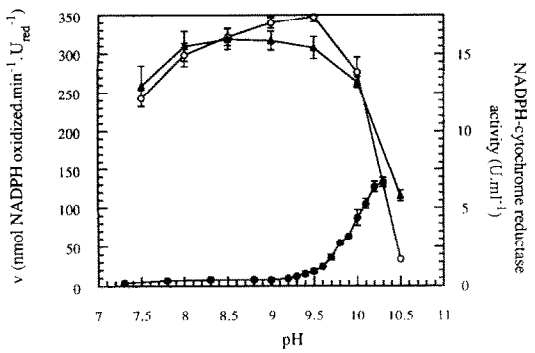

Fig. 4. pH Dependence of the NADPH-cytochrome reductase catalysed redox cycling of BQ (-●-) and tetramethyl-1,4-benzoquinone (-○-) and of the cytochrome *c* reducing activity of NADPH-cytochrome reductase (-▲-). Redox cycling activities in the presence of 1 μM BQ or 20 μM tetramethyl-1,4-benzoquinone and the cytochrome *c* reducing activity of NADPH-cytochrome reductase were determined as described in Materials and Methods. The results represent the mean ± standard error of the mean of two experiments.

follows that from pH 7.5 to 10.5 tetramethyl-1,4-benzoquinone demonstrates significant redox cycling. The activity observed parallels the activity of NADPH-cytochrome reductase, demonstrating reduced activity at higher pH values (Fig. 4). For BQ a different pattern was observed. At pH values below 9.0, BQ appeared unable to support redox cycling. However, an increase in the pH from 9.0 to 10.5 results in increased capacities for redox cycling in spite of the relative reduction in the activity of the NADPH-cytochrome reductase. Additional experiments demonstrated that the BQ-supported NADPH oxidation was accompanied by stoichiometric oxygen consumption and H_2O_2 production (Table 1), indicating that the BQ-supported NADPH oxidation at pH ≥ 9.0 indeed represents redox cycling.

The data presented in Fig. 4 also demonstrate that the redox cycling of BQ becomes significant around the pK_a value of its hydroquinone form reported to be 9.85 [22]. The pK_a of its semiquinone form has been reported to be 4.1 [22]. This result suggests that BQ redox cycles from its deprotonated hydroquinone form, not from its semiquinone form. To investigate this possibility to a further extent additional experiments were performed using HQ as a substrate for NADPH-cytochrome reductase catalysed redox cycling. The results from these experiments are presented in Fig. 5a and b. Figure 5a demonstrates that the pH dependence of the BQ- and HQ-supported redox cycling activities are similar. The HQ-supported redox cycling also becomes significant around the pK_a value of HQ, i.e. 9.85. Figure 5b demonstrates that, at pH 10.0, the NADPH-cytochrome reductase catalysed redox cycling of BQ and HQ also show a similar dependence on the substrate concentration.

Finally, the results presented in Fig. 6 demonstrate that addition of the hydroquinone to an incubation without NADPH and without NADPH-cytochrome reductase (pH = 10.0), results in a stoichiometric consumption of oxygen. This implies an oxygen-induced oxidation of the deprotonated hydroquinone.

Together the results support the conclusion that the redox cycling activities observed for BQ at pH values ≥9 can be ascribed to redox cycling of its deprotonated hydroquinone form.

DISCUSSION

In the present study the inability of BQ to support NADPH-cytochrome reductase catalysed redox cycling was investigated. Analysis of calculated molecular orbital parameters (E_{LUMO}) and the single-electron reduction potentials of these and other quinone derivatives, demonstrated that the non-redox cycling of BQ cannot be ascribed to a deviating single-electron reduction potential of this molecule. Additional experiments with BQ clearly demonstrated the two-electron reduction of this compound by NADPH-cytochrome reductase resulting in stoichiometric NADPH oxidation and formation of the hydroquinone. This result is in accordance with results from an experiment described by Nishibayashi *et al.* [23] reporting stoichiometric NADPH oxidation upon addition of BQ to a microsomal incubation. The results of the present study demonstrate that this reduction is not accompanied by oxygen consumption or H_2O_2 formation but results in formation of the HQ. Based on these observations it must be concluded that the non-redox cycling of BQ at pH 7.5 must be ascribed to the inability of

Table 1. NADPH-cytochrome reductase catalysed NADPH oxidation, H_2O_2 formation and O_2 consumption in the presence of BQ or HQ measured during the first two minutes of redox cycling at pH 10.0

Substrate	NADPH oxidation (nmol/2 min.U_{red})	H_2O_2 formation (nmol/2 min.U_{red})	O_2 consumption (nmol/2 min.U_{red})
BQ (0.5 μM)	88 ± 3	64 ± 2	80 ± 1
HQ (0.5 μM)	74 ± 6	62 ± 4	84 ± 2

NADPH oxidation, H_2O_2 formation and O_2 consumption were measured as described in Materials and Methods. The results represent the mean ± standard error of the mean ($n = 3$).

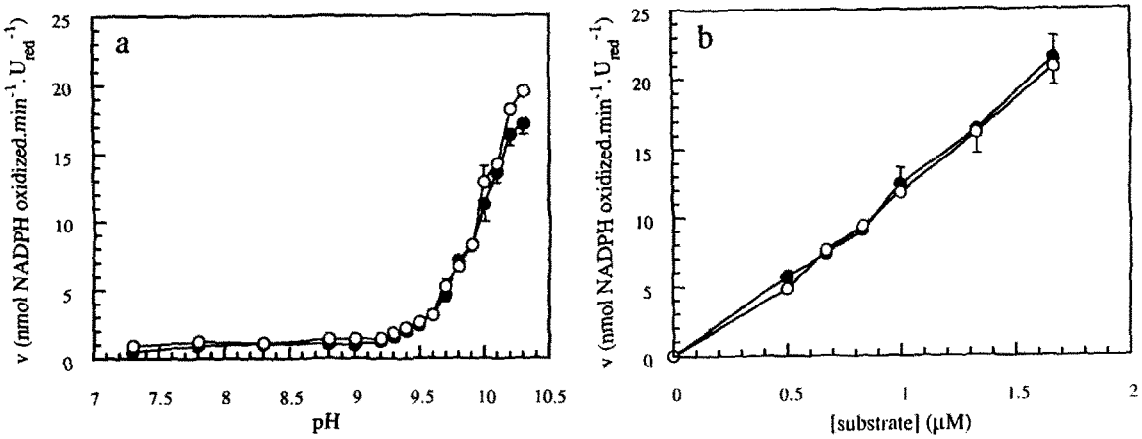

Fig. 5. pH- (a) and concentration- (b) dependent NADPH-cytochrome reductase catalysed redox cycling at pH 10.0 of BQ (-●-) and HQ (-○-). Redox cycling was measured as described in Materials and Methods. The concentration of 1,4-hydroquinone and 1,4-benzoquinone used in (a) was 1 μM. The results represent the mean ± standard error of the mean of two experiments.

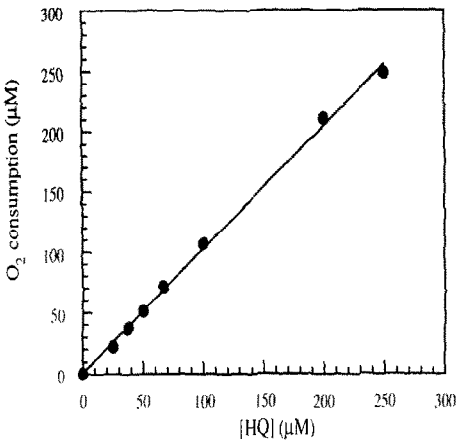

Fig. 6. Oxygen consumption in an incubation without NADPH and without NADPH-cytochrome reductase at pH = 10.0 upon addition of increasing concentration of HQ. The curve obtained fits the equation O_2 consumption = 0.86 + 1.02*[HQ], correlation coefficient = 0.999. Oxygen consumption was measured as described in Materials and Methods. The results represent the mean ± standard error of the mean of two experiments.

both its one-electron as well as its two-electron reduced form to pass their electron(s) on to molecular oxygen. Taking into account the pK_a values reported for the semiquinone and hydroquinone form of BQ of 4.1 and 9.85, respectively [22], it can be concluded that at pH 7.5 the semiquinone is present in its deprotonated form whereas the hydroquinone is present in its protonated form. Thus, the results obtained point at the inability of the deprotonated semiquinone and the protonated hydroquinone to pass electrons on to molecular oxygen. This conclusion is in line with the fact that the rate constant for the reaction of 1,4-semiquinone with molecular oxygen is at least four orders of magnitude lower than that of the tetramethyl substituted 1,4-semiquinone [9]. However, the rate constant for the protonation of the one-electron reduced 1,4-benzoquinone is high compared to the rate constant for a reaction with molecular oxygen. Comparison of the rate constant for protonation of the semiquinone, reported to be 4×10^{10}/M/sec [24], to that reported for the reaction of the semiquinone with molecular oxygen ($0.5–4.5 \times 10^4$/M/sec) [8, 10] shows that chances for protonation of the semiquinone radical are 10^6 times higher than chances for a reaction with molecular oxygen. When the protonated semiquinone is efficiently reduced by a second electron, i.e. removed from the incubation, this will shift the protonation equilibrium of the semiquinone in favor of the protonated form, even when the pH is above its pK_a. This explains why the semiquinone might become protonated and reduced by a second electron instead of passing on its electron to molecular oxygen, i.e. giving rise to redox cycling. Such a one-electron reduction of the semiquinone to the hydroquinone by NADPH-cytochrome reductase must be possible taking into account the one-electron reduction potential of the semiquinone–hydroquinone couple of +459 mV [22] and the midpoint potentials of the four one-electron steps in NADPH-cytochrome reductase, reported to be much lower, i.e. −110, −270, −290 and −365 mV [25]. In addition, disproportionation of the non-protonated semiquinone radical to result in formation of the quinone and the two-electron reduced hydroquinone might provide an additional and/or alternative route for the NADPH-cytochrome reductase initiated formation of the two-electron reduced hydroquinone.

Additional results of the present study demonstrate that with increasing pH BQ becomes capable of redox cycling. The actual pH at which this phenomenon occurs appears to be around the pK_a

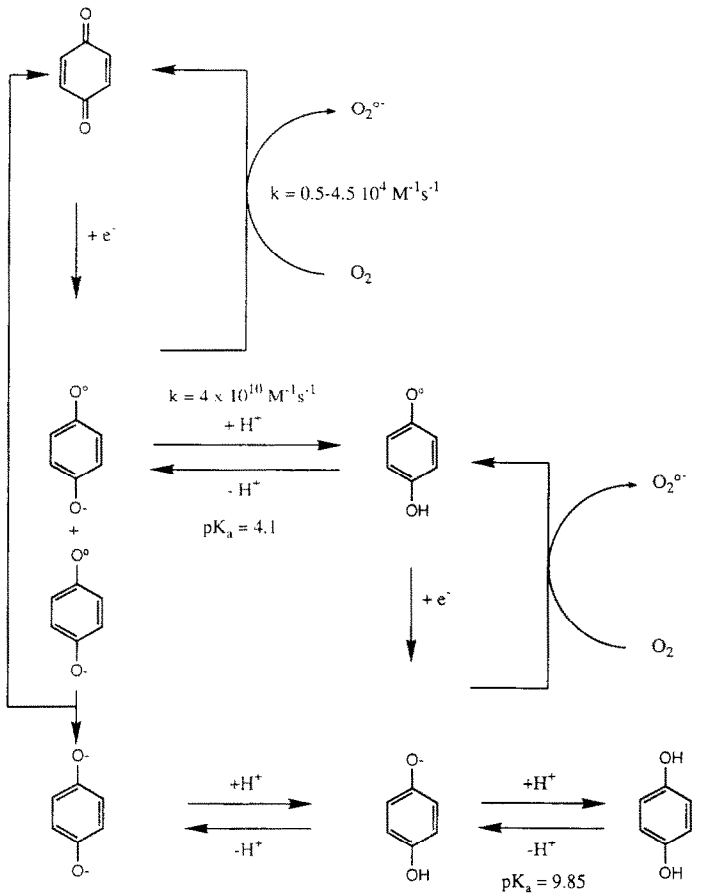

Fig. 7. Schematic presentation of the pathway for redox cycling of BQ based on the results of the present study. Kinetic data are derived from the literature [8, 10, 13]. For further explanation see text of the Discussion section.

of the hydroquinone. From this result it is concluded that the redox cycling observed for BQ at increased pH values results from redox cycling of its deprotonated hydroquinone form, present at a significant concentration when the pH of the medium reaches the pK_a of the hydroquinone. This redox cycling implies electron transfer from the deprotonated hydroquinone to molecular oxygen, a reaction hampered at neutral pH by the fact that the one-electron reduction potential for the semiquinone/hydroquinone couple might be too high, i.e. +459 mV [22], for an efficient one-electron transfer of the hydroquinone to molecular oxygen. This is because the reduction potential of the $O_2/O_2^{\cdot}$ couple has been reported to be between −155 and −330 mV [8–10, 22]. However, the reduction potential of the semiquinone/hydroquinone couple will, in analogy to what is described for the two-electron reduction potential of the quinone/hydroquinone couple, decline with increasing pH, facilitating the one-electron transfer from the deprotonated hydroquinone to molecular oxygen. The fact that addition of superoxide dismutase did not inhibit the redox cycling of the (hydro)quinone at increased pH values (data not shown) suggests that the reaction does not proceed with $O_2^{\cdot}$ as the oxidant. The stoichiometric oxygen consumption observed upon addition of the hydroquinone to an incubation without NADPH and without NADPH-cytochrome reductase further supports the O_2-catalysed oxidation of the hydroquinone, as depicted in Fig. 7.

In conclusion, the results of the present study demonstrate that the non-redox cycling of BQ at pH 7.5 is mainly due to the low rate constant for the reaction of the 1,4-semiquinone with molecular oxygen compared to the rapid protonation of the 1,4-semiquinone resulting in a form that can be efficiently reduced by a second electron and finally in the formation of the HQ. Due to its pK_a value of 9.85, at physiological pH the HQ becomes protonated which prevents redox cycling of this compound. Figure 7 schematically presents these characteristics of the BQ redox cycling. The results obtained support that under physiological conditions BQ-induced toxicity must result from its nucleophilic rather than its redox cycling capacities.

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