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ON THE MECHANISM OF ONE-ELECTRON REDUCTION OF QUINONES BY MICROSOMAL FLAVIN ENZYMES: THE KINETIC ANALYSIS BETWEEN CYTOCHROME B_5 AND MENADIONE

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Univalent oxidation-reduction reactions coupled with the menadione (MK)/menadione semiquinone ($MK^{\cdot -}$) system were investigated by using microsomal flavin enzymes. NADPH-cytochrome P-450 reductase gave a dynamic equilibrium of oxidation-reduction of cytochrome b_5 in the presence of menadione (MK), the level of which depended on the concentration of O_2 and superoxide dismutase. The data suggest that the superoxide and menadione radicals are involved as an active intermediate in this system. The overall reaction at steady state appears to be composed of four main reactions, eqs. 2-5, and eqs. 2 and 4 are in equilibrium.

KEY WORDS: One-electron reduction, menadione, superoxide, NADPH-cytochrome P-450 reductase, cytochrome b_5, redox cycling.

INTRODUCTION

It has been reported that microsomal flavoproteins, NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase catalyze a typical one-electron reduction of quinones.[1-7] Menadione (MK) is known to be good electron acceptor for NADPH-cytochrome P-450 reductase, and the MK-mediated NADPH-oxidase reaction takes place in the enzyme-MK system.[1,3,5,6] On the other hand, menadione is a very slow electron acceptor for NADH-cytochrome b_5 reductase, but O_2 consumption is greatly stimulated in the presence of cytochrome b_5[1]. This data indicates that one-electron transfer reaction takes place from reduced cytochrome b_5 to menadione. The MK-mediated NAD(P)H-oxidases have been explained by one-electron transfer from $MK^{\cdot -}$ to molecular oxygen (O_2). In these reactions menadione, which is bivalent molecule, can act as a one-electron carrier.

Yamazaki and Ohnishi[8] have indicated that a redox potentials of one-electron transfer system is useful parameters in the kinetical analysis of oxidation-reduction reactions, and the relation between one-electron redox potentials in a bivalent system and rate constants has been described.[8,9] In this paper, the studies on the reaction between menadione and cytochrome b_5 will be reported. The results are discussed on the basis of one-electron redox potentials.

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MATERIALS AND METHODS

Trypsin-solubilized NADPH-cytochrome P-450 reductase was prepared from rabbit liver microsomes according to Iyanagi and Mason[10] and Iyanagi *et al.*,[11] respectively. Lysosome-solubilized Cytochrome b_5 and NADH-cytochrome b_5 reductase were prepared from pig liver microsomes according to Iyanagi[12] and Iyanagi *et al.*,[13] respectively. Superoxide dismutase was prepared from dry seeds of *Pisum Sativum*.[14] Catalase was prepared from cow liver. Xanthine oxidase was purified caw cream according to Nakamura and Yamazaki.[15] All other materials were obtained from commercial sources at the highest available states of purity.

Absorbance measurements were carried out with a Hitachi recording spectrophotometer, Model 124, equipped with a thermostatically controlled cell compartment. Measurements of oxygen consumption and absorption change of cytochrome b_5 were measured simultaneously as described by Makino *et al.*[16] Reactions were performed at 25°C in potassium phosphate (pH 7.0), and sodium borate and carbonate-NaCl (pH 8.5). The concentration of O_2 in reaction solutions was controlled by bubbling N_2, O_2, or a mixture of both. Highly purified N_2 gas (99.9995%) obtained from a commercial source was used to maintain anaerobic conditions.

RESULTS

The reduced form of freshly prepared cytochrome b_5 from pig liver microsomes was oxidized by O_2 and rate constant k_1 was measured about $8.7\,M^{-1}\,sec^{-1}$ at pH 7.0 and $3.2\,M^{-1}$ at pH 8.5.

$$\text{Cyt } b_5^{2+} + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Cyt } b_5^{3+} + O_2^{\cdot -} \qquad (1)$$

This value might be slightly overestimated because a reaction product, $O_2^{\cdot -}$ was found to be an oxidant for reduced cytochrome b_5. Figure 1 shows that the oxidation of the reduced cytochrome b_5 was accelerated in the presence of xanthine oxidase (X.O) and xanthine (X). The oxidation occurred at a considerably high rate at pH 7.0 and 1 μM superoxide dismutase (SOD) removed the effect of xanthine oxidase. No reduction of cytochrome b_5 was observed at pH 7.0 even when the reaction was started from the fully oxidized level of cytochrome b_5. The reduction, however, became discernible at pH 8.5. This reduction was also completely inhibited by 1 μM superoxide dismutase. The rate of the reduction was proportional to the concentration of cytochrome b_5 in the oxidized form (data not shown). As the concentration of the superoxide radical could be indirectly measured using cytochrome c as an electron acceptor for the radical,[17] it was possible to calculate the value of k_{-1}. The rate constant k_{-1} was measured about $1.4 \times 10^2\,M^{-1}\,sec^{-1}$ at pH 8.5. The value for k' (Cyt $b_5^{2+} + O_2^- + 2H^+ \rightarrow$ Cyt $b_5^{3+} + H_2O_2$) was also measured about $1.4 \times 10^3\,M^{-1}\,sec^{-1}$ at pH 7.0 and $1.3 \times 10^2\,M^{-1}\,sec^{-1}$ at pH 8.5. Evidently, the superoxide radical could either oxidize or reduce cytochrome b_5. Figure 2 shows that the apparent direction of reaction 1 in the presence of a xanthine oxidase system was dependent upon the redox ratio of cytochrome b_5 previously present in reaction solutions. The redox ratio of the cytochrome b_5 at which the ratio remained unchanged was greatly dependent on pH, and at pH 7.0 the ratio was close to the fully oxidized level.

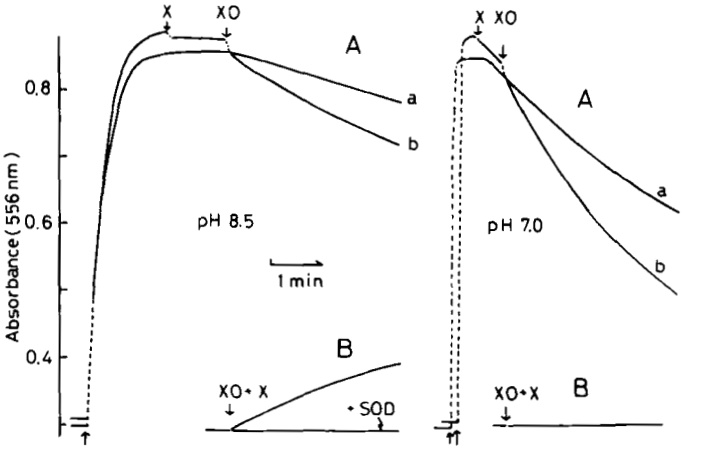


FIGURE 1 Oxidation and reduction of cytochrome b_5 occurred during the xanthine oxidase reaction. The reaction mixture contained 30 μM cytochrome b_5, 1 mM xanthine (X), 0.125 μM xanthine oxidase (X.O), and 0.2 μM catalase in an air-saturated solution. A: The autoxidation of reduced cytochrome b_5 occurred soon after it was reduced in the presence of 15.5 μM NADH and 0.1 μM NADH-cytochrome b_5 reductase (curves a). Acceleration of the oxidation was observed when the xanthine oxidase system was added (curves b). B: Reduction of cytochrome b_5 occurred at pH 8.5 but not at pH 7.0. The addition of 1 μM superoxide dismutase (SOD) removed the effect of xanthine oxidase.

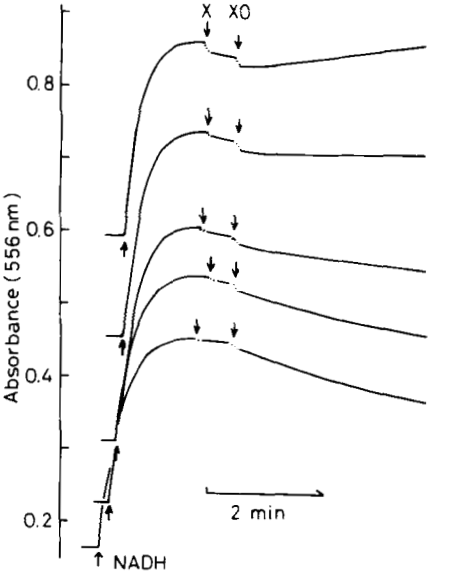


FIGURE 2 Effect of the concentration of ferricytochrome b_5 on the direction of its oxidation-reduction reactions occurred during the xanthine oxidase reaction. The reaction mixture contained 1.0 mM xanthine (X), 0.125 μM xanthine oxidase (X.O), 0.2 μM catalase, 7.6 μM NADH, 0.1 μM NADH-cytochrome b_5 reductase, and varying amounts of ferri-cytochrome b_5 (from the top; 61, 46, 30, 22, and 15 μM). Xanthine and xanthine oxidase were added after the reduction of cytochrome b_5 (15 μM) had been completed. The reaction solution was air-saturated and the value of pH was 8.5.

It has been reported by Iyanagi and Yamazaki[1,2] that microsomal NADPH-cytochrome P-450 reductase catalyzes the typical one-electron reduction of menadione to form its semiquinone which can reduce O_2 and cytochrome b_5 at considerable rates. In the presence of menadione, the reductase could catalyze complete reduction of cytochrome b_5 under anaerobic conditions[1] but under aerobic conditions the reduction reached a certain level which dependent on the concentration of O_2 (Figure 3). The increase of NADPH concentration did not affect the redox ratio of the cytochrome b_5 at steady state. There was, however, a range of menadione concentration which gave maximum reduction of the cytochrome b_5 (Figure 4). The concentration

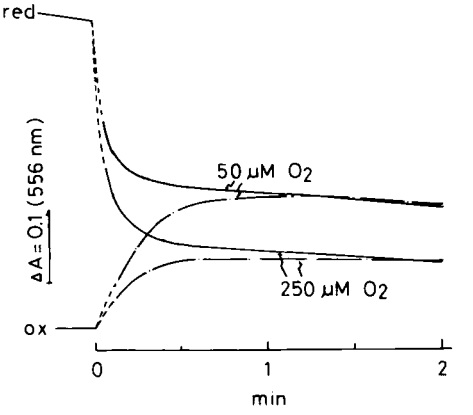


FIGURE 3 Effect of O_2 concentration on the steady state level of reduced cytochrome b_5 in the system of NADPH-cytochrome P-450 reductase. The reaction mixture (pH 7.0) contained 23.4 μM cytochrome b_5, 28 μM menadione, 50 μM NADPH, 0.1 μM NADPH-cytochrome P-450 reductase, and 0.1 μM catalase. The reaction was started by the addition of menadione. Two reactions (solid lines) were started from the reduced form of cytochrome b_5 which had been incubated in the presence of 12 μM NADH and 0.1 μM NADH-cytochrome b_5 reductase.

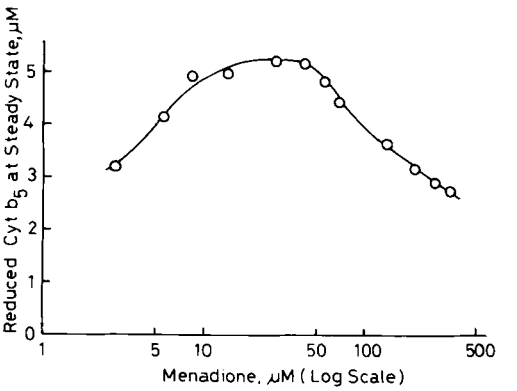


FIGURE 4 Effect of the menadione concentration on the steady state level of reduced cytochrome b_5 in the system of NADPH-cytochrome P-450 reductase. The reaction solution was air-saturated and the concentration of menadione was varied. The other condition was as described in Figure 3.

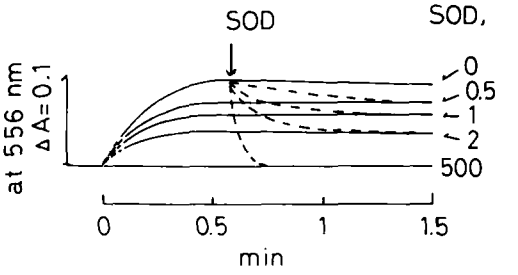


FIGURE 5 Effect of the concentration of superoxide dismutase (SOD) on the steady state level of reduced cytochrome b_5 in the system of NADPH-cytochrome P-450 reductase. The reaction solution was air-saturated and the other condition was as described in Figure 3. Broken lines show that upon addition of SOD at a time indicated by the arrow the redox ratio of cytochrome b_5 was decreased to the same level as that obtained when the SOD was previously added.

was about 10 times as high as Km for menadione. The value of Km was measured to be 3.5 μM from the dependence of the rate of menadione reduction upon concentration of menadione under anaerobic conditions. When menadione was added to the reductase system under aerobic conditions the redox ratio of cytochrome b_5 reached nearly the same level regardless of its starting state (Figure 3). The addition of superoxide dismutase after the redox ratio had reached a constant level caused rapid oxidation of the cytochrome b_5 (Figure 5). The oxidation was faster than the autoxidation occurred in the presence of superoxide dismutase only. This dramatic effect of superoxide dismutase may suggest that the reaction is at steady state in which the superoxide radical is involved as an active intermediate. As amounts of menadione and cytochrome b_5 are limited the overall reaction at steady state appears to be composed of four main reactions:

$$2MK + NADPH \xrightarrow{\text{reductase}} 2MK^{\cdot -} + NADP^+ + H^+ \quad (2)$$

$$MK^{\cdot -} + b_5^{3+} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} MK + b_5^{2+} \quad (3)$$

$$MK^{\cdot -} + O_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} MK + O_2^{\cdot -} \quad (4)$$

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{kd} O_2 + H_2O_2 \quad (5)$$

where MK and $MK^{\cdot -}$ stand for menadione and its semiquinone anion. It can be roughly said that at steady state the rates of reaction 2 and 5 are about same and reactions 3 and 4 are at a dynamic equilibrium. In other words the equilibrium of reactions 1 may be achieved in the presence of menadione as a mediator. Assuming that the rate of reaction 2 was equal to that of reaction 5 at steady the concentration of superoxide radical could be estimated using the following relation:

$$\frac{d\,NADP^+}{dt} \times 2 = 2kd(O_2^{\cdot -})^2 \quad (6)$$

Values of the "assumed" equilibrium constant of reaction 1 thus obtained at varying concentration of O_2 are listed in Table I.

It has been reported that NADH-cytochrome b_5 reductase stimulates O_2 consump-

TABLE I
Effect of the O_2 concentration on the "assumed" univalent equilibrium between O_2 and cytochrome b_5^a

O_2, μM	$O_2^{\cdot-}$, μM[b]	b_5^{2+}/b_5^{3+}	$\frac{(b_5^{2+})(O_2)}{(b_5^{3+})(O_2^{\cdot-})}$
50	0.66	0.54	41
80	0.64	0.50	62
250	0.69	0.28	100
420	0.67	0.21	130

[a]Experimental conditions are described in Figure 3.
[b]Calculated from eq. 6 and the value of $4.5 \times 10^5\ M^{-1}\ sec^{-1}$ for $2kd$.[18]

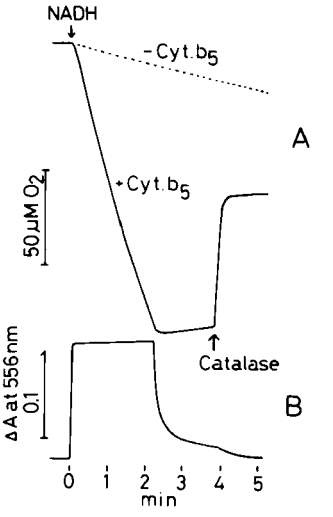
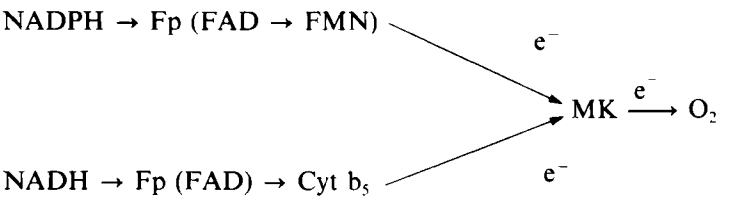


FIGURE 6 MK-mediated O_2 consumption in the NADH-cytochrome b_5 reductase-cytochrome b_5 system (A) and the redox state of cytochrome b_5 (B). Concentrations: 0.24 μM NADH-cytochrome b_5 reductase, 180 μM NADH, 20 μM menadione (MK), 7 μM cytochrome b_5 and 0.1 M potassium phosphate (pH 7.0). The reaction was started from an air-equilibrated solution. Slow O_2 consumption was observed in the absence of cytochrome b_5 (· · · · ·). Catalase (0.2 μM) was added at arrow.

tion in the presence of both menadione and cytochrome b_5^l. This result was confirmed in the present studies, as shown in Figure 6. In this system, most of the cytochrome b_5 is in the reduced state during the reaction (Figure 6,B).

DISCUSSION

Microsomal electron transport system contains two flavin enzyme, NADPH-cytochrome P-450 reductase (FAD-FMN)[10,11] and NADH-cytochrome b_5 reductase (FAD).[12,13] Both enzymes can catalyze one-electron reduction of quinones,[1-7] which are bivalent molecules. The microsomal MK-mediated O_2-consumption is summarized as the following,



NADPH-cytochrome P-450 reductase directly can catalyze one-electron reduction of menadione (MK). In the NADH-cytochrome b_5 reductase pathway, menadione (MK) accepts an electron from cytochrome b_5 and donates it to molecular oxygen.[1] These reactions are composed of elementary reactions and the univalent redox potentials of menadione and molecular oxygen are an important factor in an one-electron transfer reactions.

It seems very interesting that the NADPH-cytochrome P-450 reductase gave a dynamic equilibrium of oxidation-reduction of cytochrome b_5, the level of which dependent on the concentration of O_2 and superoxide dismutase (Figure 3). Reaction 1 is assumed to be at an equilibrium through redox reaction of menadione. This assumption is supported by the evidence that the equilibrium constant calculated in Table I is in accord with the value of k_1/k_{-1} obtained from entirely different experiments.

In the presence of superoxide dismutase the rapid oxidation of cytochrome b_5 occurs probably via two successive reactions, oxidation of cytochrome b_5 by menadione and oxidation of the semiquinone by O_2, their rate constants being measured at $10^4\,M^{-1}\,sec^{-1}$ and $5 \times 10^6\,M^{-1}\,sec^{-1}$,[17] respectively. The dynamic equilibrium of the above reaction may be schematized as shown in Figure 7. In this system the oxidation of cytochrome b_5 by menadione appears to be rate-limiting and from Figure 5 the rate constant k_{-3} can be measured approximately at $10^4\,M^{-1}\,sec^{-1}$, which is in agreement with the above value measured directly from the reaction between menadione and reduced cytochrome b_5.

From kinetic analysis of an equilibrium between *p*-benzoquinone(Q)/*p*-benzosemiquinone ($Q^{\cdot -}$) and cytochrome c/reduced cytochrome c systems, the following relation has been experimentally confirmed by Yamazaki and Ohnishi[8] and Sawada *et al*,[9] $E_0(Q/Q^{\cdot -}) - E_0(\text{Cyt } c^{3+}/\text{Cyt } c^{2+}) = -RT/F \ln k_r/k_0$. This relation is also applicable to an "assumed" equilibrium of reaction 3. The value of $3 \times 10^8\,M^{-1}\,sec^{-1}$ has been given for k_3 by Ohnishi *et al*.[17] $E_0(MK/MK^{\cdot -}) - E_0(\text{Cyt } b_5^{3+}/\text{Cyt } b_5^{2+}) = -0.20\,V - 0.01\,V = -0.21\,V$ is roughly found to be close upon

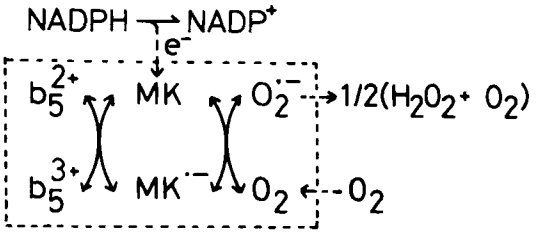


FIGURE 7 Dynamic equilibrium of oxidation and reduction of cytochrome b_5 in the presence of a NADPH-cytochrome P-450 reductase-menadione (MK) system.

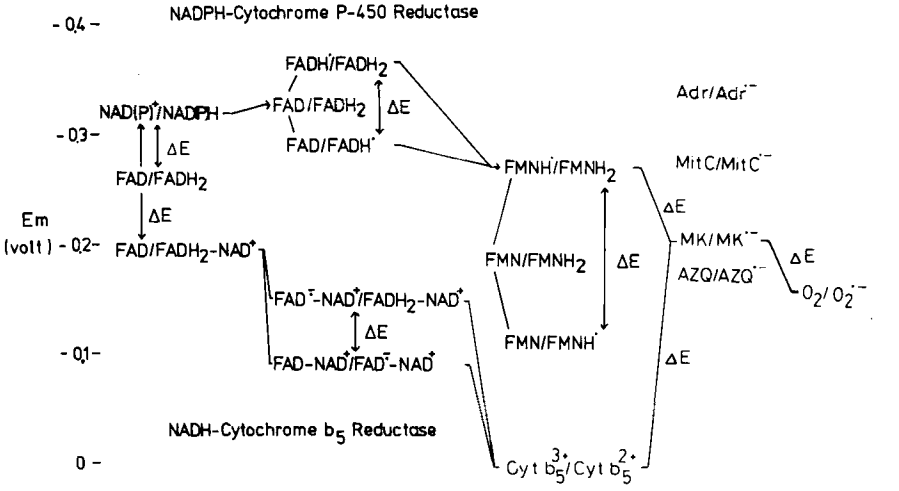


FIGURE 8 One-electron oxidation-reduction potentials of microsomal flavin enzymes, NADPH-cytochrome P-450 reductase (FAD-FMN),[11,21] NADH-cytochrome b_5 reductase (FAD),[12,13] cytochrome b_5, molecular oxygen and various quinones. Adr, Adriamycin (−341 mV);[26] Mit C, Mitomycin C (−271 mV);[7] MK, menadione (−200 mV);[19] AZQ, 2,5-diazridinyl-3,6-*bis* (carboethoxy) amino-1,4-benzoquinone (−168 mV).[7]

$-0.06\,V \times \log k_3/k_{-3} = -0.27$. Here, the values of $-0.20\,V$ (MK/MK$^{\cdot-}$) and 0.01 V (Cyt b_5^{3+}/Cyt b_5^{2+}) are cited from Ilan *et al.*[19] and Iyanagi,[12] respectively. The ratio k_3/k_{-3} at pH 8.5 was assumed to be the same at pH 7.0. In a similar method, the value of $1 \times 10^6\,M^{-1}\,sec^{-1}$ was also calculated for k_{-4}, using a values of MK/MK$^{\cdot-}$ (−0.20 V), $O_2/O_2^{\cdot-}$ (−0.16 V)[9,19] and $k_4 = 5 \times 10^6\,M^{-1}\,sec^{-1}$.

The free energy change (ΔE) between Cyt b_5^{3+}/Cyt b_5^{2+} (0.01 V) and MK/MK$^{\cdot-}$ (−0.20 V) is −0.21 V, and k_3 is greater than k_{-3}. The ΔE between MK/MK$^{\cdot-}$ and $O_2/O_2^{\cdot-}$ is −0.04 V, and k_4 is greater than k_{-4}. The electron transfer from cytochrome b_5 to O_2 is energetically unfavourable even if the menadione is present in the reaction system, as shown in Fig. 8. However, the rapid electron transfer was observed in the NADH-cytochrome b_5 reductase system (Fig. 6). In this system, the cytochrome b_5 is in the reduced state (approximately 90%) at steady state (Fig. 6,B). This makes a driving force for an electron transfer from cytochrome b_5 to MK. k_{-3} and k_4 have relatively large value, respectively. These factors can facilitate the rate of electron flow from cytochrome b_5 to molecular oxygen in the presence of menadione. In the NADPH-cytochrome P-450 reductase,[11,21,22] the redox couple, FMNH·/$FMNH_2$ = −270 mV can reduce directly various quinones with low one-electron redox potentials[5,6,11] (Fig. 8), and the reactivity is closely related to one-electron reduction potentials (Q/Q$^{\cdot-}$).[5,7]

In the physiological conditions, the oxidation-reduction state of cytochrome b_5 is in the reduced state,[23,24], and its concentration is higher than that of NADPH-cytochrome P-450 reductase.[24,25] The NADH-cytochrome b_5 reductase-Cyt b_5-MK system, therefore, can act as a major route in the redox cycling. The participate of both systems in the cells is further study.

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