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Mechanism of O_2^- generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems

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O_2^- generation in mitochondrial electron transport systems, especially the NADPH-coenzyme Q_{10} oxidoreductase system, was examined using a model system, NADPH-coenzyme Q_1-NADPH-dependent cytochrome *P*-450 reductase. One electron reduction of coenzyme Q_1 produces coenzyme Q_1^- and O_2^- during enzyme-catalyzed reduction and O_2 + coenzyme Q_1^- are in equilibrium with O_2^- + coenzyme Q_1 in the presence of enough O_2. The coenzyme Q_1^- produced can be completely eliminated by superoxide dismutase, identical to bound coenzyme Q_{10} radical produced in a succinate/fumarate couple-KCN-sub-mitochondrial system in the presence of O_2. Superoxide dismutase promotes electron transfer from reduced enzyme to coenzyme Q_1 by the rapid dismutation of O_2^- generated, thereby preventing the reduction of coenzyme Q_1 by O_2^-. The enzymatic reduction of coenzyme Q_1 to coenzyme Q_1H_2 via coenzyme Q_1^- is smoothly achieved under anaerobic conditions. The rate of coenzyme Q_1H_2 autoxidation is extremely slow, i.e., second-order constant for $[O_2][\text{coenzyme } Q_1H_2] = 1.5\ M^{-1} \cdot s^{-1}$ at 258 μM O_2, pH 7.5 and 25 °C.

Introduction

It has been reported that mitochondria isolated from various aerobic organisms produce H_2O_2 in the presence of NADH or succinate [1–4]. Mitochondrial generation of H_2O_2 is derived from succinate or is NADH-linked, and is elevated by antimycin or rotenone [1,5]. Therefore, H_2O_2 generation in mitochondrial fraction seems to be due to an autoxidizable component of the respiratory chain acting between the rotenone and antimycin-sensitive sites. Superoxide anion (O_2^-) has been established to be an essential precursor of mitochondrial H_2O_2 by using respiratory chain blockers. The experimental results with complex I (NADH-ubiquinone reductase) and complex III (ubiquinol-cytochrome *c* reductase) indicate that ubiquinone and ubiquinol are the main sources of mitochondrial H_2O_2 [6,7].

Most of the semiquinone and quinol compounds can easily be autoxidized to their corresponding quinone compounds yielding O_2^- and H_2O_2 [6]. However, the existence of the proposed ubisemiquinone in submitochondrial particles exposed to the succinate/fumarate couple in air has been reported by several workers [8–11]. Furthermore, the molar ratio of ubiquinol/total

Abbreviation: SOD, superoxide dismutase.

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ubiquinone is unexpectedly high, even though their quinone compounds were assayed under aerobic conditions [12]. Therefore, it is very important to know whether the ubiquinone radical and ubiquinol are stable under aerobic conditions.

The present work was undertaken to examine the mechanism of the two sequential univalent electron reductions of ubiquinone to ubiquinol via ubisemiquinone and generation of superoxide anion by a reduction and reoxidation cycle of ubiquinone, using the NADPH-cytochrome *P*-450 reductase system as an electron-donating system and coenzyme Q_1 as a model compound of ubiquinone. In addition, some properties of coenzyme Q_{10} radicals generated in the mitochondrial respiratory chain were studied.

Materials and Methods

Chemicals and coenzymes

Materials used in the present study were obtained from the following sources: sodium succinate, hypoxanthine and sodium fumarate, Wako Pure Chemical Industries Ltd.; NADPH, Oriental Yeast Col; mitomycin C, Kyowa Hakko Co.; vitamin K_3, Tokyo Kasei Co.; epinephrine, Sigma Co. Coenzyme Q_1 was kindly donated by Eisai Co.

Enzymes and mitochondria

NADPH-dependent cytochrome *P*-450 reductase (spec. act., 25–30 μmol ferricytochrome *c* reduced per min per mg of protein) was obtained from rat liver microsomes by the method of Omura and Takasue [13]. Mitochondrial fractions were obtained from bovine heart and submitochondrial fractions were prepared by an established method [14]. Superoxide dismutase (SOD) (bovine erythrocytes) and both catalase (bovine liver) and xanthine oxidase (milk) were obtained from Toyobo Co. and Sigma Chemical Co., respectively. Commercial catalase or xanthine oxidase was dialyzed against 3.0 l of 10 mM Tris-HCl buffer (pH 7.5) overnight, before use. Xanthine oxidase activity was determined by the method described by Roussos [15].

Incubation conditions

Unless otherwise noted, the standard incubation mixture contained and NADPH-generating system (5 mM $MgSO_4$/5 mM glucose 6-phosphate/20 μg glucose-6-phosphate dehydrogenase/ml), 0.2 mM NADPH, 0.2 units NADPH-cytochrome *P*-450 reductase/ml, 0.35 mM coenzyme Q_1 in 1.0% acetone (final concentration), and 0.1 M Tris-HCl buffer (pH 7.5) in a total volume of 0.6 ml (for ESR spectrometry), 3.0 ml (for optical absorbance measurements) and 3.8 ml (for O_2 consumption) at 25 °C. The reaction was initiated by the addition of NADPH.

Assays

Oxygen consumption was measured by a Clark-type electrode in an Instech oxygenometer (Model 102) and calculated using 258 nmol/ml for O_2 concentration in an initial incubation mixture at 25 °C. Conversion of coenzyme Q_1 to coenzyme Q_1H_2 was determined by the decrease of absorbance at 410 nm. Generation of O_2^- in the system was monitored by the oxidation of epinephrine to adrenochrome, i.e., change in absorbance at 480 nm using a reference without epinephrine [16,17]. NADPH oxidation was monitored from the decrease in absorbance at 340 nm, using the molar absorption coefficient of $6.2 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. ESR spectra were taken in a Varian E-109 X-band spectrometer at 100 kHz field modulation at 25 °C or 77 K. Simulation of ESR spectrum was performed using a Sharp MZ 80C microcomputer.

Results

NADPH oxidation and O_2 consumption during enzymatic reduction of quinone compounds

When coenzyme Q_1 or each of other quinone compounds was incubated with a fixed concentration of NADPH in the presence of NADPH-dependent cytochrome *P*-450 reductase, the system consumed O_2 at an expense of NADH. As shown in Table I, the ratio of O_2 consumed to NADPH consumed was approx. 1 in the system containing mitomycin C or vitamin K_3, while it was about 0.7 in the system containing coenzyme Q_1.

Generation of semiquinone radicals and O_2^- during enzymatic reduction of coenzyme Q_1 or vitamin K_3

To investigate the possible generation of semiquinone radicals during enzymatic reduction of

TABLE I

THE RELATION BETWEEN NADPH CONSUMPTION AND O_2 CONSUMPTION DURING REDUCTION AND OXIATION CYCLE OF SEVERAL QUINONE COMPOUNDS

Substrate [a] (0.1 mM)	NADPH consumed (μM/min)	Q_2 consumed (μM/min)
Mitomycin C	8.36	8.20
Vitamin K_3	69.20	67.74
Coenzyme Q_1	69.67	49.00

[a] The incubation mixture contained 0.1 units of NADPH-cytochrome *P*-450 reductase/ml/0.1 mM NADPH/0.1 M Tris-HCl buffer (pH 7.5) in 3.0 ml (NADPH consumption) or in 3.8 ml (O_2 consumption). The reaction was initiated by the addition of NADPH at 37°C.

coenzyme Q_1 or vitamin K_3, each compound was exposed to the NADPH-generating system in which NADPH-dependent cytochrome *P*-450 reductase was present and two measurements (O_2 consumption and O_2^- generation) were carried out in addition to ESR spectrometry. The results obtained are shown in Fig. 1A and B. ESR signals derived by coenzyme Q_1 reduction were detected in an early stage of the reaction. Signal height was maintained almost constant until 70% of the O_2 was consumed and then decreased. Aeration of the reaction mixture prolonged the period during which radical concentrations could be maintained constant (data not shown). SOD enhanced the rate of O_2 consumption, with retardation of signal appearance and suppression of maximum signal height, and also inhibited O_2^- generation monitored by adrenochrome formation. In contrast to the coenzyme Q_1 system, ESR signals derived from vitamin K_3 reduction appeared when 70% of the O_2 was consumed, and were eliminated when air was bubbled into the reaction mixture (data not shown). Furthermore, SOD did not affect O_2 consumption, signal appearance and signal heights, but inhibited O_2^- generation.

Identification of semiquinone radicals from the NADPH-cytochrome P-450 reductase-coenzyme Q_1 system

The ESR spectrum observed during the enzymatic reduction of coenzyme Q_1 at 25°C in the presence of O_2 is shown in Fig. 2(A)a. Hyperfine structure of the spectrum arises from couplings with two groups of protons, the splitting constants, $a^H_{CH_2} = 1.02$ G (2H) and $a^H_{CH_3} = 2.04$ G (3H), being confirmed by computer simulation. These constants agree well with those of the ubisemiquinone (coenzyme Q_{10}) anion radical ($a^H_{CH_2} = 1.018$ G and $a^H_{CH_3} = 2.037$ G) in ethanol at 40°C [18]. The result suggests that the number of the isoprene units attached to the quinone ring induces little change in the spin distribution in the semiquinone ring and the conformation of the methylene group, which performs a hindered rotation at an angle of about 30° between protons of

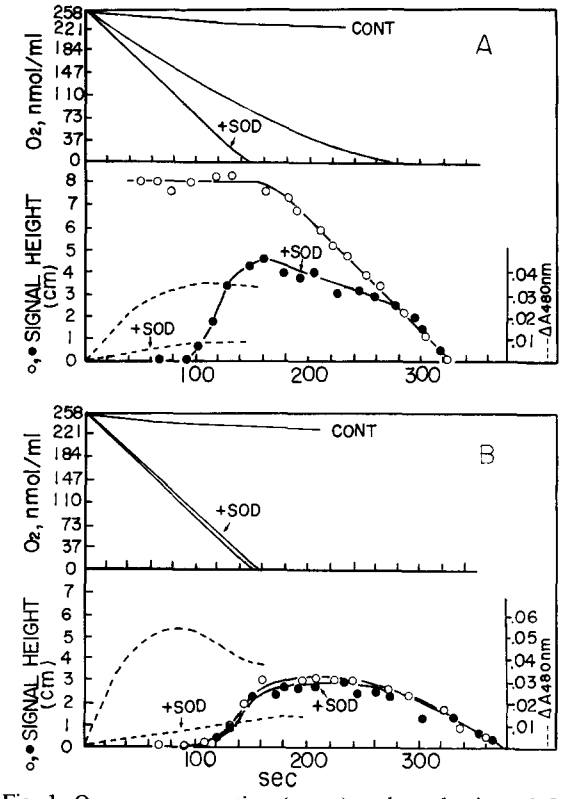

Fig. 1. Oxygen consumption (upper) and production of O_2^- and another radical (lower) during the reduction and oxidation cycle of coenzyme Q_1 (A) or vitamin K_3 (B). (A) The standard incubation mixture was used. In some cases, 0.5 μM SOD (+SOD) and coenzyme Q_1 (CONT) were added to or excluded from the standard incubation mixture, respectively, prior to the reaction. (B) Incubation conditions were as in (A) except that vitamin K_3 was used as a substrate instead of coenzyme Q_1. Conditions for ESR spectrometry were: magnetic field, 3357 G; microwave power, 12 mW; microwave frequency, about 9.41 GHz; modulation width, 0.32 G. The amplitude (cm) of the highest signal was measured at the time cited during the incubation.

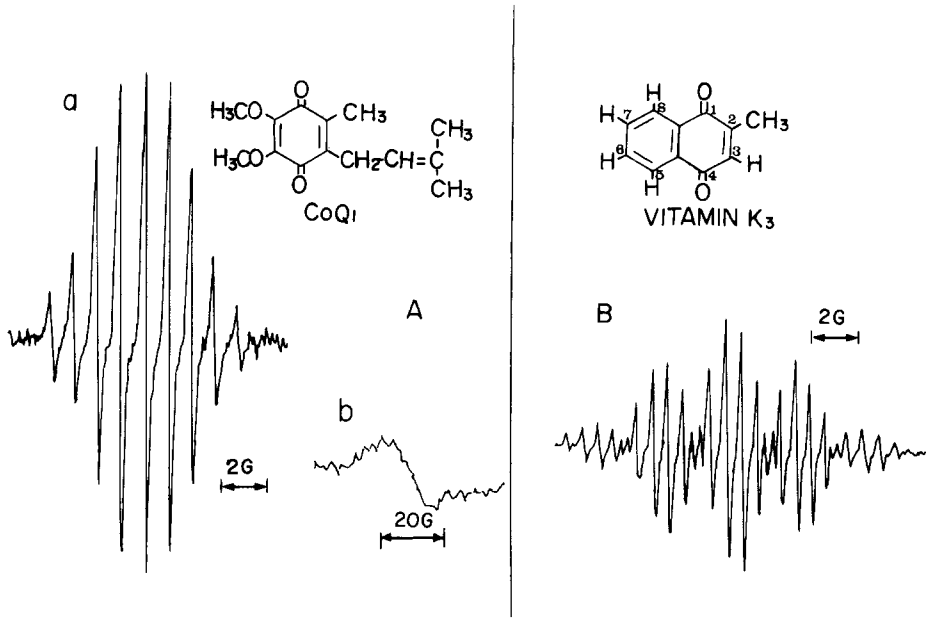

Fig. 2. ESR spectrum of coenzyme Q_1 semiquinone radical (A) or vitamin K_3 semiquinone radical (B). (A)a The standard reaction mixture was used. ESR signals were taken at 30–140 s after the addition of NADPH at 25 °C. (A)b The reaction mixture contained 1.6 units of NADPH-cytochrome *P*-450 reductase/1.6 mM coenzyme Q_1/0.8 mM NADPH, NADPH-generating system (as described in Materials and Methods) and 0.1 M Tris-HCl buffer (pH 7.5). The reaction was initiated by the addition of NADPH. At 20 s after the addition of NADPH, the reaction mixture at 25 °C was rapidly cooled down to 77 K in liquid nitrogen and used as a sample for ESR spectrometry. (B) Incubation conditions and ESR spectrometry were as in (A)a except that 0.35 mM vitamin K_3 was used as a substrate instead of coenzyme Q_1. ESR spectra were taken at the time at which most of O_2 in the reaction mixture was consumed. Conditions for ESR spectrometry (A)a were as in the legend of Fig. 1. Conditions for ESR spectrometry (A)b were: magnetic field, 3310 G; microwave power, 5 mW; microwave frequency about 9.28 GHz; modulation width, 10 G.

the methylene and quinoid plane [18]. When the temperature for ESR spectrometry was immediately decreased to 77 K, the ESR spectrum of coenzyme Q_1^- was strikingly modified, i.e., decreased signal heights and disappearance of hyperfine structure (Fig. 2(A)b).

A well-resolved ESR spectrum (Fig. 2(B)) was observed at 25 °C only after most of the O_2 in the enzymatic system containing vitamin K_3 was consumed. The hyperfine structure of the spectrum is interpreted with three groups of proton = 0.64 G (4H), as confirmed by computer simulation. These values agree well with those of the radical observed by electrochemical reduction of vitamin K_3 in 50% ethanol-buffer solution (v/v) (pH 8.0) by Fritsch et al. [19]. They pointed out that a^H_{5-8} (0.64 G) are markedly solvent-dependent and those at a^H_2 (3.01 G) and a^H_3 (2.38 G) are slightly solvent-dependent.

The properties of radicals generated in the system containing heart submitochondrial particles, succinate, fumarate and KCN

If coenzyme Q_{10} is aerobically reduced in the submitochondrial system in the presence of the succinate/fumarate couple and KCN, coenzyme Q_{10} radicals could be detected and their signals would disappear in the presence of SOD. This is similar to the behavior of the NADPH-cytochrome *P*-450 reductase-coenzyme Q_1 system in air (Fig. 2). Radicals with $g = 2.005$ have already been detected in the above submitochondrial system at 20 °C, and identified as coenzyme Q_{10}^- [11].

As shown in Fig. 3, the same radicals with $g = 2.005$ and without hyperfine splitting structure were also obtained in the submitochondrial electron transport system at 77 K and their signal heights were suppressed by about 60% in the presence of SOD, yielding radicals with $g = 2.005$.

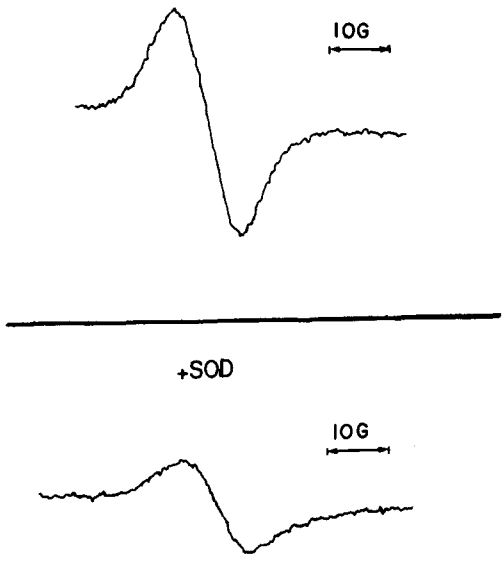

Fig. 3. ESR spectrum of submitochondria (9.1 mg of protein/ml) in the presence of 0.25 M sucrose/50 mM Tris-HCl buffer (pH 7.5)/1.0 mM KCN/9.0 mM succinate with (+SOD) or without 0.5 μM SOD (upper). The reaction was initiated by the addition of sodium succinate and the incubation mixture was then aerated for 5 min at room temperature. The reaction mixture was immediately transferred into a quartz cell, cooled down to 77 K in liquid nitrogen and used as a sample for ESR spectrometry. Conditions for ESR spectrometry were essentially the same as in the legend of Fig. 2(A)b, except that microwave power of 2 mW and modulation width of 6.3 G were used.

DeVries et al. have demonstrated that signal heights of proposed coenzyme Q_{10} radical in the succinate-fumarate-submitochondrial system including KCN are suppressed by 50% in the presence of antimycin A and suggested that the radicals detected without and with antimycin A are the coenzyme Q_{10} radical bound to coenzyme $Q_{10}H_2$-cytochrome oxido-reductase and flavin radicals in reduced succinate dehydrogenase, respectively [11]. Thus, the radicals detected in the presence of SOD seem to be flavin radicals in reduced succinate dehydrogenase.

Formation and degradation of coenzyme Q_1H_2

When coenzyme Q_1 in Tris-HCl buffer (pH 7.5) was treated with the reducing agent, $NaBH_4$, maximum absorbance at 410 nm for coenzyme Q_1 was completely abolished and little or no absorbance was detected in the visible region. Thus, the conversion of coenzyme Q_1 to coenzyme Q_1H_2 and reoxidation of coenzyme Q_1H_2 could be monitored by change in absorbance at 410 nm in the presence and absence of reducing agent, respectively. As shown in Fig. 4(A), the conversion of coenzyme Q_1 to coenzyme Q_1H_2 during enzymatic reduction is much more rapid in the N_2-bubbled system than in the aerobic system. Furthermore, SOD completely inhibited the reduction of coenzyme Q_1 to coenzyme Q_1H_2 at an early stage of

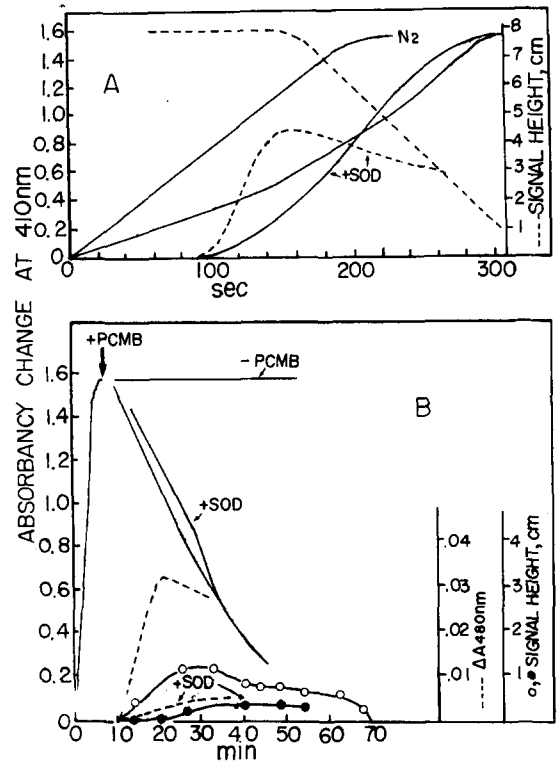

Fig. 4. Formation of coenzyme Q_1H_2 (A) and oxidations of coenzyme Q_1H_2 (B). (A) Formation of coenzyme Q_1H_2 under aerobic and anaerobic conditions. The standard reaction mixture was used and coenzyme Q_1H_2 formed was monitored by change in absorbance at 410 nm (———). Time courses for formation and disappearance of coenzyme $Q_1^{\cdot}$ in Fig. 1(A) were also depicted in this figure to compare with those for coenzyme Q_1H_2 formation. For anaerobic experiments, N_2 gas was bubbled into the reaction mixture prior to the reaction. (B) Oxidation of coenzyme Q_1H_2. The standard incubation mixture was used. 1 mM *p*-chloromercuribenzoate (PCMB) or none (−PCMB) was added to the system at the time (indicated with big arrow) and air was then bubbled for 1 min. In some cases, 0.5 μM SOD was added to the standard incubation mixture prior to the reaction (+SOD). Conditions for ESR spectrometry were the same as in the legend of Fig. 1.

the reaction, as well as the formation of coenzyme Q_1^-. The K_m value of the enzyme for coenzyme Q_1 under a N_2 atmosphere was found to be $8.85 \cdot 10^{-5}$ M by a Lineweaver-Burk plot (data not shown). To investigate the possible autoxidation of coenzyme Q_1H_2, *p*-chloromercuribenzoate, a non-competitive inhibitor of the enzyme, was added to the coenzyme Q_1-enzyme system at the time at which coenzyme Q_1 was completely reduced to coenzyme Q_1H_2, followed by bubbling air into the mixture. As shown in Fig. 4B, the conversion of

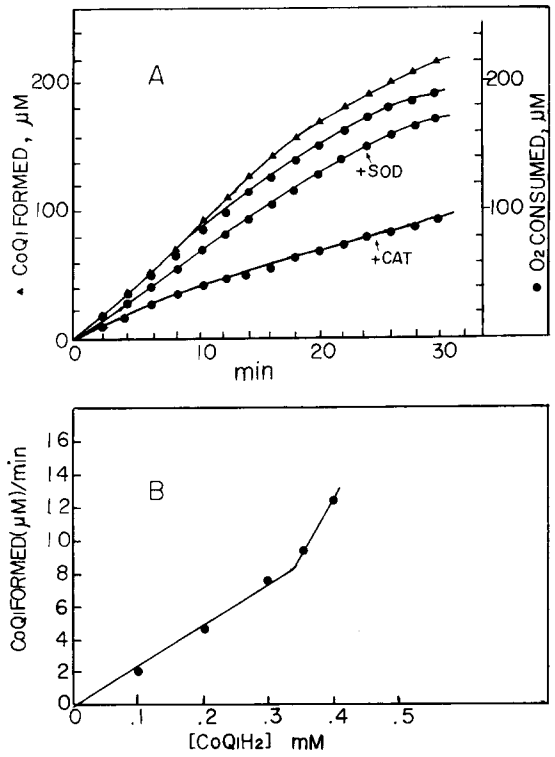

Fig. 5. Oxidation of coenzyme Q_1H_2 and O_2 uptake (A) and rate of coenzyme Q_1H_2 autoxidation (B). (A) Reaction mixtures and incubation conditions were essentially the same as in the legend of Fig. 4(B). The measurements were started just after aeration (0 time). In some cases, 20 μg of catalase/ml (+CAT) or 0.5 μM SOD (+SOD) was added to the reaction mixture prior to the reaction. The amount of coenzyme Q_1 formed was calculated using a molar absorption coefficient of 480 at 410 nm. (B) Incubation mixtures and conditions were as in the legend of Fig. 4B except that coenzyme Q_1 at a variety of concentrations was used. A concentration of coenzyme Q_1H_2 was expressed as that of coenzyme Q_1 used for the experiment, on the assumption that coenzyme Q_1 is completely reduced to coenzyme Q_1H_2 in the system.

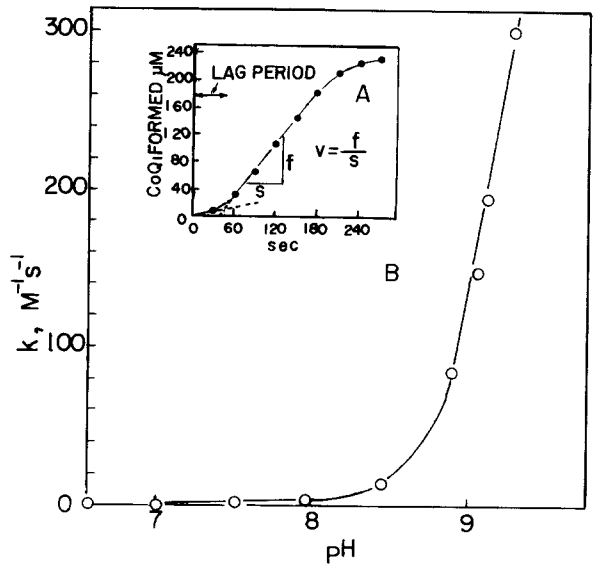

Fig. 6. The rate of coenzyme Q_1 formation (A) and the effect of pH on the second-order rate constants (B) during the autoxidation of coenzyme Q_1H_2. (A) The reaction mixture containing NADPH-generating system (as described in Materials and Methods), 1.0 mM NADPH/1.0 unit of NADPH-cytochrome *P*-450 reductase/ml per 1.75 mM coenzyme Q_1/20 mM Tris-HCl buffer (pH 7.5) in a total vol. of 3.0 ml was incubated at 25°C. When coenzyme Q_1 was completely reduced, an aliquot (0.6 ml) was taken from the reaction mixture and mixed with 2 min-aerated 0.12 M Tris-HCl buffer (pH 8.5) containing 1.0 mM *p*-chloromercuribenzoic acid (2.4 ml). Autoxidation of coenzyme Q_1H_2 was then monitored by change in absorbance at 410 nm. The pH was rechecked after experiments. *V* refers to maximum velocity of coenzyme Q_1 formed (f)/sec (s). (B) The reaction mixture for preparing coenzyme Q_1H_2 was as in (A) except that a variety of pH values was used. A second-order rate constant (*k*) was obtained from the slope (*V*), as in Fig. 6A, and calculated according to the equation described in the text. *k* value was plotted vs. pH.

coenzyme Q_1H_2 to coenzyme Q_1, monitored by change in absorbance at 410 nm, occurred slowly in the *p*-chloromercuribenzoate-treated system with the formation of O_2^- and coenzyme Q_1^-, while no change in absorbance at 410 nm occurred in the *p*-chloromercuribenzoate-untreated system. SOD inhibited O_2^- production and suppressed radical production, but did not significantly affect the conversion of coenzyme Q_1H_2 to coenzyme Q_1. With coenzyme Q_1H_2 prepared under these conditions, the conversion of coenzyme Q_1H_2 to coenzyme Q_1 was coincident with O_2 consumption for 6 min in the buffer solution containing 100–350

μM coenzyme Q_1H_2, and this exceeded O_2 consumption, even in the early stage of reaction, in the solution containing more than 350 μM coenzyme Q_1H_2. Catalase halved O_2 consumption, indicating that the system produces H_2O_2 (Fig. 5A). The second-order rate constant for the oxidation of coenzyme Q_1H_2 under aerobic conditions, $k = 1.5\ M^{-1} \cdot s^{-1}$, was calculated from the linear relationship between the initial concentrations of coenzyme Q_1H_2 and the rate of coenzyme Q_1H_2 degradation (Fig. 5(B)), according to the differential equation:

$$-\frac{d[\text{coenzyme } Q_1H_2]}{dt} = k[\text{coenzyme } Q_1H_2][O_2]$$

where $[O_2]$ is 258 μM.

Effect of pH on the autoxidation of coenzyme Q_1H_2

To investigate the rate of coenzyme Q_1H_2 autoxidation over a range of pH, an aliquot of coenzyme Q_1H_2 solution, prepared enzymatically, was added to aerated buffer solution, and the oxidation of coenzyme Q_1H_2 was followed by a change in absorbance at 410 nm. However, there is a limitation under our experimental conditins, due to the rapid autoxidation at pH higher than 9.2. A lag period with a short duration was usually observed when the reaction was carried out at pH higher than 8.2 (Fig. 6(A)). The maximum rate of autoxidation was measured as shown in Fig. 6A and a rate constant for [coenzyme Q_1H_2][O_2] was calculated according to the above-mentioned equation. As shown in Fig. 6(B), plotting the rate constant against pH gave a sigmoidal curve, indicating that deprotonation of coenzyme Q_1H_2 promotes the autoxidation. Little or no coenzyme Q_1H_2 autoxidation occurred at pH lower than 6.0.

Reduction of coenzyme Q_1 or vitamin K_3 by O_2^-

When coenzyme Q_1 was exposed to the hypoxanthine-xanthine oxidase system, radicals appeared in an early stage of the reaction and their concentrations remained almost constant until O_2^- generation ceased. SOD completely inhibited the generation of coenzyme Q_1^- in the system. These results are shown in Fig. 7, indicating that O_2^- evokes the reduction of coenzyme Q_1. Under the same conditions, except that coenzyme Q_1 was replaced with vitamin K_3, no ESR signal was detected (data not shown), presumably because of no electron transfer of O_2^- to vitamin K_3.

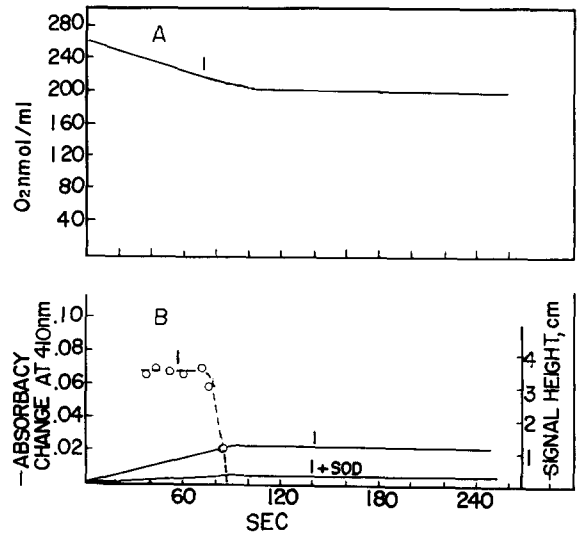

Fig. 7. Oxygen consumption (A) and formation of both coenzyme Q_1^- and coenzyme Q_1H_2 (B) from coenzyme Q_1 in a superoxide generating system. (A) The reaction mixture (1) contained 0.35 mM coenzyme Q_1/43 μM hypoxanthine/0.4 uits of xanthine oxidase/ml/0.1 M Tris-HCl buffer (pH 7.5) in a total vol. of 3.8 ml. The reaction was initiated by the addition of xanthine oxidase. (B) The reaction mixture (1) was the same as in (A) except that the total vol. was 0.6 ml (for generation of coenzyme Q_1^-) and 3.0 ml (for generation of coenzyme Q_1H_2) instead of 3.8 ml. In some cases, 0.5 μM SOD (+SOD) was added to the system prior to the reaction. Conditions for ESR spectrometry were the same as in the legend of Fig. 1.

Discussion

To investigate electron transport from NAD(P)H to coenzyme Q_{10} by NADH-ubiquinone reductase (complex I), a simple soluble system, NADPH-coenzyme Q_1-cytochrome *P*-450 reductase was used as a model.

The enzymatic reduction and the autoxidation of coenzyme Q_1 in the presence of O_2 is shown in Fig. 8. This scheme is consistent with the following findings; (1) molar ratio of less than 1.0 (0.7) for O_2 consumed to NADPH consumed, (2) production of coenzyme Q_1^- as an intermediate, (3) accumulation of coenzyme Q_1H_2 even in the presence of O_2, (4) enhancement of the rate of O_2

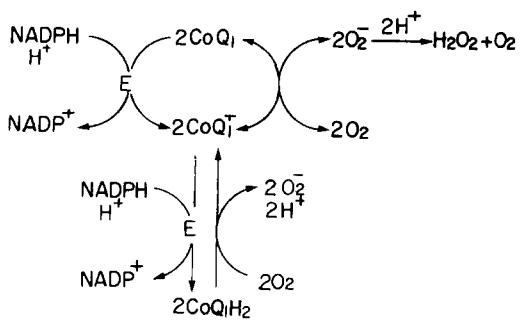

Fig. 8. Possible mechanism for reduction and oxidation cycle of coenzyme Q_1.

consumption and retardation of coenzyme Q_1^- appearance in the presence of SOD, and other results, which will be discussed below. Such a reduction and oxidation cycle of coenzyme Q_1 was strikingly different from that of vitamin K_3. This may be explained by comparing the redox potential (E_0) of the quinone/semiquinone couple with that of the O_2/O_2^- couple at the same pH.

Redox potentials, E_0 values, reported in the literature, are -0.11 to -0.15 V for coenzyme Q_{10}/coenzyme Q_{10}^- couple [20], -0.204 V [21] to -0.244 V [20] for vitamin K_3/vitamin K_3^- couple and -0.115 to -0.160 V for O_2/O_2^- couple [22]. Since pKa values of vitamin K_3^-H and coenzyme Q_{10}^-H are 4.7 ± 0.1 and 5.9 ± 0.1, respectively [23], semiquinone radicals of vitamin K_3 and coenzyme Q_{10} at pH 7.5 should be mainly in the deprotonated forms. On the assumption that the redox potential of coenzyme Q_{10}/coenzyme Q_{10}^- couple equals that of coenzyme Q_1/coenzyme Q_1^-, single electron transfer from coenzyme Q_1^- to O_2, O_2^- to coenzyme Q_1 or vitamin K_3^- to O_2 appears thermodynamically favorable, while that from O_2^- to vitamin K_3 seems to be unfavorable. Thus an equilibrium reaction (reaction 1) of general type would occur at physiological pH, where k_1 and k_2 are rate constants. Judging from the redox potentials, k_2 would be larger than k_1.

$$\text{coenzyme } Q_1^- + O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{coenzyme } Q_1 + O_2^- \qquad (1)$$

The existence of both forward and back reactions in the equilibrium (reaction 1) can be demonstrated by the results obtained with the NADPH-cytochrome-*P*-450 reductase system (one electron transfer system) and hypoxanthine-xanthine oxidase system (O_2^--generating system). The rapid dismutation of O_2^- by SOD shifts the equilibrium of reaction 1 to the right and promotes O_2 and coenzyme Q^- consumption, leaving coenzyme Q_1H_2 in the NADPH-cytochrome *P*-450 reductase system under aerobic conditions, while one electron transfer from reduced enzyme to coenzyme Q_1^- could occur smoothly under anaerobic conditions (Fig. 1(A)). Producing coenzyme Q_1H_2 from coenzyme Q_1 via coenzyme Q_1^- in the hypoxanthine-xanthine oxidase system (O_2^--generating system), which could be inhibited by SOD, supports the existence of the back reaction (reaction 1) with k_2 value of $5.0 \cdot 10^6$–$5.8 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ [23], which is larger than the rate constant of $1.8 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ for the spontaneous dismutation of O_2^- at pH 7.5 [24]. On the other hand, k_1 for the forward reaction (reaction 1) is considered to be $1.0 \cdot 10^7$–$2.0 \cdot 10^8$ $M^{-1} \cdot s^{-1}$ [23].

At physiological pH, autoxidation of coenzyme Q_1H_2 (reaction 2) ($k = 1.5$ $M^{-1} \cdot s^{-1}$) is extremely slow, compared with k_1 and k_2 of reaction 1. Furthermore, the rate of the autoxidation is not significantly influenced by SOD, even though O_2^- and CoQ_1^- can be detected. Since the mid-point potential of coenzyme Q_{10}^-/coenzyme $Q_{10}H_2$ couple (0.15 V) is significantly higher than that of coenzyme Q_{10}/coenzyme Q_{10}^- couple (-0.039 V) at pH 7.5 [11], the electron transfer of coenzyme Q_1^- to coenzyme Q_1^-, yielding coenzyme Q_1 and coenzyme Q_1H_2, appears thermodynamically favorable. Thus reaction 2 consists of two consecutive reactions, one of which is reaction 3, which appears to be the rate-determining step, and the other being reaction 4, and an additional reaction, reaction 5, for O_2^- dismutation. The sum of reactions 3–5 makes reaction 2 for overall reaction of coenzyme Q_1H_2 autoxidation. The lag period observed in the coenzyme Q_1H_2 autoxidation (Fig. 6(A)) appears to be the time required for accumulation of coenzyme Q_1^- in reaction 3.

$$\text{coenzyme } Q_1H_2 + O_2 \rightarrow H_2O_2 + \text{coenzyme } Q_1 \qquad (2)$$

$$2\text{coenzyme } Q_1H_2 + 2O_2 \leftrightharpoons 2\text{coenzyme } Q_1^- + 4H^+ + 2O_2^- \qquad (3)$$

$$2\text{coenzyme } Q_1^- + 2H^+ \rightarrow \text{coenzyme } Q_1H_2 + \text{coenzyme } Q_1 \qquad (4)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (5)$$

The redox potential of coenzyme Q_1^-/coenzyme Q_1H_2 should be lowered with increasing pH [11], thereby pushing the rate of the reaction 3 to the right side. Reaction 4 should be included in Fig. 8 for the production of coenzyme Q_1H_2 from coenzyme Q_1^- as a non-enzymatic reaction. The presence of coenzyme Q_{10}^-–coenzyme Q_{10}^-, a semiquinone pair in succinate-cytochrome *c* reductase complex from bovine heart mitochondria has been demonstrated [25].

It has been reported that, in mitochondria, coenzyme $Q_{10}H_2$ transfers its electron to the iron-sulfur protein and the resultant coenzyme Q_{10}^- transfers its electron to cytochrome *b* [7]. When submitochondrial particles were incubated with succinate, in the presence of enough air and an inhibitor of cytochrome *c* oxidase (KCN), radicals with $g = 2.005$ were detected by ESR spectrometry [11]. We have reconfirmed the production of radicals with $g = 2.005$ under similar conditions and have found a suppression of their signals in the presence of SOD, identical to the behavior of coenzyme Q_1^- generated in the NADPH-cytochrome *P*-450 reductase-coenzyme Q_1 system with or without SOD. Thus, the equilibrium reaction (reaction 6) may also occur in the mitochondrial system in the presence of enough oxygen.

$$\text{coenzyme } Q_{10}^- + O_2 \rightleftharpoons \text{coenzyme } Q_{10} + O_2^- \quad (6)$$

The radicals produced by the two systems are, however, different from each other with respect to hyperfine splitting structure, probably because of environmental differences, such as protein binding. Thus the radicals produced in the mitochondrial electron transport system are considered to be 'bound coenzyme Q_{10}^-' as proposed by DeVries et al. [11], which may transfer its electron to O_2 in the membrane, yielding O_2^-. The calculated mid-point potential of bound coenzyme Q_{10}/coenzyme Q_{10}^- couple is lower than that of the coenzyme Q_{10}/coenzyme Q_{10}^- couple [11] and coenzyme Q_{10}^- is a suitable electron donor for O_2 as well as for cytochrome *b*.

Judging from the experimental results obtained with our NADPH-cytochrome *P*-450 reductase-coenzyme Q_1 system, mitochondrial SOD may promote the rate of electron flux to coenzyme Q_{10} from reduced enzymes, such as succinate dehydrogenase and NAD(P)H-coenzyme Q_{10} reductase, by a rapid dismutation of O_2^-, thereby preventing the reduction of coenzyme Q_{10} by O_2^-.

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