A Possible Site of Superoxide Generation in the Complex I Segment of Rat Heart Mitochondria

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We searched for possible sites of superoxide generation in the complex I segment of the respiratory chain by studying both forward and reverse electron transfer reactions in isolated rat heart mitochondria. Superoxide production was monitored by measuring the release of hydrogen peroxide from mitochondria with a fluorescence spectrophotometer using the Amplex red/horseradish peroxidase system. In the forward electron transfer, a slow superoxide production in the presence of glutamate and malate was enhanced by both rotenone and piericidin A (specific inhibitors at the end of the complex I respiratory chain). Both diphenileneiodonium and ethoxyformic anhydride (inhibitors for respiratory components located upstream of the respiratory chain) inhibited the enhancement by rotenone and piericidin A.

In contrast, in reverse electron transfer driven by ATP, both diphenileneiodonium and ethoxyformic anhydride enhanced the superoxide production. Piericidin A also increased superoxide production. Rotenone increased it only in the presence of piericidin A. Our results suggest that the major site of superoxide generation is not flavin, but protein-associated ubisemiquinones which are spin-coupled with iron-sulfer cluster N2.

KEY WORDS: Heart mitochondria; complex I; superoxide; fluorescence assay of hydrogen peroxide; iron-sulfur cluster N2; ubiquinone; ubisemiquinone.

INTRODUCTION

Under physiologic conditions, mitochondria is known to convert a few percent of consumed oxygen into superoxide anions (Boveris and Chance, 1973; Lenaz *et al.*, 2002). The sites of superoxide generation are located in both complex I (Lenaz *et al.*, 2002; Barja and Herrero, 1998; Takeshige and Minakami, 1979; Herrero and Barja, 2000; Genova *et al.*, 2001) and complex III (Boveris and Chance, 1973; Zhang *et al.*, 1998; Chen *et al.*, 2003; Sun and Trumpower, 2003; Muller *et al.*, 2003). It has been recognized in recent years that the complex I segment of the electron transfer chain in both brain and heart mitochondria may be the major source of superoxide (Lenaz *et al.*, 2002; Liu *et al.*, 2002; Kudin *et al.*, 2004; Kushnaeva *et al.*, 2002; Johnson *et al.*, 2003).

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Key to abbreviations: BSA, bovine serum albumin; DBQ, decylubiquinone (2,3-dimethoxy-5-methyl-6-decylbenzoquinone); diS-C3-(5), 3,3′-dipropyl-2, 2′-thiodicarbocyanine iodide; DMSO, dimethyl sulfoxide; DPI, diphenileneiodonium; DTT, ditheiothreitol; EDTA, ethylenediaminetetraacetic acid; EFA, ethoxyformic anhydride (also known as diethyl pyrocarbonate); EGTA, ethylene glycol bis(β-aminoethyl ether); FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; Q, ubiquinone; QH_2, reduced ubiquinone; Q_i and Q_o sites, two ubiquinone binding sites in the complex III segment of the respiratory chain; ROS: reactive oxygen species; SOD, superoxide dismutase; SQ, ubisemiquinone.

A study using a heart failure model also demonstrated that the site of ROS production in this pathologic condition of the heart is complex I (Ide *et al.*, 1999, 2000).

It has been known that certain neurodegenerative disorders are related to complex I deficiency which would cause production of ROS (Ramsay and Singer, 1992; Halliwell, 1992; Beal and Bodies-Wollner, 1997; Robinson, 1998; Barrientos and Moraes, 1999; Luft, 1994). It was reported that Parkinson's disease may be related to the production of superoxide and a decreased activity of complex I (Ramsay and Singer, 1992; Mizuno *et al.*, 1987; Schapira, 1998; Umeda *et al.*, 2000; Dunnett and Bjorklund, 1999). Aging and apoptosis are also related to ROS production from mitochondria (Lenaz *et al.*, 2002; Marchetti *et al.*, 1996; Blandini *et al.*, 1998; Mancini *et al.*, 1998; Gross *et al.*, 1999; Tatton *et al.*, 1999; Chakraborti *et al.*, 1999; Nishikimi *et al.*, 2001; Fleury *et al.*, 2002). The role of mitochondrial uncoupling proteins to control superoxide production was proposed (Miwa and Brand, 2003; Brand *et al.*, 2004; Talbot *et al.*, 2004; Esteves *et al.*, 2004).

In the brain, complex I is considered to be the principal source of ROS (Liu *et al.*, 2002; Kudin *et al.*, 2004; Kushnaeva *et al.*, 2002). Since the brain and the heart are major energy-consuming organs, a significant amount of ROS would be formed from both complexes I and III in brain and heart mitocondria. As compared with our knowledge of the ROS production mechanism in complex III (Zhang *et al.*, 1998; Sun and Trumpower, 2003; Muller *et al.*, 2003), our understanding of that in complex I is still limited.

Most investigators believe that the site may be around the iron sulfur cluster N2 and ubiquinone (Lenaz *et al.*, 2002; Herrero and Barja, 2000; Genova *et al.*, 2001; Brand *et al.*, 2004; Boveris *et al.*, 1976; Lambert and Brant, 2004), but some emphasize that FMN may play an important role Liu *et al.* (2002), Kudin *et al.* (2004), Kushnaeva *et al.* (2002) Young *et al.* (2002). If the site and the mechanism of production is uncertain, then, an effort to develop a possible method of pharmacologic management to overcome adverse effects of ROS would be sidetracked. Therefore, it is important to elucidate the exact location and mechanism of superoxide generation.

In this work, we undertook a study to locate the superoxide generation site in the complex I segment of intact rat heart mitochondria. We measured hydrogen peroxide, which was produced by mitochondrial superoxide and superoxide dismutase (SOD), with a fluorescence method (Mohanty *et al.*, 1997; Furuno *et al.*, 2001). We studied the rate of hydrogen peroxide production in both forward and reverse electron transfer reactions. In the former, a mixture of glutamate and malate was used as a substrate. In the latter, ATP was used as an energy source and mucidin (also known as strobulin-A) was employed as an inhibitor for complex III respiration (Genova *et al.*, 2001) (see Fig. 1).
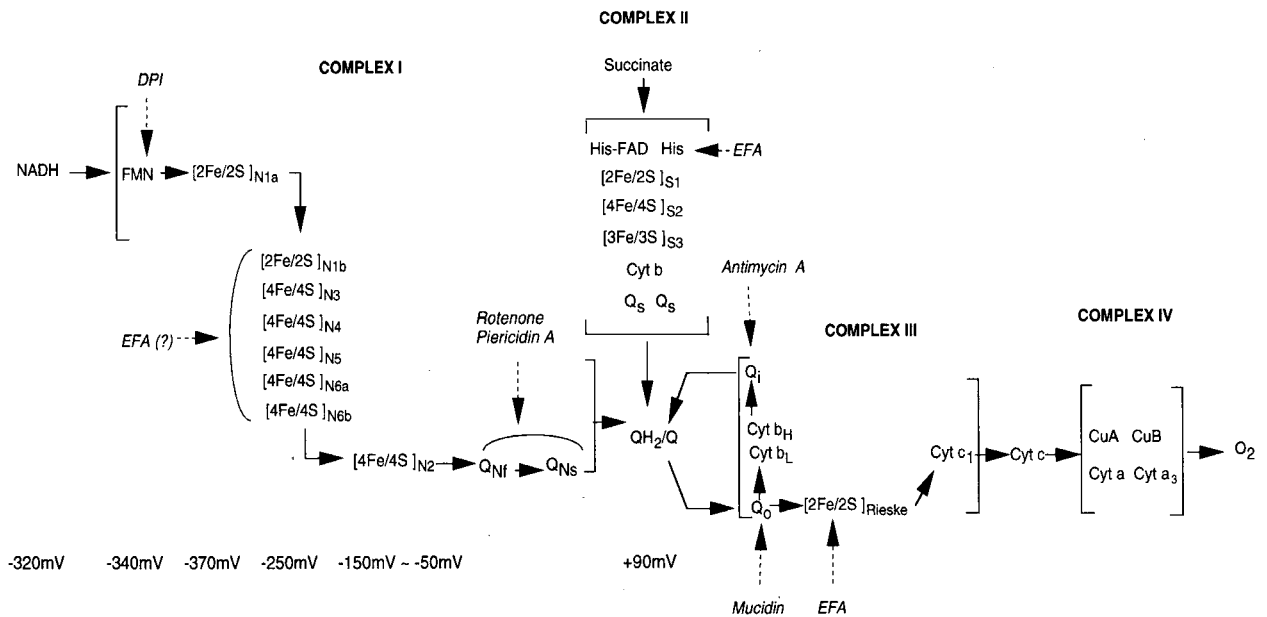

Fig. 1. Respiratory components in mammalian mitochondria, their redox potential, and the sites of action of inhibitors which were used in this study (Zhang *et al.*, 1998; Sun and Trumpower, 2003; Ohnishi, 1998).

Our strategy of identifying the site is based upon two assumptions: (i) if an inhibitor, which is specific to a respiratory component, enhanced the superoxide generation, then, the generation site should be on or before that inhibited component because accumulated electrons would leak out of the mitochondrial respiratory chain and reduce oxygen to generate superoxide; (ii) in this case, if we add the second inhibitor, which blocks any site located between the substrate and the generation site, then, the second inhibitor would decrease the superoxide generation because it restricts the electron flow to that generation site. These two assumptions lead to the following proposition: (iii) if an inhibitor increased the superoxide generation in reverse electron transfer but decreased in forward transfer, then, the inhibitor-interacting site may not be the generation site. When an inhibitor increased the generation in both forward and reverse transfer, then, this inhibitor-interacting site is the generation site. An interesting feature of our method is that a detailed kinetic result is not needed. Semiquantitative data regarding whether inhibitors increase or decrease superoxide generation would lead to the conclusion.

Since the precise sites of inhibitors as well as their mechanism of action are not fully understood, there are limitations in this method. However, by combining various inhibitors in both forward and reverse electron transfer reactions, certain useful information can be obtained as we describe in this paper.

MATERIALS AND METHODS

Chemicals

Mucidin (also called as strobulin-A) was a gift from Dr. G. Lenaz and Dr. U. Brandt, and piericidin A from Dr. T. Friedrich and Dr. W. Widger. Collagenase (Product number 034-10533) was purchased from Wako Chemicals (Japan). N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was purchased from Molecular Probes (USA). A cyanine dye 3,3′-dipropyl-2, 2′-thiodicarbocyanine iodide (diS-C3-(5)) was obtained from Kanko-Shikiso Research Institute (Japan). Horseradish peroxidase (HRP), EFA and rotenone were obtained from Sigma-Aldrich (USA). EFA was diluted with DMSO to desired concentrations and small aliquots were distributed to Eppendorf tubes and stored frozen at $-80°C$. All other chemicals were obtained from Nacalai Chemicals (Japan).

Preparation of Mitochondria

Rat heart mitochondria were prepared by combining the feature of several procedures. The basic protocol is from Blair (Blair, 1967) and Löw and Vallin (1963). The usages of a mannitol–sucrose mixture and an enzyme (a protease) come from the procedure of Chance and Hagihara (Chance and Hagihara, 1963). EDTA was replaced by EGTA to chelate high contents of Ca^{2+} in the heart preparations (Mela and Seitz, 1979). Tris was replaced by Hepes because of the low temperature coefficient of the latter buffer. Protease was replaced by collagenase (Toth *et al.*, 1986). DTT was added to protect sulfhydryl groups from oxidation during preparation. In brief: our buffer solution consists of 225 mM mannitol, 75 mM sucrose, 2.5 mM Hepes, 1 mM EGTA (pH adjusted to 7.4 by KOH). A male Whister rat (with the body weight of 200 g or less) was used for each preparation. The protocol was approved by the institutional committee for the use and care of animals. All procedures were done between 0 and 5°C. Under ether anesthesia, the heart was removed and immediately minced and washed in a rinse solution (the buffer solution plus 1 mM DTT). Then, the minced tissue is transferred into 15 mL of the rinse solution in a Ehrenmyer flask which contained 0.07% collagenase and had been bubbled with N_2 gas. The mixture was kept bubbling for 15 min. Then, we added 1 mg/mL BSA and under N_2 gas flow, it was gently homogenized with a small polytron-type homogenizer at the slowest speed until the minced muscle suspension became homogeneous. The suspension was then homogenized with a loose Teflon-glass homogenizer at 300 rpm with one stroke (one down and one up movement). It was centrifuged at $60 × g$ for 7 min. The first supernatant was saved. Twenty milliliters of the washing solution (the basic buffer solution plus 1 mg/mL BSA) was added to the precipitate, and the suspension was homogenized with a Teflon-glass homogenizer again with one stroke, and centrifuged at $300 × g$ for 7 min (this centrifugation helped to remove contamination of red blood cells). The second supernatant was collected. Then, both first and second supernatants were centrifuged at $700 × g$ for 10 min to remove cell debris. The supernatant was filtered through a porous filter paper, and the filtrate was span at $8,000 × g$ for 10 min. The surface of the precipitate was gently rinsed with the washing solution to remove a fluffy layer. Then, the precipitate was suspended with 3.6 mL of the washing solution and centrifuged again at $5,200 × g$ for 10 min. The precipitate was suspended in the same solution to make the final protein concentration of 15–20 mg/mL. Protein concentration was determined by the protein-dye binding method (Bradford, 1976) supplied by Bio-Rad (Hercules, CA). According to the work done by the Lesnefsky's group, the first supernatant and the second supernatant may be classified as the subsarcolemmal mitochondria fraction and the

intrafibrille mitochondria fraction, respectively (Chen *et al.*, 2003). In our experiments, both were mixed and regarded as rat heart mitochondria. This preparation maintained the respiratory control ratio above 6 for several hours when kept in ice.

Assay for Mitochondrial Functions

We performed all assays in this paper at 25°C using the reaction medium which was made by adding 4 mM phosphate buffer to the washing solution and the pH readjusted to 7.4 at 25°C). Oxygen consumption of mitochondria (1.5 mg protein/mL) was measured using a Clark-type oxygen electrode. The membrane potential of mitochondria (0.05 mg/mL) was measured from the fluorescence intensity of diS-C3-(5) using Hitachi Model 650-10LC, equipped with a thermoregulated cuvette holder and a stirring device at 670 nm with excitation at 622 nm (Furuno *et al.*, 2001). Both slit widths were 5 nm. When the membrane potential is generated by respiration, charged fluorescent probes are incorporated into mitochondria, thereby causing fluorescence quenching. The decrease in fluorescence is not necessarily proportional to the membrane potential, but it can be used as an estimate of the potential.

Assay for Mitochondrial H_2O_2 Generation

We assayed H_2O_2 in the Hitachi fluorescence spectrophotometer using Amplex Red and HRP (Mohanty *et al.*, 1997; Furuno *et al.*, 2001). Mitochondria (0.1 mg protein/mL) were suspended in the reaction medium containing 20 μM Amplex Red and 1 mU/mL of HRP. The change in the fluorescence intensity was measured at 590 nm with excitation at 530 nm. Both slit widths were 5 nm. The sensitivity of the instrument was calibrated by adding a known amount of H_2O_2 to the suspension.

RESULTS

Forward Electron Transfer

Figure 2 shows the production of H_2O_2 in the forward electron transfer reaction. Panel A: a slow rate of H_2O_2 production with glutamate and malate as a substrate (7.8 $\pm$ 1.2 pmoles mg^{-1} min^{-1}; standard deviation calculated from four preparations) was increased several folds by either mucidin or antimycin A, but the effect of antimycin A was stronger. When mucidin was added after antimycin A, mucidin inhibited the superoxide production caused by antimycin A (top trace).

When the order of addition was reversed, antimycin A increased the superoxide production initiated by mucidin (middle trace). Superoxide production in the presence of glutamate and malate was inhibited by EFA (bottom trace). Panel B: the rate of production was increased by rotenone, but it was inhibited by DPI (top trace) and EFA (bottom trace). Panel C: electron acceptors such as DBQ (decylubiquinone) and idebenone (the structure shown in Panel E) increased the rate of superoxide production. Panel D: both rotenone and piericidin A enhanced the superoxide generation regardless of the order of addition of these inhibitors. It was confirmed that the addition of DMSO, which was used to dissolve hydrophobic compounds, had no effect (data not shown).

Reverse Electron Transfer

Figure 3 shows that the addition of succinate built the membrane potential and quenched the fluorescent dye, diS-C3-(5). The left trace shows that mucidin, an inhibitor for the complex III respiration, dissipated the potential created by succinate respiration, but 0.5 mM ATP was able to restore it. The middle trace shows that, in the presence of mucidin, succinate could not generate a membrane potential, but ATP restored it.

The right trace shows that, in the absence of externally added succinate, ATP alone could build a membrane potential. In this case, endogenous complex II substrates and reduced ubiquinone (QH_2) in the Q-pool were used as the sources of electrons for reverse transfer. We chose this condition for our reverse electron transfer experiment for the sake of simplicity.

Figure 4 shows the experiments for H_2O_2 production in reverse electron transfer. Panel A: three traces show experiments performed in the same order as were done for membrane potential measurement (Fig. 3). Panel B shows that the addition of EFA or DPI increased the rate of superoxide production. The rate of superoxide production in the presence of mucidin (control experiment) was 22.6 $\pm$ 3.61 pmoles mg^{-1} min^{-1} (standard deviation calculated from four preparations). This includes the production of superoxide in complex III. Panel C: both idebenone and DBQ increased the rate of production. Panel D: the additions of rotenone (1 and 3 μM as shown in the right trace) did not increase the rate of superoxide production. However, piericidin A (1 μM) increased the superoxide production both in the presence of 1 μM rotenone (left trace) or in the absence of rotenone (middle trace). Only in the presence of piericidin A (1 μM), rotenone (1 μM) was able to increase the production (middle trace).
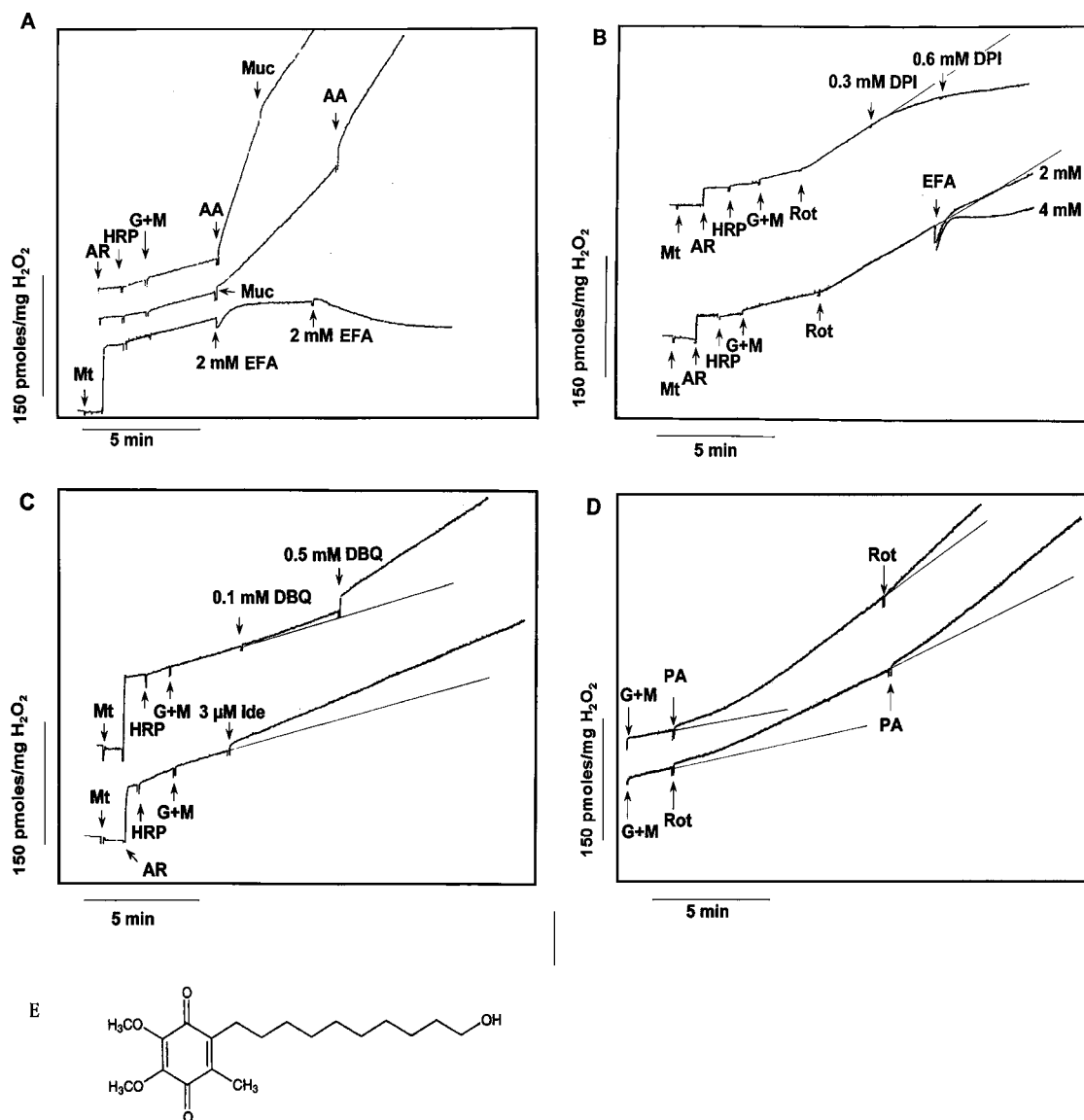
Fig. 2. Production of H_2O_2 in the forward electron transfer at 25°C as measured by fluorescence at 590 nm with the excitation at 530 nm. The reaction medium (1.5 mL, 25°C) contains 225 mM mannitol, 75 mM sucrose, 2.5 mM Hepes (pH 7.4), 4 mM phosphate, 1 mM EGTA and 1 mg/mL BSA. Experiments started with the additions of 0.1 mg/mL mitochondria, 20 μM Amplex Red (AR), 1 mU/mL horseradish peroxidase (HRP) and 3 mM glutamate +3 mM malate (G + M). Data shown here are representative traces from four separate experiments, which gave all similar results. Straight lines were added to the recorder traces in order to contrast rate changes. In some traces, the additions of mitochondria, AR and/or HRP were not shown. *Panel A:* A slow H_2O_2 production in the presence of glutamate and malate (control) was increased by either 2 μM mucidin (Muc) or 3 μM antimycin A (AA), but the effect of antimycin A was stronger. When mucidin was added after antimycin A, mucidin inhibited superoxide production initiated by antimycin A (top trace). When the order of addition was reversed, antimycin A furher increased the production of superoxide initiated by mucidin (middle trace). The superoxide production in the presence of glutamate and malate was inhibited by EFA (bottom trace). *Panel B:* The rate of production was increased by rotenone (Rot, 1 μM), but it was inhibited by DPI (0.3 and 0.9 mM (total); top trace) and EFA (2 and 4 mM; bottom trace). *Panel C:* Electron acceptors such as DBQ (decylubiquinone; 0.1 and 0.6 mM (total)) and idebenone (3 μM) increased the rate of superoxide production. *Panel D:* Each addition of 1μM rotenone (Rot) or 1 μM piericidin A (PA) enhanced the superoxide generation regardless of the order of additions. *Panel E:* The structure of idebenone.
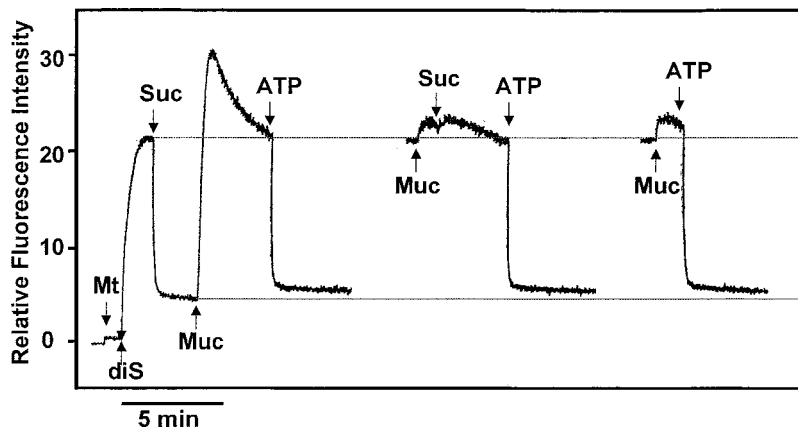
Fig. 3. Membrane potential changes as measured by fluorescence at 670 nm with the excitation at 622 nm. The downward movement corresponds to the formation of membrane potential. The reaction medium is the same as in Fig. 1. Temperature 25°C. Additions: 0.05 mg/mL mitochondria, 0.2 μg/mL diS-C3-(5), 9 mM succinate, 1.5 μM mucidin, and 0.5 mM ATP. From the left to the right: succinate was added first, and then mucidin and ATP; mucidin was added first, and then succinate and ATP; mucidin was added followed by ATP.

Reverse Electron Transfer Experiments Started in the Absence of Mucidin

In Figure 5A and B, we added succinate first, and then added other compounds (including mucidin) and recorded both superoxide production and membrane potential changes. This method was often used to study reverse electron transfer. However, we observed that succinate addition caused a high rate of H_2O_2 production (136.4 ± 16.4 pmoles mg^{-1} min^{-1}; standard deviation calculated from four preparations), but the rate was substantially reduced by (A) DPI or mucidin, and (B) rotenone or piericidin A. The reduced levels caused by these compounds were almost identical with each other even though the mechanisms of their action were different as shown by the changes in the membrane potential. For example, in Fig. 5A, DPI is a complex I inhibitor, while mucidin is a complex III inhibitor. In Fig. 5B, periecidin A is an inhibitor of reverse electron transfer, while rotenone is not in ATP-supported reverse electron transfer (see *Discussion*). These situations may make the analysis of these data complicated.

Effects of DPI and EFA on Mitochondrial Oxygen Consumption

In order to examine whether DPI or EFA interacts with intact mitochondria, its effect on NADH-supported and succinate-supported oxygen consumption of mitochondria was measured using a Clark-type oxygen electrode. We confirmed that both NADH-linked respiration

and succinate-linked respiration were inhibited by 0.5 mM EFA. However, since EFA also inhibits respiration at complex III, the oxygen electrode method cannot provide us with much information of the effect of EFA on the complex I segment. It was observed that the complex I respiration was also inhibited with 1 mM DPI, but succinate-linked respiration was not inhibited by DPI (data not shown).

DISCUSSION

Measurement of Superoxide Production in Intact Mitochondria

Different investigators use different tissues obtained from various species of animals for mitochondrial ROS study. They also choose different preparations, such as mitochondria, submitochondrial particles and isolated complex I. Even with the same preparation, methods of preparation, the degree of intactness and the purity may not be the same. Further, the assay techniques of ROS are different. Therefore, it is not straightforward to compare data obtained from different laboratories. For this study, we used intact rat heart mitochondria and assayed hydrogen peroxide production with a fluorescence technichque.

Since the mitochondrial inner-membrane is not permeable to superoxide, it is difficult to directly measure the generation of superoxide in intact mitochondria. In this study, superoxide production from intact rat heart mitochondria was continuously monitored from the
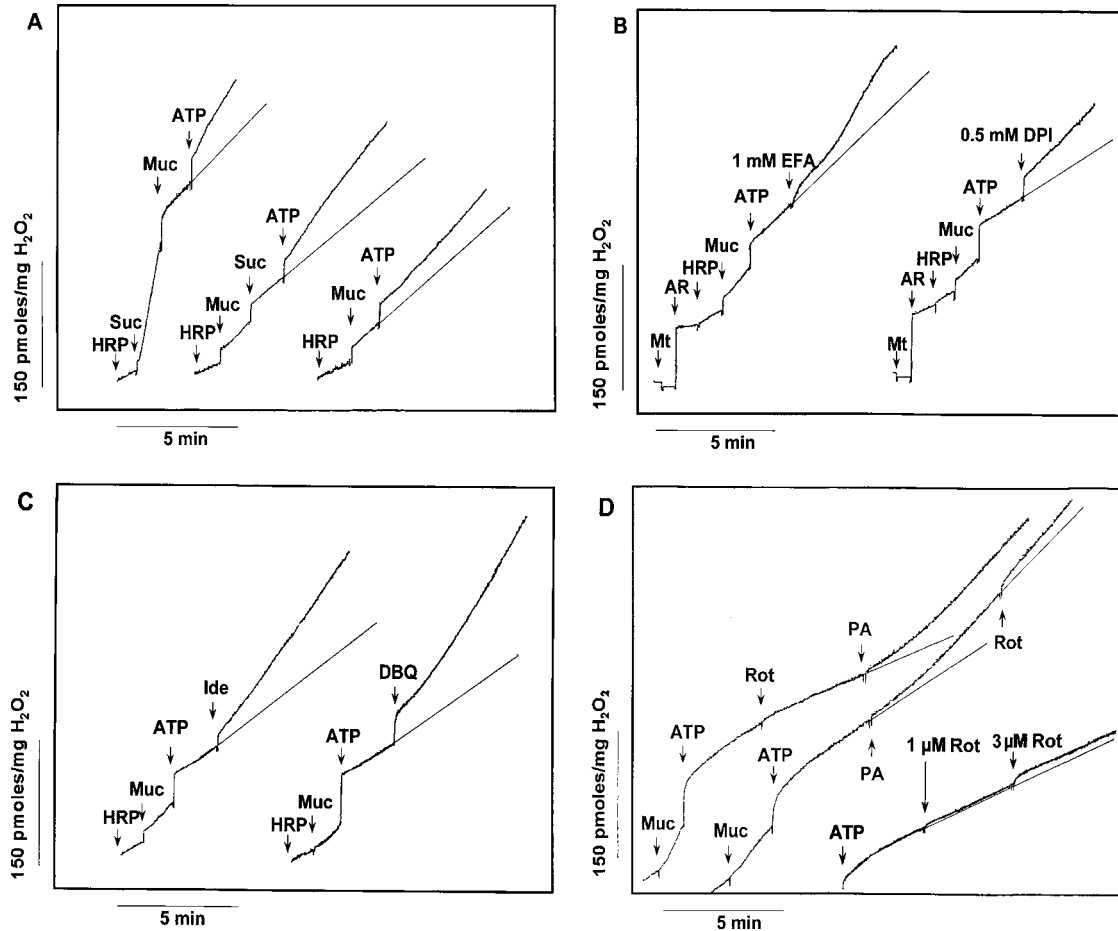
Fig. 4. Production of H_2O_2 in the reverse electron transfer at $25°C$. The experimental conditions are the same as those in Fig. 1. Additions: 0.1 mg/mL mitochondria, 20 μM Amplex Red (AR), 1 mU/mL horseradish peroxidase (HRP), 1.5 μM mucidin (Muc), and 0.5 mM ATP. Data shown here are representative traces from four separate experiments, which gave all similar results. Straight lines were added to the recorder traces in order to contrast rate changes. In some traces, the additions of mitochondria, AR and/or HRP were not shown. *Panel A:* Three traces show experiments performed in the same order as were done for membrane potential measurement (see Fig. 3). *Panel B:* Addition of 1 mM EFA or 0.5 mM DPI increased the rate of superoxide production in the reverse electron transfer initiated in the presence of ATP. *Panel C:* Both 3 μM idebenone and 0.5 mM DBQ increased the rate of production. *Panel D:* Rotenone (1 and 4 μM (total); as shown in the right trace) did not increase the rate of superoxide production. However, 1 μM piericidin A increased the superoxide production both in the presence of 1 μM rotenone (left trace) or in the absence of rotenone (middle trace). Only in the presence of piericidin A (1 μM), rotenone (1 μM) was able to increase the production (middle trace).

amount of hydrogen peroxide released from mitochondria using a fluorescence spectrophotometer. This became possible since superoxide is rapidly transformed by matrix SOD to produce permeable hydrogen peroxide. It is also known that the production of hydrogen peroxide from superoxide is stoichiometric (Boveris and Cadenas, 1975).

There are several points we have to know in using this method: (i) mucidin is an effective Q_o site inhibitor in complex III, but it also produces superoxide. The rate of superoxide production by mucidin is about 1/3 of that by antimycin A (Fig. 2A). (ii) From our data, the rate

of superoxide production in the presence of glutamate + malate (complex I substrates for forward electron transfer) was 7.8 ± 1.2 pmoles mg^{-1} min^{-1}. The rate of production for reverse electron transfer, which uses ATP as the energy source and endogeneous substrates such as reduced QH_2 (reduced form of ubiquinone pool) as the electron sources, was 22.6 ± 3.6 pmoles mg^{-1} min^{-1} (Fig. 4B–D). This is higher than the value for glutamate + malate which supported forward electron transfer, but the value includes the superoxide production caused by mucidin, which we used to inhibit forward electron transfer into
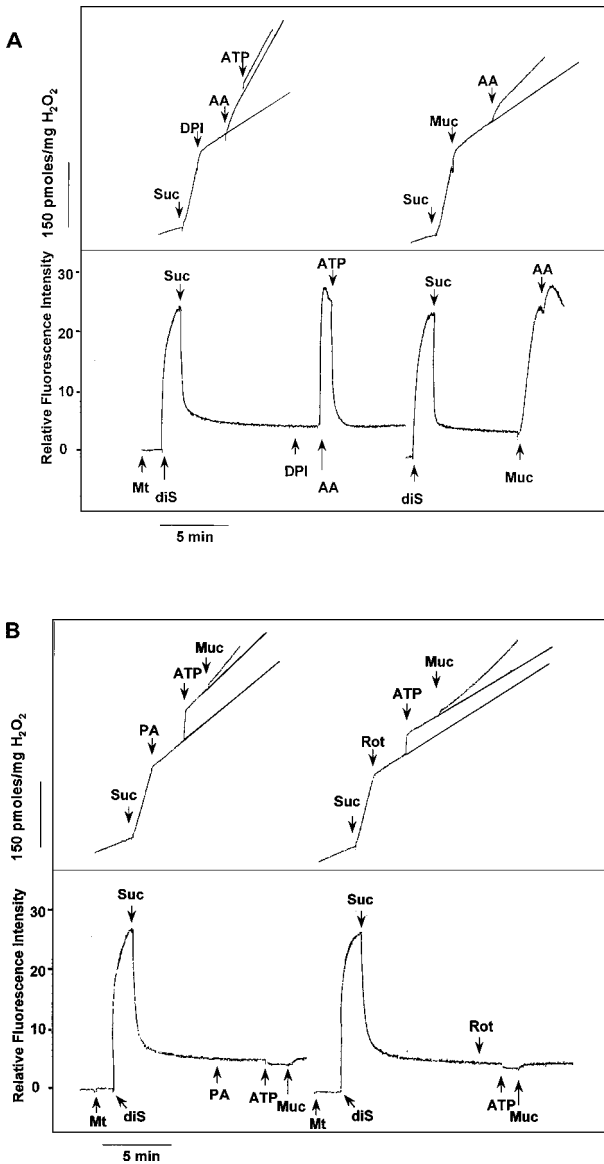
Fig. 5. (A) Superoxide production and membrane potential changes in the reverse electron transfer experiments which were started without mucidin. The conditions were the same as those in Fig. 4. *Top panel:* a high rate of superoxide production (the additions of mitochondria, AR and HRP were not shown) was inhibited by either 500 μM DPI or 2 μM mucidin (Muc). 3 μM Antimycin A (AA) increased the production. *Lower panel:* DPI did not interfere with the membrane potential (as measured by diS-C$_3$-(5)), but mucidin negated it. The order of additions and the concentration of chemicals were the same for both panels. (B) Similar experiments to those shown in Fig. 5A. *Top panel:* Both 1 μM p015 pierici-dine A (PA) and 1 μM rotenone (Rot) inhibited the succinate-induced superoxide production to a similar level, even though the mechanisms of action of these compounds are different in the reverse electron transfer. With PA, 0.5 mM ATP increased the production, while with Rot, ATP did not increase. *Lower panel:* Both PA and Rot did not change the membrane potential. Both 0.5 mM ATP and 2 μM Muc had little effects.

complex III. (iii) When succinate was used to start reverse electron transfer, we had the highest superoxide generation (136.4 $\pm$ 16.4 pmoles mg^{-1} min^{-1}), but this includes the generation from complex III through the forward transfer reaction (Fig. 5A and B). (iv) When Amplex Red and horseradish peroxidase was added to a mitochondrial suspension, superoxide generation was observed. This is caused by endogeneous substrates for both complex I and II electron transfer reactions. In some experiment, the rate of production was decreased when glutamate and malate were added (see Fig. 2C). This phenomenon seems to indicate that in this experiment, endogenous substrates for complex II had been causing an elevated reverse transfer, but the addition of glutamate + malate (complex I substrate) made the forward reaction (with a lower production rate) overide the reverse transfer reaction.

Effect of EFA

Although the inhibitor method we have employed in this paper lacks accuracy in determining the exact site, it is straight forward and provided us with some new insight. We first focused on the action of EFA. This inhibitor did not inhibit the electron transfer from NADH to ferricyanide (an artificial electron acceptor) in the flavoprotein subunit (Fp), but inhibited the electron transfer from NADH to ubiquinone (Yagi *et al.*, 1982; Vik and Hatefi, 1984). In the former case, electrons are believed to go through directly from NADH to flavin and are accepted by ferricyanide (Vinogradov, 1998). In the latter case, the site of action of EFA would be located after Fp. It is at least one of low E$_m$ iron-sulfur clusters (isopotential group including N1b, N3, N4, N5, N6a and N6b). In complex III, EFA reacted with the Rieske iron-sulfur protein through modification of ε-histidyl ligands (Ohnishi *et al.*, 1994). In the present work, we directly added EFA to a mitochondrial suspension during the experiment at 25°C. After a few minutes of a lag phase, the effect of EFA was readily observed.

The following results led us to a working hypothesis that the major superoxide generating site in intact rat heart mitochondria is around the cluster N2 and protein-bound ubisemiuinones:

1. In the forward electron transfer, EFA did not produce superoxide. Since mutagenesis analysis (Cys $\rightarrow$ Ala) in *E. coli* indicated that four ligands of cluster N2 are all cysteine (Flemming *et al.*, 2003), EFA would not interact with cluster N2. However, it has been reported that a hystidyl residue in the 49 kDa subunit, which is located near the cluster N2 but not directly liganded with the cluster, may

have a direct influence on the function of cluster N2 (Brandt *et al.*, 2003). If this is the case, EFA may react with the cluster N2. Further study is needed to clarify this point.

Another mutagenesis study indicated that the iron–sulfur cluster N5 is ligated by one histidyl and three cysteinyl residues, all of which are located in the 75 kD subunit (Yano *et al.*, 2003). Therefore, EFA may interact with at least one of low potential iron-sulfur clusters. If FMN would produce superoxide, it should do so when EFA inhibited the forward electron transfer pathway after FMN, but we did not see it.

2. In the forward electron transfer, rotenone and piericidin A, both of which are known to react with protein-associated ubisemiquinones, increased superoxide generation, but the increase was lowered by DPI or EFA. These results are consistent with our proposition for the strategy (see *Introduction*).

3. In the ATP-supported reverse electron transfer, both DPI and EFA increased the production of superoxide. This also supports our proposal that the site would be between the EFA inhibition site and the ubiquinone (see Fig. 1). However, EFA decreased the superoxide production at a level of 4 mM. Iron-sulfur clusters in complex II do not use histidyl residues as their ligands (Ohnishi, 1987). Therefore, a possibility is that EFA inhibited electron transfer in complex II at a higher concentration by modifying the histidyl residue which is involved in the substrate binding (Fig. 1). This is located at the opposite site of the flavin binding histidyl site as revealed by the X-ray structure studies on both succinate-quinone reductase (Yankovskaya *et al.*, 2003) and quinol-fumarte reductase (Iverson *et al.*, 1999; Lancaster *et al.*, 1999).

4. In the reverse electron transfer, piericidin A increased superoxide production. This suggests that the superoxide production site may be a protein-associated ubiquinone species between the N2 cluster and pool ubiquinone, namely, it could be either the fast relaxing ubisemiquinone (SQ_{Nf}) or slow relaxing ubisemiquinone (SQ_{Ns}) shown in Figs. 1 and 8 (Magnitsky *et al.*, 2002; Yano *et al.*, 2005).

5. Ubiquinone analogs which can serve as the electron acceptor in complex I, for example, idebenone (the structure shown in Fig. 2E) and DBQ, increased superoxide production in both forward and reverse electron transfer.

Effect of Mucidin

We used mucidin to inhibit the complex III electron transfer in our reverse electron transfer experiment. This inhibitor was used by the Lenaz' group (Lenaz *et al.*, 2002; Genova *et al.*, 2001) because it inhibits the Q_o site in complex III, and because it does not produce much superoxide like antimycin A (which inhibits the Q_i site). It is also important that mucidin does not inhibit complex I respiration. Fig. 2A shows that the superoxide production by mucidin is about 1/3 of that by antimycin A. It has been generally believed that ubiquinone at the Q_i site can form a relatively stable semiquinone radical (Ohnishi and Trumpower, 1980). It was suggested that ubiquinone at the Q_o site could also take a semiquinone form (De Vries *et al.*, 1981; Muller *et al.*, 2003). Our result supports these possibilities.

Figure 6 schematically depicts a hypothesis of the mechanism of how these two inhibitors may work in complex III. The observation that mucidin decreased the production of antimycin A-induced superoxide (top trace in Fig. 2A) supports the fact that mucidin interacts with the Q_o site located before the antimycin A-interacting site (Q_i site). At the concentration of mucidin used in this experiment (2 μM), a small amount of electrons must have escaped the mucidin-inhibition site, and they produced a smaller amount of superoxide when they reached the antimycin A-interacting site. The data in Fig. 2A supports the mechanism that, in complex III, the electron flow is first inhibited at the Q_o site by mucidin, then by antimycin A at the Q_i site.

Differences Between Complex I and Complex III

A difference between complexes I and III is the electron transfer mechanism. In complex III, two ubiquinone-binding sites, (Q_o and Q_i sites) are located on the opposite sides of the membrane. When QH_2 from Q pool comes in to the Q_o site, the first electron is delivered to c_1 and c cytochromes via Rieske iron-sulfur cluster, and the second electron to the Q_i site via cytochromes b_L and b_H, that was developed to the modified Q cycle (Mitchell, 1975; Crofts *et al.*, 1983). When the latter electron transfer is inhibited, by Q_i site (Ohnishi and Trumpower, 1980) or proximal Q_o site inhibitor, electrons accumulate around b cytochromes. Thereby semiquinone in the Q_o site reacts with oxygen producing superoxide.

Although we detected two different kinds of protein-associated ubiquinones in complex I (Q_{Nf} and Q_{Ns}), we do not think that electrons cycle like in complex III. Our recent study predicted that both Q_{Nf} and Q_{NS} are located on the same side of the membrane (Ohnishi *et al.*, 2005;
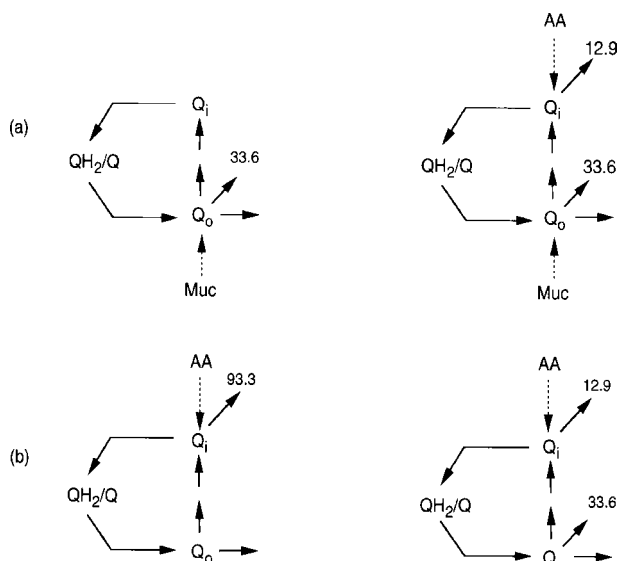
Fig. 6. Simplified schematic presentation of the production of superoxide in the complex III segment by (a) The addition of 2 μM mucidin (Muc) is shown in the left panel. Additions of mucidin followed by 3 μM antimycin A (AA) is shown in the right panel. (b) The addition of antimycin A (left). Additions of antimycin A followed by mucidin (right). Data are taken from Fig. 2A. Here, Q_o and Q_i stand for two ubisemiquinon species in complex III. Dotted arrows indicate the action of inhibitiors and solid arrows the directions of electron transfer. QH_2/Q indicate the ubiquinone pool in the membrane. For the sake of simplicity, other respiratory components, such as cytochromes and Rieske iron-sulfur cluster are omitted. Numbers in the figure represent typical values of superoxide generation for this experiment (pmoles mg^{-1} min^{-1}).

Yano *et al.*, 2005). Yet, similar to antimycin A, complex I inhibitors such as rotenone and piericidin A diminished ubisemiquinone EPR signals (Magnitsky *et al.*, 2002). How superoxide is produced by these inhibitors is not fully understood. Piericidin A is known to compete and replace protein-bound ubiquinone (Degli Esposti, 1994; Ino *et al.*, 2003). A possibility is that when a stable, protein-bound ubisemiquinone is going to be removed by piericidin A, it becomes unstable and reacts with oxygen. We need further study on this point.

Reverse Electron Transfer Experiments Performed Without Mucidin

It is essential to have coupled mitochondria to perform the reverse electron transfer, because electrons have to move against the uphill potential (endergonic reaction) using the energy supplied by the membrane potential. As shown in Fig. 3, the membrane potential can be built using succinate respiration, or in the presence of mucidin (which dissipates the potential) using ATP. Since succinate also

supports forward electron transfer by transporting electrons into complex III, we used ATP and mucidin in order to simplify the experimental system for this study.
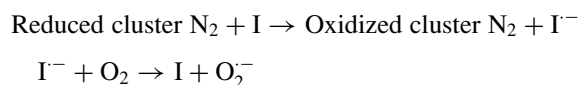
The succinate-induced superoxide production has been used as an example of ROS generation in reverse electron transfer. We would like to caution that it may produce complicated results. Figure 5A shows that a high rate of superoxide production (136.4 ± 16.4 pmoles mg^{-1} min^{-1}) was inhibited to about 1/3 of the original level either by DPI or by mucidin. However, the mechanisms of action of DPI and mucidin are entirely different. DPI inhibits the electron flow in complex I, but mucidin inhibits it in complex III. Further, the former did not interfere with the membrane potential, but the latter negated it.

Figure 5B demonstrates that both piericidine A and rotenone also inhibited the succinate-induced superoxide production to a similar level. Again, the mechanisms of action of piericidin A and rotenone may be different in the reverse electron transfer supported by ATP; the former enhanced superoxide production while the latter did not by itself. As shown in Fig. 5B, both piericidin A and rotenone did not change membrane potential. The increase of superoxide production by mucidin was caused by forward electron transfer going into complex III. In short, the electron reverse transfer experiments performed without a proper complex III inhibitor would be complicated to analyze.

Effect of Idebenone and Decylubiquinone

An inhibitor idebenone, (which is a ubiquinone-analog) produced superoxide (Figs. 2C and 4C) confirming a previous report by Genova *et al.* (2001). Since idebenone is also an electron acceptor (this is marketed as a substitute of coenzyme Q_{10} for treating mitochondrial cytopathies and neurological diseases) (Gillis *et al.*, 1994; Rustin *et al.*, 1999), it could receive electrons from cluster N2 and produce a semiquinone radical, thereby producing superoxide. Whether this drug might produce side effects based upon the ROS production should be carefully monitored.

As shown in these two figures, DBQ (analog of ubiquinone which also works as an electron acceptor), increased the superoxide production, too. These results suggest the following scheme of superoxide generation:

$$\text{Reduced cluster N}_2 + \text{I} \rightarrow \text{Oxidized cluster N}_2 + \text{I}^{\cdot -}$$

$$\text{I}^{\cdot -} + \text{O}_2 \rightarrow \text{I} + \text{O}_2^{\cdot -}$$

Here, I indicates idebenone or DBQ, which may bind to the ubiquinone binding site. If this is the case, the

same reaction may also take place in response to protein-associated ubiquinone (Q) itself, namely:

$$\text{Reduced cluster } N_2 + Q \rightarrow \text{Oxidized cluster } N_2 + Q^{\cdot -}$$

$$Q^{\cdot -} + O_2 \rightarrow Q + O_2^-$$
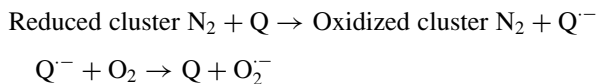
This supports our proposal that superoxide may be generated by ubisemiquinones which directly interact with iron-sulfer cluster N2. Genova *et al.* (2001) and Miyadera *et al.* (2002) also suggested this possibility.

Effect of Rotenone

Both piericidine A and rotenone are familiar specific inhibitors for complex I. While the structure of piericidin A is close to that of ubiquinone, rotenone is a much bulkier molecule consisting of five ring structures. It is well known that the mode of action of both inhibitors are different (Friedrich *et al.*, 1994; Degli Esposti *et al.*, 1994; Okun *et al.*, 1999; Ino *et al.*, 2003).

Our new observation was that rotenone did not generate superoxide production in the reverse electron transfer, while it did in the forward transfer. It was reported that rotenone has weaker inhibitory effect on the reverse electron transfer than the forward one (Grivennikova *et al.*, 1997). Grivennikova *et al.* proposed that two rotenone binding sites exist in complex I; one for the forward reaction and the other, for the reverse reaction (Grivennikova *et al.*, 1997). Another point to be considered is that rotenone may cause a conformational change on complex I (Vinogradov, 1998). Further, the activation/deactivation phenomenon seen in heart mitochondrial complex I may also be involved in the rotenone reaction (Burbaev *et al.*, 1989; Kotlyar and Vinogradov, 1990; Maklashina *et al.*, 2002; Maklashina *et al.*, 2003).

Therefore, the mechanism of action of rotenone may be complicated. Regarding this issue, it is interesting to note that both NADH and NAD^+ have been known to cause conformational changes in complex I (Belogrudov and Hatefi, 1994). Recently, Mamedova *et al.* took electron micrograph of negatively stained *E. coli* complex I and showed that the addition of NADH caused a substantial conformational change on complex I (Mamedova *et al.*, 2004) as compared with the pictures taken in the presence of NAD^+ (a condition which is commonly used for the succinate-supported electron reverse experiments in submitochondrial particles and complex I). This conformational change may have caused such a change in the binding characteristics of rotenone to the inhibition pocket(s) that, in the reverse transfer reaction, rotenone

had a much less inhibitory activity for electron transfer and did not produce superoxide.

It is noted that the previous work on the effect of rotenone was mostly on the electron transfer inhibition, but not on superoxide generation. For superoxide generation, the inhibition of electron transfer is a necessary condition, but it is not a sufficient one. We should have a sufficient degree of inhibition, and further, the site of superoxide generation must have access to oxygen.

In order to promote further discussion on this issue, speculative mechanisms concerning the actions of rotenone and piericidin A are presented in Fig. 7. In (a), two holes (labeled as F and S) represent two ubiquinone binding sites (each corresponding to fast-relaxing and slow-relaxing ubisemiquione sites (Magnitsky *et al.*, 2002)). The ubiquinone-binding pockets located near the iron-sulfur cluster N2. Semiquinone ($Q^{\cdot -}$) is stabilized when bound to the binding pockets and participate in the electron transfer as shown with arrows. (Here, protons are omitted from the illustration for the sake of simlicity.) It was proposed that the center-to-center distances between cluster N2 and F site is 12 Å, and the distance between cluster N2 and the S site is 30 Å or longer (Yano *et al.*, 2005). P and R indicate added piericidin A and rotenone molecules, respectively. These inhibitors expel bound ubisemiquinone from the pocket, making ubisemiquione to become unstable. This causes inhibition of electron transfer, and accumulated electrons would react with oxygen to generate superoxide.

Superoxide can be generated from either site, but for the sake of simplicity, only the F-site is illustrated in Fig. 7. In the forward electron transfer, the rate of superoxide production by the addition of P (shown in (b)) or R (shown in (c)) is in the same order of magnitude regardless of the order of addition. The final rates of superoxide production when both were added (d) would be the same regardless of the order of additions. Since rotenone is a bulky molecule, its mechanism of action is not identical to that of piericidin A (which is a ubiquinone analog). Both binding sites are not the same, but they may partly overlap (Degli Esposti *et al.*, 1994; Okun *et al.*, 1999). Regarding the interaction between these two inhibitors, Ino *et al.* reported that the action of rotenone on the piericidin A-induced inhibition is similar to that by DPI (which reacts with FMN, the site located way before the piericidin A site) (Ino *et al.*, 2003).

In the reverse electron transfer, ubisemiquinones are also bound to the pocket and stabilize themselves (a). When piericidin A is added (as indicated in (b)), it stimulated superoxide production by the same mechanism as in forward transfer. If rotenone is added after piericidin A, it also enhances the production (d). A difference from the forward reaction is that when rotenone alone is added
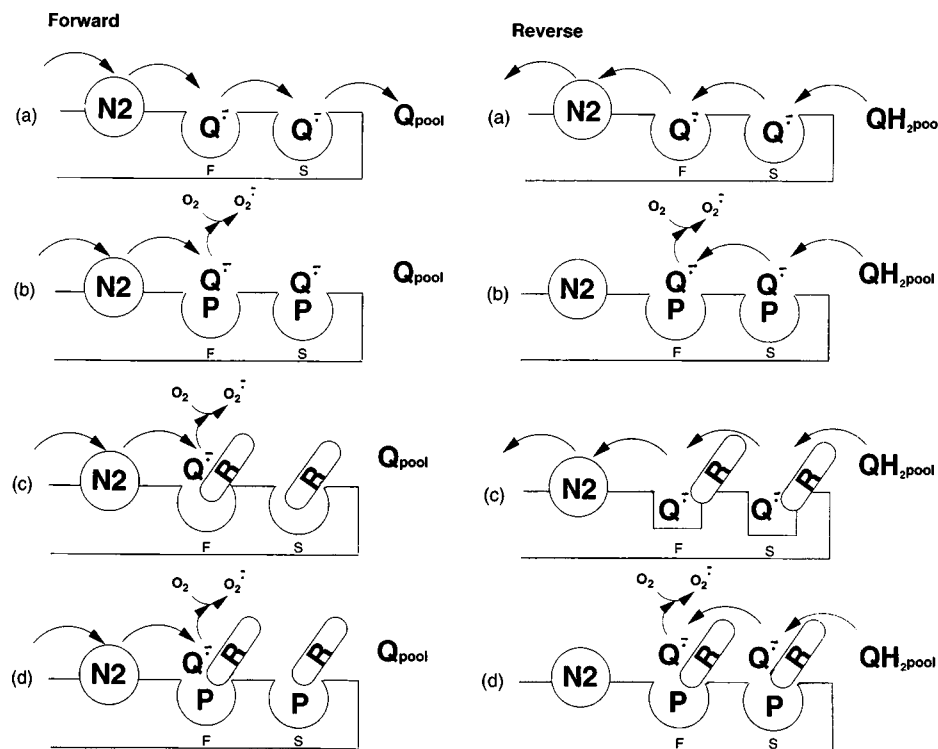
Fig. 7. Schematic presentation of simplified mechanisms of superoxide production in the complex I segment by pericidine A (P) and rotenone (R). Left four panels represent forward electron transfer, and right four panels reverse electron transfer. In both panels, (a) indicates no addition; (b) Pericidine A is added; (c) rotenone is added; and (d) Both piericidin A and rotenone are added. See details in the text.

(c), it did not generate superoxide. In (c), this situation is depicted by a change of the round hole to a square hole in order to represent some kind of conformational change of the ubiquinone-binding pocket(s).

However, if piericidin A is added after rotenone, it produced superoxide, perhaps because it helped bringing the structure back to the original conformation. Further investigation is required to test the model.

On the Flavin Theory

According to our results, two inhibitors, rotenone and piericidin A, generated superoxide in both forward and reverse transfer. Based upon our proposition made in *Introduction*, the ubiquinone binding sites seem to be the major superoxide generation site.

We cannot still completely rule out the possibility that FMN may produce superoxide. It is well-known that the flavin moiety of xanthine oxidase generates superoxide (Enroth *et al.*, 2000). A number of studies proposed that FMN is a possible site of superoxide generation in complex I (Liu *et al.*, 2002; Kushnaeva *et al.*,

2002; Vinogradov, 1998; Turrens and Boveris, 1980). In our experiments, DPI enhanced the generation of superoxide in the reverse electron transfer (Fig. 4B), which may support this possibility. It has been believed that the redox mid-point potential ($E_{m,7}$) of the superoxide/oxygen couple was -0.33 V, but it was corrected to -0.14 V (Petlicki and van de Ven, 1998; Muller, 2000). Since the midpoint potential of non-covalently bound flavin (FMN) is around -0.34 V, it was natural to accept from the old value for superoxide that the FMN radical would reduce oxygen to generate superoxide. With the previous value for the superoxide anion, it was harder to consider that cluster N2 or ubisemiquinone could be the superoxide generation site because much higher energy is required to donate electrons to oxygen.

It is interesting to note that the corrected value for superoxide could provide us with the justification that the cluster N2-semiquinone region could be the site of superoxide generation. In complex III, the redox mid-point potential ($E_{m,7}$) for the semiquionone/quinone couple at the Q_o site, which produces superoxide, is estimated to be -0.16 V (Sun and Trumpower, 2003). This value is very close to the corrected value for the superoxide/oxygen
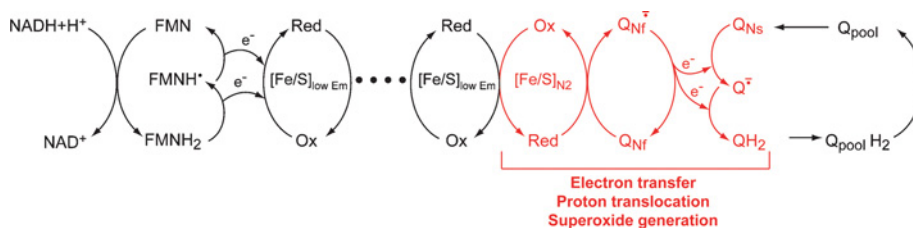
Fig. 8. Schematic presentation of putative mechanism of electron transfer in complex I (Magnitsky *et al.*, 2002a; Yano *et al.*, 2004). At neutral pH, FMN has five forms based upon the state of protonation and electron reduction (Sled *et al.*, 1994), but for the sake of simplicity, only three are shown. ($n = 2e^-$) indicates two electron transfer reaction, and ($n = e^-$) $\times$ 2 means two cycles of one electron transfer reaction. Q_{Nf} represents the fast relaxing ubisemiquinone radical and Q_{Ns} the slow relaxing ubisemiquione radical, respectively (Magnitsky *et al.*, 2002a; Yano *et al.*, 2004; Ohnishi *et al.*, 2004) [Fe/S]$_{low\ Em}$ indicates several low potential iron-sulfur clusters. The region consisting of iron-sulfur N2 and ubisemiquinones play central roles in electron transfer, proton translocation and superoxide generation in complex I.

couple. The redox mid-point potential for the cluster N2 is around -0.15 to -0.05 V (Ohnishi, 1998). Therefore, when fully reduced, cluster N2 and/or ubisemiquinones (SQ$_{Nf}$ and SQ$_{Ns}$ in Fig. 8) could reduce oxygen to superoxide anions.

The Central Role of the Cluster N2-Ubisemiquinone Region

Our results suggest that, in intact rat heart mitochondria, the major site of superoxide generation may not be FMN. In complex I, one flavin (FMN) and at least eight iron-sulfur clusters are involved in electron transfer. It appears that FMN and seven iron-sulfur clusters (low potential iron-sulfur clusters; see Fig. 1) are well protected from reacting with oxygen. This may be related to the fact that so many protein subunits (46) are found in bovine heart complex I. Some of these proteins may serve as a protective wrapping to prevent the respiratory components from reacting with oxygen (Vinogradov, 1998; Ragan *et al.*, 1986; Ragan, 1987).

The only exception may be cluster N2. Based upon observations that the mid-point potential of cluster N2 was pH dependent, it was hypothesized that this cluster may serve as the coupling site between electron transfer and proton transport in complex I (Ingledew and Ohnishi, 1980). This requires that the region of cluster N2 and protein-bound ubisemiquinones must be accessible to H$^+$ and water. Hence, this region is also accessible to oxygen, and the possibility of reducing oxygen to superoxide would also occur. Our results seem to indicate that cluster N2 and bound semiquinones may play key roles in the function of complex I, namely, electron transfer, proton transport and superoxide generation (Fig. 8). Further investigation is required to test this possibility.

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