Production of superoxide anion (O$_2^-$), measured as the chemiluminescence of the 2-methyl-6-($p$-methoxyphenyl)-3,7-dihydroimidazo[1,2-$a$]pyrazin-3-one hydrochloride (MCLA)-O$_2^-$ adduct, was observed during electron transfer from succinate to cytochrome $c$ by reconstituted succinate-cytochrome $c$ reductase-phospholipid vesicles replenished with succinate dehydrogenase. Addition of carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone or detergent to the reconstituted reductase-phospholipid vesicles abolished O$_2^-$ production, suggesting that O$_2^-$ generation is caused by the membrane potential generated during electron transfer through the cytochrome $bc_1$ complex. Production of O$_2^-$ was also observed during electron transfer from succinate to cytochrome $c$ by antimycin-treated reductase, in which ~99.7% of the reductase activity was inhibited. The rate of O$_2^-$ production was closely related to the rate of antimycin-insensitive cytochrome $c$ reduction. Factors affecting antimycin-insensitive reduction of cytochrome $c$ also affected O$_2^-$ production and vice versa. When the oxygen concentration in the system was decreased, the rate of O$_2^-$ production and cytochrome $c$ reduction by antimycin-treated reductase decreased. When the concentrations of MCLA and cytochrome $c$ were increased, the rate of O$_2^-$ production and cytochrome $c$ reduction by antimycin-treated reductase increased. The rate of antimycin-insensitive cytochrome $c$ reduction was sensitive to Q$_{10}^-$ site inhibitors such as 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole. These results indicate that generation of O$_2^-$ during the oxidation of ubiquinol by the cytochrome $bc_1$ complex results from a leakage of the second electron of ubiquinol from its Q cycle electron transfer pathway to interact with oxygen. The electron-leaking site is located at the reduced cytochrome $b_{562}$ or ubisemiquinone of the Q$_{10}^-$ site because addition of MCLA to antimycin-treated cytochrome $bc_1$ complex, in the presence of catalytic amounts of succinate-cytochrome $c$ reductase, delayed cytochrome $b$ reduction by succinate. In the presence of oxidized cytochrome $c$, purified succinate dehydrogenase also catalyzed oxidation of succinate to generate O$_2^-$ When succinate dehydrogenase was reconstituted with the $bc_1$ particles to form succinate-cytochrome $c$ reductase, the production of O$_2^-$ diminished. These results suggest that reduced FAD of succinate dehydrogenase is the electron donor for oxygen to produce O$_2^-$ in the absence of their immediate electron acceptor and in the presence of cytochrome $c$.

During mitochondrial respiration, there is a continuous release of electrons from their normal pathway to molecular oxygen (O$_2$) to form superoxide anion (O$_2^-$) (1–3). O$_2^-$ subsequently dismutates to H$_2$O$_2$ spontaneously or by the action of superoxide dismutases (4). Isolated mitochondria in state 4 generate 0.6–1.0 nmol of H$_2$O$_2$/min/mg of protein, accounting for ~2% of O$_2$ uptake under physiological conditions (5). Production of O$_2^-$ during mitochondrial respiration is closely related to mitochondrial coupling efficiency.

Reactions in two segments of the respiratory chain are responsible for univalent reduction of dioxygen to O$_2^-$. The one located in NADH-Q$^+$ reductase is cyanide-insensitive. Production of O$_2^-$ is probably via autoxidation of the reduced flavin mononucleotide of NADH dehydrogenase (6, 7). The other is located in the cytochrome $bc_1$ complex (ubiquinol-cytochrome $c$ reductase). Two redox components of the cytochrome $bc_1$ complex, ubisemiquinone (8) and reduced cytochrome $b_{562}$ (9), have been suggested as the autoxidizable factors causing O$_2^-$ production.

To date, most information concerning mitochondrial O$_2^-$ generation has been obtained from studies with intact heart mitochondria and electron transfer inhibitors. Production of O$_2^-$ in intact mitochondria is difficult to measure because O$_2^-$ has a very short half-life (2) and cannot pass outward through the inner membrane. However, since heart mitochondria contain superoxide dismutase, but no catalase, and H$_2$O$_2$ is a relatively stable species that readily penetrates the mitochondrial membrane, production of O$_2^-$ in intact heart mitochondria is generally determined by measuring H$_2$O$_2$ concentration in the suspending medium (6). Studies of O$_2^-$ formation by purified electron transfer complexes should yield less ambiguous results, especially when production of O$_2^-$ is directly measured by chemiluminescence of the MCLA-O$_2^-$ adduct (10).

The availability of highly purified bovine heart mitochondrial succinate-cytochrome $c$ reductase complex (11) and its subcomplexes, such as succinate-Q reductase (11), the cytochrome $bc_1$ complex (16), succinate dehydrogenase (16), membrane-anchoring proteins of succinate-Q reductase (QPs) (17), and the $bc_1$ particles (QPs plus the cytochrome $bc_1$ complex) (18), in our laboratory enabled us to systematically study O$_2^-$ generation in this region of the mitochondrial electron transfer chain. Purified bovine heart succinate-cytochrome $c$ reductase complex (11), which catalyzes electron transfer from succinate to cytochrome $c$, is composed of succinate-Q reductase and the cytochrome $bc_1$ complex. Succinate-Q reductase catalyzes electron transfer from succinate to ubiquinone, whereas the cytochrome $bc_1$ complex catalyzes electron transfer from ubiquinol to cytochrome $c$. The cytochrome $bc_1$ complex, which contains four redox centers (cytochromes $b_{560}$, $b_{562}$, and $e_1$ and the Rieske iron-sulfur cluster),

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The abbreviations used are: Q, ubiquinone; MCLA, 2-methyl-6-($p$-methoxyphenyl)-3,7-dihydroimidazo[1,2-$a$]pyrazin-3-one hydrochloride; QPs, membrane-anchoring proteins of succinate-Q reductase; XO, xanthine oxidase; PL, phospholipid; FCCP, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazine.
has recently been crystallized (12), and its structure was determined at 2.9-Å resolution (13). It is generally accepted that proton and electron transfer in the cytochrome bc₁ complex follows the Q cycle mechanism (14, 15). Succinate-Q reductase is composed of a two-subunit succinate dehydrogenase (16) and a three-subunit membrane anchoring protein (QPs) (17). Four redox components of the succinate-cytochrome c reductase segment of the respiratory chain (reduced FAD, ubiquinol, ubiquisemiquinone, and reduced cytochrome b₅₆₅) have been suggested as the electron donors for O₂. Reduced FAD is in succinate dehydrogenase; ubiquinol is produced by succinate-Q reductase; and ubiquisemiquinone and reduced cytochrome b₅₆₅ are generated during the catalytic cycle of the cytochrome bc₁ complex. Herein we report the production of O₂ during electron transfer through succinate-cytochrome c reductase, succinate-Q reductase, and succinate dehydrogenase under various conditions.

EXPERIMENTAL PROCEDURES

Asolectin was obtained from Associate Concentrates (Woodsfield, New York) and purified according to Sone et al. (19). Horse heart cytochrome c (type II) and cytochrome oxidase, superoxide dismutase, and catalase were from Sigma. Antimycin A was from U. S. Biochemical Corp. MCLA was a gift from Dr. Anraku (University of Tokyo, Tokyo, Japan).

Bovine heart mitochondrial succinate-cytochrome c reductase (11), succinate-Q reductase (11), succinate dehydrogenase (16), the cytochrome bc₁ complex (16), the bc₁ particles (16), and QPs (17) were prepared and assayed according to the reported methods. Protein-photospholipid vesicles were prepared by the cholate dialysis method (21, 22). Spectral measurements were carried out with a Shimadzu UV-2101PC spectrophotometer at room temperature.

O₂ generation was determined by measuring the chemiluminescence of MCLA-O₂ (10). An assay mixture (1 ml) containing 50 mM Tris-Cl, pH 7.8, 0.1 mM EDTA, 4 mM MCLA, 7 mM sodium succinate, and 10 μM cytochrome c was treated with an appropriate amount of enzyme solution for 1 min. O₂ generation was measured with a Lumac/3M Biocounter (Model M2010). O₂ generation is expressed in XO units. One XO unit is defined as the chemiluminescence generated by 1 unit of xanthine oxidase in 1 min, which equals 4.3 × 10⁹ counts from a Lumac/3M Biocounter (Model M2010). For comparison, the reaction was measured with a Lumac/3M Biocounter (Model M2010A). O₂ generation is expressed in XO units. One XO unit is defined as the chemiluminescence generated by 1 unit of xanthine oxidase in 1 min, which equals 4.3 × 10⁹ counts from a Lumac/3M Biocounter (Model M2010). O₂ production was inactivated before it could be re-associated with QPs, in the latter. In the absence of antimycin the succinate-Q reductase-phospholipid vesicle system decreased the rate of O₂ production by 50%, suggesting that the membrane potential generated during electron transfer through the cytochrome bc₁ complex region induces electron leakage, from the mitochondrial system can be explained by the presence of antimycin and endogenous cytochrome c in the latter. In the mitochondrial system, the presence of protonophores causes the disruption of membrane potential, thus stimulating the oxidation of endogenous cytochrome c by cytochrome c oxidase to generate the oxidized cytochrome c needed for the generation of O₂ from reduced Q. In the case of reconstituted succinate-cytochrome c reductase-PL vesicles at a 3:1 molar ratio (succinate dehydrogenase/succinate-cytochrome c reductase) and succinate-cytochrome c reductase activities. Whereas addition of the protonophore FCCP to the reductase-PL vesicles enhanced succinate-cytochrome c reductase activity only slightly, it increased ubiquinol-cytochrome c reductase activity by 7-fold. This indicates that the succinate-Q reductase region, but not the cytochrome bc₁ complex, is inactivated in the reductase-PL vesicles. The inactivated component was identified as succinate dehydrogenase because addition of purified, reconstitutively active succinate dehydrogenase to the FCCP-treated reductase-PL vesicles increased antimycin-sensitive succinate-cytochrome c reductase activity to about the same level as that of the starting reductase. Inactivation of succinate dehydrogenase is due to the labile nature of this enzyme in soluble form under aerobic conditions (16). Succinate dehydrogenase was detached from the QPs-cytochrome bc₁ complex upon addition of a mixture of 2% sodium cholate/α-solucin during the first step of reductase-PL vesicle preparation. The high detergent concentration, used to ensure complete dispersion of phospholipid during the preparation of reductase-PL vesicles, apparently caused solubilization of succinate dehydrogenase from its anchoring proteins (QPs). Soluble succinate dehydrogenase was inactivated before it could be re-associated with QPs, in the phospholipid bilayer, during the subsequent dialysis step.

Oxidation of succinate by succinate-cytochrome c reductase-PL vesicles, replenished with a 3-fold molar excess of succinate dehydrogenase, produced 2.45 XO units of O₂/mg of protein. Addition of FCCP or detergent to the succinate dehydrogenase-replenished reductase-PL vesicles decreased the rate of O₂ production by 50%, suggesting that the membrane potential generated during electron transfer through the cytochrome bc₁ complex region induces electron leakage, from the normal pathway to react with O₂ to form O₂⁻. The remaining O₂⁻ production observed with FCCP-treated reductase-PL vesicles was due to the excess succinate dehydrogenase in the reconstituting system and not to incomplete disruption of protein vesicles by FCCP or detergent. Production of O₂⁻ by soluble succinate dehydrogenase is discussed below.

It has been reported that the production of H₂O₂ in rat or pigeon heart mitochondria supplemented with antimycin was stimulated up to 13-fold by addition of protonophores (20). The difference in the protonophore sensitivity between the succinate-cytochrome c reductase-photospholipid vesicle system and the mitochondrial system can be explained by the presence of antimycin and endogenous cytochrome c in the latter. In the mitochondrial system, the presence of protonophores causes the disruption of membrane potential, thus stimulating the oxidation of endogenous cytochrome c by cytochrome c oxidase to generate the oxidized cytochrome c needed for the generation of O₂⁻ from reduced Q. In the case of reconstituted succinate-

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**Table I**

| Additions                                | O production* |
|------------------------------------------|---------------|
| SCR                                      | XO units/mg protein |
| ([SCR-PL vesicle] + SDH)                 | 0             |
| ([SCR-PL vesicle] + SDH + FCCP)          | 2.45          |
| ([SCR-PL vesicle] + SDH + KDOC)          | 1.34          |
| SCR + PL + SDH + KDOC                    | 1.31          |
| SCR + TTFA                               | 0.07          |
| SCR + antimycin                          | 0.71          |

* XO units are defined under “Experimental Procedures.”

**SCR,** succinate-cytochrome c reductase; SDH, purified succinate dehydrogenase; KDOC, potassium deoxycholate; TTFA, thieno[3,2-b]furoate.

**([SCR-PL vesicle] + SDH) indicates that purified, reconstitutively active succinate dehydrogenase was added to the succinate-cytochrome c reductase-PL vesicle at a 3:1 molar ratio (succinate dehydrogenase/succinate-cytochrome c reductase).**
cytochrome c reductase vesicles, disruption of membrane potential by addition of protonophores will simply stimulate the activity of succinate-cytochrome c reductase to reduce cytochrome c.

Production of \( \text{O}_2^\cdot \) by Antimycin-treated Succinate-Cytochrome c Reductase—The most unambiguous way to confirm membrane potential-induced \( \text{O}_2^\cdot \) production by the cytochrome \( bc_1 \) complex is to detect \( \text{O}_2^\cdot \) production by protein-PL vesicles formed from purified cytochrome \( bc_1 \) complex and phospholipid. When purified cytochrome \( bc_1 \) complex was embedded in phospholipid vesicles, the resulting cytochrome \( bc_1 \)-PL vesicles showed an oxidation control ratio of 11 with an \( H^+/e^- \) ratio of 2. However, detection of \( \text{O}_2^\cdot \) production by these vesicles was difficult because the rate of non-enzymatic production of \( \text{O}_2^\cdot \) from ubiquinol (substrate) by cytochrome c (acceptor) was high compared with that of the enzymatic production. The finding that during oxidation of succinate by antimycin A inhibited succinate-cytochrome c reductase \( \text{O}_2^\cdot \) is produced (Table I) provides us a model system to study generation of \( \text{O}_2^\cdot \) in the cytochrome \( bc_1 \) complex region of the respiratory chain.

When detergent-dispersed succinate-cytochrome c reductase was treated with antimycin, 99.7% of its activity was inhibited, and 0.71 XO units of \( \text{O}_2^\cdot \) were produced per mg of protein. Antimycin is known to block transfer of the second electron of ubiquinol from cytochrome \( b_{661} \) to ubiquinone to form ubisemiquinone at the \( Q \) site, according to the Q cycle mechanism (Fig. 1). Therefore, the reduction of cytochrome c observed with antimycin-inhibited reductase must result from the first electron of ubiquinol being transferred to cytochrome c via the iron-sulfur protein and cytochrome \( c_1 \). The second electron of ubiquinol in antimycin-treated reductase probably leaks out of its normal electron transfer pathway at reduced cytochrome \( b_{661} \) or ubisemiquinone at the \( Q \) site and reacts with \( O_2 \) to produce \( \text{O}_2^\cdot \). This is the case, one would expect the rate of \( \text{O}_2^\cdot \) production to be closely related to the rate of electron transfer to cytochrome \( c \) in the antimycin-treated reductase. In other words, factors affecting the rate of \( \text{O}_2^\cdot \) production should also affect the rate of antimycin-insensitive cytochrome c reduction and vice versa. Since \( O_2 \) is the reactant for \( \text{O}_2^\cdot \) formation and MCLA reacts with \( \text{O}_2^\cdot \) to produce the chemiluminescence compound MCLA-\( \text{O}_2^\cdot \), their involvement in \( \text{O}_2^\cdot \) production is expected. Cytochrome \( c \), on the other hand, is the electron acceptor for the first electron of ubiquinol via the iron-sulfur protein and cytochrome \( c_1 \). The relationship between \( \text{O}_2^\cdot \) production and cytochrome c reduction by antimycin-treated reductase can be studied by examining the effect of MCLA, \( O_2 \), and cytochrome c on the rate of \( \text{O}_2^\cdot \) production. It should be noted that in the antimycin-treated reductase, MCLA not only facilitated the detection of \( \text{O}_2^\cdot \) but also had a great effect on stimulation of \( \text{O}_2^\cdot \) production. A similar stimulating effect on \( \text{O}_2^\cdot \) production was not observed in the soluble xanthine oxidase system. Addition of MCLA has no effect on cytochrome c reduction by \( \text{O}_2^\cdot \) generated by xanthine oxidase in the presence of catalase (4).

Effect of Concentrations of MCLA, \( O_2 \), and Cytochrome c on the Rate of \( \text{O}_2^\cdot \) Production and Cytochrome c Reduction by Antimycin-treated Succinate-Cytochrome c Reductase—Fig. 2 shows the effect of MCLA concentration on the rate of \( \text{O}_2^\cdot \) production and cytochrome c reduction by antimycin-treated succinate-cytochrome c reductase. When succinate was added to antimycin-inhibited reductase in the presence of varying concentrations of MCLA, the rate of \( \text{O}_2^\cdot \) generation increased as the MCLA concentration increased. Maximum production of \( \text{O}_2^\cdot \) was observed when the MCLA concentration reached 2 \( \mu M \); higher MCLA concentrations caused an apparent decrease in \( \text{O}_2^\cdot \) production. This apparent decrease is due to quenching of the chemiluminescence of MCLA-\( \text{O}_2^\cdot \) by higher concentrations of MCLA (yellow color) and not to a decrease in the rate of \( \text{O}_2^\cdot \) production. Since cytochrome c acts both as the first electron acceptor and the scavenger of superoxide, the reduction of cytochrome c should be twice as much as the MCLA-\( \text{O}_2^\cdot \) adduct formation if the measured chemiluminescence could stoichiometrically represent the latter. This relation was only observed when a low concentration of MCLA was used (Fig. 2). The quenching of the chemiluminescence of MCLA-\( \text{O}_2^\cdot \) by high concentrations of MCLA was also observed with \( \text{O}_2^\cdot \) generated by the xanthine oxidase system (Fig. 3). The amount of chemiluminescence generated by a given amount of xanthine oxidase increased as the MCLA concentration increased, reached a
maximum (at 1.2 μM), and then decreased. This result confirms that higher concentrations of MCLA cause quenching of chemiluminescence. It should be noted that MCLA at the concentrations used had neither an inhibitory nor an activating effect on xanthine oxidase when the activity was followed by product formation spectrophotometrically or cytochrome c reduction in the presence of catalase (4). When cytochrome c reduction by antimycin-inhibited succinate-cytochrome c reductase was determined in the presence of varying concentrations of MCLA, using succinate as substrate, the rate of reduction increased when the MCLA concentration was increased, reaching a maximum at an MCLA concentration of 15 μM. This increase can be explained by assuming that MCLA serves as an electron sink for O₂ by forming MCLA-O₂. It pulls the second electron of ubiquinol from reduced cytochrome b_{red} or ubiquinol at the Q₁ site, thus increasing the rate of O₂ production and facilitating transfer of the first electron of ubiquinol to cytochrome c via the iron-sulfur protein and cytochrome c₁. This explanation is supported by the observation that MCLA-induced antimycin-insensitive cytochrome c reduction was sensitive to Q₁ site inhibitors such as 5-undecyl-6-hydroxy-4,7-dioxobenzothiazole (Fig. 4).

Since O₂ is converted to H₂O₂ by superoxide dismutase, it was of interest to see whether or not the rate of O₂ generation and cytochrome c reduction by antimycin-treated succinate-cytochrome c reductase is affected by addition of superoxide dismutase. To our surprise, addition of superoxide dismutase did not enhance the antimycin-sensitive cytochrome c reduction activity. Apparently, the O₂ generation site is in a hydrophobic environment easily accessible to MCLA, but not to superoxide dismutase. On the other hand, addition of superoxide dismutase to the antimycin A- and MCLA-treated reductase greatly reduced the chemiluminescence of the system, suggesting that superoxide dismutase is able to catalyze dismutation of O₂⁺ in the form of MCLA-O₂⁻. Addition of superoxide dismutase to the xanthine oxidase system did not stimulate the
oxidation of hypoxanthine, but decreased the chemiluminescence of MCLA-O$_2^*$, as expected, indicating that MCLA-O$_2^*$ can serve as a substrate for superoxide dismutase.

Fig. 5 shows the effect of O$_2^*$ concentration on the rate of O$_2^*$ production and antimycin-insensitive cytochrome c reduction by antimycin-treated succinate-cytochrome c reductase. When the O$_2^*$ concentration was decreased, the rate of O$_2^*$ production and cytochrome c reduction decreased. When O$_2^*$ was completely depleted, neither O$_2^*$ production nor antimycin-insensitive cytochrome c reduction was observed. This again demonstrates that cytochrome c reduction correlates with O$_2^*$ production by antimycin-treated succinate-cytochrome c reductase.

Fig. 6 shows the effect of cytochrome c concentration on O$_2^*$ production and cytochrome c reduction by antimycin-treated succinate-cytochrome c reductase. When varying amounts of cytochrome c were added to antimycin-treated succinate-cytochrome c reductase in the presence of 4 $\mu$M MCLA, O$_2^*$ production increased as the concentration of cytochrome c increased, up to 2 $\mu$M. When the cytochrome c concentration was higher than 2 $\mu$M, O$_2^*$ production decreased. This decrease results from the quenching of the chemiluminescence of MCLA-O$_2^*$ by cytochrome c. On the other hand, the rate of cytochrome c reduction catalyzed by antimycin-inhibited reductase increased as the concentration of cytochrome c increased. The apparent $K_m$ for cytochrome c for antimycin-treated succinate-cytochrome c reductase in the presence of MCLA was calculated to be 40 $\mu$M, similar to that obtained for the uninhibited reductase, indicating that binding of cytochrome c to cytochrome b$_1$ is not affected by antimycin or MCLA. Although cytochrome c is an O$_2^*$ scavenger (10), it is less effective than MCLA in scavenging O$_2^*$ produced in hydrophobic environments or in a protein-bound form.

**Identification of the O$_2^*$ Generation Site in the Cytochrome b$_1$ Complex**—By limiting the rate of electron input to the cytochrome b$_1$ complex, either by partial inhibition of succinate-Q reductase activity in succinate-cytochrome c reductase preparations by malonate or by the addition of limiting amounts of succinate-cytochrome c reductase to purified cytochrome b$_1$ complex, the sequence of reduction of cytochromes b and c$_1$ can be determined by conventional spectrophotometric methods. When succinate was added to purified cytochrome b$_1$ complex in the presence of catalytic amounts of purified succinate-cytochrome c reductase, the reduction of cytochrome b$_1$ occurred at about the same time as the reduction of cytochrome c$_1$. When the cytochrome b$_1$ complex was treated with antimycin, the reduction of cytochrome b$_1$ occurred before the reduction of cytochrome c$_1$. Addition of MCLA to the antimycin-inhibited cytochrome b$_1$ complex resulted in cytochrome c$_1$ being reduced before cytochrome b$_1$. This delayed reduction of cytochrome b$_1$ further supports the hypothesis that the second electron of ubiquinol leaks out to react with oxygen to form O$_2^*$ trapped as the MCLA-O$_2^*$ adduct, before it reduces cytochrome b$_1$.

**Production of O$_2^*$ by Purified Succinate-Ubiquinone Reductase**—The observation that ubiquinol reduces O$_2$ to O$_2^*$ in the presence of cytochrome c encouraged us to investigate the production of O$_2^*$ by succinate-Q reductase. Purified succinate-Q reductase, in the presence and absence of cytochrome c, produced 0.18 and 0.05 XO units of O$_2$/mg of protein, respectively (Table II). Succinate-Q reductase supplemented with Q$_2$ in the presence and absence of cytochrome c produced 9.88 and 0.05 XO units of O$_2$/mg of protein, respectively. Production of O$_2^*$ by succinate-Q reductase in the presence of cytochrome c and Q$_2$ was diminished upon addition of its electron acceptor, the cytochrome b$_1$ complex. This is in line with the observation that electron transfer through soluble succinate-cytochrome c reductase does not generate O$_2^*$. Apparently, active succinate-cytochrome c reductase is formed from succinate-Q reductase by addition of the cytochrome b$_1$ complex. These results indicate that electron transfer through succinate-Q reductase in the absence of the cytochrome b$_1$ complex produces O$_2$ through the non-enzymatic oxidation of ubiquinol in the presence of cytochrome c. The production of O$_2^*$ through oxidation of ubiquinol by cytochrome c may proceed by a sequence in which ubiquinol gives its high potential electron to cytochrome c to generate a low potential semiquinone that reduces O$_2$ to O$_2^*$. This suggestion is supported by the fact that no O$_2^*$ is formed from ubiquinol in the presence of reduced cytochrome c.

**Production of O$_2^*$ by Purified Succinate Dehydrogenase**—Since O$_2^*$ production was observed during succinate oxidation by the reconstituted succinate-cytochrome c reductase system in the presence of excess succinate dehydrogenase (Table I), O$_2^*$ production by succinate dehydrogenase under various conditions was investigated. The rate of O$_2^*$ production by soluble succinate dehydrogenase increased from 0.07 to 0.49 XO units/mg of protein upon addition of oxidized cytochrome c (Table III). Addition of the b$_1$ particles (QPs + the cytochrome b$_1$ complex), but not the cytochrome b$_1$ complex, drastically decreased the rate of O$_2^*$ production by reconstitutively active succinate dehydrogenase in the presence of cytochrome c. The difference between the b$_1$ particles and the cytochrome b$_1$ complex is that the former contains QPs, which mediate electron transfer between Q and succinate dehydrogenase. Thus, addition of the b$_1$ particles to reconstitutively active succinate dehydrogenase reconstitutes succinate-cytochrome c reductase. Although the rate of O$_2^*$ production by reconstitutively inactive succinate dehydrogenate was the same as that of the reconstitutively active enzyme, addition of the b$_1$ particles to the reconstitutively inactive succinate dehydrogenate did not decrease O$_2^*$ production. These results indicate that reduced flavin in succinate dehydrogenase, in the absence of an immediate electron transfer partner, gives a high potential electron to cytochrome c through iron-sulfur clusters to generate a low potential flavin semiquinone radical, which in turn reduces O$_2$ to form O$_2^*$. Ferricyanide can replace cytochrome c effectively in the generation of O$_2^*$ catalyzed by succinate dehydrogenase (Table III) or succinate-Q reductase (Table II). At the same concentration used, ferricyanide is more effective than cytochrome c in stimulating O$_2^*$ formation in succinate-Q reductase than in succinate dehydrogenase.

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**REFERENCES**

1. Loschen, G., Azzi, A., and Flohe, L. (1973) FEBS Lett. 33, 84–88
2. Loschen, G., Azzi, A., Richter, C., and Flohe, L. (1974) FEBS Lett. 42, 68–72
3. Nohl, H., and Hegner, D. (1978) Biochem. J. 134, 707–714
4. McCord, J. M., and Fridovich, I. (1970) J. Biol. Chem. 244, 6049–6055
5. Boveris, A., Oshino, N., and Chance, B. (1972) Biochem. J. 128, 617–630
6. Turrens, J. F., and Boveris, A. (1980) Biochim. Biophys. Acta 62, 191–202
7. Cadenas, E., Boveris, A., Ragan, C. L., and Stoppani, A. O. M. (1977) Arch. Biochem. Biophys. 180, 244–257
8. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) Arch. Biochem. Biophys. 237, 408–414
9. Nohl, H., and Jordan, W. (1986) Biochem. Biophys. Res. Commun. 138, 533–539
10. Nakano, M. (1990) Methods Enzymol. 186, 585–591
11. Yu, L., and Yu, C.-A. (1982) J. Biol. Chem. 257, 2016–2021
12. Yu, C.-A., Xia, J. Z., Kachurin, A. M., Yu, L., Kim, H., and Deisenhofer, J. (1990) Biochim. Biophys. Acta 1275, 47–53
13. Xia, D., Yu, C.-A., Kim, H., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1997) Science 277, 60–66
14. Mitchell, P. (1975) J. Theor. Biol. 62, 327–337
15. Trumpower, B. L. (1990) J. Biol. Chem. 265, 11409–11412
16. Yu, L., and Yu, C.-A. (1980) Biochim. Biophys. Acta 501, 409–420
17. Yu, L., Xu, X.-X., Haley, P. E., and Yu, C.-A. (1987) J. Biol. Chem. 262, 1137–1143
18. Yu, C.-A., Yu, L., and King, T. E. (1974) J. Biol. Chem. 249, 4905–4910
19. Sone, N., Yoshiida, M., Hiraiz, H., and Kagawa, Y. (1977) J. Biochem. (Tokyo) 81, 519–526
20. Cadenas, E., and Boveris, A. (1980) Biochem. J. 186, 31–37
21. Miki, T., Miki, M., and Orii, Y. (1994) J. Biochem. 116, 1827–1833
22. Beattie, D. S., and Villalobo, A. (1982) J. Biol. Chem. 257, 14745–14752
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