

# Myxothiazol Induces H<sub>2</sub>O<sub>2</sub> Production from Mitochondrial Respiratory Chain

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**Interruption of electron flow at the quinone-reducing center (Q<sub>i</sub>) of complex III of the mitochondrial respiratory chain results in superoxide production. Unstable semiquinone bound in quinol-oxidizing center (Q<sub>o</sub>) of complex III is thought to be the sole source of electrons for oxygen reduction; however, the unambiguous evidence is lacking. We investigated the effects of complex III inhibitors antimycin, myxothiazol, and stigmatellin on generation of H<sub>2</sub>O<sub>2</sub> in rat heart and brain mitochondria. In the absence of antimycin A, myxothiazol stimulated H<sub>2</sub>O<sub>2</sub> production by mitochondria oxidizing malate, succinate, or  $\alpha$ -glycerophosphate. Stigmatellin inhibited H<sub>2</sub>O<sub>2</sub> production induced by myxothiazol. Myxothiazol-induced H<sub>2</sub>O<sub>2</sub> production was dependent on the succinate/fumarate ratio but in a manner different from H<sub>2</sub>O<sub>2</sub> generation induced by antimycin A. We conclude that myxothiazol-induced H<sub>2</sub>O<sub>2</sub> originates from a site located in the complex III Q<sub>o</sub> center but different from the site of H<sub>2</sub>O<sub>2</sub> production inducible by antimycin A.** © 2001 Academic Press

**Key Words:** mitochondria, complex III, reactive oxygen species, hydrogen peroxide, superoxide, myxothiazol, stigmatellin.

Mitochondrial production of reactive oxygen species (ROS) is thought to play an important role in many pathologies, including heart and brain injury due to ischemia/reperfusion (1, 2), and in apoptotic cell death (3, 4). Therefore, a detailed understanding of the mechanism of ROS production by mitochondria is important and could aid in the development of new strategies for the prevention of cell death.

The primary species of ROS produced by mitochondria is superoxide (5–9), which quickly dismutates

Abbreviations used: BSA, bovine serum albumin; EGTA, ethylene glycol-bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid; FCCP, *p*-trifluoromethoxycarbonylcyanide phenylhydrazine.

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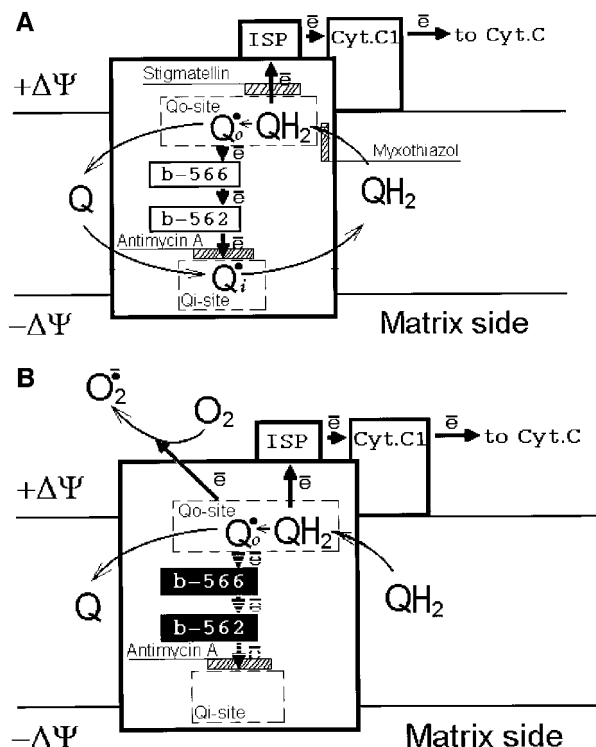
forming H<sub>2</sub>O<sub>2</sub> (10). Multiple sites of ROS generation have been identified in the respiratory chain of mammalian mitochondria (see (11) for a recent review). The present study focuses on the site located in the quinol-oxidizing center (Q<sub>o</sub>) of the Complex III (bc<sub>1</sub> complex) of the mitochondrial electron transport chain (Fig. 1). The one-electron donor responsible for the superoxide formation is believed to be an unstable semiquinone (7, 12–14) formed in the Q<sub>o</sub> center. According to the Q-cycle mechanism of electron transport in the bc<sub>1</sub> complex, an unstable semiquinone is formed in center Q<sub>o</sub> due to the sequential, bifurcated reaction of quinol oxidation (15, 16) (Fig. 1).

The formation of semiquinone and hence, superoxide production can be modulated by specific inhibitors of the bc<sub>1</sub> complex. According to the Q-cycle hypothesis, inhibitors acting at the quinone reducing center (Q<sub>i</sub>), e.g., antimycin A, stimulate superoxide formation by inhibiting semiquinone oxidation (Fig. 1B), whereas inhibitors of the Q<sub>o</sub> site, e.g., such as myxothiazol, inhibit superoxide production by preventing semiquinone formation (11, 15, 16) (Fig. 1A).

The semiquinone proposed to be present in the Q<sub>o</sub> center has, however, never been detected (11, 17). The effects of specific inhibitors on mitochondrial ROS production are therefore important for identification of both the site and the source of superoxide production in the bc<sub>1</sub> complex. In this study, we conducted a comparison between the effects of three different bc<sub>1</sub> complex inhibitors, antimycin A, myxothiazol and stigmatellin, on mitochondrial ROS production. The results indicate that myxothiazol-induced H<sub>2</sub>O<sub>2</sub> originates from a site located in the complex III Q<sub>o</sub> center but different from the site of H<sub>2</sub>O<sub>2</sub> production inducible with antimycin A.

## MATERIALS AND METHODS

*Isolation of heart and brain mitochondria.* All animal experiments were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. For preparation of heart mitochondria, a male Sprague–Dawley rat (120–140 g body weight) was euthanized, the heart excised and placed into ice-cold isolation medium (Medium



**FIG. 1.** The scheme of electron transport and superoxide production in *bc1* complex. (A) The simplified reaction scheme and sites of action of inhibitors of *bc1* complex are demonstrated. Quinol is oxidized at the  $Q_o$ -site of the complex. The oxidation occurs in a bifurcated reaction. The first electron is transferred to a high potential chain consisting of the iron sulfur protein (ISP, or Rieske protein), cytochrome  $c_1$  and cytochrome  $c$  and cytochrome oxidase (not shown), leaving a semiquinone at the  $Q_o$ -site. The semiquinone formed is unstable, and undetectable during normal turnover. This semiquinone donates the second electron to the low potential chain consisting of two cytochrome  $b$  hemes (cyt b566 and cyt b562), which serve as a pathway through which electrons are transferred from semiquinone at the  $Q_o$ -site to the  $Q_i$ -site, at which quinone is reduced to quinol. To provide the two electrons at the  $Q_i$ -site required for reduction of quinone, the  $Q_o$ -site oxidizes two equivalents of quinol in successive turnovers. The first electron at the  $Q_i$ -site generates a relatively stable semiquinone that is reduced to quinol by the second electron. Antimycin A interrupts the transfer of the second electron to  $Q_i$ -site, Stigmatellin prevents the transfer of first electron to ISP, and myxothiazol prevents the binding of quinol at the  $Q_o$ -site (see (16) for details). (B) The hypothetical mechanism of superoxide formation at the  $Q_o$ -site in the presence of antimycin A is shown. The inhibitor prevents the transfer of the second electron to  $Q_i$ -site, thus switching off the low potential chain. This leads to the accumulation of unstable semiquinone at  $Q_o$ -site and increases the probability of its side reaction with oxygen. See (11) for details.

A) composed of 225 mM mannitol, 75 mM sucrose, 5 mM Hepes (pH 7.4), 1 mM EGTA, and 1 mg ml<sup>-1</sup> BSA. Cooled heart muscle, purified from fat and tendons, was minced and homogenized for 2 min with a Teflon pestle in a glass (Pyrex) homogenizer; the ratio of tissue to isolation medium being 1 to 20 (weight to volume). After the first centrifugation (10 min, 600g), the supernatant was decanted and then centrifuged at 10,000g for 10 min. The supernatant and the light fraction of the pellet were discarded. The pellet was resuspended in 15 ml of isolation medium not containing EGTA (medium B). Mitochondria were centrifuged at 10,000g for 10 min and the

pellet was resuspended in 100  $\mu$ l of medium B. The final suspension of mitochondria (50–60 mg protein ml<sup>-1</sup>) was stored on ice.

Synaptosomal plus nonsynaptosomal rat brain mitochondria were isolated exactly as described in (18). Two rat forebrains were used for each mitochondria isolation procedure.

To estimate the functional quality of mitochondrial preparations, acceptor control index (ACI) was determined. For these experiments, incubation medium (see below) was supplemented with 1 mM MgCl<sub>2</sub>, 5 mM glutamate, and 5 mM malate. Oxygen consumption was recorded with a Clark-type oxygen electrode. The State 3 respiration was initiated by the addition of 400  $\mu$ M of ADP to the mitochondrial suspension, and was terminated with 2  $\mu$ M oligomycin, an inhibitor of the ATP synthetase. The ratio of respiration rate in State 3 to that in the presence of oligomycin (State 4) was defined as ACI. Only mitochondria with an ACI of greater than 7 were used in our experiments.

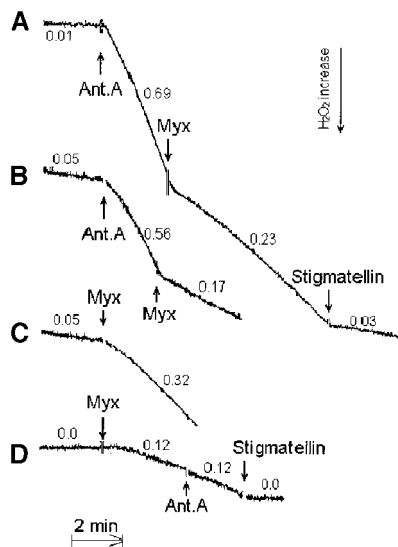
*Experimental conditions and incubation medium composition.* In all experiments, the incubation medium contained 125 mM KCl, 20 mM Hepes (pH 7.0), and 1 mM KH<sub>2</sub>PO<sub>4</sub> and was maintained at 37°C. The concentration of mitochondria was 0.25 mg protein ml<sup>-1</sup> through all the experiments.

The concentrations of myxothiazol and stigmatellin that inhibited electron flow through the *bc1* complex of the electron transport chain were determined by measuring the effect of these inhibitors on mitochondrial respiration in the presence of 5 mM succinate, 4  $\mu$ M rotenone, and 0.5  $\mu$ M FCCP. Greater than 98% inhibition of respiration was obtained at 0.20  $\mu$ M myxothiazol and 0.05  $\mu$ M stigmatellin, respectively.

*Measurement of hydrogen peroxide.* The production of reactive oxygen species was measured as H<sub>2</sub>O<sub>2</sub> production by mitochondria employing a classical scopoletin plus horseradish peroxidase method (5, 12, 14, 19, 20). The fluorescence of scopoletin was measured with a Perkin–Elmer fluorescence spectrometer. The excitation and emission wavelengths were 388 nm and 460 nm, respectively. The concentration of scopoletin was 1100 pmol ml<sup>-1</sup> and that of peroxidase was 7.5 U ml<sup>-1</sup> through all the experiments. The scopoletin fluorescence was calibrated by adding aliquots of a H<sub>2</sub>O<sub>2</sub> standard solution to the suspension of mitochondria in the absence of respiratory substrates. The concentration of commercial 30% H<sub>2</sub>O<sub>2</sub> solution was calculated from light absorbance at 240 nm employing  $E_{240}^{240} = 43.6$ ; the stock solution was diluted to 100  $\mu$ M with water and used for calibration immediately. Under the conditions used in our experiments, the relationship between H<sub>2</sub>O<sub>2</sub> concentration and scopoletin fluorescence was linear up to 800 pmol ml<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub>, and was unaffected by the presence of mitochondria. All the measurements of mitochondrial H<sub>2</sub>O<sub>2</sub> production were performed within the linear range of the scopoletin response. Hydrogen peroxide production rates are expressed as nmol H<sub>2</sub>O<sub>2</sub>  $\times$  min<sup>-1</sup>  $\times$  mg<sup>-1</sup> protein.

*NADH-ubiquinone-reductase assay.* Mitochondria (0.25 mg ml<sup>-1</sup>) were permeabilized by osmotic shock in incubation medium diluted 1:10 with distilled, deionized H<sub>2</sub>O in the presence of 80  $\mu$ M exogenous NADH and 2 mM KCN (37°C). The oxidation of NADH was initiated by the addition of 100  $\mu$ M decylubiquinone (DcQ). The decrease in NADH fluorescence was followed for 4 min, and the initial rate of NADH fluorescence decay was calculated. NADH fluorescence was calibrated by sequential additions of NADH to the medium containing mitochondria in the absence of DcQ, and the rate of NADH oxidation was expressed in nmol NADH min<sup>-1</sup> mg<sup>-1</sup> protein. No detectable NADH oxidation was observed in the absence of DcQ.

*Chemicals and reagents.* Oligomycin, myxothiazol, stigmatellin, antimycin A1, and rotenone were dissolved in ethanol, and scopoletin was dissolved in DMSO. All reagents and ethanol were tested and exhibited no interference with H<sub>2</sub>O<sub>2</sub> assay at the concentrations used in our experiments. All the reagents were purchased from Sigma (U.S.A.).



**FIG. 2.** Effects of myxothiazol and stigmatellin on  $\text{H}_2\text{O}_2$  production by heart mitochondria oxidizing various substrates. Incubation medium and other conditions were as described under Materials and Methods. Incubation medium ( $37^\circ\text{C}$ ) was supplemented with either 5 mM malate (A), 5 mM succinate (B, C), or 10 mM  $\alpha$ -glycerophosphate (D) and 25 nM FCCP (A-D) prior to the addition of isolated cardiac mitochondria ( $0.25 \text{ mg protein ml}^{-1}$ ). Hydrogen peroxide generation was monitored fluorometrically at 388 nm excitation and 460 nm emission wavelengths using the scopoletin and horseradish peroxidase method described under Materials and Methods. Other additions to the mitochondrial suspensions were  $2 \mu\text{M}$  antimycin A1 (Ant. A),  $0.2 \mu\text{M}$  myxothiazol (Myx) in A and  $1.0 \mu\text{M}$  Myx in B-D, and  $2.4 \mu\text{M}$  stigmatellin. Slope values represent rates of  $\text{H}_2\text{O}_2$  production expressed in  $\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  protein.

## RESULTS

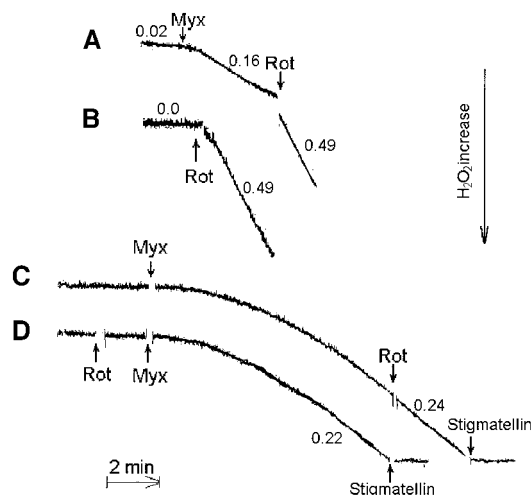
Myxothiazol has been reported to inhibit mitochondrial superoxide (21) and  $\text{H}_2\text{O}_2$  production (7, 12, 13, 20, 22). The results shown in Fig. 2 confirm and expand upon these observations. In the presence of the NADH-linked substrate malate, little  $\text{H}_2\text{O}_2$  was generated by isolated rat heart mitochondria until antimycin A was added (Fig. 2A). The subsequent addition of  $0.2 \mu\text{M}$  myxothiazol inhibited  $\text{H}_2\text{O}_2$  production by approximately 65%. An incomplete inhibition of  $\text{H}_2\text{O}_2$  production by myxothiazol at an even higher concentration of  $1.0 \mu\text{M}$  was also observed in the presence of FADH-linked substrate succinate (Fig. 2B) or  $\alpha$ -glycerophosphate (not shown). The protonophore uncoupler FCCP was present in these experiments to eliminate any contribution of reversed electron flow through Complex I to the production of  $\text{H}_2\text{O}_2$ . The residual  $\text{H}_2\text{O}_2$  production observed in the presence of myxothiazol was inhibited by the alternative Complex III inhibitor stigmatellin ( $2.4 \mu\text{M}$ ) (Figs. 2A and 2D). When the same experiments were conducted with isolated brain mitochondria, similar results were obtained (not shown).

Surprisingly, myxothiazol appeared to induce  $\text{H}_2\text{O}_2$  production when added in the absence of antimycin.

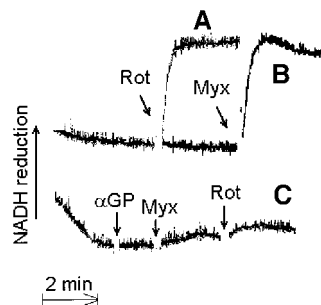
Figures 2C and 2D show that  $\text{H}_2\text{O}_2$  production was induced by the addition of myxothiazol to suspensions of heart mitochondria oxidizing succinate or  $\alpha$ -glycerophosphate. Myxothiazol-induced  $\text{H}_2\text{O}_2$  production was not affected by the subsequent addition of antimycin A but was completely inhibited by stigmatellin (Fig. 2D).

Figure 3 demonstrates that the site of  $\text{H}_2\text{O}_2$  production in the presence of myxothiazol is located in the respiratory chain downstream of Complex I. With mitochondria oxidizing malate, the Complex I inhibitor rotenone stimulates  $\text{H}_2\text{O}_2$  generation likely at the level of the iron-sulfur center at the NADH dehydrogenase (8, 11). Rotenone stimulated malate-dependent  $\text{H}_2\text{O}_2$  generation to identical rates in the presence and absence of myxothiazol (Figs. 3A and 3B). However, rotenone was without effect in the presence of the FAD-linked oxidizable substrate  $\alpha$ -glycerophosphate that donates electrons directly to ubiquinone downstream of Complex I (Fig. 3D), whereas myxothiazol induced  $\text{H}_2\text{O}_2$  production (Figs. 3C and 3D).

Figure 4 shows the effects of myxothiazol and rotenone on the level of reduction of pyridine nucleotides in mitochondria. Both rotenone and myxothiazol induced the reduction of pyridine nucleotides to almost identical level (Figs. 4A and 4B) with malate as substrate. With  $\alpha$ -glycerophosphate, pyridine nucleotides were essentially oxidized and both myxothiazol and rotenone were without effect (Fig. 4C). However the rate of  $\text{H}_2\text{O}_2$  production induced by myxothiazol was similar both with malate and with  $\alpha$ -glycerophosphate (Fig. 3).



**FIG. 3.** Effects of myxothiazol and rotenone on the rate of  $\text{H}_2\text{O}_2$  production. Incubation medium and other conditions were as in Fig. 2. Incubation medium was supplemented either with 5 mM malate (curves A and B) or with 10 mM  $\alpha$ -glycerophosphate (curves C and D) added before mitochondria. Additions: Myx,  $1 \mu\text{M}$  myxothiazol; Rot,  $2 \mu\text{M}$  rotenone; stigmatellin,  $2.4 \mu\text{M}$  stigmatellin. Slope values represent rates of  $\text{H}_2\text{O}_2$  production expressed in  $\text{nmol} \times \text{min}^{-1} \times \text{mg}^{-1}$  protein.



**FIG. 4.** Effects of myxothiazol and rotenone on the level of reduction of intramitochondrial pyridine nucleotides. Incubation medium and other conditions were as in Fig. 3. Incubation medium was supplemented either with 5 mM malate (curves A and B) or with 10 mM  $\alpha$ -glycerophosphate (curve C) added before mitochondria. Mitochondrial pyridine nucleotides fluorescence was measured at 346 nm excitation and 460 nm emission wavelengths. Additions: Myx, 1  $\mu$ M myxothiazol; Rot, 2  $\mu$ M rotenone;  $\alpha$ -GP, 10 mM  $\alpha$ -glycerophosphate.

High concentrations of both myxothiazol and a closely related analog of stigmatellin have been reported to inhibit Complex I (23). Although we used these inhibitors at significantly lower concentration, we decided to determine if there is an inhibition (if any) of Complex I under our conditions. We measured NADH-ubiquinone reductase activity of heart mitochondria as described under Materials and Methods in the presence of various concentrations of myxothiazol, stigmatellin, and rotenone. Myxothiazol produced no apparent inhibition at 0.2  $\mu$ M and 13% at 1  $\mu$ M, whereas 2.4  $\mu$ M stigmatellin was without any effect on the rate of NADH oxidation. Rotenone inhibited NADH-ubiquinone reductase by 92% at 0.4  $\mu$ M.

Figure 5 demonstrates that myxothiazol-induced production of  $H_2O_2$  exerts different dependence on the ratio of fumarate to succinate than that induced with antimycin. As expected [13], antimycin A-induced  $H_2O_2$  production curve was bell-shaped with the maximum  $H_2O_2$  generation rate at the 10:1 ratio of fumarate to succinate. However,  $H_2O_2$  production induced by myxothiazol declined continuously with an increase in fumarate/succinate ratio.

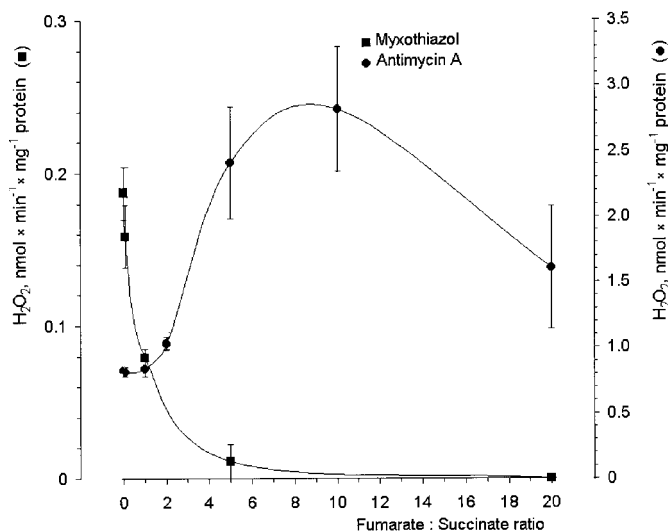
## DISCUSSION

The hypothesis that semiquinone in  $Q_o$  center is the donor of electrons for the reduction of oxygen to superoxide in bc1 complex is based on three sets of arguments. First, it has long been known (7, 24) that quinone of inner mitochondrial membrane is necessary for antimycin A-induced superoxide production in bc1 complex. Second, the effects of specific  $Q_i$  site inhibitor antimycin and the inhibitor of  $Q_o$  site myxothiazol on the superoxide production may be best explained in the framework of the "classical" Q-cycle model of electron transport in bc1 complex, as it was demonstrated in several studies (7, 12, 13). According to this model,

antimycin increases the steady-state concentration of unstable semiquinone at center  $Q_o$  by inhibiting its oxidation by cytochromes *b* (Fig. 1B). Myxothiazol inhibits semiquinone formation at center  $Q_o$  by simply displacing quinol at its binding site (Fig. 1A; see (11, 15)).

Therefore, antimycin A should, and was repeatedly shown to stimulate superoxide production in bc1 complex, whereas myxothiazol should prevent and inhibit it. Indeed, myxothiazol was reported to inhibit superoxide production in mammalian mitochondria (7, 12, 13, 20–22). However, in the study of Turrens *et al.* (12), myxothiazol did not completely suppress the rate of antimycin-induced  $H_2O_2$  production. These results are therefore similar to those reported here (Fig. 2B). The aspect of our results that is significantly different from those previously reported is that myxothiazol not only incompletely inhibits  $H_2O_2$  production but actually partially stimulates  $H_2O_2$  generation. Our finding that antimycin does not affect  $H_2O_2$  production when added after myxothiazol (Fig. 2D) indicates that myxothiazol stimulates  $H_2O_2$  production at a site proximal to the site in electron flow that is inhibited by antimycin. These results are not unique to heart mitochondria as similar effects of both myxothiazol and stigmatellin were also observed with isolated brain mitochondria.

Earlier, myxothiazol was shown to stimulate  $H_2O_2$  production in insect mitochondria, where  $\alpha$ -glycerophosphate dehydrogenase and not bc1 complex was



**FIG. 5.** Redox dependence of  $H_2O_2$  production in rat heart mitochondria. Incubation medium and other conditions were as in Fig. 2. Incubation medium was supplemented with 2 mM succinate and various (0–40 mM) concentrations of fumarate. After 2 min of incubation, mitochondria were permeabilized with alamethicin (80  $\mu$ g/mg protein). Myxothiazol (0.5  $\mu$ M) or antimycin A1 (2  $\mu$ M) was added 3 min after alamethicin, and  $H_2O_2$  production was measured as described under Materials and Methods. Data points represent an average from 3–4 experiments and the error bars show standard errors.

postulated to be the site of H<sub>2</sub>O<sub>2</sub> production (25, 26). Moreover, myxothiazol was recently shown to inhibit Complex I (23). The ability of myxothiazol to inhibit Complex I can potentially complicate interpretation of results as Complex I is apparently also a source of ROS production (11). Several observations indicate that Complex I is not the source of H<sub>2</sub>O<sub>2</sub> production observed in the presence of myxothiazol in our experiments. Myxothiazol-induced H<sub>2</sub>O<sub>2</sub> production was observed with substrates for succinate and  $\alpha$ -glycerophosphate dehydrogenases (Figs. 2 and 3), enzymes that have not been reported to be affected by myxothiazol. Furthermore, in our experiments myxothiazol induced H<sub>2</sub>O<sub>2</sub> production at concentrations that have negligible effects on Complex I (NADH-ubiquinone reductase) activity. In addition, the observation that the Complex I inhibitor rotenone stimulates malate-dependent H<sub>2</sub>O<sub>2</sub> in the presence of myxothiazol (Fig. 3) indicates that the site of H<sub>2</sub>O<sub>2</sub> production in Complex I is located upstream of that induced by myxothiazol. Finally, Additional evidence favoring localization of myxothiazol-induced H<sub>2</sub>O<sub>2</sub> production to the bc1 complex is provided by the observation that stigmatellin completely inhibited H<sub>2</sub>O<sub>2</sub> production induced by either myxothiazol (Figs. 2 and 3) or antimycin. This inhibitor can be considered to be specific toward center Q<sub>o</sub> of bc1 complex (15).

Data presented in Fig. 5 indicate that the electron donor for myxothiazol-induced H<sub>2</sub>O<sub>2</sub> production may be different than the semiquinone accumulated in the presence of antimycin A. It was previously shown with submitochondrial particles that superoxide and H<sub>2</sub>O<sub>2</sub> production in the presence of antimycin A depend on the redox poise of the respiratory chain, as commonly established by adjusting the ratio of the concentrations of fumarate:succinate. Antimycin-induced ROS production curve was bell-shaped with the maximum observed at a 5:1 fumarate to succinate ratio (13). We also obtained a bell-shaped curve for H<sub>2</sub>O<sub>2</sub> production in the presence of antimycin (Fig. 5), however, at a slightly higher 10:1 fumarate:succinate ratio. Under the same experimental conditions, the dependence of myxothiazol-induced H<sub>2</sub>O<sub>2</sub> production on the ratio of fumarate to succinate was clearly different, with the rate of production declining continuously with the increase in the ratio (Fig. 5).

In summary, our results indicate that the site of H<sub>2</sub>O<sub>2</sub> production in the presence of myxothiazol is the Q<sub>o</sub> site of the bc1 complex. According to the most well supported hypothesis on the organization of electron transport and binding of inhibitors and quinone within the bc1 complex (16), semiquinone cannot exist at this center. Hence, semiquinone may not formally be considered as a source of ROS without making several *ad hoc* assumptions about structure and functioning of Q<sub>o</sub> site and/or the site of binding of myxothiazol. Therefore, although the presented data allow us to assign this new H<sub>2</sub>O<sub>2</sub> producing site to center Q<sub>o</sub> of bc1 com-

plex, the nature of electron donor for H<sub>2</sub>O<sub>2</sub> production at Q<sub>o</sub> site has yet to be revealed.

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