THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 31, pp. 21649–21654, August 1, 2008

The Mechanism of Mitochondrial Superoxide Production by the Cytochrome bc_1 Complex*

Received for publication, April 28, 2008 Published, JBC Papers in Press, June 3, 2008, DOI 10.1074/jbc.M803236200

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Production of reactive oxygen species (ROS) by the mitochondrial respiratory chain is considered to be one of the major causes of degenerative processes associated with oxidative stress. Mitochondrial ROS has also been shown to be involved in cellular signaling. It is generally assumed that ubisemiquinone formed at the ubiquinol oxidation center of the cytochrome bc_1 complex is one of two sources of electrons for superoxide formation in mitochondria. Here we show that superoxide formation at the ubiquinol oxidation center of the membrane-bound or purified cytochrome bc_1 complex is stimulated by the presence of oxidized ubiquinone indicating that in a reverse reaction the electron is transferred onto oxygen from reduced cytochrome b_L via ubiquinone rather than during the forward ubiquinone cycle reaction. In fact, from mechanistic studies it seems unlikely that during normal catalysis the ubisemiquinone intermediate reaches significant occupancies at the ubiquinol oxidation site. We conclude that cytochrome bc_1 complex-linked ROS production is primarily promoted by a partially oxidized rather than by a fully reduced ubiquinone pool. The resulting mechanism of ROS production offers a straightforward explanation of how the redox state of the ubiquinone pool could play a central role in mitochondrial redox signaling.

The mitochondrial respiratory chain is not only the main source of ATP in eukaryotic cells, but it is also responsible for the production of deleterious reactive oxygen species (ROS)[2] (1). ROS have been implicated in apoptosis, cellular injury during ischemia and reperfusion, and the aging process as well as in the pathophysiology of several neurodegenerative diseases including Parkinson, Huntington, and Alzheimer diseases (2, 3). More recently, it has been recognized that ROS from mitochondrial sources is also involved in cellular signaling (4). Within the respiratory chain, complex I (NADH:ubiquinone oxidoreductase) and the cytochrome bc_1 complex (complex III, ubiquinol:cytochrome *c* oxidoreductase) were identified as the main sources of superoxide anion radicals ($O_2^{\cdot-}$; Fig. 1). In complex I, superoxide was shown to be produced primarily by the oxidation of reduced flavine or flavine semiquinone (5, 6).

It has been shown a long time ago (7, 8) that superoxide is formed at the ubiquinol oxidation center (Q_o site, center P) of the cytochrome bc_1 complex. The rate of superoxide formation is strongly increased under conditions of so-called oxidant-induced reduction, *i.e.* in the presence of the specific center N inhibitor antimycin A, sufficient amounts of reducing equivalents, and an oxidized downstream respiratory chain. According to the general scheme of the protonmotive Q cycle operating in the cytochrome bc_1 complex, ubisemiquinone is formed during ubiquinol oxidation (9). However, more recent mechanistic studies aimed at understanding the strict control of the Q cycle suggest that the bifurcated ubiquinol oxidation at center P occurs in a quasiconcerted reaction (10–13). In fact, formation of semiquinone ($Q^{\cdot-}$) associated with center P was difficult to show experimentally, and the occupancy for this redox intermediate generated under special conditions is rather low (14–16). This implies that ubisemiquinone is formed only very transiently at center P, if at all, and never accumulates to significant amounts in the functional enzyme. Therefore, the assumption generally adopted in the ROS field that oxygen reacts with semiquinone formed as a reaction intermediate of the protonmotive Q cycle to form superoxide is probably not suffcient. Here we show with membrane-bound and purified detergent-solubilized enzyme that superoxide formation is maximal at center P of the cytochrome bc_1 complex if the ubiquinone pool is partially oxidized. This implies a mechanism by which superoxide is formed in a reverse reaction via oxidation of reduced heme b_L (high potential cytochrome *b*) and with ubiquinone acting as a redox mediator.

EXPERIMENTAL PROCEDURES

Sample Preparation—Submitochondrial particles (SMPs) from bovine heart mitochondria were prepared as described by Okun *et al.* (17). The preparation used in the experiments had a protein concentration of 26.8 mg/ml, and the heme content was 17.2 μM cytochrome *b* and 19.8 μM heme a + a_3. Cytochrome bc_1 complex was purified from bovine heart mitochondria as described by Engel *et al.* (18). The concentration of cytochrome *b* was determined spectroscopically using $\epsilon_{562-575} = 28.5\ mM^{-1}\ cm^{-1}$. Protein concentrations were determined according to a modified Lowry protocol. The reduced form of decylubiquinone (DBH) was produced by dithionite reduction of the oxidized form (DBQ) according to a protocol of Wan *et al.* (19).

* This work was supported by the Cluster of Excellence EXC115 "Macromolecular Complexes" of the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: ROS, reactive oxygen species; cytochrome b_H, high potential cytochrome *b*; cytochrome b_L, low potential cytochrome *b*; DBH, *n*-decylubiquinol; DBQ, *n*-decylubiquinone; HRP, horseradish peroxidase; Q, ubiquinone; SMP, submitochondrial particle; SOD, superoxide dismutase.

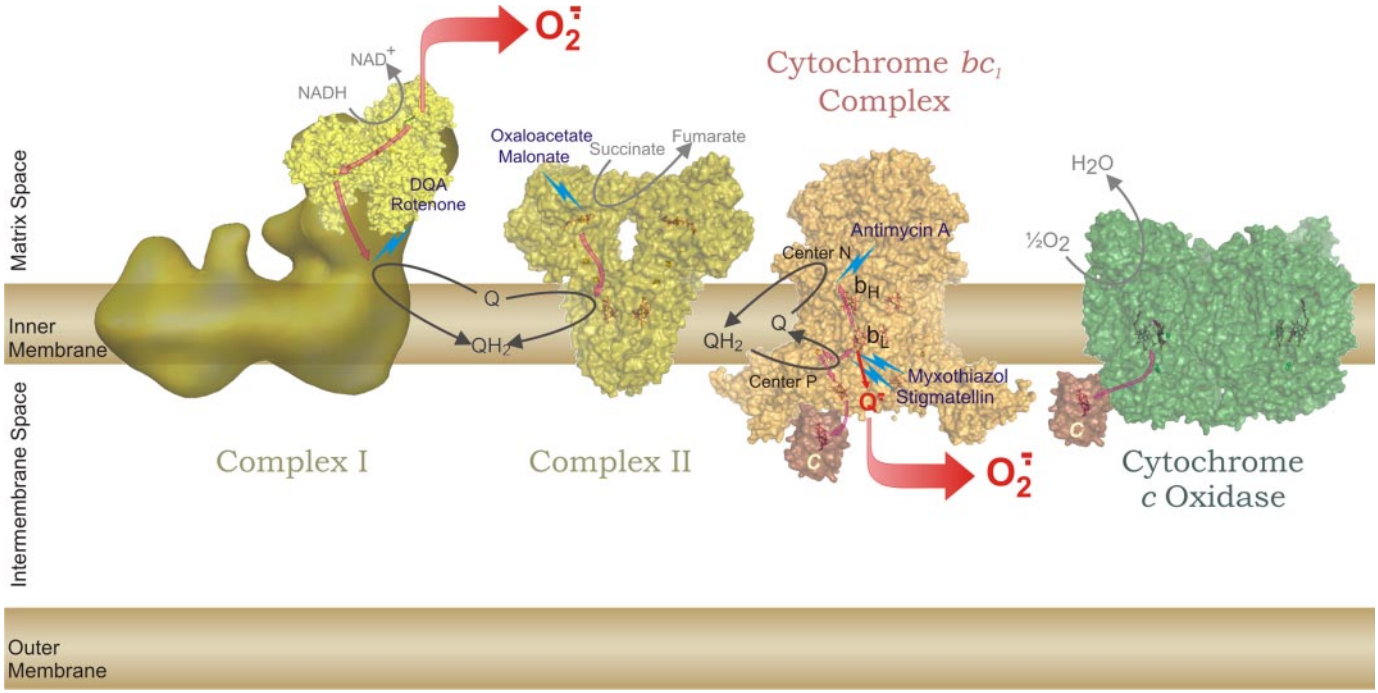

FIGURE 1. **Superoxide generation by the respiratory chain.** Complex I and the cytochrome bc_1 complex of the mitochondrial respiratory chain are generally regarded as the main sources of superoxide anion radicals. In complex I, superoxide is primarily produced at the bound flavine facing the matrix side, whereas in the cytochrome bc_1 complex superoxide is formed at the ubiquinol oxidation site (Q_o site, center P) facing the intermembrane space. The sites where the inhibitors bind that are mentioned in the text are indicated. b_L, heme b_L; b_H, heme b_H; *c*, cytochrome *c*; QH_2, ubiquinol.

The concentration of the ethanolic solution was determined spectrophotometrically using ϵ_{290}: 4.2 mM^{-1} cm^{-1}.

Measurement of ROS Production—ROS were detected by the Amplex Red/HRP (horseradish peroxidase) assay (Molecular Probes, Leiden, Netherlands) in a SpectraMax Plus microplate reader (Molecular Devices) at 30 °C (20). 0.1 mg/ml SMPs were used in a reaction mixture containing 50 μM Amplex Red, 0.1 unit/ml HRP, 75 mM sodium phosphate, pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$, and (where necessary) 100 units/ml superoxide dismutase (Cu,Zn-SOD).

ROS production of purified cytochrome bc_1 complex was measured at the isosbestic point of cytochrome *c* at 540 nm in the presence of 100 units/ml SOD, 50–100 μM cytochrome *c*, and 200 μM DBH. In some experiments, the oxidation level of the Q pool was adjusted by the application of known mixtures of DBH and DBQ at a total ubiquinone concentration of 200 μM. For activation, the enzyme was mixed with 75% phosphatidylcholine (99%, Sigma type III-E), 20% phosphatidylethanolamine (98%, Sigma type IV-S), and 5% cardiolipin (>80%, bovine heart) dissolved in 1.7% sodium cholate and 2.4% *n*-octyl-β-D-glucopyranoside at a molar ratio of about 1:3000. Because the ϵ_{563} could not be used for the calculation of the H_2O_2 generation, the assay was calibrated with known H_2O_2 concentrations.

Inhibition of Succinate Oxidase—Inhibition of succinate oxidase by malonate and oxalacetate was determined at 25 °C with an Oxygraph-2k (Oroboros), chamber volumes of 2 ml, using 54 μg of SMP and 5 mM succinate in 75 mM sodium phosphate pH 7.4, 1 mM EDTA, 1 mM $MgCl_2O$.

Statistics—All experiments were performed in triplicate and data are given ±S.D.

RESULTS

Partial Inhibition of Complex II Stimulates Complex III-dependent ROS Production—First we analyzed the effect of different respiratory chain inhibitors on the production of ROS by submitochondrial particles prepared from bovine heart mitochondria (Fig. 2). We employed the HRP/Amplex Red assay to monitor ROS generation. This assay detects hydrogen peroxide (H_2O_2) that is formed by spontaneous dismutation of superoxide, the primary ROS species generated by the electron transfer chain. The addition of SOD to the assay had almost no effect on the rate of H_2O_2 generation indicating that the SMP preparation contained a sufficient amount of endogenous SOD (21). Yet, if NADH was used as a substrate, it was necessary to add SOD to reduce the substrate-dependent background rate of the assay.

With NADH-consuming SMP (Fig. 2*A*), the basal rate of H_2O_2 generation was increased approximately 2-fold following the addition of the complex I inhibitors *n*-decylquinazolineamine or rotenone due to increased ROS production by complex I. When we added antimycin A, an inhibitor of center N of the cytochrome bc_1 complex, ROS production was more than 4 times faster than the basal rate. Addition of the center P inhibitor stigmatellin alone or in the presence of antimycin A reduced the rate back to the "rotenone level." Also addition of rotenone abolished most of the increase induced by antimycin A. Inhibition of center P-linked ROS production was significantly less efficient with myxothiazol than with stigmatellin (not shown). This is in agreement with recent findings that the bc_1 complex can produce some superoxide in the presence of the proximal center P inhibitors like myxothiazol, whereas dis-

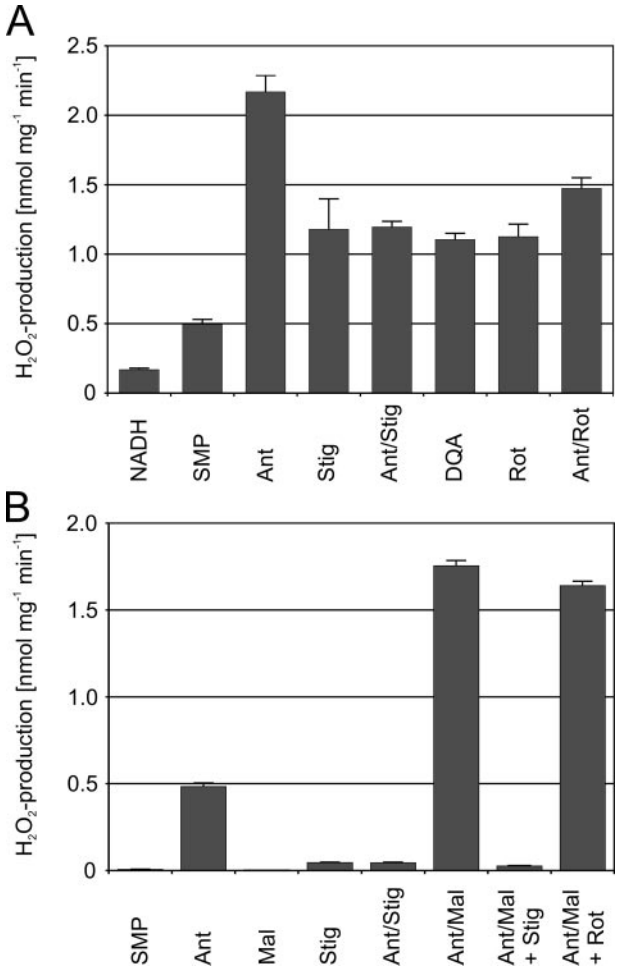

FIGURE 2. **Effect of different respiratory chain inhibitors on the mitochondrial ROS production.** The respiratory chain of bovine heart mitochondria was fueled by either 0.5 mM NADH (*A*) or 10 mM succinate (*B*), and the H_2O_2 production was determined in the absence (*SMP*) or presence of different complex I (*Rot* = 5 μM rotenone, 5 μM *n*-decylquinazolineamine (*DQA*)), complex II (*Mal* = 2.5 mM malonate), or complex III (*Stig* = 10 μM stigmatellin; *Ant* = 10 μM antimycin A) inhibitors. In *A*, the NADH-dependent background (no SMP) of the Amplex Red/HRP assay is also shown.

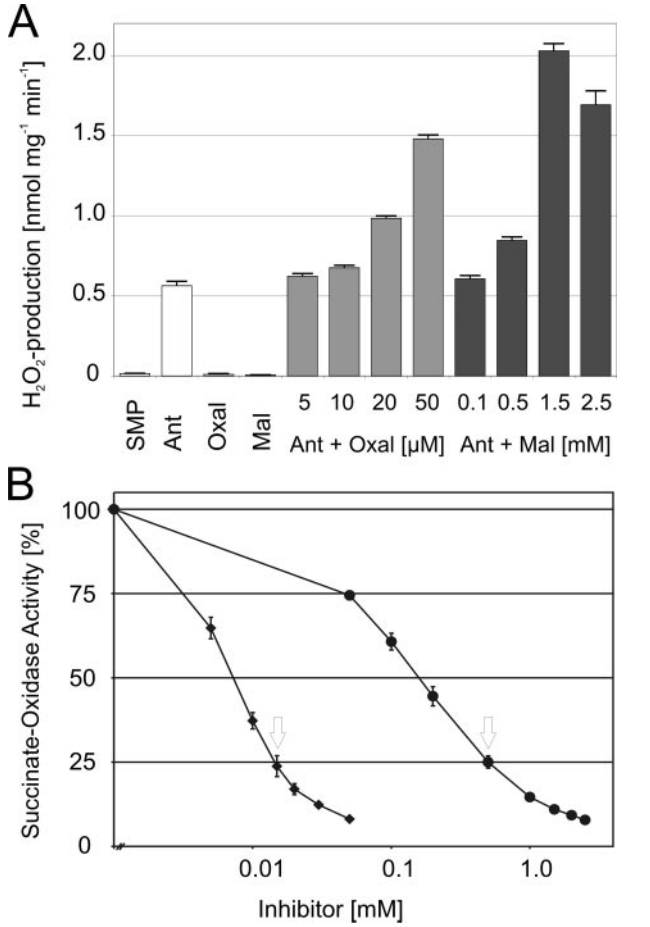

FIGURE 3. **Effect of complex II inhibitors on the antimycin-induced ROS production.** *A*, SMPs were fueled with succinate. The effect of different concentrations of the complex II inhibitors (malonate (*Mal*); oxalacetate (*Oxal*)) in the absence or presence of 10 μM antimycin A (*Ant*) was analyzed. Surprisingly, inhibition of complex II promoted complex III-dependent H_2O_2 production. 50 μM oxalacetate and 2.5 mM malonate alone had no effect. *B*, inhibition of the succinate oxidase activity of SMPs by malonate (●) and oxalacetate (◆), respectively. Approximate threshold concentrations for increased ROS production is marked by *arrows*.

tal center P inhibitors like stigmatellin completely abolish ROS generation (22). It should be noted that in contrast to recently published results by Kudin *et al.* (23), we did not observe inhibition of the horseradish peroxidase by stigmatellin (not shown).

Succinate-dependent ROS generation was negligible in the absence of respiratory chain inhibitors (Fig. 2*B*) and complex I inhibitors like rotenone (not shown), and the complex II inhibitor malonate (Fig. 2*B*) had no effect. In contrast, antimycin A markedly stimulated superoxide production (Fig. 2*B*). We could assign this ROS production unambiguously to the cytochrome bc_1 complex, because it was almost fully suppressed by the addition of the center P inhibitor stigmatellin. Unexpectedly, however, succinate-dependent ROS production in the presence of antimycin A was increased more than 3-fold by the complex II inhibitor malonate (Fig. 2*B*). This increase was abolished by the addition of stigmatellin but was essentially unaffected by the complex I inhibitor rotenone (Fig. 2*B*) suggesting that still all ROS was produced by center P under these conditions. However, a marked increase of ROS production was observed if approximately 75% of the succinate oxidase activity was inhibited by malonate or oxalacetate, another inhibitor of complex II (Fig. 3). As direct involvement of complex II and reverse electron flow into complex I could be excluded, it seemed that partially oxidizing the ubiquinone pool by limiting its re-reduction by complex II stimulated ROS production by center P of the cytochrome bc_1 complex.

Maximal ROS Production by the Cytochrome bc_1 Complex Is Observed if the Q Pool Is Partially Oxidized—To test whether indeed partial oxidation of the ubiquinone pool stimulated ROS production we next measured the dependence of the ROS production by antimycin-inhibited cytochrome bc_1 complex on the ubiquinone/ubiquinol ratio. With DBH the rate of antimycin-induced ROS generation by SMP was much higher than with NADH or succinate as substrates but was also efficiently suppressed by center P inhibitors (Fig. 4*A*). When we analyzed the intial rates of ROS production with a preset ubiquinol: ubiquinone ratio, we found that the rate of ROS production increased proportionally until a maximal value was reached at

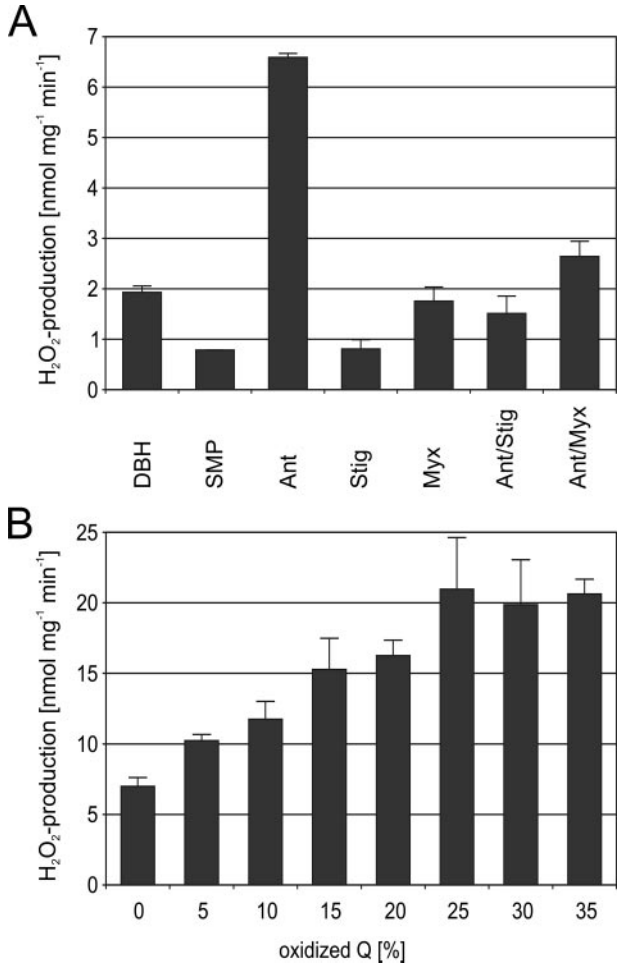

FIGURE 4. **ROS generation at the Q_o site of membrane-bound complex III is increased by a partially oxidized Q pool.** *A*, ROS generation of SMPs with 100 μM DBH as substrate in the presence of different complex III inhibitors (*Myx* = 10 μM myxothiazol, for other additions see Fig. 2). *B*, initial rates of H_2O_2 generation of DBH-fueled, antimycin-inhibited SMPs at different DBH:DBQ ratios (total concentration was kept constant at 100 μM). The initial rate is maximal, when the Q pool is 25–30% oxidized.

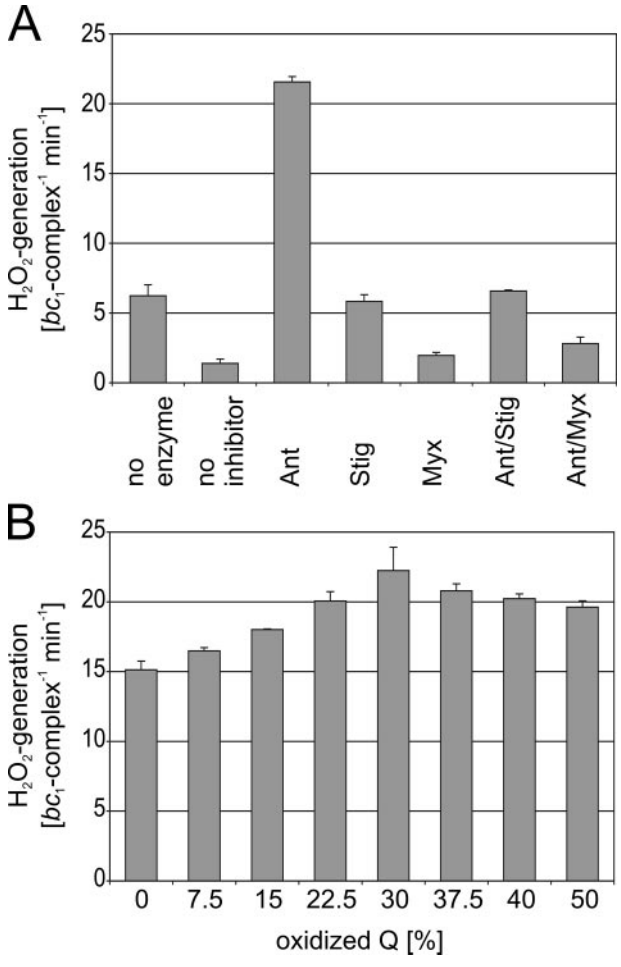

FIGURE 5. **H_2O_2 generation of lipid-activated purified bc_1 complex.** *A*, H_2O_2 generation of lipid-activated purified bc_1 complex fueled by 200 μM DBH in the presence of center P and center N inhibitors. *B*, H_2O_2 generation of antimycin-inhibited bc_1 complex at different DBH:DBQ ratios (total concentration of 200 μM, % oxidized refers to DBQ). As with SMPs, a partially oxidized Q pool promoted ROS production. In this presentation, the unspecific background was subtracted from each column.

about 25–30% ubiquinone (Fig. 4*B*). This maximal rate was 3 times the rate observed with DBH alone.

To exclude any interference from other mitochondrial components, we finally tested whether we could observe the same dependence on the DBQ/DBH ratio with purified lipid-reactivated cytochrome bc_1 complex. The presence of detergent resulted in a higher background activity, but still most of the ROS production was abolished by center P inhibitors. The residual rate was consistently somewhat higher with stigmatellin than in the presence of myxothiazol, but this clearly depended on the presence of cytochrome *c*. We observed an antimycin A-induced increase in superoxide production that was reduced to background levels by center P inhibitors (Fig. 5*A*). The reduction of the rate by center P inhibitors c indicated that as in SMP the antimycin-related increase was mainly due to superoxide production at the ubiquinol oxidation side. The maximal rates of ~0.35 H_2O_2 s^{-1} or 0.7 $O_2^{\cdot-}$ s^{-1} were in good agreement with the antimycin-induced superoxide generation of ~1 s^{-1} by purified cytochrome bc_1 complexes determined by other groups (22, 24, 25). Sun and Trumpower (26) and Forquer *et al.* (27) found higher rates, but these results might be misleading because the method used by Sun and Trumpower (cytochrome *c* reduction ± SOD) even gave high rates for the uninhibited complexes from yeast and bovine heart, whereas Forquer *et al.* used SMPs, in which a contribution of complex I-dependent ROS production to the antimycin-induced rate seems likely (see Fig. 2*A*). It should be noted that the high cytochrome *c* concentrations, a known superoxide scavenger, required in this assay also may have lead to some underestimation of the ROS production rates. At any rate, confirming our results with SMP, the antimycin-induced ROS production of purified bc_1 complex increased with the DBQ/DBH ratio (Fig. 5*B*). Again, maximal stimulation of the initial rate was observed at a DBQ content of about 25%. In contrast, a further decrease of the already low basal rate of ROS generation by the uninhibited or myxothiazol-inhibited cytochrome bc_1 complex was observed with increasing DBQ concentrations (not shown).

DISCUSSION

Analyzing the mechanism of superoxide formation at center P of the cytochrome bc_1 complex is prerequisite for understanding the importance of this reaction in mitochondrial oxi-

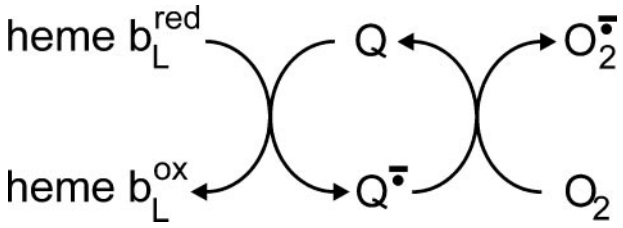

FIGURE 6. **Proposed mechanism of superoxide formation at center P of the cytochrome bc_1 complex by reverse electron transfer from heme b_L.**

dative stress and redox signaling. Here we show that the rate of ROS production by cytochrome *bc*1 complex is maximal if the ubiquinone pool is partially oxidized. Moving from a fully reduced to a 25% oxidized ubiquinone pool resulted in an approximately 3-fold increase in superoxide formation by membrane-bound cytochrome bc_1 complex in the presence of the center N inhibitor antimycin A. Consistent with early observations that ROS production in the presence of antimycin A is maximal only in the presence of malonate (28), we also observed a 3- to 4-fold stimulation of succinate-supported ROS generation if we inhibited >75% complex II activity by the addition of oxalacetate or malonate. We propose that oxidized ubiquinone supports superoxide formation at center P by acting as a redox mediator to transfer electrons from reduced heme b_L onto oxygen in a reverse reaction of center P (Fig. 6).

This proposal is quite different from the generally assumed mechanism of ROS formation according to which the semiquinone intermediate is the electron donor for oxygen that is formed during normal turnover at center P of the cytochrome bc_1 complex. It has to be stressed however that it remains unclear whether ubiquinol oxidation at center P occurs via a sequential mechanism with semiquinone as a true intermediate (12) or a concerted mechanism avoiding semiquinone formation during normal turnover (10, 11, 29). Recently direct detection of center P semiquinone radicals by EPR spectroscopy in the antimycin-inhibited complex at high pH (16) and in cytochrome bc_1 complex from a heme b_H knock-out mutant (15) were reported. Although these findings indicate that center P can be tweaked to form and stabilize semiquinone at low occupancy, they do not prove that this semiquinone is a genuine intermediate during normal turnover, nor do they show that it is the reductant for molecular oxygen responsible for superoxide generation of the cytochrome bc_1 complex. Although this is conceded by Zhang *et al.* (15), Kramer and colleagues argue that a semiquinone is in fact a transient intermediate during normal turnover that is destabilized under normal conditions to limit ROS generation (16, 27). When judging the significance of the finding that a semiquinone can be detected in center P under very special conditions, it should be noted that our results show that the basal rate of ROS production was much higher and the stimulatory effect by oxidized ubiquinone was much less pronounced when we used purified cytochrome bc_1 complex (Fig. 5). This may suggest that other mechanisms like the direct electron transfer from a semiquinone onto oxygen may contribute much more to ROS formation in the detergent-solubilized state with less hydrophobic quinone analogues than if the reaction were studied in the native environment of the mitochondrial membrane in the presence of endogenous ubiquinone 10 (Figs. 3 and 4).

At any rate, even if a semiquinone intermediate at center P formed during normal turnover may act as a source of electrons for superoxide formation, the occupancy of this state and thereby the resulting rate of ROS production is expected to be significant only under conditions of the so-called "oxidant-induced reduction" (30), *i.e.* a highly reduced ubiquinone pool, a largely oxidized downstream respiratory chain, and in the absence of antimycin A, a very high membrane potential. Although such a scenario may be considered for the rather extreme pathological condition, when tissues experience ischemia followed by reperfusion, it is difficult to envision other physiological situations meeting these criteria, especially for the generation of ROS involved in redox signaling. A very high membrane potential for example would inevitably slow down cytochrome *c* oxidase resulting in a high reduction state for cytochrome *c*, thus counteracting ROS production at center P by this mechanism.

If most of the superoxide would be formed by reverse electron transfer at center P as proposed here, maximal rates of ROS production are expected under quite different conditions, namely with a partially oxidized Q pool to provide sufficient amounts of the mediator and a moderately high membrane potential to increase the fraction of reduced heme b_L. This is a physiological scenario that seems much more typical for active mitochondria. Thus it seems likely that *in vivo* most of the superoxide formation at center P of the cytochrome bc_1 complex occurs via the reverse electron mechanism proposed here.

In fact, the redox state of the ubiquinone pool varies considerably depending on the organ analyzed over the entire range from mostly oxidized to almost fully reduced (31) offering an explanation as to why different tissues are affected differentially by mitochondrial ROS production. This is of particular interest because ROS production by the cytochrome bc_1 complex has been implicated to play a major role in hypoxia-induced redox signaling and hypoxia-inducible factor 1α stabilization (4, 32). In organs like lung, brain, and heart, where the ubiquinone pool is normally too oxidized for efficient ROS generation, already moderate oxygen limitation could directly increase the fraction of reduced ubiquinone to the critical level of about 75% ubiquinol. This would result in a burst of ROS production setting off the hypoxia defense signaling cascade, thus providing a mechanism for hypoxia-induced cardiac preconditioning (33). Indeed exposure of rats to systemic mild hypoxia for several hours has been shown to markedly increase the fraction of oxidized ubiquinone (34). A similar adaptive response has also been shown to occur in liver, kidney, and testis, although in these organs the state of ubiquinone reduction is under normal conditions far above the level required for maximal ROS production (34). In these tissues, adaptation could be mediated by regulatory mechanisms acting at the level of cytochrome *c* oxidase; it has been shown for astrocytes that hypoxia induces a shift between isoform 1 and isoform 2 of subunit IV of cytochrome oxidase abolishing allosteric inhibition by ATP (35). This could lower the level of ubiquinol reduction to the critical level of approximately 75% ubiquinol, whereas under normal conditions limitation of the electron flow through the respira-

tory chain at the level of cytochrome *c* oxidase would keep the ubiquinone pool in a highly reduced state.

It follows from these scenarios that it would depend on the Q pool redox state of a given tissue under normal conditions whether the ROS response is induced by increasing or decreasing the fraction of reduced ubiquinone until it reaches the critical level of 75% ubiquinol. Although further studies will be needed to prove and understand theses regulatory scenarios in detail, it seems clear that the proposed mechanism of cytochrome bc_1 complex-dependent ROS production via modulation of the redox state of the ubiquinone pool could contribute significantly to the control of mitochondrial redox signaling.

Acknowledgments—We thank Ilka Siebels for excellent technical assistance and Hermann Schägger and Ilka Wittig for critically reading the manuscript.

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