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Superoxide anion generation by the cytochrome bc_1 complex

Jian Sun and Bernard L. Trumpower*

Department of Biochemistry, Dartmouth Medical School, 7200 Vail, Hanover, NH 03755, USA

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Abstract

We have measured the rates of superoxide anion generation by cytochrome bc_1 complexes isolated from bovine heart and yeast mitochondria and by cytochrome bc_1 complexes from yeast mutants in which the midpoint potentials of the cytochrome *b* hemes and the Rieske iron–sulfur cluster were altered by mutations in those proteins. With all of the bc_1 complexes the rate of superoxide anion production was greatest in the absence of bc_1 inhibitor and ranged from 3% to 5% of the rate of cytochrome *c* reduction. Stigmatellin, an inhibitor that binds to the ubiquinol oxidation site in the bc_1 complex, eliminated superoxide anion formation, while myxothiazol, another inhibitor of ubiquinol oxidation, allowed superoxide anion formation at a low rate. Antimycin, an inhibitor that binds to the ubiquinone reduction site in the bc_1 complex, also allowed superoxide anion formation and at a slightly greater rate than myxothiazol. Changes in the midpoint potentials of the cytochrome *b* hemes had no significant effect on the rate of cytochrome *c* reduction and only a small effect on the rate of superoxide anion formation. A mutation in the Rieske iron–sulfur protein that lowers its midpoint potential from +285 to +220 mV caused the rate of superoxide anion to decline in parallel with a decline in cytochrome *c* reductase activity. These results indicate that superoxide anion is formed by similar mechanisms in mammalian and yeast bc_1 complexes. The results also show that changes in the midpoint potentials of the redox components that accept electrons during ubiquinol oxidation have only small effects on the formation of superoxide anion, except to the extent that they affect the activity of the enzyme.
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Keywords: Superoxide anion; Cytochrome bc_1 complex; Mitochondria; Midpoint potential; Antimycin; Stigmatellin; Myxothiazol

Respiration is linked to the generation of reactive oxygen species, which have been implicated in the aging process as well as in a variety of pathological conditions [1–4]. Mitochondria have long been considered as the major site of intracellular reactive oxygen radical generation [5,6]. The superoxide anion radical, $O_2^{\cdot -}$, is the first molecular species in the univalent pathway of oxygen reduction, and is predominantly generated in the mitochondrial electron transport chain by the autoxidation of a semiquinone species [7–10].

There are two principal sites in the mitochondrial electron transport chain that are responsible for the partial reduction of oxygen to superoxide anion. One is located in the NADH[1] dehydrogenase complex, which probably produces superoxide anion through autoxidation of the flavin semiquinone of NADH dehydrogenase [11]. The other site is located in the cytochrome bc_1 complex, which produces ubisemiquinone [11].

Most previous studies concerning mitochondrial superoxide anion generation have been conducted with intact mitochondria or sub-mitochondrial particles. The quantitative analysis of the superoxide anion radical produced in mitochondria or sub-mitochondrial particles is restricted by the inaccessibility of the organelle interior to most compounds normally used to detect superoxide anion and by the instability of the radical itself. In addition, the information on superoxide anion generation obtained from intact mitochondria is somewhat ambiguous due to manganese superoxide dismutase in mitochondria.

To understand the parameters that affect superoxide anion formation by the cytochrome bc_1 complex, we have examined superoxide anion generation in purified cytochrome bc_1 complexes from bovine heart mito-

* Corresponding author. Fax: 1-603-650-1389.
E-mail address: trumpower@dartmouth.edu (B.L. Trumpower).

[1] *Abbreviations used:* ROS, reactive oxygen species; DFP, diisopropylfluorophosphate; SOD, superoxide dismutase; CuZnSOD, copper–zinc superoxide dismutase; MnSOD, manganese superoxide dismutase.

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chondria, wild type yeast, and yeast with mutations in cytochrome *b* and the Rieske iron–sulfur protein. We report that the enzymes from these two species appear to form superoxide anion by similar mechanisms. We also show that superoxide anion formation is not significantly altered by changes in the midpoint potentials of the redox components that accept electrons during the bifurcated oxidation of ubiquinol, except to the extent that the changes in midpoint potentials cause changes in activity of the enzyme.

Experimental procedures

Materials

Yeast extract and peptone were from Difco. Nitrogen base without amino acids but with ammonium sulfate was from US Biological. Dodecylmaltoside was obtained from Roche Molecular Biochemicals. DEAE-Biogel A was obtained from Bio-Rad Laboratories. Diisopropylfluorophosphate, decyl ubiquinone, dithionite, catalase from bovine liver, copper–zinc superoxide dismutase from bovine erythrocytes, manganese superoxide dismutase, antimycin, myxothiazol, and horse heart cytochrome *c* were purchased from Sigma. Stigmatellin was from Fluka. Rabbit polyclonal antibodies to yeast MnSOD were a gift from Dr. Val Culotta (Johns Hopkins University).

Purification of cytochrome bc_1 complexes

Bovine heart mitochondria were a gift from Dr. C. A. Yu (Oklahoma State University). The yeast strains containing mutations in the cytochrome *b* gene, BE924/76 and W30C, and the KM91 parental strain were obtained from Dr. Anne-Marie Colson (Université Catholique de Louvain La Neuve, Belgium). These yeast were grown in 80 liters of yeast extract/peptone/dextrose medium. The *rip1* deletion yeast strain [12] complemented with a wild-type copy of the *RIP1* gene and the same strain complemented with the *RIP1* gene carrying a *Y185F* mutation [13] were grown in 80 liters of tryptophan dropout minimal medium plus dextrose.

Yeast cells were harvested by centrifugation and washed once with distilled water and once with disruption buffer (100 mM Tris, 150 mM potassium acetate, 5 mM $MgCl_2$, 250 mM sorbitol, and 1 mM dithiothreitol, pH 8.0). The washed yeast cells were resuspended with disruption buffer to obtain a thick suspension and frozen by slowly pouring the suspension as a thin stream into liquid nitrogen. The frozen yeast cells were blended in liquid nitrogen in a stainless steel blender for a total of five times at 1 min intervals.

The cell powder was thawed in a warm water bath with the addition of 1 mM DFP and centrifuged at 3000*g* for 15 min. The supernatant was collected and the pellet was washed once with disruption buffer and sedimented again at 3000*g* for 15 min. The supernatants were combined and centrifuged at 20,000*g* for 30 min. The mitochondrial membranes were washed twice with 50 mM Tris acetate, 400 mM mannitol, and 2 mM EDTA, pH 7.4, and then washed once with 50 mM Tris acetate, 150 mM potassium acetate, and 2 mM EDTA, pH 7.4. Mitochondrial membranes were resuspended with 50 mM Tris acetate, 150 mM potassium acetate, 2 mM EDTA, pH 7.4, and 50% glycerol with the addition of 1 mM DFP and stored at −20 °C.

Cytochrome bc_1 complexes were isolated from bovine heart and yeast mitochondria [14] and concentrated to 30–40 μM cytochrome *b* by centrifugal filtration, using Amicon Centriprep YM-30 tubes. Cytochrome *b* concentration was determined from the spectrum of dithionite reduced versus ferricyanide-oxidized enzyme with an extinction coefficient of 25 mM^{-1} cm^{-1} at 563–578 nm [15]. Western blotting showed that there was no MnSOD in the purified bc_1 complexes.

Measurement of superoxide anion formation

Superoxide anion generation by the purified cytochrome bc_1 complexes was measured at room temperature in an assay buffer containing 50 mM potassium phosphate, 250 mM sucrose, 0.2 mM EDTA, 0.01% Tween 20, pH 7.4, 50 U/ml catalase, and 40 μM cytochrome *c*, with and without 50 U/ml CuZnSOD. To test the effects of inhibitors on superoxide anion formation, 25 nM antimycin, 25 nM stigmatellin or 25 nM myxothiazol was added to the assay. Cytochrome bc_1 complex was added into the cuvette to a final concentration of 2.5 nM and if inhibitor was added, equilibrated by stirring for 1 min. The reaction was started by adding 50 μM decyl-ubiquinol and reduction of cytochrome *c* was monitored in an Aminco DW-2a spectrophotometer at 550 versus 539 nm in dual wavelength mode. Data were collected and analyzed using an Online Instrument Systems Inc. computer interface and software. The rate of cytochrome *c* reduction was calculated with an extinction coefficient for reduced cytochrome *c* of 21.5 mM^{-1} cm^{-1} at 550–539 nm [16]. Each activity assay was performed in triplicate and the stated activities are averages of the three assays.

In this assay, the rate of formation of superoxide anion is equal to the SOD-sensitive rate of cytochrome *c* reduction, measured in the absence and presence of CuZnSOD. At pH 7.0, the rate constant of the SOD catalyzed $O_2^{\cdot -}$ dismutation to H_2O_2 and O_2 is approximately 2×10^9 M^{-1} s^{-1}, which is much faster than that of superoxide–cytochrome *c* reduction ($k \sim 10^6$ M^{-1} s^{-1}) [17,18]. Control experiments were performed, which

established that addition of larger amounts of SOD to the assay did not increase the SOD-sensitive rate of cytochrome *c* reduction. The decomposition of H_2O_2 to H_2O and O_2 catalyzed by catalase is a first-order reaction ($k \sim 10^7$ M^{-1} s^{-1}), the rate of which is proportional to H_2O_2 [19]. Catalase facilitates the superoxide dismutation reaction forwards by decomposing H_2O_2.

The bc_1 complex of bovine heart was highly purified and contained no cytochrome *c* oxidase. However, the bc_1 complex of yeast was contaminated with cytochrome *c* oxidase. Control experiments with the yeast bc_1 complex in the presence or absence of cyanide showed no difference in the MnSOD sensitive cytochrome *c* reduction rate, which indicated that cytochrome *c* oxidase had no effect on the formation or detection of superoxide anion.

Superoxide anion generation by the bc_1 complexes from wild-type yeast and from the BE924/76 and W30C yeast strains was also measured by following the formation of fluorescent adduct upon oxidation of dihydroethidium by superoxide anion [20]. In this assay, 50 nM bc_1 complex was mixed with 50 μM dihydroethidium and 250 μg/ml calf thymus DNA in assay buffer containing 50 mM potassium phosphate, 250 mM sucrose, 0.2 mM EDTA, and 0.01% Tween 20, pH 7.4, with and without 50 U/ml CuZnSOD. The reaction was started by injecting 50 μM decyl-ubiquinol into the cuvette. The rate of superoxide anion dependent fluorescence emission upon oxidation of dihydroethidium was monitored with a Hitachi F-3010 fluorescence spectrofluorometer at an emission wavelength of 571 nm with excitation at 480 nm, a 3 nm excitation bandpass, 20 nm emission bandpass, and a 2 s response time. The superoxide anion dependent fluorescence intensity increased in a linear manner for 5 min and the rate was calculated from the slope of the resulting line. Each measurement was made in triplicate and averaged.

Results

Sources of superoxide anion generation in the cytochrome bc_1 complex

The proton-motive Q cycle, shown in Fig. 1, describes the pathway of electron transfer among the redox groups of the cytochrome bc_1 complex [21,22]. When ubiquinol is oxidized, the two electrons enter the bc_1 complex by a divergent pathway in which one electron is transferred to the Rieske iron–sulfur protein while the second electron is transferred to cytochrome *b* (reaction 1 in Fig. 1). Subsequently, the reduced Rieske cluster oscillates to within electron transfer distance of cytochrome c_1, allowing electron transfer from the iron–sulfur cluster to the c_1 heme (reaction 2, Fig. 1). Two protons are released from center P coincident with

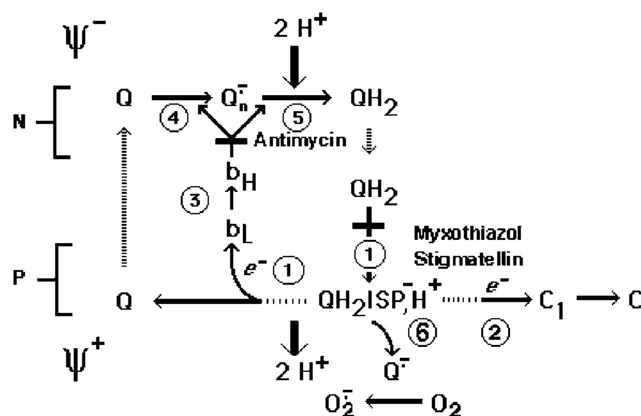

Fig. 1. Mechanism of electron transfer through the cytochrome bc_1 complex. The scheme shows the pathway of electron transfer from ubiquinol to cytochrome *c* through the redox centers of the cytochrome bc_1 complex. The circled numbers designate electron transfer reactions. Dashed arrows designate movement of ubiquinol (QH_2) or ubiquinone (Q) between center P on the positive side of the membrane and center N on the negative side of membrane and movement of the iron–sulfur protein between cytochrome *b* and cytochrome c_1. Solid black bars indicate sites of inhibition by antimycin, myxothiazol, and stigmatellin. In reaction 1 ubiquinol oxidation delivers two electrons divergently to the Rieske iron–sulfur cluster and the b_L heme, two protons are released, and the resulting ubiquinone leaves center P. In reaction 2 the reduced Rieske cluster oscillates to within electron transfer distance of cytochrome c_1, resulting in electron transfer from the iron–sulfur cluster to the c_1 heme. In reaction 3 an electron is transferred from the b_L to b_H heme, which in turn reduces ubiquinone to ubisemiquinone (reaction 4). Following oxidation of a second ubiquinol at center P and reduction of the *b* cytochromes, the b_H heme reduces ubisemiquinone to ubiquinol (reaction 5), accompanied by uptake of two protons at center N. In reaction 6 the bifurcated oxidation of ubiquinol is disrupted so that one electron is transferred to the Rieske iron–sulfur cluster and an unstable semiquinone anion is formed and reacts with oxygen to form superoxide anion.

ubiquinol oxidation. The electron that is transferred from ubiquinol to cytochrome *b* passes essentially instantaneously from the b_L to b_H heme (reaction 3), which in turn reduces ubiquinone to ubisemiquinone (reaction 4). Following oxidation of a second ubiquinol at center P and reduction of the *b* cytochromes, the b_H heme reduces ubisemiquinone to ubiquinol (reaction 5), accompanied by uptake of two protons at center N.

Recently revised estimates indicate that the potential for the one electron reduction of oxygen to superoxide anion is approximately −140 mV ([23], see also [24]). The oxidation of ubiquinol at center P is the only reaction in the Q cycle that transfers electrons at sufficiently low potential to reduce oxygen to superoxide anion. Oxidation of ubiquinol at center P involves an unstable semiquinone anion, with an estimated midpoint potential of −160 mV or lower for the semiquinone/quinone couple, depending on whether oxidation of ubiquinol is a concerted [25,26] or sequential reaction [27]. This semiquinone is a sufficiently strong reductant to reduce the b_L heme, which has a midpoint potential of −30 mV

in mammalian [28] and yeast [29] mitochondria, and the autoxidation of this semiquinone is thought to be the source of superoxide anion [10,30,31].

Although a semiquinone intermediate is also formed during reduction of ubiquinone at center N, this semiquinone is relatively stable. In the bovine bc_1 complex, the potentials of the ubiquinone/semiquinone and semiquinone/ubiquinol couples at center N have been estimated to be +21 and +99 mV, respectively [32]. The thermodynamic properties of this semiquinone thus pose a significant kinetic barrier to the one electron reduction of oxygen to superoxide anion.

Inhibitors such as stigmatellin and myxothiazol, that inhibit ubiquinol oxidation at center P, would be expected to also inhibit superoxide anion formation. Below we show that stigmatellin does inhibit superoxide anion formation but myxothiazol does not and offer an explanation for this difference. Anything that compromises the fidelity of the bifurcated transfer of electrons during ubiquinol oxidation may divert electrons from the semiquinone to oxygen out of the normal respiration sequence, as shown by reaction 6 in Fig. 1. Antimycin is an inhibitor of the bc_1 complex that blocks reoxidation of cytochrome *b* by ubiquinone [22] and is known to enhance superoxide anion formation [30]. This effect of antimycin is expected, since the presence of reduced cytochrome *b* would interfere with the bifurcated electron transfer and divert an electron from the semiquinone to oxygen. Below we show that antimycin promotes superoxide anion formation by the purified cytochrome bc_1 complex, but at a rate that is not much greater than that promoted by myxothiazol. We discuss possible reasons for the relatively similar rates of formation of superoxide anion in the presence of these two inhibitors.

Superoxide anion generation by cytochrome bc_1 complexes from bovine heart and yeast

We compared the superoxide anion generation by cytochrome bc_1 complexes purified from bovine heart and yeast mitochondria. The bovine heart cytochrome bc_1 complex catalyzes the reduction of cytochrome *c* by ubiquinol at a rate of $375\,s^{-1}$ (Fig. 2A). This rate decreased to $355\,s^{-1}$ when superoxide dismutase was included in the assay. This indicates that the bovine bc_1 complex forms superoxide anion at a rate of $20\,s^{-1}$, which is equivalent to 5.3% of the rate of cytochrome *c* reduction. Antimycin inhibited the cytochrome *c* reduction rate by approximately 98%, to $9.03\,s^{-1}$ (Fig. 2B). Addition of SOD further decreased the antimycin-resistant rate of cytochrome *c* reduction to $5.19\,s^{-1}$, which indicates that the purified bovine bc_1 complex forms superoxide anion at a rate of $3.84\,s^{-1}$ in the presence of antimycin (Fig. 2B). Stigmatellin is a somewhat less effective inhibitor of the bovine bc_1 complex than antimycin and reduced the rate of cytochrome *c* reduction to about $13.8\,s^{-1}$. However, SOD had no observable effect on the stigmatellin-resistant reduction rate, indicating that this center P inhibitor does not allow formation of superoxide anion (Fig. 2B).

The effects of myxothiazol, another center P inhibitor, differ notably from stigmatellin. Myxothiazol inhibited the cytochrome *c* reduction rate to $8.95\,s^{-1}$, essentially the same as antimycin (Fig. 2B). However, SOD further decreased the myxothiazol-resistant rate to $7.26\,s^{-1}$, indicating that this center P inhibitor allows formation of superoxide anion by the bovine enzyme at a rate of $1.7\,s^{-1}$.

The cytochrome *c* reductase activity of the yeast bc_1 complex, $232\,s^{-1}$, is lower than that of the bovine

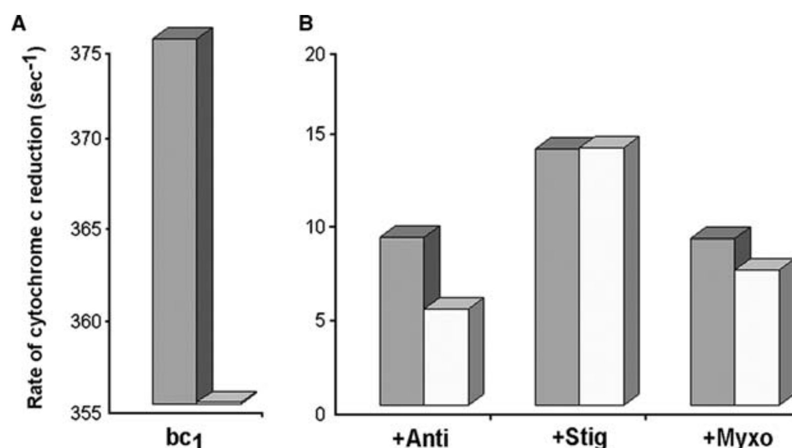

Fig. 2. Superoxide anion generation by the cytochrome bc_1 complex from bovine heart mitochondria. Panel A shows the ubiquinol–cytochrome *c* reductase activity of cytochrome bc_1 complex from bovine heart mitochondria and the effect of superoxide dismutase on the rate of cytochrome *c* reduction. The rate in the absence of SOD is indicated by the solid bar. The decrease in rate when SOD is included in the assay is taken as the rate of superoxide anion formation. Panel B shows the effects of antimycin, stigmatellin, and myxothiazol on the activity of the cytochrome bc_1 complex from bovine heart mitochondria measured in the absence (solid bars) and presence (open bars) of superoxide dismutase.

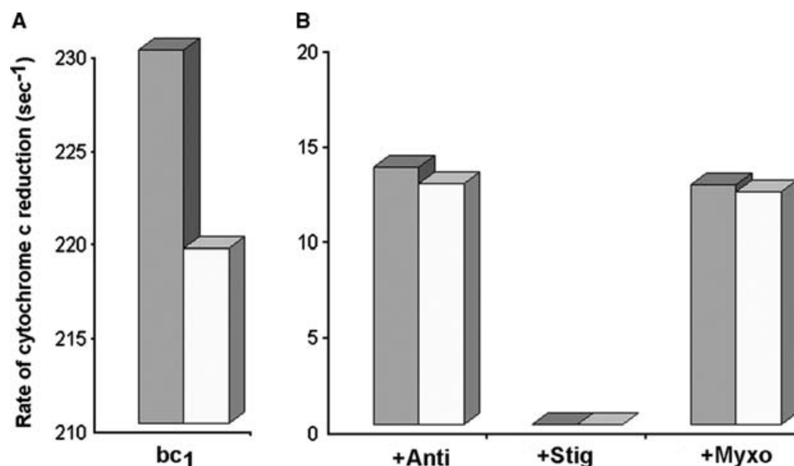

Fig. 3. Superoxide anion generation by the cytochrome bc_1 complex from wild type yeast. Panel A shows the ubiquinol–cytochrome c reductase activity of cytochrome bc_1 complex from yeast mitochondria without (solid bar) and with (open bar) superoxide dismutase in the assay. Panel B shows the effects of antimycin, stigmatellin, and myxothiazol on activity of the cytochrome bc_1 complex from yeast mitochondria measured in the absence (solid bars) and presence (open bars) of superoxide dismutase.

enzyme (Fig. 3A), as was noted in earlier comparisons of the two enzymes [14]. The SOD-sensitive rate of cytochrome c reduction by the yeast bc_1 complex was $12.3\,s^{-1}$, which is 5.3% of the rate of cytochrome c reduction (Fig. 3A). The rate of superoxide anion formation, expressed as a percentage of the cytochrome c reduction rate, is thus identical with the yeast and bovine enzymes.

The bc_1 inhibitors also had similar effects on superoxide anion generation by the yeast bc_1 complex as with the bovine enzyme (Fig. 3B). With antimycin, the catalytic activity of the yeast enzyme was reduced to $13.5\,s^{-1}$ and the SOD-sensitive rate in the presence of this inhibitor was $0.84\,s^{-1}$ (Fig. 3B). Stigmatellin fully inhibited the cytochrome c reductase activity of the yeast enzyme and thus there was no superoxide anion formed in the presence of this center P inhibitor. Myxothiazol reduced the activity of the yeast enzyme to $12.6\,s^{-1}$ and allowed superoxide anion generation at a rate of $0.37\,s^{-1}$ (Fig. 3B).

Superoxide anion generation by cytochrome bc_1 complexes with altered cytochrome b midpoint potentials

The stability of semiquinone is directly related to the potentials of the two semiquinone couples [33]. The increment between the midpoint potential of the b_L heme of cytochrome b ($E_{m,7} = -30$ mV) and the iron–sulfur cluster in the Rieske protein ($E_{m,7} = +285$ mV) is sufficiently large that any semiquinone species that is formed would be relatively unstable. We thus examined the rates of formation of superoxide anion by cytochrome bc_1 complexes in which the midpoint potential of the b_L heme is higher and the midpoint potential of the Rieske protein is lower than in the bc_1 complex from wild-type yeast. The midpoint potential of the b_H heme will affect the redox status and thus the apparent potential of the b_L heme under conditions of continuous bc_1 turnover. We thus also examined the rates of formation of superoxide anion by a bc_1 complex in which the midpoint potential of the b_H heme is lower than that in wild-type yeast.

The midpoint potentials of the b_L and b_H hemes in the bc_1 complex from wild-type yeast are −30 and +120 mV, respectively. The yeast strains BE924/76 and W30C each contain mutations in the cytochrome b gene that affect the midpoint potential of one of the b hemes. The midpoint potential of the b_L heme in the BE924/76 yeast mutant is +30 mV while that of the b_H heme in the W30C mutant is +60 mV (T. Merbitz-Zahradnik and B. L. Trumpower, unpublished results). As shown in Figs. 4A and C, these changes in midpoint potential do not significantly affect the cytochrome c reductase activities of the bc_1 complexes. The activity of the enzyme from the BE924/76 strain was $217\,s^{-1}$ and the activity of the enzyme from the W30C strain was $196\,s^{-1}$. The SOD-sensitive rates of cytochrome c reduction were 8.5 and $7.3\,s^{-1}$ with the enzymes from the BE924/76 (Fig. 4A) and W30C (Fig. 4C) strains, respectively. These rates of superoxide anion formation correspond to 3.9% and 3.7% of the rates of cytochrome c reduction. These rates of superoxide anion formation, expressed as a percentage of the rate of cytochrome c reduction, are slightly lower than the 5.3% observed with the enzyme from wild-type yeast.

As with the bc_1 complex from the wild-type yeast, both antimycin and myxothiazol allowed superoxide anion formation by the bc_1 complexes with altered cytochrome b midpoint potentials. The rates of superoxide anion formation by the bc_1 complex from the BE924/76 mutant in the presence of antimycin and myxothiazol were 2.4 and $1.5\,s^{-1}$, respectively (Fig. 4B).

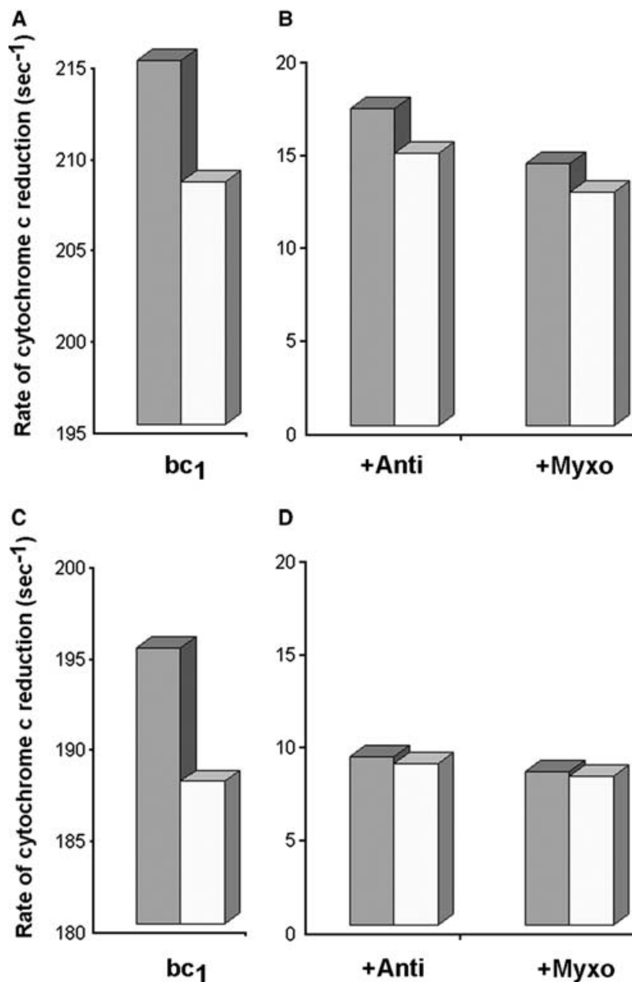

Fig. 4. Superoxide anion generation by bc_1 complexes from yeast with mutations in cytochrome *b*. Panel A illustrates the cytochrome *c* reduction rates obtained with yeast mutant BE924/76 without (solid bars) and with (open bars) superoxide dismutase in the assay. Panel B illustrates the cytochrome *c* reduction rates obtained with yeast mutant BE924/76 in the presence of antimycin and myxothiazol without (solid bars) and with (open bars) superoxide dismutase in the assay. Panel C illustrates the cytochrome *c* reduction rates obtained with yeast mutant W30C without (solid bar) and with (open bar) superoxide dismutase in the assay. Panel D illustrates the cytochrome *c* reduction rates obtained with yeast mutant W30C in the presence of antimycin and myxothiazol, without (solid bars) and with (open bars) superoxide dismutase in the assay.

The rates of superoxide anion formation by the bc_1 complex from the W30C mutant in the presence of antimycin and myxothiazol were 0.35 and $0.26\,s^{-1}$, respectively (Fig. 4D). The rates of superoxide anion formation are lower in the presence of myxothiazol than in the presence of antimycin in the bc_1 complexes with mutations that affect the midpoint potentials of the *b* hemes, as was observed with the enzyme from wild-type yeast. Stigmatellin fully inhibited the cytochrome *c* reductase activity of the bc_1 complexes from both mutants and thus did not support superoxide anion formation (data not shown).

Superoxide anion generation by a cytochrome bc_1 complex with altered Rieske iron–sulfur protein midpoint potential

The midpoint potential of the Rieske iron–sulfur protein is lowered from +285 to +220 mV by a Y185F mutation in the protein [13]. The cytochrome *c* reductase activities of the bc_1 complex from the *rip1* deletion strain complemented with a wild-type copy of the *RIP1* gene and complemented with a copy of the *RIP1* gene carrying a Y185F mutation were 121 and $28.2\,s^{-1}$, respectively (Figs. 5A and C). The decline in catalytic activity of the enzyme with the iron–sulfur protein mutation agrees with previous findings that the activity of the bc_1 complex is extensively influenced by the midpoint potential of the iron–sulfur protein [13]. The SOD-sensitive rates of cytochrome *c* reduction were 6.5 and $0.79\,s^{-1}$ with the two yeast strains complemented with wild-type and mutated iron–sulfur protein. These rates correspond to 5.4% and 2.8% of the rates of cytochrome *c* reduction. These results indicate that the absolute rate of superoxide anion formation decreases as the activity of the bc_1 complex decreases, and the rate of superoxide anion formation, expressed as a percentage of the rate of cytochrome *c* reduction, also decreases as the midpoint potential of the iron–sulfur protein decreases.

In the presence of antimycin, the rates of superoxide anion formation by the enzymes from the two yeast strains complemented with wild-type and mutated iron–sulfur protein were 1.25 and $0.41\,s^{-1}$, respectively (Figs. 5B and D). In the presence of myxothiazol, the superoxide anion generation rates by these two strains were 0.40 and $0.20\,s^{-1}$. As with the other yeast strains, stigmatellin fully inhibited the cytochrome *c* reductase activity and thus did not support superoxide anion formation (data not shown).

Superoxide anion generation by the bc_1 complex in the absence of cytochrome c

Measuring the decrease in rate of cytochrome *c* reduction in the presence of superoxide dismutase measures the rate of oxygen radical formation under conditions of continuous turnover of the bc_1 complex. However, the rate of superoxide anion formation is slow relative to the rate of cytochrome *c* reduction and amounts to only about 5% of the latter rate. It is thus difficult to evaluate any changes in the rate of superoxide anion formation, as in the cytochrome bc_1 complexes with mutations that change the midpoint potential of cytochrome *b*, against the high background rate of cytochrome *c* reduction.

As an alternative measure of superoxide anion formation we followed the superoxide anion dependent

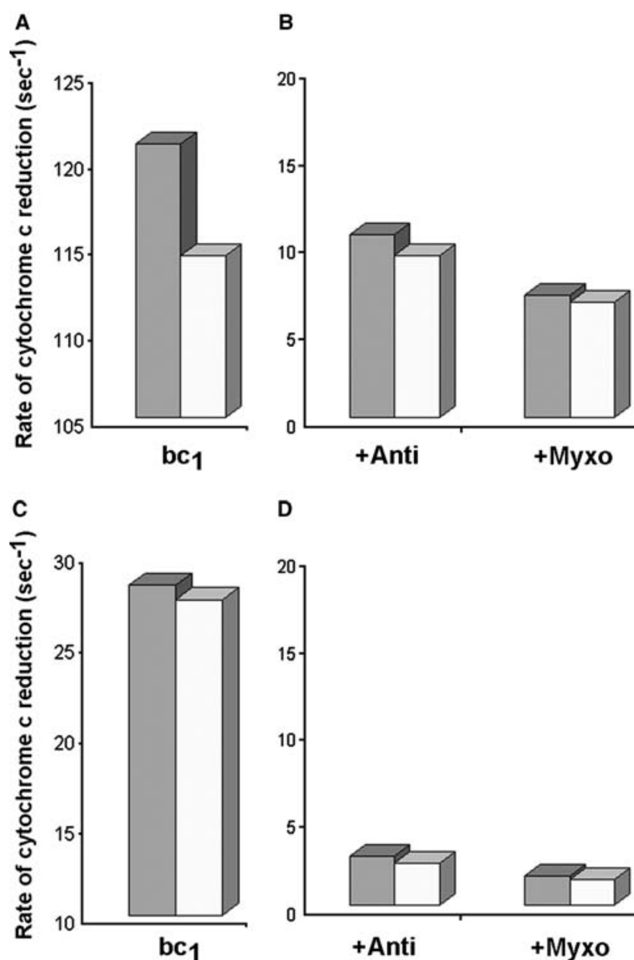

Fig. 5. Superoxide anion generation by the bc_1 complex from a yeast with a mutation in the Rieske iron–sulfur protein. Panel A shows the cytochrome c reduction rates obtained with the bc_1 complex from wild-type yeast without (solid bar) and with (open bar) superoxide dismutase in the assay. Panel B shows the cytochrome c reduction rates obtained with the bc_1 complex from wild-type yeast in the presence of antimycin and myxothiazol without (solid bars) and with (open bars) superoxide dismutase in the assay. Panel C shows the cytochrome c reduction rates obtained with the bc_1 complex from yeast with a Y185F mutation in the Rieske iron–sulfur protein. Panel D shows the cytochrome c reduction rates obtained with the bc_1 complex from yeast with a Y185F mutation in the Rieske iron–sulfur protein in the presence of antimycin and myxothiazol without (solid bars) and with (open bars) superoxide dismutase in the assay.

oxidation of dihydroethidium by the bc_1 complex from wild-type yeast and by the enzymes with mutations that change the midpoint potential of cytochrome b. As can be seen in Fig. 6, the rate of superoxide anion formation by the bc_1 complexes with cytochrome b mutations is significantly lower than that with the enzyme from wild-type yeast. Control experiments in the presence of CuZnSOD confirmed that the fluorescence increase was solely due to superoxide anion. These results agree with the apparent decrease in superoxide anion formation by these bc_1 complexes in the presence of cytochrome c (Fig. 4).

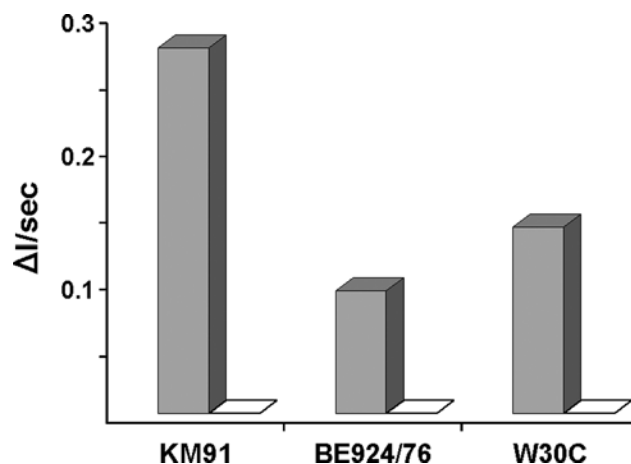

Fig. 6. Superoxide anion generation by the bc_1 complex in the absence of cytochrome c. Superoxide anion generation by the bc_1 complexes from wild-type yeast and from the BE924/76 and W30C yeast strains was measured by following the formation of fluorescent adduct upon oxidation of dihydroethidium by superoxide anion. The solid bars show the rate of fluorescence increase in the absence of CuZnSOD and the open bars show the rate in the presence of CuZnSOD.

Discussion

Superoxide anion is a cause or consequence of various pathologies in humans and has also been implicated in the aging process. To understand the parameters that affect superoxide anion formation by the cytochrome bc_1 complex, it would be advantageous to use site-directed mutagenesis and studies on the purified bc_1 complex to examine how changes in the enzyme affect superoxide anion formation. Although the bc_1 complex can be isolated from mammalian mitochondria, it is difficult to genetically manipulate those species from which the enzyme can be isolated in sufficient quantities for biochemical characterization. The budding yeast *Saccharomyces cerevisiae* is easy to manipulate genetically and sufficient quantities of the bc_1 complex can be isolated from this yeast for biochemical studies. We thus compared superoxide anion generation by cytochrome bc_1 complexes purified from bovine heart and yeast mitochondria to determine whether the enzyme from yeast might be a suitable model for the mammalian enzyme. The enzyme from the former is essentially identical to that from humans.

There have been numerous studies on the formation of superoxide anion by isolated mitochondria and many of these have led to the view that the bc_1 complex is the major source of superoxide anion in mitochondria. There have been two previous studies with the purified enzyme from bovine heart [8,34] and two with the enzyme from yeast mitochondria [9,35]. However, there have been no previous attempts to compare superoxide anion formation by the enzymes from the two species.

With enzymes from both species we found that superoxide anion is formed at the highest rate in the absence of any inhibitors and at a rate equivalent to approximately 5% of the rate of cytochrome *c* reduction. Antimycin, myxothiazol, and stigmatellin, inhibitors of the bc_1 complex, had essentially identical effects on superoxide anion formation by the enzymes from bovine and yeast mitochondria. With both enzymes antimycin and myxothiazol allowed superoxide anion formation, while stigmatellin prevented it, and the rate of superoxide anion formation in the presence of antimycin was slightly greater than that in the presence of myxothiazol. From these results we conclude that the bc_1 complexes from bovine heart and yeast mitochondria form superoxide anion by identical mechanisms.

The effects of antimycin are consistent with a mechanism of superoxide formation in which oxidation of ubiquinol at center P forms an unstable ubisemiquinone that reacts aberrantly with oxygen instead of reducing the b_L heme of cytochrome *b*. By blocking reoxidation of cytochrome *b* antimycin eliminates one of the electron acceptors for ubiquinol, while the other, the Rieske iron–sulfur cluster, is still available. Consequently, while one electron from ubiquinol oxidation follows its normal pathway through the iron–sulfur cluster to cytochrome c_1, the second is transferred to oxygen, instead of the b_L heme, to form superoxide anion (reaction 6 in Fig. 1).

Inhibitors that block ubiquinol oxidation at center P might be expected to likewise block superoxide anion formation. In an early study in which superoxide anion formation by sub-mitochondrial particles was monitored indirectly by an EPR visible superoxide scavenger, it was reported that antimycin promotes and myxothiazol prevents the formation of superoxide anion [36]. Our findings with purified bc_1 complexes show that myxothiazol allows superoxide anion formation, although at a rate lower than that with antimycin. This is consistent with the previous study with the yeast bc_1 complex which showed that myxothiazol only partially eliminated the formation of superoxide anion that was induced by antimycin [9]. The failure to detect superoxide anion by the paramagnetic EPR probe may be due to insensitivity of the method.

Stigmatellin blocked superoxide anion formation in bovine and yeast bc_1 complexes, even though it did not fully inhibit cytochrome *c* reductase activity of the former enzyme. The differential effect of stigmatellin and myxothiazol on superoxide anion formation may result from the difference in how these two inhibitors bind to center P. The crystal structures of the bovine and yeast bc_1 complexes show that stigmatellin binds to cytochrome *b* and the Rieske iron–sulfur protein and forms a hydrogen bond to the histidine residue on the Rieske protein that is a ligand to the iron–sulfur cluster [37,38]. Stigmatellin competes with ubiquinol and prevents ubiquinol from forming a hydrogen bond to the Rieske protein that is required for electron transfer to the iron–sulfur cluster. Myxothiazol binds closer to the b_L heme and does not form a hydrogen bond to the Rieske

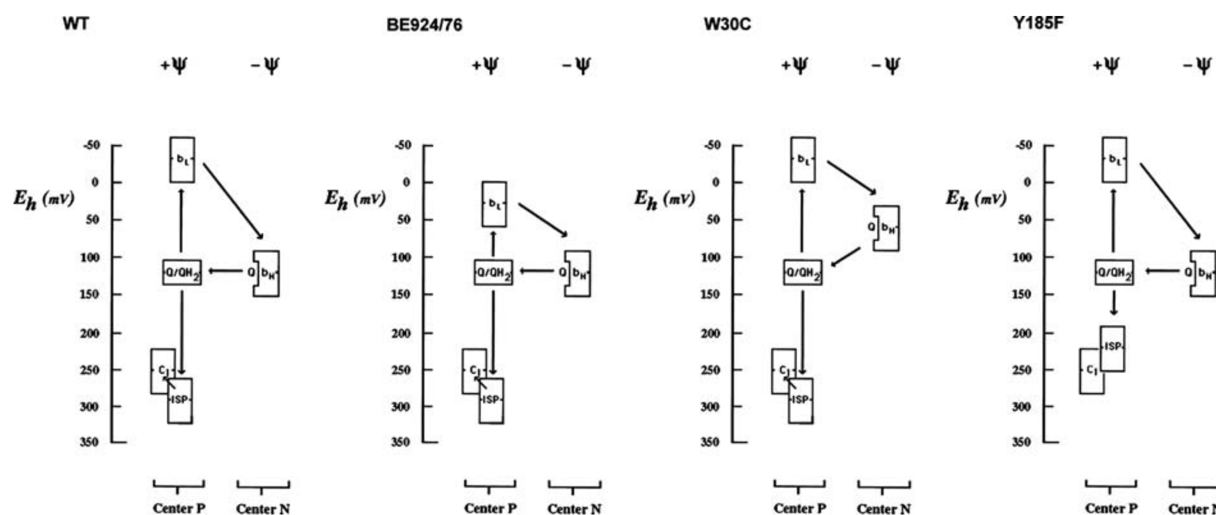

Fig. 7. Thermodynamic profiles of the Q cycle in bc_1 complexes with altered midpoint potentials of cytochrome *b* and the Rieske iron–sulfur protein. The figure illustrates the thermodynamic relationship between the redox components of the cytochrome bc_1 complex in wild-type yeast (left), cytochrome *b* mutant *BE924/76*, cytochrome *b* mutant *W30C*, and Rieske iron–sulfur protein mutant Y185F. The redox groups are arranged vertically according to their oxidation–reduction potentials, with the center of the boxes positioned vertically at the midpoint potentials of the redox groups. The open boxes depict the approximate range of potentials spanned by the redox components as their oxidation–reduction status varies in response to changes in the rates of electron transfer. The midpoint potentials in the bc_1 complex from the wild-type yeast are cytochrome b_L, −30 mV, cytochrome b_H, +120 mV, cytochrome c_1, +240 mV, and Rieske iron–sulfur protein, +285 mV. In the cytochrome *b* mutant, BE924/76, the midpoint potential of the heme b_L is +30 mV. In the cytochrome *b* mutant, W30C, the midpoint potential of the heme b_H is +60 mV. The midpoint potential of the iron–sulfur cluster in the Rieske iron–sulfur protein mutant, Y185F, is +220 mV.

histidine residue [37]. Ubiquinol can thus access the Rieske cluster but not the b_L heme in the presence of myxothiazol, thus allowing superoxide anion formation in the presence of this center P inhibitor. Muller et al. [35] came to the same conclusion regarding differential accessibility of the Rieske iron–sulfur protein to ubiquinol in the presence of myxothiazol and stigmatellin.

Lowering the rate of cytochrome *c* reduction by changing the midpoint potential of the Rieske iron–sulfur protein has the expected effect of lowering the rate of superoxide anion production. The lower midpoint potential of the Rieske protein with the Y185F mutation also lowers the potential increment and thus the driving force for electron transfer between ubiquinol and one of its electron acceptors by 60 mV as illustrated in Fig. 7. This causes the rate of superoxide anion formation, expressed as a percentage of the cytochrome *c* reduction rate to drop by almost half, from 5.4% to 2.8% of the cytochrome *c* reduction rate. A similar, but somewhat smaller effect, was observed when the potential increment was decreased by a 60 mV increase in the midpoint potential of the b_L heme, in which case the rate of superoxide anion decreased to 3.9% of the cytochrome *c* reduction rate.

Interestingly, lowering the midpoint potential of the b_H heme had an almost identical effect on superoxide anion production as did raising the midpoint potential of the b_L heme. The rate of superoxide anion by the bc_1 complex from the W30C mutant decreased to 3.7% of the cytochrome *c* reduction rate. In this case, however, the decrease in relative rate of superoxide anion production cannot be attributed to a change in the driving force for ubiquinol oxidation. We suggest that the change in midpoint potential of the b_H heme increases the rate of electron transfer out of the *b* hemes to the quinone pool at center N due to the increased driving force for electron transfer between the b_H heme and ubiquinone (Fig. 7). This would lower the steady-state redox poise of the *b* hemes under conditions of continuous turnover of the bc_1 complex, thus facilitating electron transfer to the *b* hemes, instead of to oxygen.

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