Supplemental Data

Assay to measure superoxide anion formation- To minimize the phospholipid enhancement of the chemical production of superoxide we added 2 mM $MgCl_2$ to the reaction medium to minimize the binding of cytochrome *c* to the phospholipid vesicles. We also used a relatively low concentration of cytochrome *c* (7.5 μM) in the assay that both reduces the rate of the chemical reaction and minimizes the optical interference with the fluorescence assay. In addition, we selected an excitation wavelength that coincides with an isosbestic point in the reduced-oxidized difference spectrum of cytochrome *c* (526 nm) and an emission wavelength well above the absorption peak of reduced cytochrome c (590 nm). Finally, we took advantage of the fact that the isolated bc_1 complex contains small amounts of cytochrome *c* oxidase. In the absence of CN^-, that is normally added to inhibit cytochrome oxidase, the rate of cytochrome *c* oxidation by the oxidase is increased as the concentration of reduced cytochrome *c* rises until cytochrome *c* reaches a steady state of reduction. At this point there is minimal fluorescence interference from cytochrome *c* reduction, and since most of the cytochrome *c* is already reduced, the rate of the chemical reaction is relatively slow compared to the rate of cytochrome *c* reduction by the bc_1 complex, which is not affected as strongly by the concentration of cytochrome *c*.

An example of this assay is shown in Fig. S1. First, the reagents of the reaction mixture, 2 mM $MgCl_2$, 100 units SOD, 1 unit HRP, 7.5 μM cytochrome *c* and 50 μM DBH were added to a 2 ml suspension of the phospholipid reconstituted bc_1 complex in the dialysis buffer (8.35 nM final concentration). The reaction mixture was incubated for 5 min to allow the system to approach a steady state of cytochrome *c* reduction. Then the Amplex ultraRed reagent dissolved in DMSO was added to a concentration of 16.6 μM and the fluorescence was monitored for ~ 5 min until the rate stabilized as the system reaches a steady state. Stigmatellin was then added to inhibit superoxide generation by the bc_1 complex, and the fluorescence was monitored further (~ 3 min) to obtain the rate of the chemical reaction. Finally, an aliquot of hydrogen peroxide (0.6 nmoles) was added to calibrate the signal. The difference between the rate of hydrogen peroxide formation before the addition of stigmatellin and the rate of the chemical reaction after the addition is the rate of hydrogen peroxide formation by the bc_1 complex, which is half the rate of superoxide formation. Many reagents (e.g. nigericin, antimycin, stigmatellin; see Fig. S1) have a direct effect on the fluorescence, but the rate that is measured after the addition of the reagent is not related to these effects.

Supplemental Figure Legend

Figure S1 Determination of the rate of superoxide generation by the reconstituted bc_1 complex. Phospholipid-reconstituted bc_1 complex was suspended in 2 ml of the dialysis K^+-buffer (8.35 pmoles/ml). The suspension was supplemented with 2 mM $MgCl_2$, 7.5 μM cytochrome c, 100 units SOD, and 1 unit HRP. The reaction was started by the addition of 50 μM DBH; after 5 min the Amplex-ultraRed reagent (16.6 μM) was added and the rate of the fluorescence increase (excitation, 526 nm; emission, 590 nm) was followed for another 5 min; at that time stigmatellin (20 nM) was added to obtain the rate of the chemical reaction. Finally, 600 pmoles of H_2O_2 was added for calibration of the signal. The rate of superoxide generation by the bc_1 complex was calculated from the difference between the rates before and after stigmatellin addition, which was calculated from the slopes of the traces, indicated by the dashed lines.

Figure S1