Investigating the Mechanism behind Antimycin Induced Oxidation and Heterogenous Titration of Cytochrome b_H of the Cytocrhome bc_1 Complex

Karen Chiang and Edward A. Berry

INTRODUCTION

Background on the BC_1 complex (cyt bc1):

The bc_1 complex is the middle part of the mitochrondrial respiratory chain. It oxidizes ubiquinol and reduces cyt c. Part of the energy released by this exergonic reaction is utilized to translocate protons across the mitochrondria membrane, storing energy in the proton gradient. The bc_1 complex is part of the machinery which builds up the proton gradient, pumping electrons "uphill" against the gradient to store more energy in the gradient. The energy of the gradient can be used by other systems, like ATP synthase, to do useful work like ATP synthesis.

The mechanism by which cyt bc_1 couples electron transfer to proton translocation is probably that described by the "protonmotive Q cycle" of Peter Mitchell (Reference 1). In this mechanism cyt bc_1 has two active sites where Q reacts. Quinol (QH_2) is oxidized, releasing protons, at the Qo site near the outside surface of the membrane. One of the two electrons is used to reduce cyt c, providing the driving force for the reaction. The other electron crosses the membrane and reduces Q, which proton uptake at the Qi site closes to the inside surface of the membrane. The two cytochrome b hemes serves as an electron wire to bring electrons from the Qo site, where they are produced by QH_2 oxidation, to the Qi site. The mitochronia membrane contains about 20 Q/QH_2 molecules per cyt bc_1. The excess of Q is called the "Q pool". Since the b cytochromes are connected to both Qo site and Qi site, there are two pathways by which cyt b reacts with Q pool; the normal reaction at the Qi site and the reverse of the normal reaction at the Qo site. Specific inhibitors have been found which blocks these reactions; stigmatellin blocks the reaction at the Qo site and antimycin or funiculosin block the reaction at the Qi site. QH_2 can reduce cyt b in the presence of either of these inhibitors, but if both inhibitors are present QH_2 can not reduce cyt b.

The obervation to be explained (Ref 1):

1. Cyt bc_1 containing endogenous ubqunione is poised at +100mV.
Cyt b_H is seen to be partially reduced eventhough the redox potential is not low enough to reduce cyt b_H. (To be precise, cyt b_H is more reduced than you would predict based on its midpoint potential (Em = 50mV) and the redox potential (100mV)).
Adding antimycin causes cyt b to be oxidized.

2. In anaerobic redox titrations with redox level of cyt b_H is monitored as a function of redox potential, part of cyt b_H titrates with a midpoint potential of +150mV. With Q-depleted cyt bc_1 or in the presence of antimycin, all of cyt b_H titrates with the potential of +50 mV.

Explanation 1 (Kinetic explanation, reference 1-3): Cyt b is oxidized and Q site is empty. QH_2 binds at the Qi site and transfers one electron to cyt b. Now we have cyt b reduced and semi-quinone (QH*) bound at the Qi site. The Qi site stablizes QH* (the site is designed to bind QH*

very tightly ie: stablizes QH*). This pulls the reaction forward. This explains why cyt b is reduced.

$b^{+3} \rightarrow b^{+3}$ QH2 $\rightarrow$ $\underline{b^{+2}\ QH^*}$

Now we add antimycin. Antimycin can not bind when Q is in the site but everytime Q dissociates the site is available for antimycin to bind. As QH* can't dissociates except by reversal of the reaction, ie taking the electron back from cyt b. Therefore whenever the site is empty cyt b is oxidized. And antimycin can only bind when the site is empty, which is only when cyt b is oxidized.

$b^{+3} \rightarrow b^{+3}$ QH2 $\rightarrow b^{+2}$ QH*

A |

$\underline{b^{+3}A}$

Once antimycin binds, it stays for a long time. Therefore most of the Q has antimycin bound and site cyt b oxidized. Cyt b is not in equilibrium with the Q pool.

Explanation 2 (thermodynamic, reference 4-5): In the presence of Q at 100mV, some redox form of Q is bound at the Qo site and changes the effective midpoint potential of cyt b to make it easier to reduce. Therefore, cyt b is more reduced than we calculated. Antimycin binds at the Qo site (known fact) and displaces Q. (Antimycin binds much more tightly). Antimycin does not affect Em of cyt b so cyt b becomes hard to reduce again so electrons go back to Q pool. Cyt b is actually in equalibrium with the Q pool, only the Em changes. This would probably be due to some sort of redox interactions (e.g. coulombic or allosteric) between cyt b_H and the species at the Qi site, as proposed in Reference 4-5.

What do the two explanations have in common?

- Both explanations depend on ubiquinone.
- In both explanations, antimycin works by displacing ubiquinone. What are the differences between the two explanations?
- Explanation 2 assumes all the redox centers are at equilibrium with Q/QH_2 (surely true at long time scale).
- Explanation 1 assumes cytochrome b_{562} equilbrates only by the special mechanism described above (which is very fast because this is the enzymatic reaction, so this should hold at a short time scale).

How can we differentiates these two explanations?

Funiculosin is another inhibitor that binds at the Qi site like antimycin. Funiculosin raises the midpoint potential of cyt b_H, making it easier to reduce. Explanation 1 should work the same with funiculosin or antimycin, because it can only bind when the site it empty. So it traps the complex with cyt b_H oxidized, just like antimycin. According to the assumption of explanation 1, cyt b_H cannot equilbrate with the Q pool when the inhibitor is bound, so the increase midpoint potential is irrelevant.

However, according to explanation 2, cyt b_H equilibrates with the Q pool so the increased Em should result in further reduction in cyt b_H, regardless of the mechanism. Therefore if we repeat the antimycin induced oxidation experiment but used funiculosin instead of antimycin, explanation 2 predicts we should see funiulosin induced reduction instead of oxidation. Explanation 1 predicts we should see oxidation in both cases.

MATERIALS AND METHODS

In the original experiment (Ref 1), an anaerobic cuvette is used with platinum electrode to measure the redox potential. Redox mediators were used to bring the Q pool into equilibrium with the electrode. We avoided this complicated set up by using another enzyme, Complex II, to bring the Q pool into equilbrium with the succinate/fumerate couple. Succinate and fumerate were added at known concentrations to define the redox poise. The level of cyt b reduction was monitored by taking spectra, which were fit with a linear combination of standard spectra to see how much cyt b was reduced. After waiting for cyt b to equilbriate with succinate and fumerate, antimycin or funiculosin were added and more spectra were taken to monitor the change in redox level of the cytochromes. Specific detials are given in the figure legends.

RESULTS AND DICUSSION

1. Standard spectra for analyzing redox level of the cytchromes. These spectra were already available in the lab from previous work. Figure 1 shows the spectrum of oxidized bovine cyt bc_1 complex and difference spectra of the three cytochromes in cyt bc1 (cyt c_1, cyt b_H, cyt b_L). Cyt b_H has a peak at 562 nm, so reduction of cyt b_H causes increase of absorption at 562 nm. Any spectrum of cyt bc1 in any redox state can be fit by a linear combination of these three spectra. The amount of the different spectra of each cytochrome require for the fit depends on how much of that cytochrome is reduced. A program (Scaned) was available in the lab to perfrom the fit and print out the results in a table.

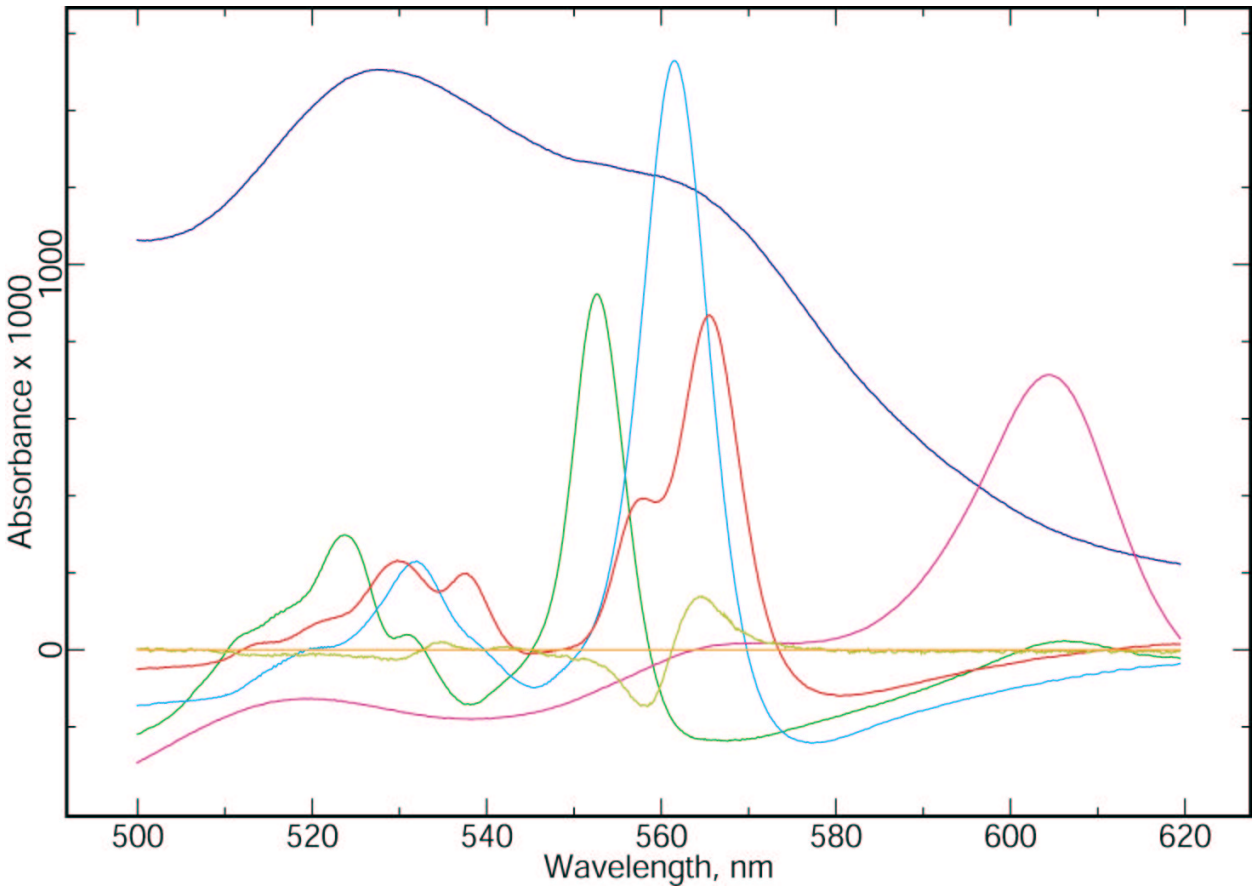

Figure 1. The spectra of oxidized cyt bc_1 (dark blue) and reduced minus oxidized difference spectra of cytochromes c_1 (green), b_H (light blue), b_L (red). These are the basis spectra used to fit the experimental spectra to determine the redox level of cytochromes. The difference spectrum induced by adding antimycin to reduced cyt bc_1 (yellow) was also included in the fitting spectra in the experiment of Figures 2-4, and the funiculosin difference spectrum (not shown) was used in the experiment of Figures 5-7.

2. Antimycin induced oxidation (Experiment 071109). Cyt bc_1 was incubated in a silica cuvette with stigmatellin, Complex II, and 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzohydroquinone (DBH) as described in the legend of Figure 2. Succinate and fumerate were added to final concentrations of 144.4 uM and spectra were recorded at intervals until quasi-equilibrium was reached. Then antimycin was added in excess over the bc_1 complex and further spectra were recorded at intervals. Figure 3 shows the spectra before and after adding antimycin, and Figure 5 shows difference spectra obtained by substracting the last spectrum before antimycin from the first three spectra after antimycin. Figure 4 shows the redox levels of the cytochromes at each stage of the experiment. Antimycin was added where indicated.

As can be seen in Figures 3 and 5, cytochrome b was only slightly reduced (5%) under our incubation conditions. After of addition of antimycin it went fully oxidized. The difference spectra of Figure 5 clearly shows the spectra change induced by antimycin is due to oxidation of cyt b_H (through at 562 nm), complicated by the continued reduction of cyt c_1 (peak at 553 nm). Thus we were able to reproduced the antimycin reduced oxidation. This result is not completely satisfactory due to the small size of the change, but this is simply due to the fact that only small amount of cyt b was reduced to start with. A more satisfactory result can probably be obtained by using a higher ratio of succinate to fumerate or waiting longer for the system to reach equilibrium. So cyt b is more reduced before adding antimycin. However the basic phenonmenon, antimycin induced oxidation was demonstrated. Succinate added prior to spectrum 18 produced a slight re-reduction of cyt b_H then DT added where indicated produced full reduction of cyt b_H.

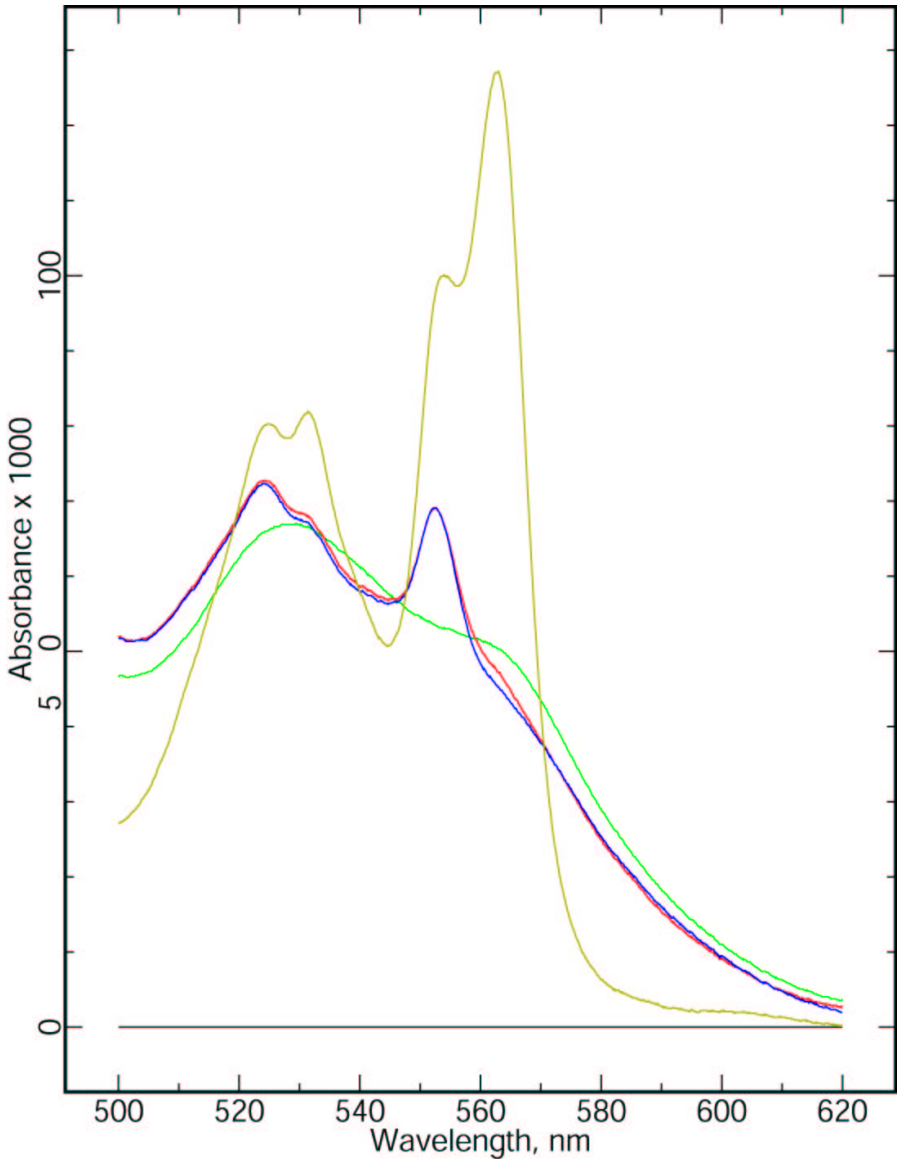

Figure 2. Absolute spectra of the reaction mixed at different stages of the experiment. The green trace is the initial spectrum of the fully oxidized sample. The yellow trace is the fully reduced sample after adding DT. Red is after equilibration before adding antimycin. Blue is after adding antimycin. Notice the difference between the red and blue traces is mainly a decrease in absorbance around 562 nm, suggesting that cyt b_H is oxidized. This is confirmed by the spectra analysis in Figure 3 and seen more clearly in the difference spectra of Figure 4.

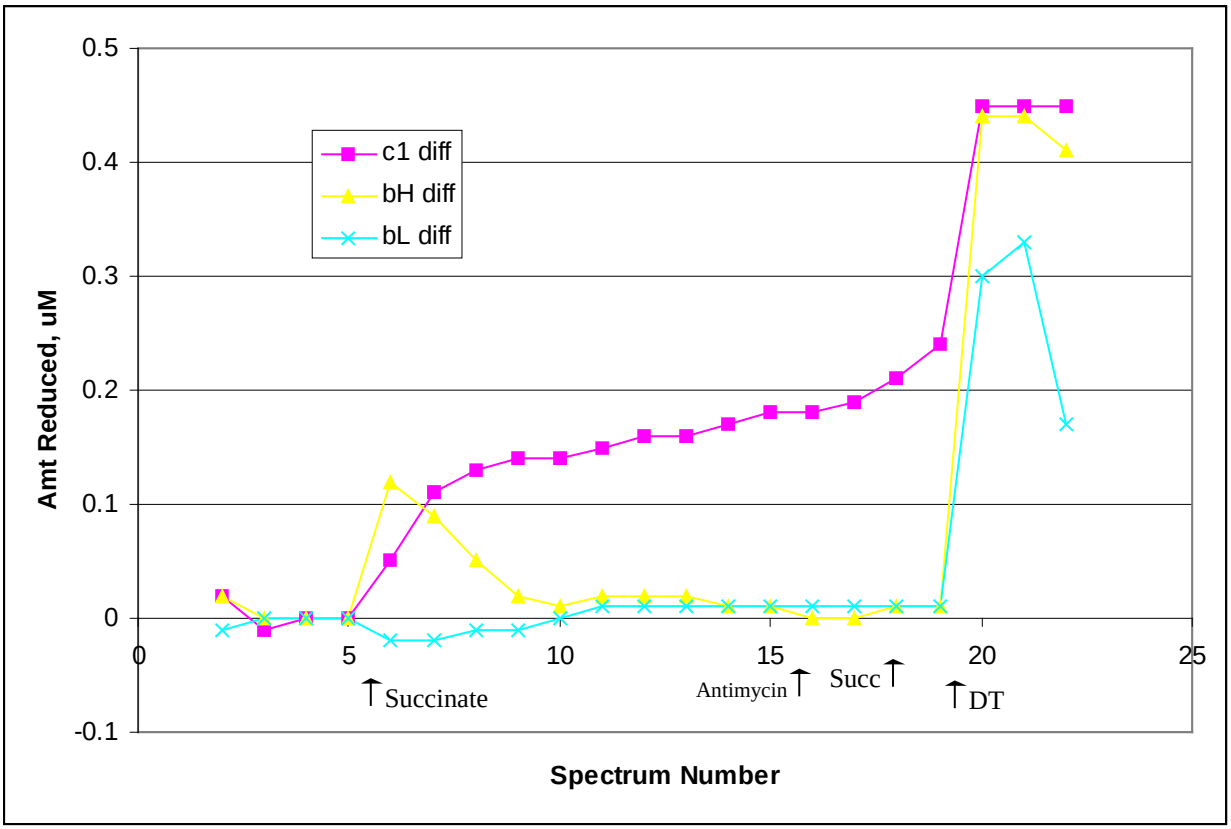

Figure 3. The effect of antimycin added to bc_1 complex poised with succinate (expt071109). Spectra was taken at different times during the experiment were analyzed to determine the amount of each cytochrome reduced. These results are plotted against spectrum number (the first spectrum was buffer baseline, not shown). For the second spectrum, bovine cyt bc_1 complex (1.8 uM) was added to a cuvette containing 20 mM KMOPS, 100 mM NaCl, 0.5 mM EDTA, 0.1 g/L dodecyl maltoside. Stigmatellin (11 uM) and Complex II (0.3 uM) were added before spectra 3 and 4. Succinate (22 uM), DBH (111.1 uM), and fumerate (2.2 uM) were added before spectra 6, 11, and 14. Antimycin (11 uM) was added before spectra 16, succinate (2.7mM) before spectra 18, and DT before spectra 20. Note that cyt b_H (yellow) went partially reduced initially, as in the "triphasic" reduction of (Reference 8), then reoxidized almost completely by spectra 15. After antimycin was added in spectra 16, cyt b_H was completely oxidized. Succinate reduced a little bit cyt b_H then DT gave full reduction. Cyt c_1 (magenta) was reduced gradually after adding succinate and leveled off but continued to drift reduced; fully reduction was obtained after adding DT. Cyt b_L (blue) hovered around the fully oxidized level until it went briefly reduced after DT immediately started to reoxidized. Spectra 5, 15, 16, and 21 are plotted in Figure 2. Control experiment where cyt bc_1 was omitted showed no significant change in the spectral region 550-560 nm upon adding succinate (the heme of Complex II is not reduced by succinate).

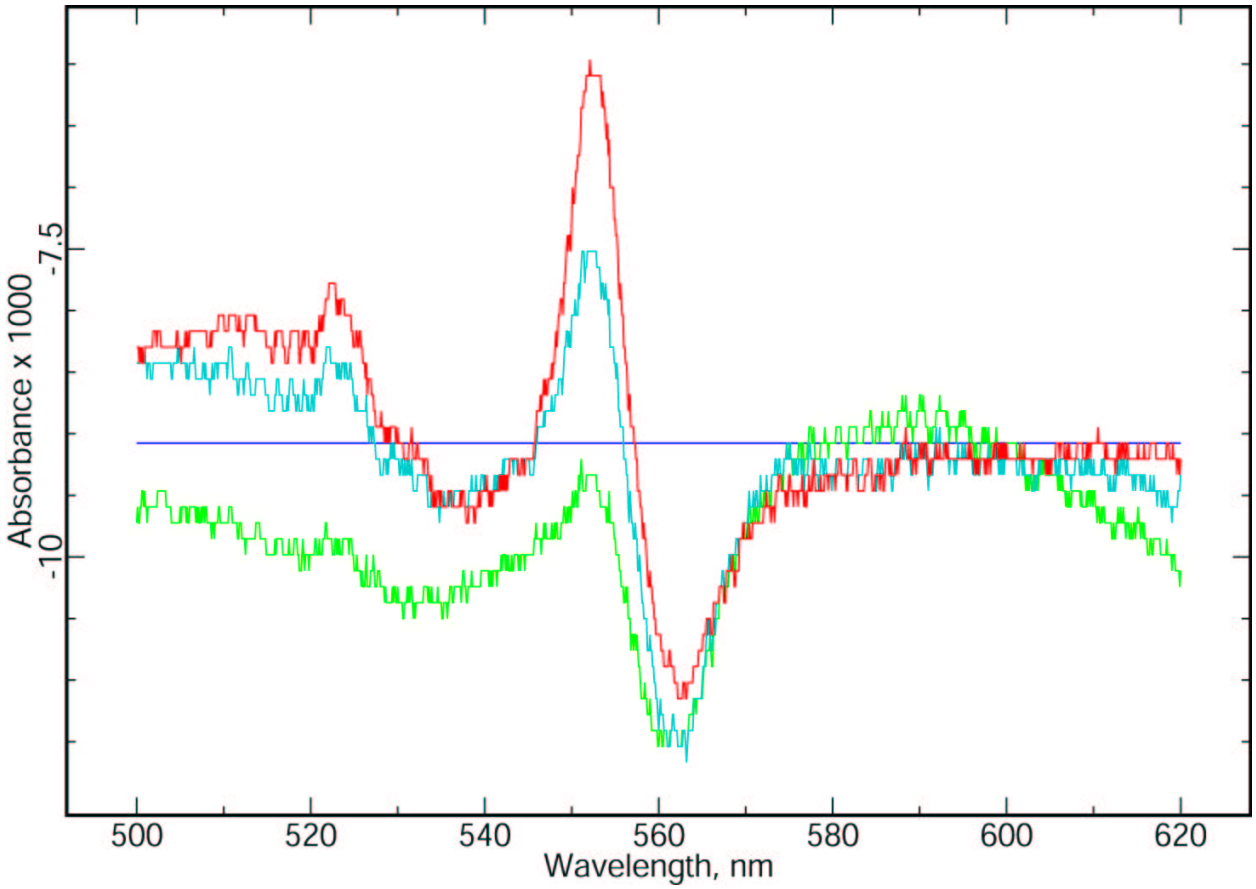

Figure 4. Difference spectra showing the change upon adding antimycin.
The blue spectrum (spectrum 15, before antimycin) was subtracted from all to show the changes. The green spectrum (16) is the first spectrum after antimycin followed by light blue (17) and red (18). The decrease in absorbance at 562 nm, which occurred immediately upon adding antimycin, is due to oxidation of cyt b_H. The increase at 553 nm is due to continued slow reduction of cyt c_1.

3. Experiment 2 was repeated with funiculosin instead of antimycin (Experiment 071116). Cyt bc_1 was incubated in a silica cuvette with stigmatellin, Complex II, and DBH as described in the legend of Figure 6. Succinate and fumerate were added to final concentrations of 113 uM and spectra were recorded at intervals until quasi-equilibrium was reached. Then funicolusin was added in excess over the bc_1 complex and further spectra were recorded at intervals. Figure 5 shows the spectra before and after adding funiculosin as well as the initial and DT-reduced spectra, and Figure 7 shows difference spectra obtained by subtracting the last spectrum before funiculosin from the first three spectra after funiculosin. Figure 6 shows the redox levels of the cytochromes at each stage of the experiment. Funiculosin was added where indicated.

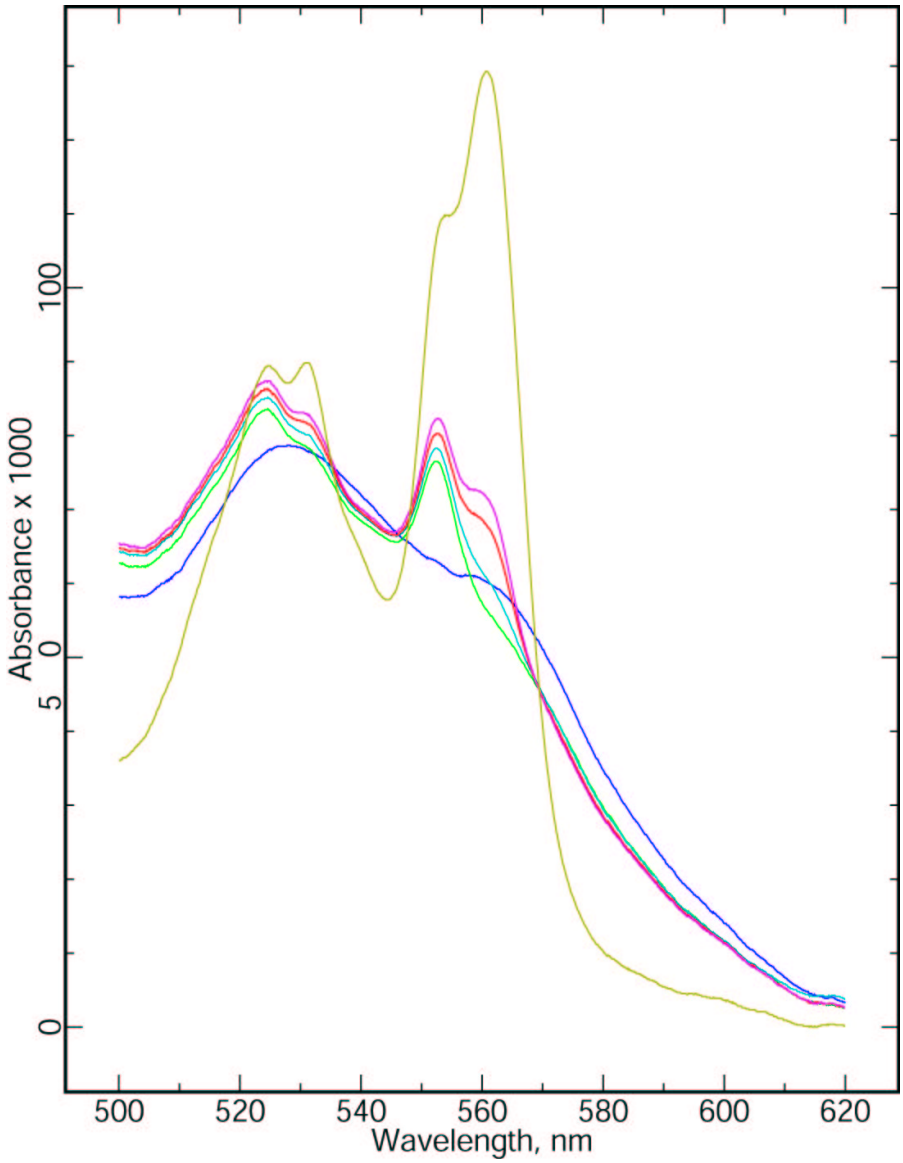

Figure 5. Absolute spectra of cyt bc_1 before and after adding funiculosin. The blue trace is the initial spectrum of the fully oxidized sample. The yellow trace is the fully reduced sample after adding DT. Green is after equilibration before adding funiculosin. Light blue, red, and magenta are the first three spectra after adding funiculosin. Notice the increase of absorbance around 562 nm, indicating that cyt b_H is going reduced. This is confirmed by the spectral analysis in Figure 6 and seen more clearly in the difference spectra of Figure 7.

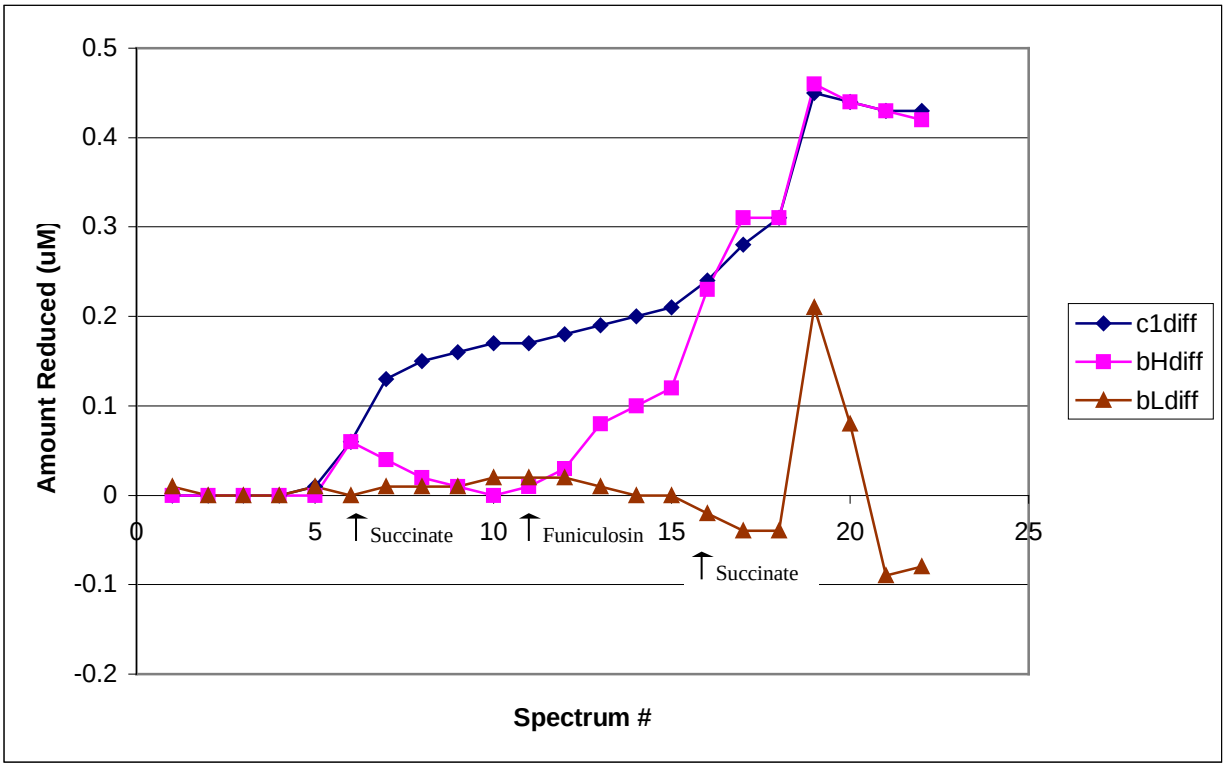

Figure 6: Effect of funiculosin addition to bc_1 complex poised with succinate/fumerate (experiment 071116).

Spectra was taken at different times during the experiment were analyzed to determine the amount of each cytochrome reduced. These results are plotted against spectrum number (the first spectrum was buffer baseline). For the second spectrum, bovine cyt bc_1 complex (1.8 uM) was added to a cuvette containing 20 mM KMOPS, 100 mM NaCl, 0.5 mM EDTA, 0.1 g/L dodecyl maltoside. Stigmatellin (11 uM) and Complex II (0.3 uM) were added before spectra 3 and 4. DBH (111.1 uM), succinate (22 uM), and fumerate (2.2 mM) were added before spectra 5, 6, and 10. Funicolusin (6 uM) was added before spectra 12, succinate (2.7mM) before spectra 16, and DT before spectra 19. Note that cyt b_H (magenta) went partially reduced initially, as in the "triphasic" reduction of (Reference 8), then reoxidized almost completely by spectra 12. After funicolusin was added in spectra 12, cyt b_H was completely oxidized. Succinate reduced a little bit cyt b_H then DT gave full reduction. Cyt c_1 (blue) was reduced gradually after adding succinate and leveled off but continued to drift reduced; fully reduction was obtained after adding DT. Cyt b_L (brown) hovered around the fully oxidized level until it went briefly reduced after DT immediately started to reoxidized. Spectra 4, 11-14, and 19 are plotted in Figure 5.

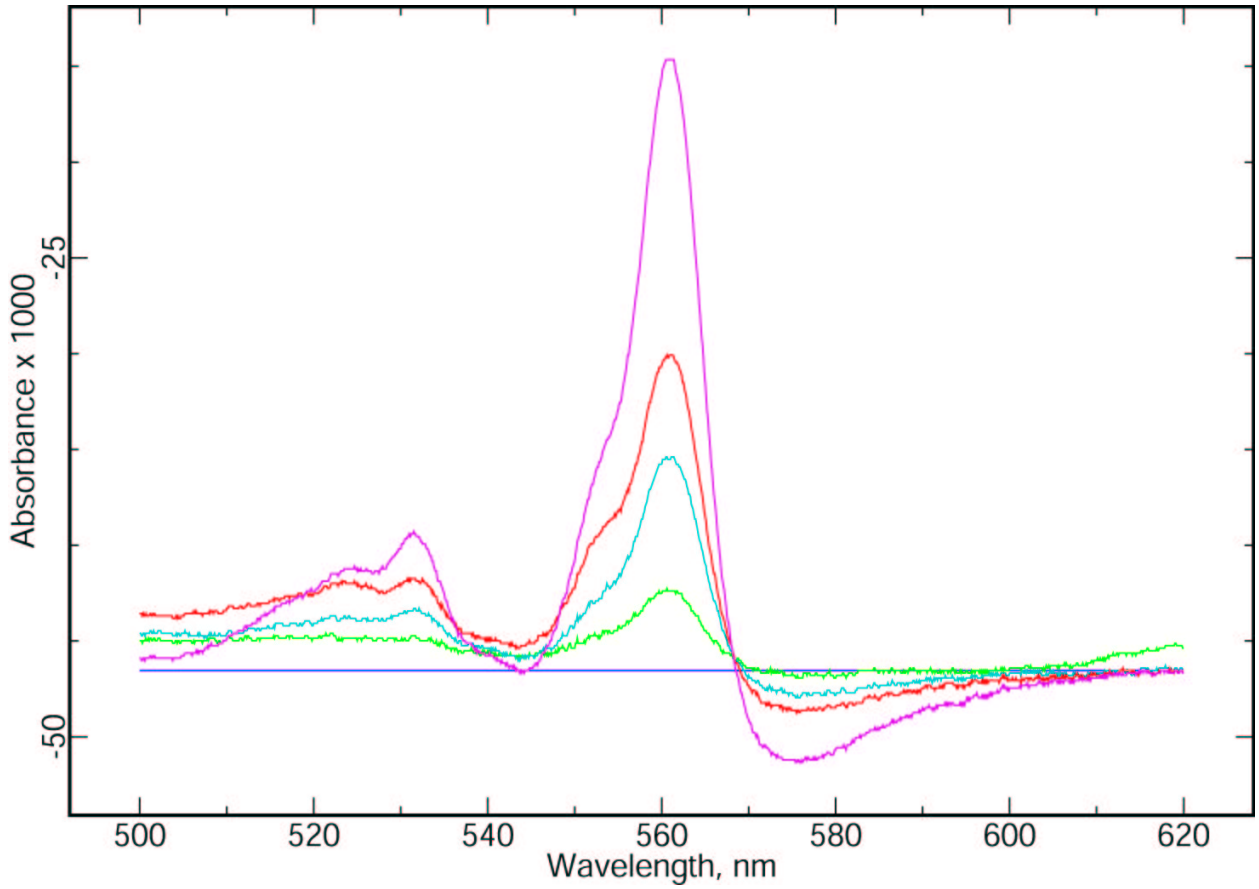

Figure 7. Difference spectra showing the change upon adding funiculosin.
The blue spectrum (spectrum 11, before funiculosin) was subtracted from all to show the changes. The green spectrum (12) is the first spectrum after funiculosin followed by light blue (13), red (15), and purple (16). The increase in absorbance at 562 nm, which occurred immediately upon adding funiculosin, is due to reduction of cyt b_H.

CONCLUSION

In the Introduction, two explanations were described for the phenomenon of antimycin-induced oxidation of cyt b_H. According to the kinetic explanation, funiculosin should have the same effect as antimycin, that is it should induce oxidation. However, according to the thermodynamic explanation, funiculosin should cause further reduction of cyt b_H. As shown in Figures 5-7, on the time scale we are examining, funiculosin caused further reduction. Therefore, on this time scale, the equilibrium explanation seems to correctly predict the results. This does not however mean that the kinetic explanation is invalid, and it seems quite likely that it may dominate on a short time scale. Further experiments should be done, perhaps using a stopped-flow spectrophotometer to record data on a millisecond time scale. If the kinetic explanation is valid, when we add funiculosin, we expect to see cyt b go oxidized initially as funiculosin traps the bc_1 complex in the oxidized state. Then it will go reduced again as cyt b gradually equilibrates with the Q pool by other mechanisms (direct reaction with the Q/QH_2 couple). However it is important to note, that the antimycin induced oxidation was observed on our time scale. Since we know cyt b_H equilibrates nonspecifically on our time scale (since it goes reduced with funiculosin), we still need the thermodynamic explanation (explanation 2 in the introduction) to explain our results. In addition, it could be assumed that in redox titrations (see introduction) where the redox state is monitored as a function of redox potential, cyt b_H equilibrates with the mediators. Thus, it can be concluded that the effective midpoint potential of cyt b_H is higher when some form of quinone ($Q/QH^*/QH_2$) is bound then when antimycin is bound when the site is empty, as proposed by Explanation 2 in the introduction and References 4-5.

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