Spectral Resolution of Four Cytochrome b Components in Mitochondria

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Abstract: The effects of the inhibitors antimycin, 2-heptyl-4-hydroxyquinoline N-oxide (NOQNO) and 2-nonyl 4-hydroxyquinoline N-oxide (NQNO) on the cytochrome b-c_1 region spectrum of submitochondrial particles are compared with those of a new inhibitor 2-ω-cyclohexyl pentyl 3-hydroxy 1,4-naphthoquinone. Dithiothreitol without electron mediators can cause selective reduction of part of the cytochromes b. Evidence for at least four cytochromes b is presented.

INTRODUCTION

The light absorption by mitochondrial inner membrane between 557 and 570 nm at room temperature is due to two or more b cytochromes (1). The partial resolution of the spectrum into its components has been accomplished by use of low temperature, ATP, electron mediators in solutions of preset potentials, and selective photoalteration (1-10). Although at room temperature this spectrum has been resolved into three peaks at 558, 561 and 566 nm, the apparent independent variation of the 558 and 566 peak was explained by the effect of shifting the 561 nm peak two nmeters to the red because of a unique antimycin A perturbation (7-9), explaining the existence of three peaks in terms of only two chromatophores.

In this paper, by using a new inhibitor 2-ω-cyclohexylpentyl 3-hydroxy 1,4-naphthoquinone (C-OH-NQ) and a new reducing system (dithiothreitol without electron mediators), we further resolve this spectrum.

METHODS AND MATERIALS

Heavy beef heart mitochondria were isolated according to Hatefi and Lester (11), and ETPH was isolated by the Lee and Ernster procedure (12). Mitochondrially orientated particles (MOP) were isolated according

to Hansen and Smith (13) with significant modifications in that the isolation and storage medium was 0.50 M sucrose, 20 mM histidine; 1 mM ATP, 15 mM magnesium acetate, 5 mM EDTA, and 1 mM succinate (SHAMES) neutralized to pH 7.6 with NaOH. All spectra were measured on an Aminco DW-2 spectrophotometer at room temperature in 0.45 M sucrose 50 mM glycylglycine 12 mM MgO pH 8.0 (SGM8).

RESULTS AND DISCUSSION

Recent studies in our laboratory with the new inhibitor, C-OH-NQ have revealed that C-OH-NQ is similar to antimycin A, HOQNO and NQNO in that all cause cytochrome c_1 oxidation and increased cytochrome b reduction in aerobic mitochondrial particles oxidizing NADH, succinate or duroquinol (14). C-OH-NQ differed from the other inhibitors in that the cytochrome b peak with C-OH-NQ added was unusually narrow and did not shift from 562 nm to longer wavelengths. In Figure 1 we compare the effects of C-OH-NQ on the cytochromes b spectrum with those caused by antimycin A and by HOQNO. Chromatophores with peaks at 558 nm and 567 nm can be more reduced by succinate in the presence of HOQNO than in the presence of C-OH-NQ. Similar effects are seen if antimycin A is used in place of HOQNO (figure 1D) with the exception that the absorption from 563 through 568 nm is stronger with antimycin A, especially at 564 nm. That the difference between the effects of C-OH-NQ and antimycin A between 563 and 568 nm are caused by effects on at least two different chromatophores neither of which is cytochrome b_{561} (b_K) is shown in Figures 1B and 1C. C-OH-NQ addition to the reduced particle previously inhibited only by antimycin A causes loss of adsorption throughout the cytochrome b region but especially at 558 and 566 nm (peaks normally associated with b_T). The addition of antimycin A to the reduced particle previously inhibited by C-OH-NQ, however causes the increased reduction at 564 nm, a previously unreported peak. For neither spectrum is there any evidence of a red shift of cytochrome b_{561} (Figure 1C) or its reversal (Figure 1B).

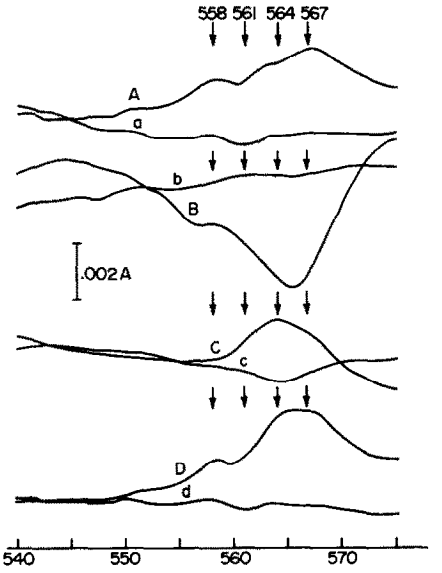

Figure 1.
a. Each cuvette contained 0.84 mg MOP and 1 µmole NADH in 1 ml.
A. 40 µg HOQNO or 3.9 µg C-OH-NQ were added respectively to the sample or the reference cuvettes.
b. Each cuvette contained 1.5 mg MOP, 0.47 µg antimycin A and 0.9 µmoles NADH in 1 ml.
B. 3.9 µg C-OH-NQ was added to the sample cuvette.
c. Each cuvette contained 1.5 mg MOP, 5.3 µg C-OH-NQ and 0.9 µmoles NADH in 1 ml.
C. 0.52 µg antimycin A was added to the sample cuvette.
d. Each cuvette contained 0.84 mg MOP in 1 ml.
D. 0.52 µg antimycin A plus 1 µmole NADH or 3.9 µg C-OH-NQ plus 1 µmole NADH were added to the sample and reference cuvettes respectively.

Since the difference between spectrum 1C and spectrum 1B (or the sum of 1C and 1B after 1B has been inverted), when corrected for protein, produced a spectrum like 1D, the two spectral changes caused by antimycin A, one of which is preventable by C-OH-NQ_1, must be able to occur concurrently. Spectrum 1C is not due to a modification of the components of 1B. Hence two spectral components with peaks at 564 and at 567 nm are present. We propose that the peaks previously seen between 565 and 566 are in fact composit peaks.

Generally spectral studies of inhibitor action are performed with the electron source reducing the electron transport chain at a point more electronegative than the site of inhibition. For the studies of figure 2,

the initial point of reduction is more electropositive than the site of inhibition, perhaps at cytochrome c_1 which is exposed in inside-out particles (15) such as the ETPH (Lee) (16). Dithiothreitol in the presence of CN^- can reduce virtually all of cytochrome c_1 and some of the cytochrome b peaks between 561 and 564 nm independent of the presence or absence of either antimycin A or NQNO. Even though cytochrome c_1 is virtually totally reduced and coupling sites 2 and 3 blocked the subsequent addition of succinate can produce a spectrum the characteristics of which are similar to that of cytochrome b_T. According to Wikström (1) the reduction of a component between the cytochromes b and cytochrome c_1 prevents cytochrome$_T$ reduction. The exact shape of this succinate minus dithiothreitol spectrum is dependent on the presence or absence of either antimycin A or NQNO (Figures 2C and 2E). The remaining dithionite reducible spectrum (2D) contains no distinct peaks. That the total dithionite reducible spectrum is independent of the presence or absence of dithiothreitol shows that dithiothreitol does not alter any of the cytochromes b. (unpublished data)

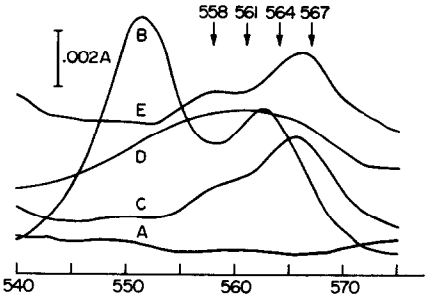

Figure 2.

A. Each cuvette contained 0.66 mg ETPH in 1 ml.

B. 2.4 µmoles NaCN and 0.52 µg antimycin A were added to the sample cuvette. The sample cuvette was then reduced with 10 µmoles dithiothreitol.

C. The reference cuvette was treated similar to the sample cuvette for B. 10 µmoles sodium succinate was added to the sample cuvette.

D. Solid dithionite was added to the reference cuvette and the cuvettes switched.

E. Same as for C, except 26 µg NQNO was used in place of antimycin A.

Since the cytochromes b reduced by dithiothreitol in the presence of either antimycin A or NQNO are distinct from longer wavelength cytochromes b, a more complete analysis of the difference in the effects of antimycin A and NQNO on the cytochrome b can be made. According to Brandon et al. (8) the only difference between the inhibitions of antimycin A and a quinoline N oxide is that antimycin A shifts cytochrome b_{561} two nmeters to the red. If so, the comparison of spectra of quinoline N oxide inhibited particles with those of antimycin A inhibited particles in the presence of identical reducing environments should show either no difference if cytochrome b_{561} is not reduced or a peak at 558 nm equal in height to the depth of a trough at 565.5 nm independent of the type of reducing environment. As shown in figure 3 such is not the case. In the presence of only dithiothreitol, the NQNO verses antimycin spectra has a peak between 558 and 561 nm several times stronger than the slight trough at 567 nm. In the presence of all three reductants, the peak, which has now shifted to 558 nm and becomes narrower, is approximately half as high as the depth of the trough which has broadened and is now at 566 nm. The effects of quinoline N oxides and antimycin A on the cytochromes b

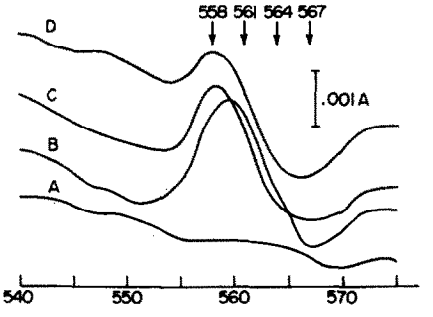

Figure 3.
A. Each cuvette contained 1.32 mg ETPH and 10 µmoles dithiothreitol. 26 µg NQNO or 0.68 µg antimycin A (each in 5 µl methanol) were added to the sample or reference cuvettes respectively.
B. 10 µmoles sodium succinate was added to each cuvette.
C. Solid dithionite was added to each cuvette.

spectrum differ by at least two effects. We therefore propose that there are four distinct components in cytochrome b with peaks at room temperature at 558, 561-62, 564 and 567 nm. The chromophore at 562 nm described by Davis et al. (9) would constitute a fifth element.

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