EFFECTS OF GENERAL ANESTHETICS ON CYTOCHROME *c* OXIDASE

A Thesis

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by

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I. Abstract

Studies were conducted to determine the effects of n-alcohols as general anesthetic on the enzyme, cytochrome *c* oxidase. Results from these studies indicate the following: n-alcohols inhibit the activity of cytochrome *c* oxidase through hydrophobic interactions; the inhibition of cytochrome *c* oxidase by a 1:2 n-butanol/buffer mixture is reversible; the amount required for a 50% inhibition of cytochrome *c* oxidase by n-butanol is 10 times greater than that of physiological concentrations; and n-butanol decreases the rate of reduction of cytochrome *c* oxidase by NADH\PMS, but does not affect the UV-VIS spectra or the ligand binding rates.

II. Introduction

2.1 Cytochrome c Oxidase and Functions

Cytochrome c oxidase, the terminal inner mitochondrial respiratory chain enzyme, is responsible for catalyzing the reduction of O_2 to H_2O utilizing electrons from cytochrome *c*. This enzyme also serves as a CO deoxygenase[1]. In addition to acting as a catalyst and deoxygenase, cytochrome *c* oxidase (complex IV) is important in energy conservation. This enzyme, along with NADH ubiquinone oxidoreductase (complex I) and ubiquinol-ferricytochrome c oxidoreductase (complex III), creates a redox-linked electrochemical proton gradient across the mitochondrial membrane by the reduction of various species. The energy stored in this proton gradient is eventually used by ATPase to synthesize ATP[2].

The reduction of O_2 occurs when electrons obtained from

cytochrome *c* are transferred from a non-ligand binding heme (cyt a), which works in conjunction with a copper atom (Cu_A), to the ligand binding cite-which also consists of a heme (cyt a_3) and a copper atom (Cu_B) . The binuclear center is responsible for the binding and activation of O_2 and also binds other external ligands, such as CO[1]. Some studies report an additional copper atom (Cu_x), whose function is unknown. Both Zn and Mg atoms are also present[1].

Cytochrome *c* oxidase has been found to be inhibited by general anesthetics. Although the main function of general anesthetics is to block nerve endings, they have also been found to inhibit microtubule polymerization, to affect calcium transport and binding abilities, to protect red blood cells from hemolysis, and to inhibit mitochondrial electron transport[3]. It has not been agreed upon whether or not this inhibition occurs due to a partitioning of the anesthetic into the lipid bilayer or a direct inhibition of the protein itself[4]. The aim of this project was to investigate the effect of general anesthetics on the structure and function of cytochrome *c* oxidase.

2.1 Goals

To accomplish this goal the following projects were undertaken: 1) activity assays were conducted to determine the activity of the enzyme in the presence and absence of n-alcohols[5], and the reversibility of these effects; 2) spectroscopic studies were conducted to determine the effects of the n-alcohols on the

structure of the enzyme; and 3) time resolved flash photolysis studies were conducted to determine the rebinding ability of CO to the enzyme in the presence of n-butanol.

III. Experimental

3.1 Materials

Cytochrome *c* oxidase was extracted from the left ventricles of bovine hearts. Cytochrome *c* was obtained from Sigma at >99% purity. Sodium phosphate dibasic and monobasic salts were obtained from Fisher Scientific. Cholate, Tween 20, and dithionite were obtained from Sigma. Samples of n-hexanol were obtained from the Dave Deamer's laboratory and samples of n-pentanol were obtained from the UCSC stock room.

3.2 Methods employed

3.2a Isolation of Cytochrome c Oxidase

Cytochrome *c* oxidase was isolated from bovine hearts using the method of Yoshikawa et al[6]. This first step involved isolation of submitochondrial particles by several homogenization and centrifugation steps. To do this the fat and gristle were cut away from bovine hearts. The hearts were cut into small 1-2 cm cubes, using the left ventricles first until a total of 1.8 kg was obtained. This was divided into two 900 g portions which were treated in the same manner. From this point on every thing was done in the cold room at 4°C or on ice.

Mince each portion of the hearts in a Waring Blender and add 300 mL of 0.2 M Na-phosphate, pH 7.4 buffer and add 1.8 L deionized water. Homogenize this mixture at medium speed for 3 minutes. Centrifuge the homogenized mixture for 40 minutes at 5300 rpm at 4°C. If a lot of fat is present after the centrifugation, use cheese cloth to filter the supernatant. Save the supernatant in a 4 L plastic beaker. Resuspend the pellets in 150 mL of 0.2 M Na-phosphate, pH 7.4 and 1.35 L of deionized water. Homogenize this mixture at medium speed for 3 minutes and centrifuge for 40 minutes at 5300 rpm at 4°C. Combine these two supernatants and mix thoroughly. Split this mixture into two 4 L beakers with a large stir bar and place in a tub of ice. Adjust the pH of each solution to 5.3 with 2 N of acetic acid, then centrifuge for 45 minutes at 5400 rpm. Discard the sup and resuspend the pellet in no more than 290 mL of 0.2 M Na-phosphate, pH 7.4 buffer. Homogenize this mixture at the lowest setting for 30 seconds. Slowly adjust the pH to 7.4 with 3 M NaOH. Place this mixture in a 1000 mL beaker covered with parafilm, saran wrap and aluminum foil and store overnight in the cold room in a bucket of ice.

The next step involved solubilizing the protein using cholate and Tween 20 as detergents, while simultaneously purifying cytochrome *c* oxidase using multiple steps of fractional ammonium sulfate precipitation. Measure the volume of suspension. Calculate the volume of KH particles and add enough 0.2 M Na-phosphate, pH 7.4 buffer to obtain a mixture of 1:1 KH particles and buffer and adjust the pH of this mixture to 7.4 with 3 M NaOH.

Measure out 575 mL of the suspension for further use and save any remaining at -60°C for future preparations.

Add 50 mL of the 40% cholate stock solution to the suspension and take to 35% saturation with 122.5 g of ammonium sulfate immediately after adding the cholate. Adjust the pH of this suspension to 7.4 with 3 M NaOH and let it stand on ice for 25 minutes while stirring. Then centrifuge the suspension for 20 minutes at 17000 rpm at 4°C. Discard the precipitate and add enough ammonium sulfate to take the supernatant to 50% saturation. Centrifuge this solution for 20 minutes at 1700 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in 200 mL of 0.1 M Na-phosphate, 0.5% cholate solution for a total volume of 220 mL. Dialyze this solution in 3-4 L of 0.04 M Na-phosphate, pH 7.4 for 90 minutes. Centrifuge the dialyzed solution for 100 minutes at 18500 rpm at 4°C. Decant the supernatent very carefully removing any fat and discard.

Resuspend the precipitate in a total volume of 200 mL 0.1 M Na-phosphate, 2% cholate solution with a small hand homogenizer on ice until all particles disappear. After homogenizing pour the suspension into a 400 mL beaker on ice. Take the suspension to 25% saturation with 28.8 g of ammonium sulfate and adjust its pH to 7.4 with 3 M NaOH. Let the suspension stand for 30 minutes with no stirring, then centrifuge for 10 minutes at 17000 rpm at 4°C. Discard the precipitate and record the volume of the supernatant. Take the supernatant to a 45% saturation and centrifuge for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in a total volume of 200 mL 0.1 M Na-phosphate, 0.5% cholate solution. Take this suspension to 25% saturation with 28.8 g ammonium sulfate and allow it to stand for 30 minutes with no stirring. Centrifuge this suspension for 10 minutes at 17000 rpm at 4°C. Discard the precipitate and take the supernatant to 40% saturation with ammonium sulfate. Centrifuge this suspension for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in a total volume of 130 mL 0.1 M Na-phosphate, 0.5% cholate solution and use 18.7 g ammonium sulfate to take this suspension to a 25% saturation. Allow the suspension to stand for 30 minutes with no stirring and centrifuge at 17000 rpm at 4°C for 10 minutes. Discard the precipitate and take the supernatant to 35% saturation with ammonium sulfate. Centrifuge for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in a total volume of 200 mL 0.1 M Na-phosphate, 0.75% Tween 20 solution. Do not let the suspension stand and take it to a 25% saturation with 28.8 g ammonium sulfate. Centrifuge for 10 minutes at 17000 rpm at 4°C. Filter the supernatant obtained and adjust to 25% saturation with ammonium sulfate, discarding the precipitate. Centrifuge the suspension for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in a total volume of 100 mL 0.1 M Na-phosphate, 0.5% Tween 20 and check the pH. Store overnight in a beaker covered with parafilm and

aluminum foil on ice in the cold room.

Check the pH of the suspension (should be about 7.4) and take to 25% saturation with ammonium sulfate. Centrifuge the suspension for 10 minutes at 17000 rpm at 4°C. Discard the precipitate and filter the supernatant with glass wool. Take the supernatant to 35% saturation with ammonium sulfate and centrifuge for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in a total volume of 80 mL 0.1 M Na-phosphate, 0.5% Tween 20 solution. Take this suspension to 25% saturation with 11.5 g ammonium sulfate and centrifuge for 10 minutes at 17000 rpm at 4°C. Discard the precipitate and take the supernatant to 33% saturation with ammonium sulfate. Centrifuge the suspension for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in a total volume of 80 mL 0.1 M Na-phosphate, 0.5% Tween 20 solution. Take the suspension to a 25% saturation with 11.5 g ammonium sulfate and centrifuge for 10 minutes at 17000 rpm at 4°C.

Discard the supernatant and resuspend the precipitate in as little volume of 0.1 M Na-phosphate, pH 7.4 buffer as possible. Dialyze this suspension in 3-4 L 0.1 M Na-phosphate, pH 7.4 buffer overnight in the cold room.

Centrifuge the dialyzed suspension at 18000 rpm for 30 minutes at 4°C. Pipette the supernatant and place in an amicon concentrator and discard the precipitate . Concentrate under pressure to a volume of about 4-5 mLs. Pellet the concentrated

suspension by pipetting small drops into liquid nitrogen and placed in eppendorf tubes. The pellets were 1.5 mM cytochrome *c* oxidase, and were stored at -80°C until used.

3.2b Preparation of cytochrome c oxidase for activity assays

A cytochrome *c* oxidase stock solution was prepared by diluting 10 μL of the concentrated enzyme into enough of the 0.1 M Na-phosphate, pH 7.4, buffer to obtain a 1 μM solution.

3.2c Preparation of n-alcohol solutions

Saturated stock solutions of n-butanol, n-pentanol, and n-hexanol were prepared by adding 1 mL of each of the alcohols per 5 mL of 0.1 M Na-phosphate, pH 6.00, buffer. Each of these stock solutions were further diluted in the same buffer to form various percent saturations ranging from 5% to 50%.

3.2d Preparation of reduced cytochrome c Solution

Cytochrome *c* was prepared by placing 3 or 4 scoops (enough to make the solution a very deep red color) into about 0.5 to 0.7 mL of 0.1% NaCl. This solution was reduced using a small amount of dithionite powder (which makes it turn a lighter red). The solution was then ran over a G-25 column, equilibrated with about 100 mL of 0.1% NaCl. The solution of cytochrome *c* was eluted with a 0.1% NaCl solution and collected in eppendorf tubes. The most concentrated portion (the darkest part of the solution) was

collected into a separate tube and used for the activity assay. The concentration of the solution was measured at 550 nm, the maximum peak of the reduced cytochrome *c*, in a Hewlet Packard (HP) UV/VIS spectrophotometer using an extinction coefficient of 30.4 $mM^{-1}cm^{-1}$ at 550 nm. Twenty μL of the sample in 2.0 mL of 0.1 M Na-phosphate, pH 6.00, were placed in a 1 cm path length cuvette. Dithionite was subsequently added to see if any oxidation had occurred in the column. If there was a significant change in absorbance, the cytochrome *c* solution was prepared again in the same fashion. The solution was capped and stored on ice to prevent reoxidation from occurring.

3.2e Activity assays

The activity of cytochrome *c* oxidase was followed by observing the oxidation of reduced cytochrome *c* at 550 nm, the maximum absorbance for reduced cytochrome *c*, using the HP spectrophotometer. Control samples to be tested were prepared by adding 20 μL of the 1 μM solution of cytochrome *c* oxidase, enough cytochrome *c* to make its concentration 15 μM, and enough 0.1 M Na-phosphate, pH 6.00, buffer to make a solution whose total volume was 3 mL. Each of the n-alcohol solutions were prepared by adding 20 μL of the 1 μM cytochrome *c* oxidase and enough cytochrome *c* to make its concentration 15 μM to a n-alcohol solution at a certain fractional concentration. For each assay, the buffer or n-alcohol/buffer mixture was placed in a 1 cm path length cell. Then the cytochrome *c* oxidase was added and allowed to incubate for 2

minutes so that it had time to interact with the n-alcohol. Subsequently the cytochrome *c* was added and a scan was taken immediately. The activity was determined for the cytochrome *c* oxidase as the control and in the presence of varying percent saturations for each of the alcohols. This process was also used to determine if the inhibitory effects of n-butanol were reversible. A 1:2 n-butanol/0.1 M Na-phosphate, pH 6.00, buffer mixture was prepared and its activity was assessed as described above. This solution was then further diluted into 4 mL of the same buffer and its activity assessed again. Assays for a control of cytochrome *c* oxidase were carried out in the same way.

3.2f Spectroscopic studies

In order to determine the effect of the n-alcohols on the spectroscopic properties of cytochrome *c* oxidase, spectroscopic studies were conducted in which the absorbance was measured versus wavelength from 190 nm to 820 nm. The samples were prepared by adding 10-20 μL of stock cytochrome *c* oxidase to a total volume of 3 mL 0.1 M Na-phosphate, pH 6.00, buffer for the control or a 1:2 n-butanol/buffer solution. The solutions were then deoxygenated by passing nitrogen gas over them for about one hour. Both of the solutions were reduced by adding 10 μL of NADH and 2 μL of PMS, which had both been previously deoxygenated with nitrogen gas, to the solutions. Repeated scans were taken until fully reduced species were present. The CO-complexes were made by passing CO over the reduced enzyme solutions for about 15 to 20 minutes.

Spectra were obtained for each of the CO-bound species.

Since it was noted that it took longer to fully reduce the cytochrome *c* oxidase solution that had been inhibited by n-butanol, spectra for both a control solution and a 1:2 n-butanol/0.1 M Na-phosphate, pH 6.00, solution were collected as a function of time. The solutions were prepared as noted above and scans were taken in 15 second intervals immediately after the addition of NADH and PMS until each solution was fully reduced.

3.2g Photometric studies

Time resolved flash photolysis experiments were conducted using reduced cytochrome *c* oxidase bound to CO in the presence and absence of n-butanol to determine how its presence affects the enzyme's ligand binding ability. The time resolved spectra were collected using an optical multichannel spectrometric analyzer system. An oxidized enzyme solution (ca. 10 μL) was deoxygenated for about 1 hour with nitrogen, then fully reduced using 15 μL NADH and 2 μL PMS. The reduced cytochrome *c* oxidase-CO complex was obtained by passing CO over the solution for about 30 minutes. The 1:9 n-butanol/ buffer cytochrome *c* oxidase solutions were prepared in an analogous way. The time-resolved spectra were collected between 40 ns to 126 ms after CO photolysis at 75 time points

IV. Results

4.1 Results from Activity Assays

When observing the oxidation of cytochrome *c* by cytochrome *c* oxidase at 550 nm in the activity assays, a distinct decrease may be seen in the activity of cytochrome c oxidase in increasing concentrations of the n-alcohols as seen in Figure 1a. This is more evident in Figure 1b where the log of the initial absorbance at 550 nm minus the final absorbance at 550 nm is plotted verses time. The greater the slope, the greater the rate of activity in the enzyme. The rate constants for each of the different ratios of n-alcohols were used to calculate the percent inhibition of cytochrome c oxidase. Figure 2 shows the percent inhibition versus the concentration of each of the n-alcohols (2a) and the percent inhibition verses the percent saturation of each of the n-alcohols (2b).

4.2 Results from Spectroscopic Studies

Results from the spectroscopic studies may be found in Figure 3. The spectra for the oxidized, reduced, and reduced CO-bound species of cytochrome *c* oxidase in the presence and absence of a 1:2 n-butanol/buffer mixture in both the Soret and alpha regions are shown. The difference spectra for the reduced minus oxidized and the reduced minus the reduced CO-bound cytochrome *c* oxidase are represented in Figure 4 in both the presence and absence of a 1:2 n-butanol/buffer mixture in the Soret and alpha regions. Figure 5

depicts the shift from the oxidized to reduced species of cytochrome *c* oxidase in the presence and absence of a 1:2 n-butanol/buffer mixture with respect to time in the Soret and alpha regions.

4.3 Results from Time Resolved Flash Photolysis

The results from the time resolved flash photolysis experiments are represented in Figure 6. Two distinct processes, with apparent lifetimes of 1.57 μs and 9.60 ms, were obtained in the spectra of the fully reduced cytochrome CO-bound complex in the presence of n-butanol. The same two lifetimes were obtained in the absence of the inhibitor.

V. Discussion

5.1 Activity Assays

From the activity assays each of the n-alcohols were found to inhibit cytochrome *c* oxidase to some extent. When the percent inhibition was plotted versus concentration, it was seen that at the same concentrations the inhibition of n-hexanol was greater than that of n-pentanol, which was greater than that of n-butanol. However when the percent inhibition was plotted versus percent saturation, it was found that each of these alcohols had about the same inhibitory effects at the same percent saturations. Since it is known that an increase in chain length results in a decrease in water solubility and an increase in lipid solubility, the

difference in these two plots indicates hydrophobic interactions between the n-alcohols and the cytochrome *c* oxidase. Even though these n-alcohols produce anesthetic effects by non-specific interactions at the target sites, anesthesia potency can be related to lipid solubility. The activity assays also indicate the inhibitory effects caused by n-butanol are completely reversible.

5.2 Spectroscopic Studies

From the spectroscopic studies, the only change that occurred in the spectra of cytochrome *c* oxidase when inhibited by n-butanol in a 1:2 mixture was a very slight increase in absorbance of the cytochrome *c* oxidase. This indicates the n-butanol does not have a significant effect on the structure of this enzyme. However, it was found that it takes longer to fully reduce cytochrome *c* oxidase that has been inhibited by a 1:2 butanol/buffer mixture. This indicates some type of interference with the electron transfer, although the exact mechanism is not known.

5.3 Time Resolved Flash Photolysis

The results from the photolysis studies indicate that the presence of a 1:9 n-butanol/buffer mixture does not affect the CO recombination. However since this particular study was done at a lower concentration of n-butanol, these results may not be comparable to those from the spectroscopic studies conducted with a 1:2 butanol/buffer mixture.

VI. Conclusions

We do not know whether the effects of the anesthetics are on the lipid or the protein portion of the cytochrome *c* oxidase or both. The results from the activity assays indicate that the hydrophobic interactions occur between the n-alcohols and the enzyme. These could be lipid sites or alternatively hydrophobic sites in the protein portion. The effects of n-butanol on the electron transfer indicates that the protein itself, namely the redox centers, are affected. Analogous studies on cytochrome *c* oxidase that have been depleted of lipids may help resolve the relative importance of the inter actions of the n-alcohols with the protein versus the lipids.

VI. Acknowledgements

I would like to thank Dr. Olof Einarsdottir and members of her laboratory, especially Artur, for all the knowledge and experience they shared with me will working on this project. I would like to thank the SURF (Summer Undergraduate Research Fellowship) Program and National Science Foundation for funding this project. I especially want to thank both the Chancellor's Scholar Program and the NASA Minorities in Science, Engineering, and Applied Mathematics Program for funding and encouraging me to participate in undergraduate research projects. Last, but definitely not least, I would like to thank my advisor and mentor Dr. Paul A. Flowers for working with me for the duration.

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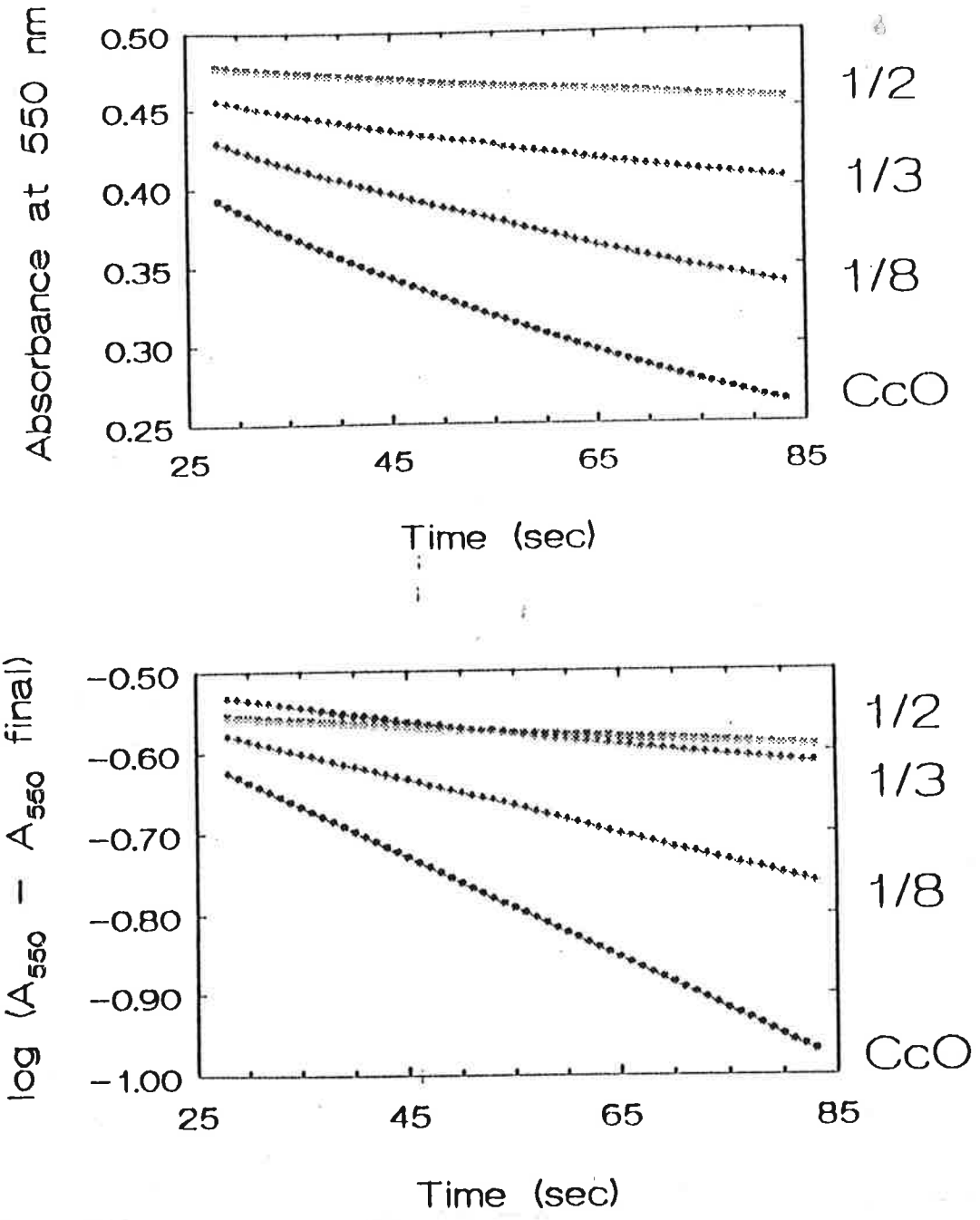
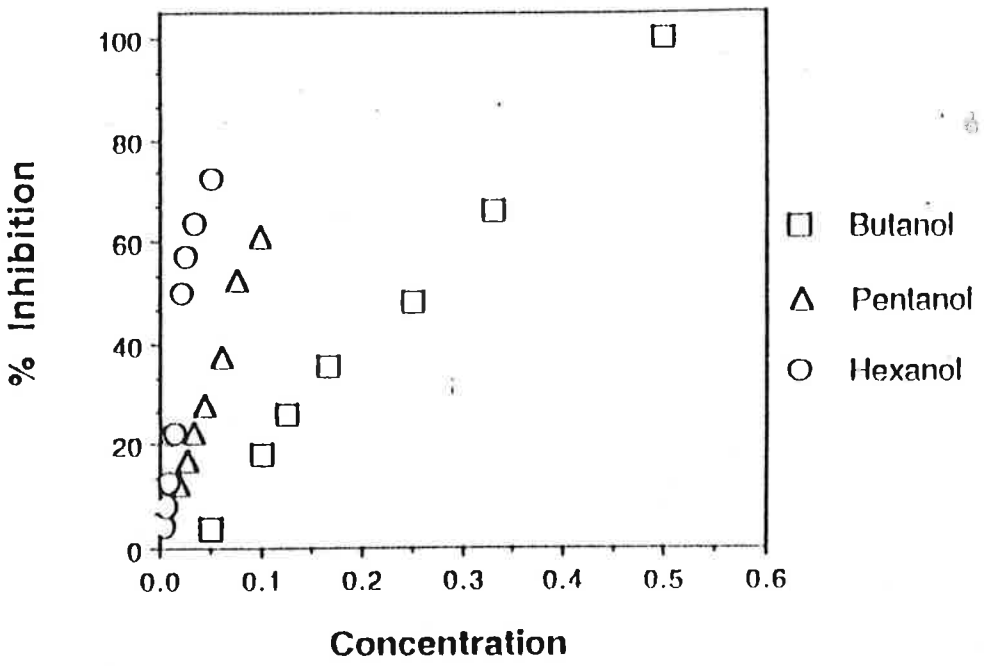


Figure 1 Spectra from activity assays of(a) absorbance at 550 nm verses time and (b) log of (A_{550} - A_{550}) verses time for the control and varying concentrations of n-butanol.



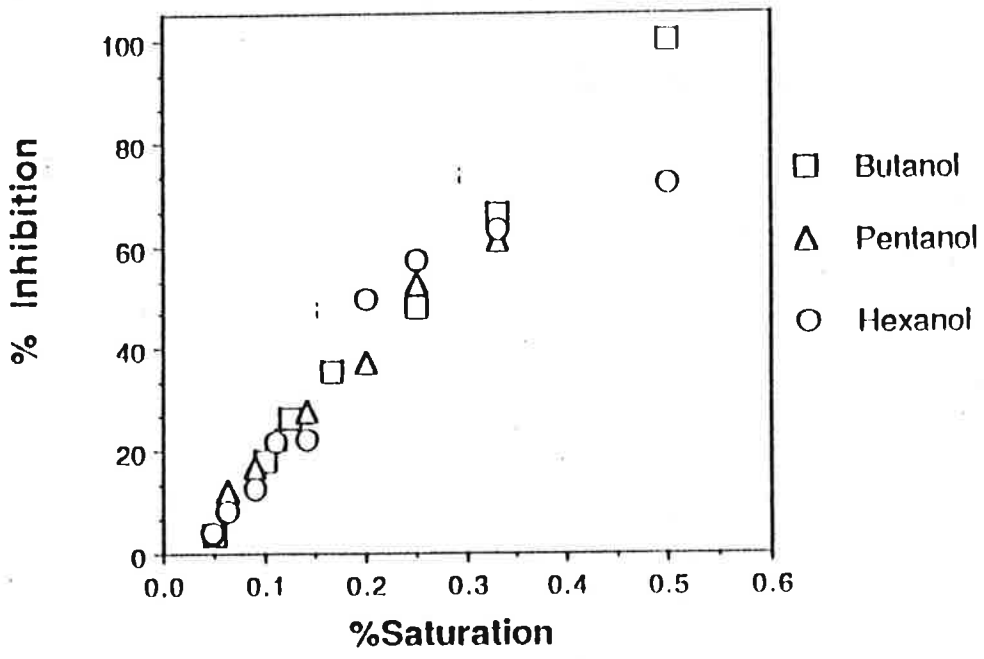


Figure 2 Comparisons of plots of data received from activity assays as (a) percent inhibition verses concentration and (b) percent inhibition verses percent saturation of n-butanol, n-pentanol, and n-hexanol.

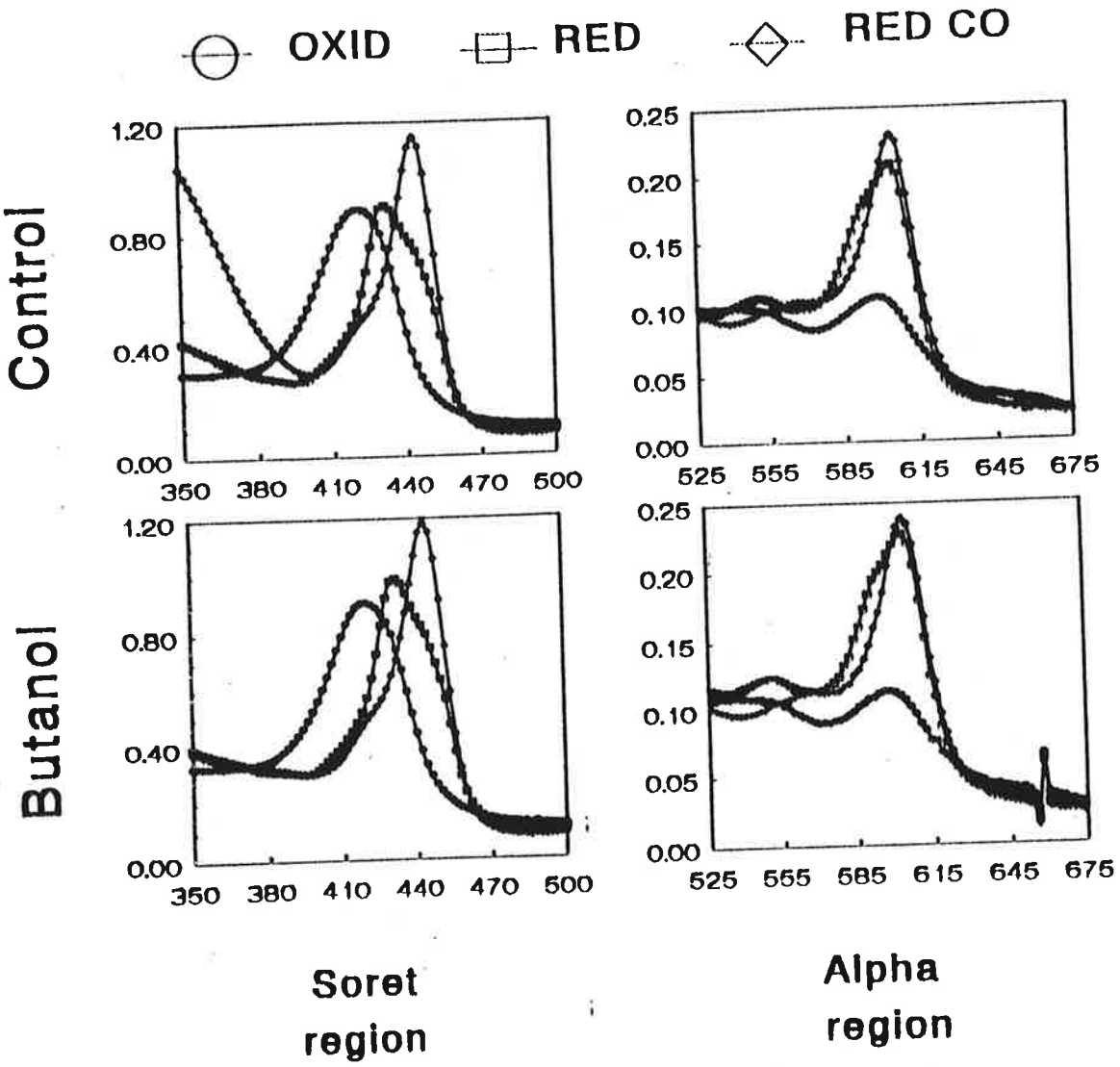


Figure 3 Spectra of oxidized, fully reduced, and fully reduced bound to CO species of cytochrome c oxidase for (a) the control enzyme in the soret region, (b) the control enzyme in the alpha region, (c) the enzyme in n-butanol/buffer solution in the soret region, and (d) the enzyme in the n-butanol/buffer solution in the alpha region.

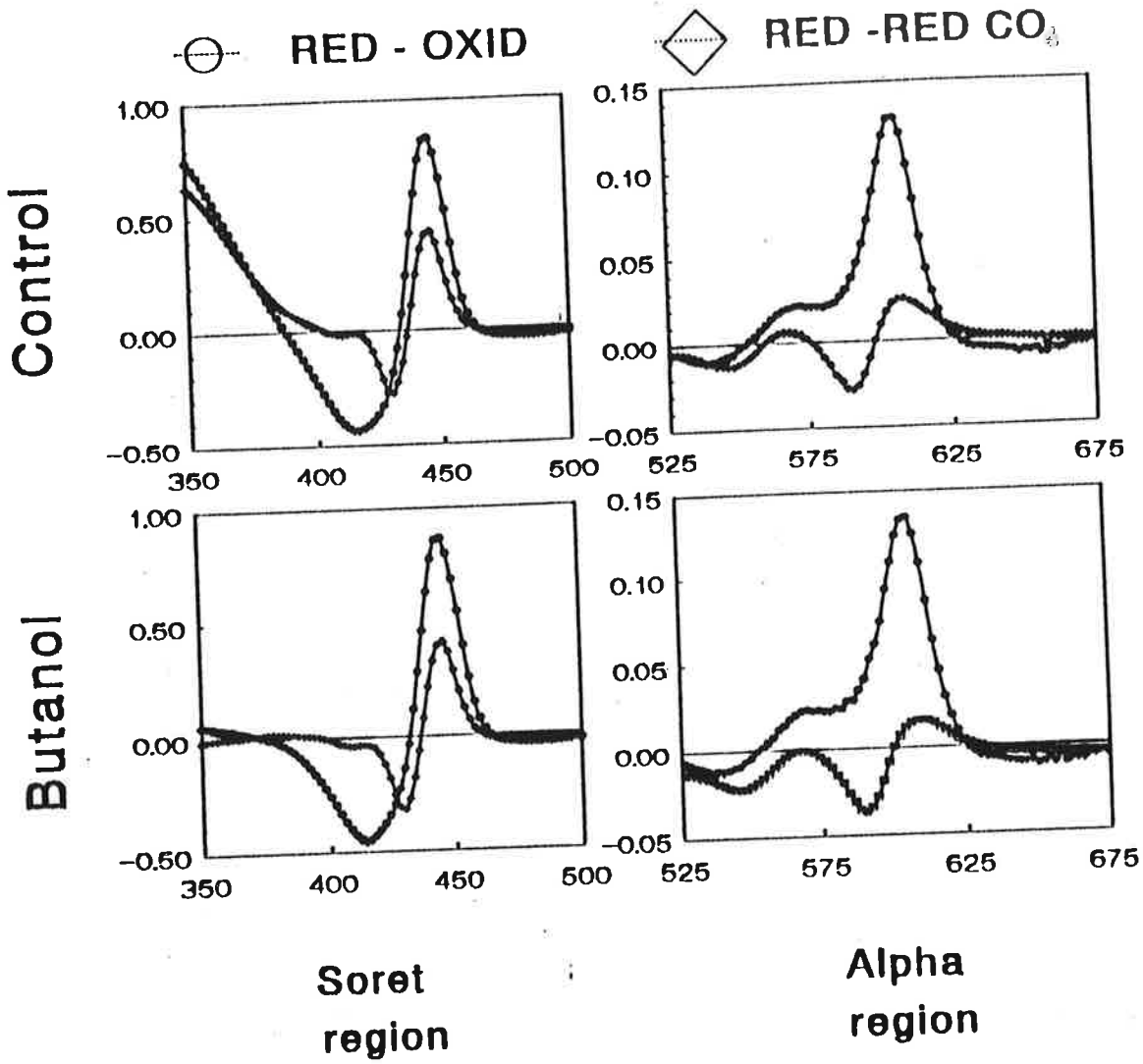


Figure 4 Difference spectra for the reduced - oxidized and reduced - reduced bound to CO cytochrome c oxidase for (a) the control enzyme in the soret region, (b) the control enzyme in the alpha region, (c) the enzyme in the n-butanol/buffer mixture in the soret region, and (d) the enzyme in the butanol/buffer mixture in the alpha region.

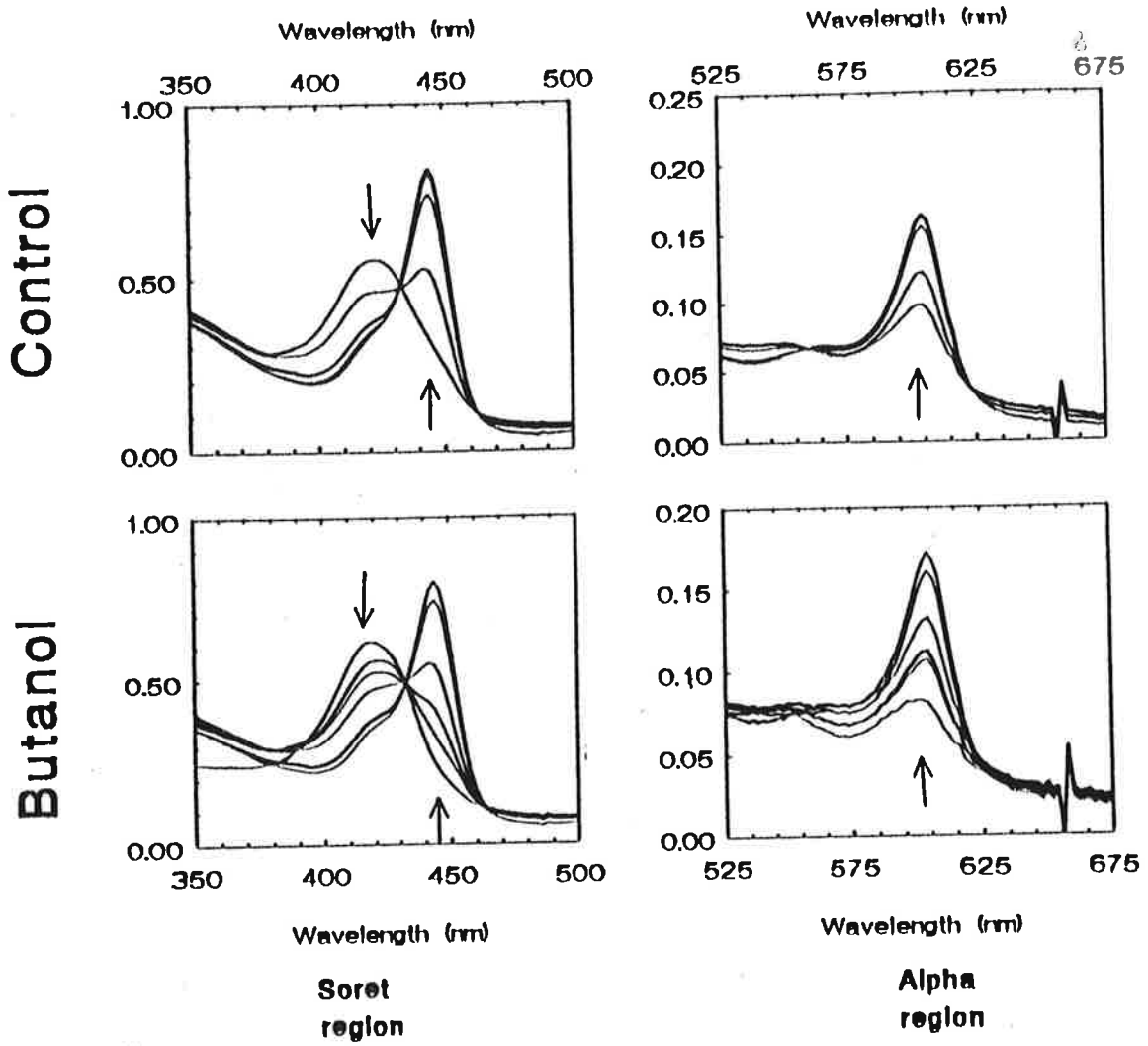


Figure 5 Spectra representing the shift from a oxidized to a fully reduced species for (a) the control enzyme in the soret region, (b) the control enzyme in the soret region, (c) the enzyme in the n-butanol/buffer mixture in the soret region, and (d) the enzyme in the n-butanol/buffer mixture in the alpha region. The spectra represent 30 second intervals beginning at 30 seconds after the injection of NADH/PMS.

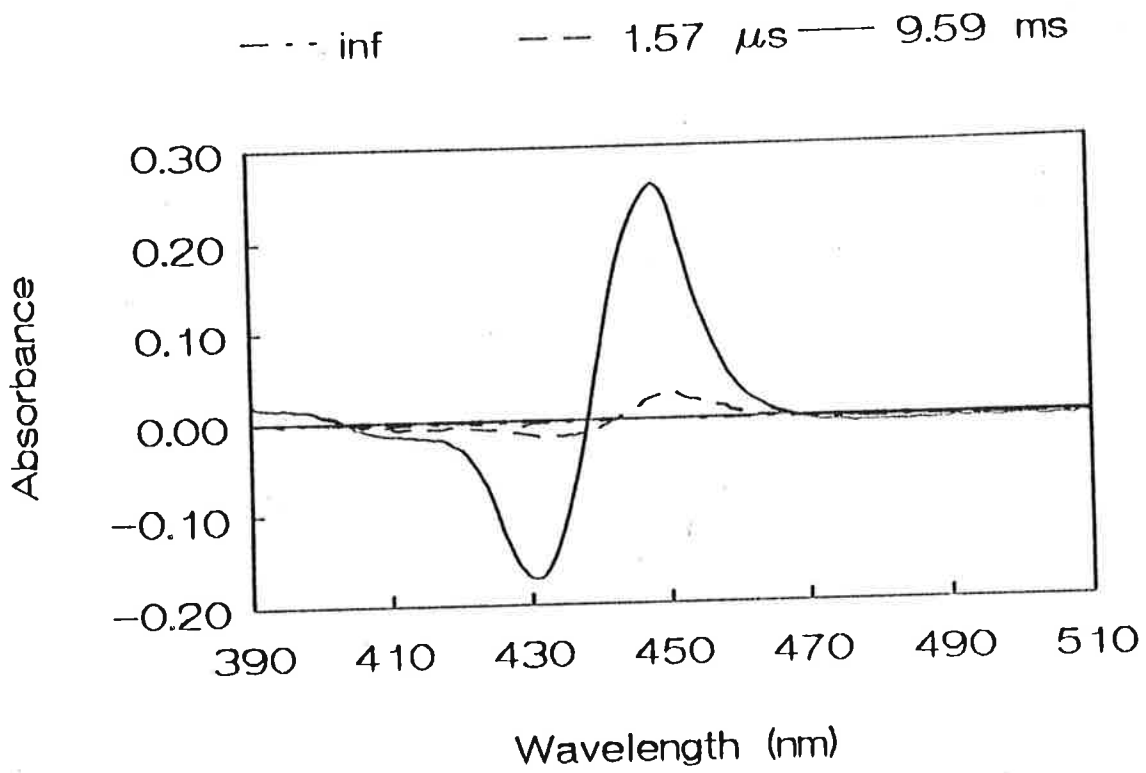


Figure 6 Difference spectra representing the two different species obtained from the time resolved flash photolysis of fully reduced cytochrome c oxidase bound to CO.