CYTOCHROME C-BASED DETECTION OF SUPEROXIDE

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Abstract

Superoxide (O_2^-) is a free radical produced by the one-electron reduction of oxygen. It is a side product of the reduction of oxygen to water, but also produced by immune cells as a chemical weapon to fight infection. There is evidence linking superoxide production to neurodegenerative and autoimmune diseases in humans. Among the methods of superoxide detection and quantitation, the most reliable is by the reduction of cytochrome c, observed spectrophotometrically. However, this method suffers from low sensitivity. To address the issue, we suggest attaching a fluorescent label, fluorescence being dependent on the redox state of cytochrome c. As fluorescence is a much more sensitive spectroscopic method compared to absorbance measurements, we assume that the modified cytochrome c will afford better sensitivity in the detection of superoxide. Wild type and a few mutated cytochromes c are used for modification. We perform spectroscopic studies in order to choose the best probe for superoxide detection. Then, fluorescently modified cytochrome c will be used in model and cellular superoxide-generating systems and the sensitivity of this approach will be compared with the absorbance measurements.

Goal

The goal of our research is to construct a probe for superoxide based on cytochrome c. Cytochrome c was chosen as the candidate for the probe because its reaction with superoxide is fast and specific. The reduction of cytochrome c can be observed through the change of its optical absorption spectrum; however, spectrophotometry is not sensitive enough for the detection of low fluxes of superoxide. Instead, we propose to use fluorescence, which is more sensitive. The dye we have chosen is carboxy-X-rhodamine, with excitation at 584 nm and emission at 600 nm. We suggested that the attached dye would change its fluorescence depending on the oxidation state of cyt. c, as oxidized cyt. c is paramagnetic (Fe^{3+}) and reduced is diamagnetic (low spin Fe^{2+}).

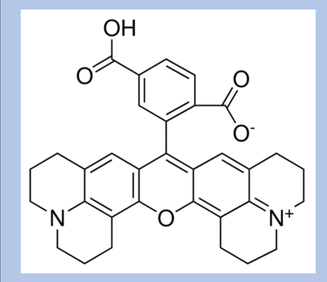

Fig 1 Structure of Carboxy-X-Rhodamine

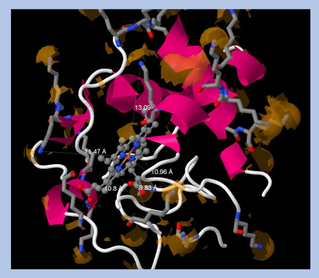

Fig 2 3D Structure of cytochrome c

Making Cytochrome c Mutants

Horse cytochrome c contains 19 lysines in its amino acid sequence. Based on 3-D structure of cyt. c, four lysines closest to heme (lys13, 27, 72 and 79) were chosen for mutation to cysteine. As no free cysteines are present in cytochrome c, the one introduced would be the only point available for modification by a cysteine-specific carboxy-X-rhodamine derivative. Using PCR with mutagenic primers and a plasmid containing wild-type horse cyt. c as a template, we created 4 different mutants, each having one of the lysine codons replaced with a cysteine codon. Overlap extension PCR was used to introduce the mutation at the desired position. Restriction enzymes were employed to replace the wild-type cyt. c with the mutated cyt. C and insert it into the pBT (hcc) plasmid that was further used to transform *E. coli.* Work on getting the cells to express mutated cyt. c is currently underway

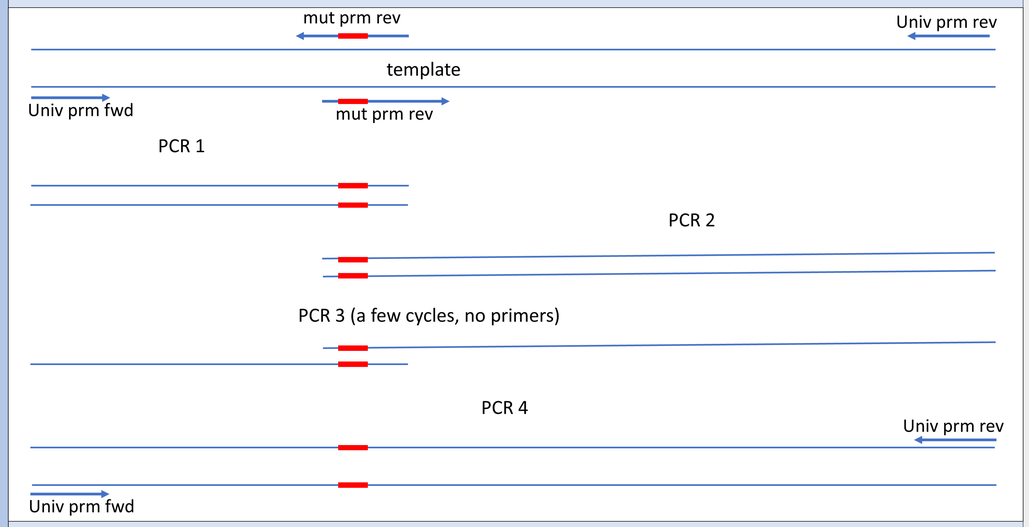

Fig 3 Schematic detailing the overlap PCR technique that was used for making cytochrome c mutants.

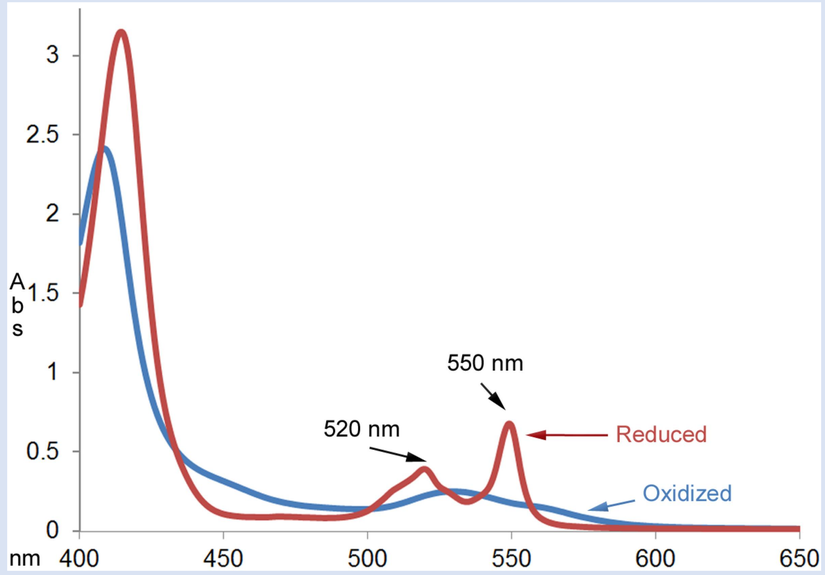

Fig 4 Optical spectrum of cytochrome c displaying the difference in spectra between its reduced and oxidized forms.

Modification of Wild Type Cytochrome c

Fluorescent label was attached to commercial wild-type cytochrome c by slowly infusing it with 5(6)-carboxy-X-rhodamine-N-succinimidyl ester by adding 1mL of dye to 10 mL of cytochrome c via syringe pump at a flow rate of 2.00 µl/min. The reaction mixture was concentrated using a centrifuge filter, allowing for excess dye to filter out. The labeled cytochrome c was then run through gel filtration Sephadex G-25 column to separate it from any remaining unbound dye. Five fractions were collected then had their optical spectra recorded to determine the dye to cytochrome c ratio. With all five fractions shown to be approximately the same, the fractions were combined. Potassium ferricyanide, $K_3Fe(CN)_6$ was added to the cytochrome c to convert it to its oxidized form, and then the cytochrome c was put through dialysis overnight. The dialyzed cytochrome c was then run through ion exchange CM-Sephadex column, resulting in the collection of 49 fractions. All fractions had their absorbance and fluorescence spectra recorded. Based on the results of the optical spectra, the fractions were combined into four samples and concentrated using centrifuge filter. Optical and fluorescent spectra were recorded for the four samples to determine which should be used for kinetics analysis. Commercial cytochrome c was used for recording the reduction of cytochrome c in the presence of superoxide by spectrophotometry. Cytochrome c was added to superoxide-generating system consisting of hypoxanthine and xanthine oxidase in sodium phosphate buffer pH 7.5. Trace without xanthine oxidase was run as a blank. After recording the blank, xanthine oxidase was added and the kinetics of the reduction of cytochrome c was recorded. Then, fluorescence was used to measure the reduction rate of cytochrome using the modified cytochrome c. Fluorescence was tested at various concentrations to show that the increase in the intensity of the fluorescence was not dependent on concentration of cytochrome c.

Results

Rate of superoxide production by optical measurements, using differential extinction coefficient at 550 nm ($21.1 \times 10^3\ M^{-1}\ cm^{-1}$): 1.17 nM/s.

Rate of superoxide production by fluorescence measurements: 1.07 nM/s.

The rates measured by spectrophotometry and by fluorescence are the same, validating the fluorescence method.

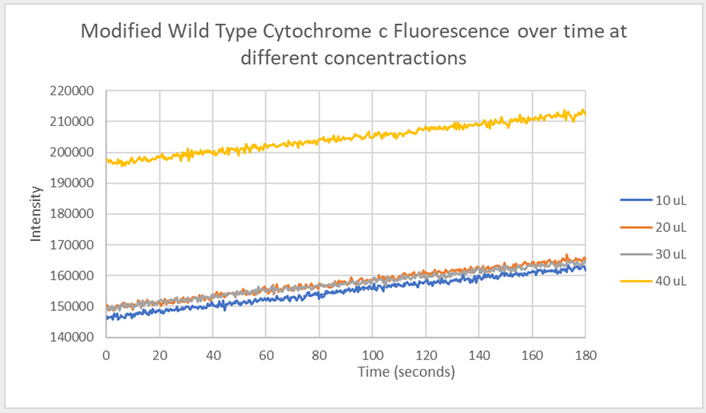

Fig 5 A graph showing the change in fluorescence intensity over time as modified cytochrome c is reduced by superoxide. Tests were carried out at four different concentrations which were determined to have the same slope, showing that the change in fluorescence did not depend on cytochrome c concentration.