Generation of a protein based fluorescent reactive oxygen species sensor

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Abstract

The long- term goal of the project is to develop an environmentally safe, sensitive method for the detection of reactive oxygen species. The current hypothesis is that the biosynthetic incorporation of [2,5-H_2]-phenylalanine into the UV sensitive fluorescent variant Tyr66Phe of the green fluorescent protein will allow the sensing of reactive oxygen species by fluorescence.

Introduction.

Reactive oxygen species such as superoxide($\cdot O_2$) and hydroxyl radical ($\cdot OH$) are highly reactive and are responsible for extensive cellular damage [1]. Biological molecules are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutase and catalases. However a deficiency in these enzymes has been linked to various diseases such as heart disease, cancer, Alzheimer's disease and lateral sclerosis. The aim of this research is to test the hypothesis that the biosynthetic incorporation of [2,5-H_2]-phenylalanine into proteins will facilitate the detection of radical oxygen species via the free radical oxidation of [2,5-H_2]-phenylalanine to phenylalanine. A mutant variant Tyr66Phe (Y66F) of Green fluorescent protein (GFP) [2] was used to test this hypothesis. Tyr66 is an essential component for the formation of the chromophore of GFP and mutation of Tyr66Phe will result in a blue shift in the fluorescence [3]. However, when [2,5-H_2]phenylalanine is incorporated biosynthetically to this mutant GFP it will become non fluorescent and in the presence of superoxide the conversion to Phe will occur and GFP will become fluorescent again. In the presence of superoxide the [2,5-H_2]-phenylalanine-incorporated protein changes from a non fluorescent to a fluorescent protein whereas the Y66F mutant protein does not. This result indicates that Green Fluorescent Protein can be used as a fluorescent reactive oxygen species sensor.

Experiment.

Site directed mutagenesis

Mutagenesis of Y66F was carried out using the Quickchange mutagenesis kit from Stratagen. The following primers were used: forward 5'-GTCACTACTTTCTCTTTTGGTGTTCAATGCTTTTCCCG;-Reverse 5'CGGGAAAAGCATTGAACACCAAAAGAGAAAAGTAGTGAC. Sequences were verified at the Protein Nucleic Acid Laboratory (PNACL) at Washington University in St. Louis. Strain NK6024, which is a phenylalanine auxotroph, was obtained from the Yale University Genetic Stock Centre.

Protein Expression:

NK6024 cells harboring Pglogfpuv were used to label with [2,5-H_2]-Phe. A single colony was incubated in Luria-Bertani (LB, 5 ml) medium with ampicilin (0.1 mg/ml) and tetracycline (.25 mg/ml) and grown for 8-10 hours. The culture was diluted (1:100) with freshly prepared minimal medium (100 ml) and incubated at 32°C overnight. This was further diluted to 600 ml minimal medium and incubated at 32°C and grown to an OD_{600} of 1 by continuous shaking using a platform shaker. The cells were harvested and washed twice with 0.9% NaCl (250 ml each) and transferred to fresh minimal medium containing [2,5-H_2]-Phe (1 mM) and catalase (0.01 mg/ml) and incubated for 10 minutes. The protein was induced by adding arabinose (20%) and allowed to grow for three hours. Cells were harvested by centrifugation and the cell pellet was frozen.

Protein purification:

The protein was then isolated from the bacterial pellet by first resuspending it in TE buffer (pH 8, 20 ml), sonicating for approximately one minute and centrifuging at 17,000 rpm for 30 min at 4°C. The supernatant thus obtained was applied to a HiTrap Q (Amersham Biosciences) and the chromatogram developed using a NaCl gradient (20% over 120 ml). using an Akta prime FPLC system. The fractions containing GFP were pooled and concentrated by ammonium sulfate precipitation. Sufficient 3 M $(NH_4)_2SO_4$ was added to adjust the final salt concentration to 1.3 M and was further purified using.

a HiTrap butyl FF (Amersham Biosciences) column. The protein was eluted with a gradient of 80% over 120ml of buffer TE. The fractions were analyzed using SDS page. This method yielded sufficiently pure protein for further analysis.

UV absorbance

The UV absorption spectra of the proteins were recorded between 200-500 nm on a Cary-Eclipse UV/ Vis spectrophotometer at room temperature. For protein concentration determinations an extinction coefficient of 22015 $M^{-1}cm^{-1}$ was used.

Fluorescence

Fluorescent spectra were recorded in buffer TE on a Carry-eclipse fluorescence spectrophotometer at 20°C by using an excitation wavelength of 350 nm. The slits were set at 5nm.

Preparation of superoxide

Generation of superoxide was carried out according to the method of Fee and Bull[3] under N_2 and was stored at -20^{0}C.

Result and discussion

The initial goal of this research is to show that [2,5-H_2]phenylalanine could be biosynthetically incoperated in to proteins in *E.coli*. Birch reduction was used to synthesis [2,5-H_2]-phenylalanine from phenylalanine. Site directed mutagenesis to insert a Phe in the Y66 position was carried out using the plasmid pGlo(Bio-Rad), which has gfp_{uv} under control of arabinose inducible prometer and the plasmid was transformed into the Phe-auxotrophic strain NK6024.Similar labeling protocol which is used for PapD (Fridden et.al 2003) was used for the Incorporation of [2,5-H_2]-phenylalanine and the protein was purified to near homogeneity. The UV absorption spectra of WT GFP, MUT Y66F and the [2,5-H_2]-phenylalanine labeled protein are shown in Fig1. The absence of the chromaphore at 350nm is clearly visible in the [2, 5-H_2]-phenylalanine labeled protein. 500 µl each of the Y66F protein and the [2, 5-H_2]-phenylalanine labeled protein were mixed

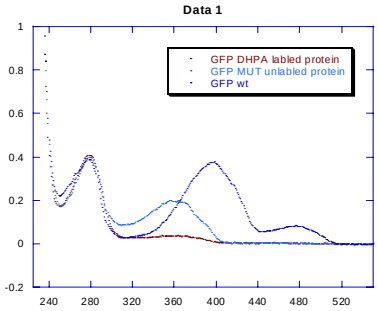

Fig 1 UV absorption spectra for GFP wt, Y66F and DHPA labled proteins

Separately with 50 µl of superoxide under N_2. After 15 minutes of incubation the fluorescence

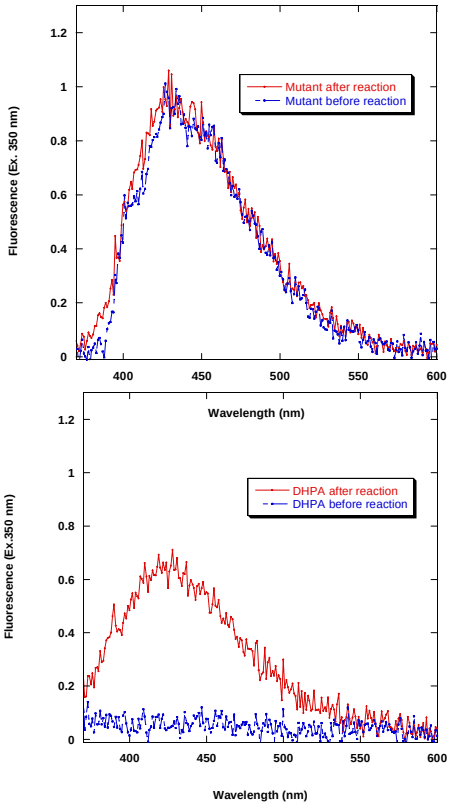

Fig 2 Fluorescence data (EX at 350nm) for Y66F (Top) and [2,5-H_2]-phenylalanine labled protein (bottom)

Fluorescence spectra were recorded as shown in Fig. 2. Control experiments for buffer and DMSO/DMF were also carried out. The fluorescence data [4] for the Y66F protein (Peak max at 427 nm) did not show any significant difference either in the presence or in the absence of superoxide, however the increase in fluorescence for the [2,5-H_2]-phenylalanine-labeled protein in the presence of superoxide was clearly visible.

Conclusions

The increase in fluorescence in the presence of superoxide in the [2, 5-H_2]-phenylalanine-labeled GFP is the initial step toward fabrication of the protein based fluorescent superoxide sensor.

Acknowledgements

This work was supported from an ACS PRF grant (JGB).

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