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Specificity analysis of site-directed mutated glucose indicator proteins

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Honors Thesis

Specificity Analysis of Site-Directed Mutated Glucose Indicator Proteins

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INTRODUCTION

Diabetes Mellitus is the center of focus for many healthcare problems. It affects 16 million people in the United States and over 100 million worldwide[1]. Finger stick testing restricts maintaining strict levels of blood glucose concentration. Invasive techniques involve drawing the blood and hence the sample can be drawn only once daily or less often. Due to this limitation insulin dosage is not optimally coordinated with blood glucose levels and complications continue to arise. Hence it is more important to develop noninvasive technologies for continuous blood glucose monitoring. A variety of glucose biosensors have been developed, including a trilayer coated probe[2], microdialysis probes[3] and amperometric sensors[4].

Most biosensors use enzymes, which provide the desired analyte specificity, but are often not appropriate for noninvasive detection because they lack an intrinsic signal transduction mechanism[5]. The goal of this project is to create a glucose indicator protein by integrating an optical signal transduction function directly into a glucose binding protein (GBP). The strategy is to incorporate fluorescent reporter groups into a GBP in such a manner that the spatial separation between the two fluorescent moieties changes upon glucose binding, thus generating a signal for optical detection. Fluorescence resonance energy transfer (FRET) is to be capitalized on, utilizing green fluorescent proteins (GFPs) that have been used for a number of bioassays[6,7,8].

BACKGROUND

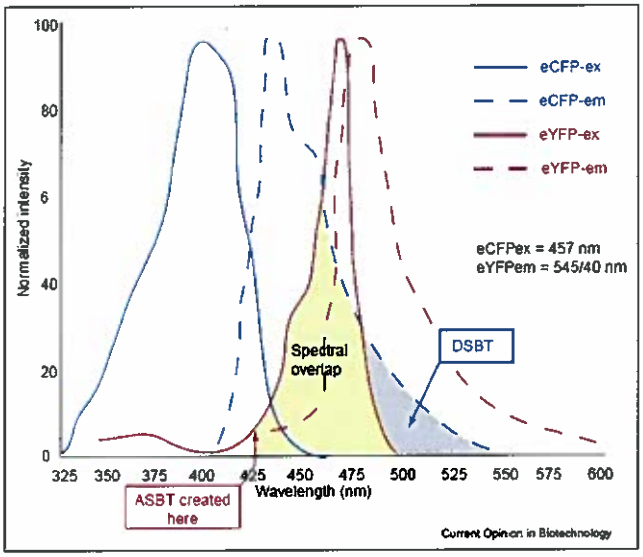
- Fluorescence Resonance Energy Transfer

FRET is unique in generating optical signals sensitive to molecular conformation, association, and separation within the 1–10 nm range. When the resonance energy is transferred from one excited fluorophore that acts as a donor to another fluorophore which functions as an acceptor, FRET occurs. This process takes place *via* a short range of dipole-dipole coupling[9].

The main criteria for the occurrence of FRET are a large spectral overlap between the donor and the acceptor, a favorable dipole–dipole orientation, a proximity of 1–10 nm, and a large enough quantum yield of both fluorescent proteins. When FRET occurs, the fluorescence of an acceptor will increase due to the resonance energy transferred from the donor, whereas the donor's fluorescence will be quenched. This also causes an increase in the fluorescence lifetime of the donor[10].

The cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) when paired together create a good combination for FRET. CFP is bright and permits accurate ratiometric measurement of both donor and acceptor GFPs by a variety of fluorescence detectors. The efficient energy transfer in the CFP–YFP pair leads to a larger change in the donor CFP emission[11].

Figure 1: CFP-YFP pairing for FRET



Sufficient spectral overlap is seen between eCFP and eYFP. Spectral bleed does occur: ASBT- acceptor spectral bleed-through & DSBT - donor spectral bleed-through[11].

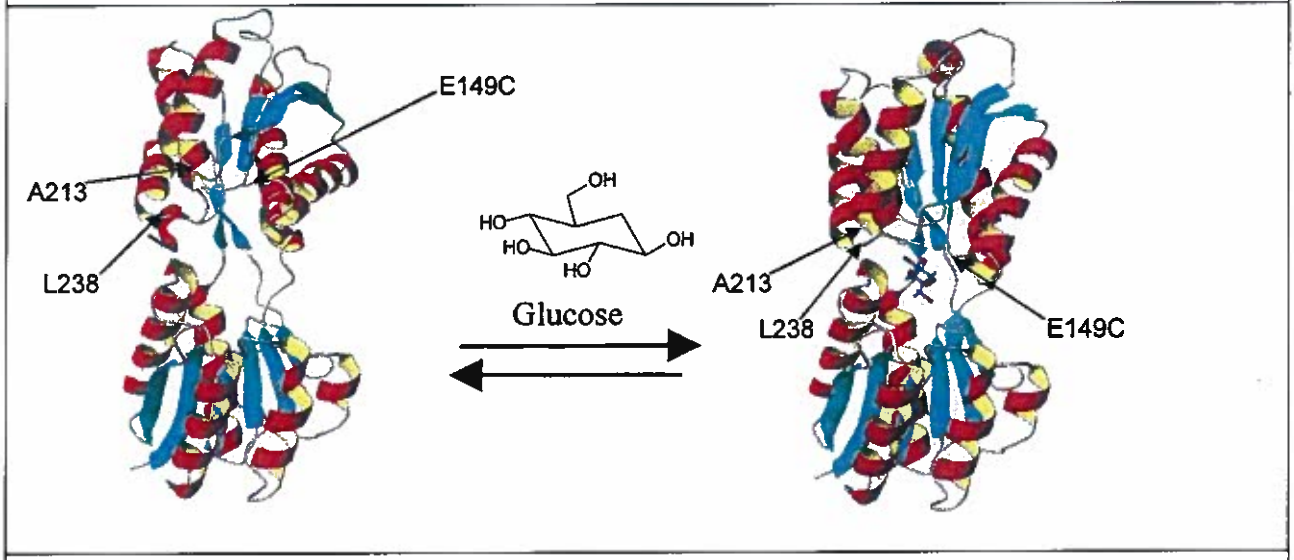
- Glucose Binding Proteins

The GBP used in this study is highly specific for both glucose and galactose because of its unique molecular structure[14] . The periplasmic space of *E. Coli* contains a family of ligand binding proteins that form part of the chemotaxis and nutrient-uptake systems and are specific for a wide variety of ligands. This GBP is one of them. This protein has a hinge region that constitutes a sugar binding site. The two domains in this monomeric protein are connected by three strands, referred to as a hinge region[12]. The cleft between the two lobes forms the sugar binding site. The two domains bend and twist around the hinge region and close around the ligand during the sugar binding[6].

Each of the two domains contains a β-sheet packed between α-helices. The binding cleft is between the two hinged domains, which are connected by three short segments of amino acid chain). Binding of

glucose to GBP causes a large amplitude conformational change that (31° hinge movement) and the two domains close to entrap glucose. When site-specifically labeled with an environmentally sensitive fluorophore, glucose/galactose binding protein (GGBP) generates a signal that can be used to quantify glucose. Once glucose binds to the protein, a rearrangement of the flap region located on one side of the hinge α-sheet occurs, giving rise to a conformational change. This makes the protein potentially useful for monitoring glucose in diabetics[13].

Figure 2: Open and closed structure of GGBP



The 34-kDa protein has two large domains connected by a hinge region. On glucose binding a large conformational change (31°) occurs, bringing the two domains together and entrapping glucose[14]

By utilizing this glucose binding-induced conformational change, glucose indicator proteins were constructed by tagging the GBP with two fluorescent proteins so that a FRET signal can be generated for detection when glucose binds to the Glucose Indicator Protein (GIP)[13]. A panel of GIPs

was constructed through site-directed mutagenesis that manipulates the glucose detection range of the GIPs and in this study the specificity of these GIPs for glucose was characterized.

MATERIALS & METHODS

The project involved three major parts:

Task 1: Expression of mutated GIPs

i. 500ml of LB (Luria Bertani)(25g/L) was prepared and autoclaved.

ii. 10µl of ampicillin (100mg/ml) and 100ul of glucose (100x) was added to 10mL of LB. This was inoculated with 10µl of recombinant cell *E. coli* DH5α that had been processed and contained the fusion protein.

iii. The cells were allowed to grow overnight with shaking at 225rpm and 37°C.

iv. 4ml of the overnight culture was transferred a 1 L flask containing 400ml LB, 100µl ampicillin and 4ml glucose stock (100x). This culture was grown till its OD_{600} reached 0.6 (≈2hrs) with shaking at 225rpm and 37°C

v. 200µl of Isopropyl-*β*-D-thiogalactopyranoside (IPTG, Boehringer Mannheim, German) (final concentration: 2mM) was added to 100 ml of LB. Samples were taken every hour till the cells reached a mid-exponential growth phase, i.e., its OD_{600} was 2.5-3.0 (≈8hours) with shaking at 225rpm and 37°C

vi. The cells were collected by centrifugation at 4,000 x *g* for 15 minutes at 4°C.

vii. The cell pellet was then weighed and extraction solution (Tris Buffer with 1mM PMSF) was added at 5ml/g wet weight of the cells

viii. The cells were homogenized in the extraction solution.

ix. The cells were placed in a centrifuge tube and spun at 13,000 rpm for 10min at 4°C

x. The supernatant was filtered through a 0.45 μm low protein binding membrane filter and the stored.

Task 2: Extract and purify the protein

Reagents:

Extraction buffer: 20mM Tris-HCl (Fisher), pH 7.5, 500mM NaCl (Fluka, Biochemica) and 1mM phenylmethylsulphonyl fluoride (PMSF) (Sigma)

Equilibration buffer: 30mL of 20mM Tris-HCl, pH 7.5

Wash buffer: Tris-HCl:20mM, NaCl: 500mM, Imidazole (Sigma-Aldrich): 40mM. Adjust pH=7.5 using 10N NaOH

Elution buffer: Tris-HCl:20mM, NaCl:500mM, Imidazole:500mM. Adjust pH=7.5 using 10N NaOH

Sugar free dialysis buffer: 20 mM Tris-HCl, 5 mM dithioerythritol (DTE) (Gold Biotechnology), 150mM NaCl, and 1 mM CaCl2.

i. An IMAC (immobilized metal affinity chromatography) column filled with 3mL of Ni-NTA agarose (Qiagen, CA) was used to purify both the GBP and the fusion protein which has YFP and GFP tagged to it.

ii. The column was washed with 15ml of distilled water at a flow rate 4ml/min and then equilibrated with 30mL of 20mM Tris-HCl, pH 7.5 at 4ml/min.

iii. The column was loaded with 2.5mL of 0.1M $NiSO_4$ (Fisher Scientific) at a flow rate of 1ml/min. The column was checked to make sure there was no air bubble.

iv. The cell lysate was applied to the column at 1ml/min.

v. After loading ≈10 bed volumes of washing buffer (30ml) was used to wash the column to remove any nonspecifically bound proteins at the rate of 4ml/min

vi. 30mL of Elution Buffer @1ml/min eluted both $(His)_6$-tagged GBP and YFP-GBP from the column.

vii. A fraction collector (BioRad) was used to collect 1mL fractions in Eppendorf tubes. The fractions were stored at 4C.

viii. After elution, the column was washed with 30mL of 50mM EDTA (GIBCO) in 20mM Tris HCl, pH 7.5 at 4ml/min followed by 50 ml of distilled water (dH_2O) at a flow rate of 4ml/min. The column was then stored at 4C after filling it with 20% Ethanol

ix. The absorption at 280nm for each fraction was determined, and the fractions with highest absorption (protein content) were pooled together. Elution buffer was used as a blank for the measurement.

x. The resulting proteins were then dialyzed against four exchanges of 50 volumes of final sugar free dialysis buffer. The dialysis column was placed in 500ml of buffer. The buffer was stirred at 4 °C using a magnetic stirrer for 2hrs, change buffer. And continue dialysis overnight. At the end, store the fractions at 4 °C for short term.

Task 3: Measure FRET of the GIP to various sugars using a luminescence spectrometer (Perkin Elmer)

i. The glucose binding assays were performed by titrating sugars to GIPs and FRET signals were measured using a luminescence spectrophotometer by exciting the proteins at 433 nm and monitored at 476 nm (for enhanced cyan fluorescent protein- ECFP) and 526 nm (for enhanced yellow fluorescent protein EYFP), respectively.

ii. Three different concentrations of sugars were used for the assay: 1, 10 and 100mM. The proteins were titrated with the sugars, incubated at room temperature for 10 minutes and then FRET signals were measured.

iii. For specificity assay, the following sugars were tested: glucose, galactose, fructose, ribose, arabinose, sorbitol, melebiose, xylose, and trehalose

RESULTS

The experiment was repeated three times to get triplicate data sets. When the protein was excited at 433nm wavelength, the resulting emissions were detected at 476nm and 526 nm for CFP and YFP respectively. The intensity ratio for these emissions: ECFP/EYFP was averaged and analyzed. Five different proteins were used:

CoYo – Control,with no mutation

Cys – Protein with amino acid at 16^{th} position changed to Cysteine

i-Cys_i – pH insensitive protein with amino acid at 16^{th} position changed to Cysteine

Thr – Protein with amino acid at 16^{th} position changed to Threonine

i-Thr_i – pH insensitive protein with amino acid at 16^{th} position changed to Threonine

The following table summarizes the average intensity ratios for the five different proteins for each of the nine different sugars at the varying molar concentrations. These different sugars were chosen because they are metabolized in the human body. Glucose was the center of focus and the other sugars helped compare if the proteins retained specificity towards glucose compared to other sugars.

Table 1: ECFP/EYFP ratio for sugars						
Sugar	Conc (mM)	C_oY_o	Cys	i-Cys_i	Thr	i-Thr_i
Glucose	1	1.847 ± 0.028	1.680 ± 0.011	2.272 ± 0.062	1.929 ± 1.881	2.266 ± 0.052
	10	1.771 ± 0.024	1.650 ± 0.005	2.267 ± 0.049	1.869 ± 1.807	2.199 ± 0.032
	100	1.735 ± 0.002	1.643 ± 0.039	2.214 ± 0.039	1.806 ± 1.713	2.115 ± 0.031
Galactose	1	1.814 ± 0.043	1.696 ± 0.005	2.319 ± 0.071	1.891 ± 0.021	2.243 ± 0.067
	10	1.774 ± 0.041	1.691 ± 0.010	2.307 ± 0.049	1.799 ± 0.052	2.164 ± 0.036
	100	1.698 ± 0.011	1.648 ± 0.007	2.247 ± 0.024	1.722 ± 0.047	2.089 ± 0.022
Fructose	1	1.897 ± 0.020	1.773 ± 0.026	2.441 ± 0.059	1.905 ± 0.082	2.339 ± 0.072
	10	1.895 ± 0.022	1.781 ± 0.016	2.443 ± 0.059	1.899 ± 0.081	2.302 ± 0.024
	100	1.860 ± 0.017	1.727 ± 0.012	2.384 ± 0.032	1.852 ± 0.076	2.257 ± 0.029
Ribose	1	1.890 ± 0.023	1.801 ± 0.003	2.470 ± 0.035	1.914 ± 0.043	2.328 ± 0.055
	10	1.894 ± 0.037	1.801 ± 0.024	2.448 ± 0.014	1.940 ± 0.060	2.328 ± 0.047
	100	1.846 ± 0.054	1.742 ± 0.016	2.376 ± 0.031	1.843 ± 0.052	2.226 ± 0.033
Arabinose	1	1.909 ± 0.023	1.768 ± 0.017	2.455 ± 0.036	1.918 ± 0.078	2.319 ± 0.041
	10	1.877 ± 0.025	1.771 ± 0.026	2.459 ± 0.052	1.898 ± 0.073	2.314 ± 0.055
	100	1.736 ± 0.048	1.686 ± 0.059	2.326 ± 0.045	1.806 ± 0.037	2.145 ± 0.049
Sorbitol	1	1.875 ± 0.015	1.810 ± 0.033	2.462 ± 0.038	1.929 ± 0.066	2.304 ± 0.060
	10	1.890 ± 0.032	1.787 ± 0.025	2.485 ± 0.025	1.926 ± 0.068	2.279 ± 0.051
	100	1.876 ± 0.027	1.775 ± 0.037	2.417 ± 0.017	1.912 ± 0.063	2.248 ± 0.096
Melbiose	1	1.906 ± 0.037	1.787 ± 0.026	2.437 ± 0.052	1.908 ± 0.071	2.339 ± 0.053
	10	1.907 ± 0.051	1.801 ± 0.023	2.434 ± 0.052	1.916 ± 0.072	2.331 ± 0.041
	100	1.871 ± 0.045	1.756 ± 0.030	2.407 ± 0.049	1.888 ± 0.094	2.292 ± 0.045
Xylose	1	1.923 ± 0.036	1.779 ± 0.008	2.440 ± 0.032	1.941 ± 0.063	2.338 ± 0.065
	10	1.904 ± 0.028	1.781 ± 0.019	2.415 ± 0.047	1.934 ± 0.068	2.361 ± 0.067
	100	1.864 ± 0.029	1.740 ± 0.025	2.362 ± 0.049	1.868 ± 0.050	2.254 ± 0.050

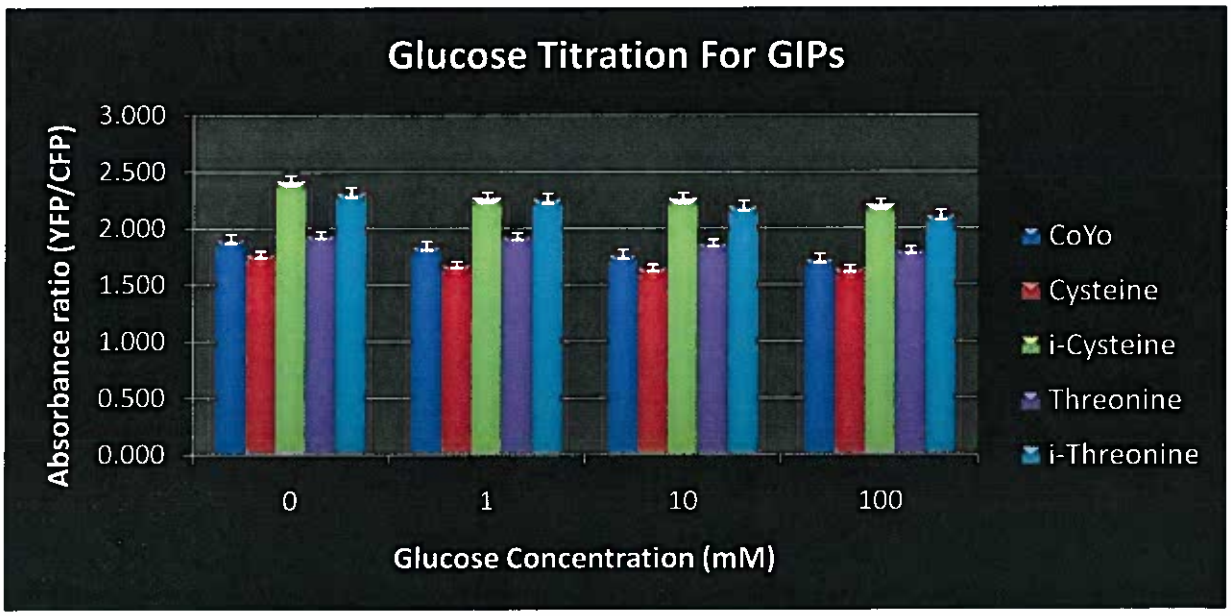
Trehalose	1	1.925 ± 0.001	1.755 ± 0.009	2.419 ± 0.028	1.996 ± 0.005	2.301 ± 0.057
	10	1.927 ± 0.004	1.760 ± 0.005	2.399 ± 0.026	1.968 ± 0.035	2.300 ± 0.059
	100	1.871 ± 0.041	1.707 ± 0.030	2.305 ± 0.035	1.939 ± 0.011	2.222 ± 0.077

Symbols: C_oY_o (a control GIP); several GIPs were constructed by using a mutated GBP which 16th amino acid residue is replaced by cysteine (GIP-Cys) and threonine (GIP-thr). The subscript "i" represents a pH insensitive GIP in which a pH insensitive YFP serves as an acceptor for generating a FRET signal for glucose detection.

DISCUSSION

A graph was plotted for glucose titration for the five different proteins:

Graph 1: Glucose titration for GIP's



The graph shows the average intensity measurement for glucose titrations for the different proteins. A trend is seen where absorbance decreases with increase in concentration

T-tests were conducted to see if the specificity for binding to glucose/galactose is retained after the site-directed mutagenesis. T-values were calculated with respect to glucose and then for a n=2, if the t-value was less than 2.57 (from t-table) then the binding was not significant and vice-versa

Table 2: T-values for n=2 as compared to glucose					
	CoYo	Cysteine	i-Cysteine	Threonine	i-Threonine
Galactose	0.549	1.268	1.593	1.232	0.513
Fructose	3.295	5.894	7.421	0.501	2.474
Ribose	2.948	6.278	6.146	0.772	2.112
Arabinose	1.042	3.211	3.948	0.130	1.065
Sorbitol	3.336	10.028	8.642	1.742	2.081
Melbiose	3.657	8.199	9.635	1.129	3.211
Xylose	3.513	7.186	6.005	1.259	2.653
Trehalose	3.781	4.684	3.586	2.929	1.846

Comparing these t-values to the critical value of t=2.57 for a two-tailed test with a significance of 0.05 the sugars were split into two categories, one displaying significant difference and the other displaying insignificant difference with respect to glucose binding

Table 3: Results from t-test		
Protein	**Sugars displaying significant difference w.r.t glucose in binding**	**Sugars not displaying significant difference w.r.t glucose in binding**
CoYo	Fructose, Ribose, Sorbitol, Melbiose, Xylose, Trehalose	Galactose, Arabinose
Cys	Fructose, Arabinose, Ribose, Sorbitol, Melbiose, Xylose, Trehalose	Galactose
iCysi	Fructose, Ribose, Sorbitol, Melbiose, Xylose, Trehalose	Galactose, Arabinose
Thr	Trehalose	Galactose, Fructose, Ribose, Sorbitol, Melbiose, Xylose, Trehalose
iThri	Melbiose, Xylose	Galactose, Fructose, Ribose, Sorbitol, Trehalose

The results show that the mutated proteins retain specificity of binding towards glucose/galactose

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