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Fluorescence-based sensing of glucose using engineered glucose/galactose-binding protein: A comparison of fluorescence resonance energy transfer and environmentally sensitive dye labelling strategies

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Abstract

Fluorescence-based glucose sensors using glucose-binding protein (GBP) as the receptor have employed fluorescence resonance energy transfer (FRET) and environmentally sensitive dyes, but with widely varying sensitivity. We therefore compared signal changes in (a) a FRET system constructed by transglutaminase-mediated N-terminal attachment of Alexa Fluor 488/555 as donor and QSY 7 as acceptor at Cys 152 or 182 mutations with (b) GBP labelled with the environmentally sensitive dye badan at C152 or 182. Both FRET systems had a small maximal fluorescence change at saturating glucose (7% and 16%), badan attached at C152 was associated with a 300% maximal fluorescence increase with glucose, though with badan at C182 there was no change. We conclude that glucose sensing based on GBP and FRET does not produce a larger enough signal change for clinical use; both the nature of the environmentally sensitive dye and its site of conjugation seem important for maximum signal change; badan-GBP152C has a large glucose-induced fluorescence change, suitable for development as a glucose sensor.

Keywords: Glucose sensing; Glucose/galactose-binding protein; Fluorescence resonance energy transfer; Fluorescence; Dye; Badan

New approaches to monitoring glucose in patients with diabetes mellitus are urgently needed for several reasons, including the requirement for technologies that can be adapted to continuous measurement or as non-invasive or semi-invasive devices [1]. Commercially available continuous glucose sensors are based on amperometric enzyme electrodes [2] or microdialysis systems [3] and though such devices have given significant clinical benefit to date, they continue to suffer limitations such as suboptimal accuracy, output drift and the need for frequent calibration against finger-prick blood glucose tests [1]. The performance of glucose sensors also will need to be improved if sensors eventually are to be coupled to insulin pumps for closed-loop control of insulin delivery (an 'artificial endocrine pancreas') [4].

When considering alternative technologies for glucose sensing, fluorescence is an attractive option because of high sensitivity, the absence of electrochemical interference in vivo, the ability to record intensity and lifetime, and the potential (with near infrared light) for non-invasive monitoring [5,6]. Amongst the molecular receptors for glucose that have been used in fluorescence-based glucose assays and sensors are the lectin concanavalin A [7], enzymes such as glucose oxidase [8], glucose dehydrogenase [9] and hexokinase [10], and boronic acid derivatives [11]. The glucose/galactose-binding protein (GBP) from *Escherichia coli* and other bacteria has served as a glucose detector in several reports of fluorescence-based sensors [12–19] and is notable for the conformational change that occurs on glucose binding that, with associated changes in fluores-

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cence of site-specifically attached fluorophores, can be transduced into a signal of glucose concentration.

Glucose-induced fluorescence changes in GBP have been detected by two main strategies, one is the labelling with environmentally sensitive dyes [12–17] and the other is by a fluorescence resonance energy transfer (FRET)-based signal change, using site-specific labelling with donor and acceptor at different sites on the protein [18,19]. It is unclear what determines the wide variation in the sensitivity of glucose sensing with these approaches and most GBP systems are reported to have zero to <30% fluorescence change [14,15,18] and very few about 300% [12].

Sensitive detection of glucose changes is important clinically, and the purpose of the present research therefore was to compare glucose-induced fluorescence changes using FRET and environmental sensing in the same study.

For FRET labelling, we report the usefulness of a recently introduced method for site-specific attachment of probes at the N or C-terminus: transglutaminase (TGase) catalysed conjugation of a fluorescently-labelled primary amine (cadaverine, in this case) to a glutamine-containing tag introduced by recombinant DNA techniques [20–22]. A non-fluorescent acceptor (QSY7) was attached to a thiol group, introduced by mutation to a cysteine residue at either of two sites near the binding site of glucose. For environmental sensing, we covalently coupled the dye 6-bromoacetyl-2-dimethylaminonaphthalene (badan) to the thiol group of cysteine mutations at one of the two positions, 152 and 182.

Materials and methods

The pTZ18U-mglB vector [13] containing the GBP gene was a kind gift from Dr. S. D'Auria. The plasmid pET303/CT-His vector was purchased from Invitrogen (Paisley, UK). *E. coli* DH5α cells were used as host cells for plasmid proliferation. LB media supplemented by antibiotics (50 μg/ml of kanamycin or 100 μg/ml of ampicillin) were employed to grow cells. *E. coli BL21(DE3)* was from BD Biosciences (Franklin Lakes, NJ, USA). All restriction enzymes were purchased from either New England Biolabs (Hitchen, UK) or Fermentas (York, UK). High fidelity DNA polymerase, Pfu Ultra, and the Quick-change site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA, USA). The rapid DNA ligation kit and long-template polymerase chain reaction (PCR) enzyme (Expand) were from Roche Applied Science (Basel, Switzerland). The kit used for plasmid extraction and Ni-NTA agarose was from Qiagen (West Sussex, UK) and the kit used to purify PCR products or restriction reactions was from Qbiogene (Morgan Irvine, CA, USA). Pig liver TGase was purchased from Sigma–Aldrich (St. Louis, USA) and the fluorescent probe Alexa fluor 555 cadaverine, Aexa fluor 488 cadaverine, acceptor QSY 7 maleimide, along with Tris(2-carboxyethyl)phosphine (TCEP) were from Invitrogen. All other chemicals were of molecular biology grade and purchased from Sigma–Aldrich.

FRET labelling. TGase catalyses an acyl transfer reaction between the γ-carboxylamide group of glutamine (Gln) and various primary amines [23]. For donor labelling of GBP, a short Gln-containing substrate recognition sequence for TGase (Pro-Lys-Pro-Gln-Gln-Phe-Met) was introduced at the N-terminus, the chimeric protein expressed and cadaverine fluorescently labelled with either Alexa fluor 555 or Alexa fluor 488 attached to a Gln residue in the tag, mediated by TGase. QSY7 was coupled to the thiol of a cysteine mutation at position 152 or 182. The strategy is shown in Fig. 1.

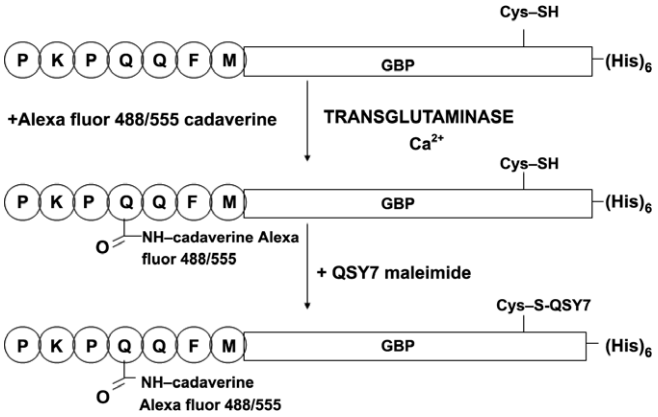

Fig. 1. Diagramatic representation of the strategy for construction of the FRET system. Alexa Fluor 488 or 555 cadaverince was covalently conjugated to glucose-binding protein (GBP) engineered with a Gln (Q)-containing sequence at the N terminus, catalysed by TGase. Cysteine residue at position 152/182 for linking to QSY7 maleimide.

Construction of the expression vector pET303-GBP with Gln tag. The GBP gene (mglB) was isolated from the plasmid pTZ18U-mglB by PCR. The forward primer, containing the sequence for the TGase-tag (underlined), was TAGC*TCTAGA*ATGCCAAAACCTCAGCAGTTTATGGC TGATACTCGCATTGGTGT and the reverse primer was GCAT*CTC GAG*TTTCTTGCTGAATTCAGCCAGGTTGTCTTTATCTACGCC. The forward and reverse primers were designed such that the 'stop' codon of the GBP gene was removed. Restriction sites XbaI (forward primer) and XhoI (reverse primer) were also introduced. The PCR enzyme 'Expand' was used for amplification with 18 cycles and annealing temperature of 70 °C for 30 s. The PCR product was digested with XbaI and XhoI and ligated into the XbaI and XhoI digested pET303/CT-His vector using a Rapid DNA ligation kit. The ligation reaction was then transformed into competent *E. coli* DH5α cells and the resulting pET303-GBP plasmid was isolated from these cells. The plasmid DNA was then sequenced to confirm the correct clone.

Construction of H152C mutant of GBP. pET303-GBP vector was used as template for the construction of H152C mutant. Site-directed mutagenesis was performed using the Quick-change mutagenesis kit. The DNA sequencing data verified the presence of desired point mutation.

Expression and purification of the Gln sequence-tagged GBP protein. A single colony of *E. coli* BL21(DE3) transformed with the pET303-GBP plasmid was inoculated into 50 ml of LB media containing 100 μg/ml of ampicillin. The culture was grown overnight at 37 °C and then added to 500 ml of the same media for an additional 3–4 h at 37 °C. Expression of the protein was induced by adding IPTG (isopropyl-2-D-thiogalactopyranoside) to a final concentration of 1 mM and the culture was incubated at 37 °C for an additional 4 h. Bacterial cells were harvested by centrifugation at 8000 rpm and the pellet was dissolved in lysis buffer (50 mM NaH_2PO_4 + 300 mM NaCl + 10 mM imidazole, pH 8.0). Lysozyme at 1 mg/ml was added to these cells and incubated on ice for 30 min, followed by sonication. The cell extract was clarified by centrifugation at 14,000 rpm and the supernatant collected. Affinity chromatography was performed in a glass column packed with 5 ml Ni-NTA agarose. The column was washed with buffer (50 mM NaH_2PO_4 + 300 mM NaCl + 20 mM imidazole, pH 8.0) and the protein then eluted with buffer consisting of 50 mM NaH_2PO_4 + 300 mM NaCl + 250 mM imidazole). Positive fractions of 1 ml were collected by recording the optical density and pooled. The purity of the Gln sequence tagged GBP was determined by SDS–PAGE using 10% acrylamide gels that were viewed by Coomassie blue staining.

Fluorophore labelling. The Gln-tagged protein was dialysed against 100 mM Tris–HCl buffer pH 7.5. To label the protein with Alexa flour 488/555 cadaverine at the N-terminus via TGase, a tenfold molar excess of dye-cadaverine (1 mM) was added to the protein (50 μM) together with

0.5 U of TGase in Tris buffer containing 10 mM $CaCl_2$ in a 100 μl reaction. The enzymatic reaction was allowed to proceed overnight at 25 °C. Unreacted dye was removed by G-25 gel-filtration using phosphate-buffered saline pH 7.4. For subsequent labelling of the Alexa 488/555-linked GBP with non-fluorescent acceptor at position 182 (cysteine), a tenfold molar excess of QSY 7 maleimide (200 μM) was added to 20 μM protein in phosphate-buffered saline pH 7.4 with 5 mM TCEP, incubated overnight at 4 °C and free dye removed by G-25 gel-filtration chromatography. To label protein with badan, 50 μM protein was first treated with 5 mM TCEP in phosphate-buffered saline pH 7.4, and then a tenfold excess of dye (500 μM) was added and incubated overnight at room temperature, after which it was purified on a G-25 gel filtration column.

Fluorescence measurements. Steady-state fluorescence measurements were recorded on a Perkin–Elmer LS50B fluorimeter (Perkin–Elmer Instruments, Beaconsfield, UK). The excitation wavelength was set at 295 nm for excitation of GBP tryptophan residues (emission maximum 350 nm) and 488/555 nm for Alexa Fluor 488/555 (for Alexa fluor 488 emission maximum 495 nm and for Alexa Fluor 555 emission maximum 565 nm). The excitation of QSY7 was at 560 nm. The excitation and emission of badan was at 400 and 550 nm, respectively. All data were obtained at room temperature using quartz cuvettes with sample volume of 100 μl. The labelled protein was incubated with increasing amounts of D-glucose for 15–20 min at room temperature before fluorescence was recorded.

The binding constant K_d was calculated from one-site saturation binding isotherms, using Prism 4 software (GraphPad, San Diego, CA, USA).

Results

Alexa Fluor/QSY 7 labelled GBP for FRET-based glucose sensing

The expression in *E. coli* BL21(DE3) of GBP tagged at the N-terminus by a nucleotide sequence coding for a 7-amino acid substrate recognition peptide for TGase and mutated at residues 182 and 152 was assessed by SDS–PAGE. The chimeric GBP migrated at approximately the 34 kDa position, the expected size of $(His)_6$-tagged GBP.

Fig. 2A shows that the fluorescence emission spectrum of GBP labelled at the N terminus with Alexa Fluor 555 was similar to that of dual-labelled GBP with Alexa Fluor 555 at the N-terminus and the non-fluorescent acceptor QSY 7 conjugated at cys 182 (emission maximum 555 nm). Free Alexa Fluor 555 dye had a fluorescence emission spectrum with a maximum which was slightly red shifted at 563 nm. Addition of glucose caused a maximal 7% increase in the fluorescence (maximum at 10 μM, K_d 0.1 μM) (Fig. 2B), consistent with a decrease in FRET between the Alexa Fluor 555 donor and the QSY 7 acceptor.

In an attempt to improve the signal change and increase K_d we studied another FRET pair: Alexa Fluor 488 attached at the N-terminus and QSY 7 attached to Cys introduced by mutation of His at position 152. The H152C mutant will be expected to have a weaker glucose binding since it replaces the H152 residue which forms hydrogen bond with the O6 oxygen of glucose. As with the M182C mutant, the fluorescence emission spectra for free Alexa Fluor 488, Alexa-GBP and Alexa-GBP-QSY 7 were similar. Addition of glucose caused a maximal 16% increase in the fluorescence intensity but with similar K_d (0.1 μM) to the Alexa-M182C QSY 7 mutant (Fig. 3).

Badan-labelled GBP

The GBP mutants M182C and H152C were labelled with the environmentally sensitive fluorophore, badan. The absorption spectra of H152C-badan and M182C-badan conjugates showed a peak at 280 nm, corresponding to the tryptophan residues of GBP and a peak in the 380–410 nm, indicating the conjugated badan. The fluorescence intensity increased by 300% with the addition of glucose to H152C-badan (maximum at 50 μM and K_d 2.35 μM) while the change in the fluorescence of M182C-badan was negligible (Fig. 4A and B).

Discussion

In this study, we compared glucose-induced fluorescence changes of dye-labelled GBP, using strategies based on FRET (with two donor–acceptor pairs) and with environmentally sensitive dye conjugated at two positions near the binding site. Both FRET-based systems resulted in a comparatively small change in fluorescence intensity (7% for Alexa Fluor 555-M182C-QSY 7 and 16% for Alexa Fluor 488-H152C-QSY 7). The environmentally sensitive dye badan attached at C152 produced a maximum 300%

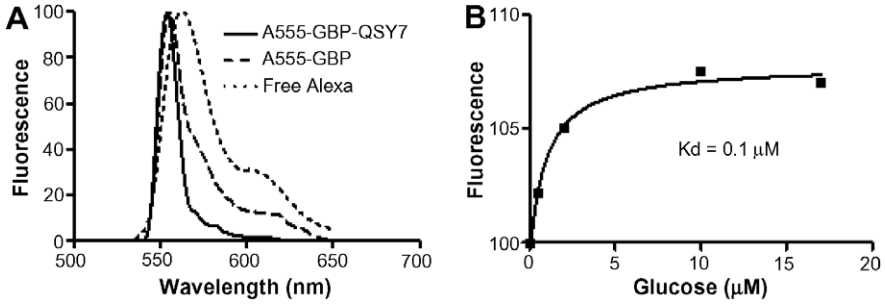

Fig. 2. (A) Fluorescence emission spectra of A555-GBP (dashed line) and A555-GBP-QSY7 (solid line) and free Alexa Flour 555 dye (dotted line); excitation wavelength 555 nm. Fluorescence normalized to maximum intensity = 100. (B) Change in fluorescence intensity of Alexa Fluor 555-M182C-QSY 7 at increasing glucose concentrations. Fluorescence at zero glucose concentration = 100.

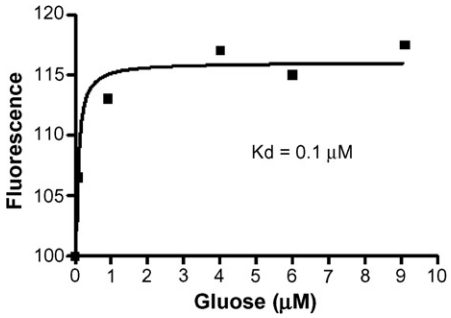

Fig. 3. Change in fluorescence intensity of Alexa Fluor 488-H152C-QSY 7 at increasing glucose concentrations. Fluorescence at zero glucose concentration = 100.

fluorescence change with glucose addition, but negligible change when attached at C182.

The conformational alterations in GBP that accompanies glucose binding to GBP forms the likely basis of fluorescence change by FRET and environmentally sensitive labelling [12,24]. GBP has a single polypeptide chain that folds into two domains connected by a hinge. Ligand binding at a site between the domains results in closing round the glucose [12]. The glucose-induced increase in fluorescence that we showed with the two Alex Fluor-GBP-QSY 7 derivatives indicates an increase in the distance between the N-terminal donor label and the non-fluorescent acceptor near the binding site (decreased FRET). This is consistent with GBP being a Type I binding protein, where the termini are located at the proximal ends of the two lobes [25,26]. The substrate-induced hinge-twist motion is therefore expected to move the termini further apart, and the N-terminus further from the binding centre, causing a decrease in FRET. In agreement with this, Fehr et al. [19] noted that FRET decreased and fluorescence increased when glucose was added to GBP labelled at either terminus with different variants of green fluorescent protein.

The small change in FRET-mediated signal with addition of glucose is consistent with the previous donor–acceptor labelled GBP derivatives, which have used two different variants of green fluorescent protein as FRET pairs, one at the N and one at the C terminus [18,19]. In our study, we used for the first time in glucose sensing the recently introduced technique for N- or C-terminal site-specific labelling of proteins of TGase-catalysed conjugation of probes at the termini [20–22]. This method offers potential advantages for biosensor construction, such as mild conditions (preserving biological function of the receptor) and highly specific labelling (directed only at the Gln at the terminus).

A number of different environmentally sensitive dyes have been used before for glucose sensing by GBP, with changes in fluorescence varying from zero [15] to fourfold [12]. The magnitude of the signal change seems to be not solely due to the site of dye attachment since labelling at position 152 with Acrylodan and IAEDANS (5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulphonic acid) was associated with zero fluorescence response to glucose [15] but labelling at the same site with IANBD (*N*-((2-iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole) was reported to cause a fourfold change [12]. We are not aware of previous conjugation of badan to GBP. We found that badan-GBP was associated with a fourfold glucose-induced fluorescence change at position 152, though badan linked at position 182 showed negligible fluorescence change. GBP labelled with IANBD at C152 is also associated with a fourfold change in fluorescence on addition of glucose [12]. Therefore, both the nature of the dye and the site of conjugation seem important for maximum signal change.

For clinical in vivo glucose detection the sensor must operate in the region of 0.5–20 mM or greater [1]. Approaches to increasing the K_d in fluorescence-based glucose detectors include engineering mutations which will weaken the glucose binding, but none of the mutants we studied (which were near the binding site) had a K_d greater than 2.35 μM. The alternative strategy that we have demonstrated previously of entrapping the detecting protein in a matrix such as silica sol gel [10] and/or encapsulating the sensor in a covering membrane which restricts glucose diffusion [10] are likely to be better options for increasing the K_d into the range useful for clinical in vivo operation.

We conclude that glucose detection by GBP based on FRET is unlikely to be sufficiently sensitive, whereas detection based on labelling near the binding site with certain environmentally sensitive dyes such as badan can produce

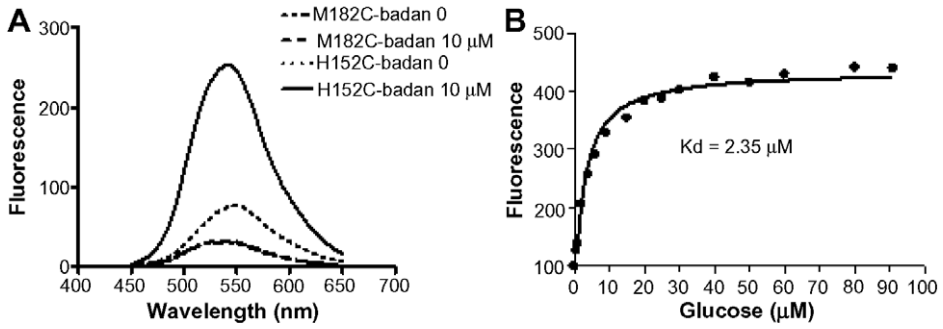

Fig. 4. (A) Fluorescence emission spectra of GBP-badan conjugates with and without glucose. Spectra for M182C at 0 and 10 μM were superimposable. (B) Change in fluorescence intensity of badan-H152C mutant at increasing glucose concentrations. Fluorescence at zero glucose concentration = 100.

at least a fourfold change in signal, suitable for development of a clinical glucose sensor.

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