Engineering Biosensors by Introducing Fluorescent Allosteric Signal Transducers: Construction of a Novel Glucose Sensor

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Abstract: The development of biosensors based on genetically engineered proteins offers many potential advantages to sensors that rely on natural proteins only. Here we present how protein engineering techniques can be used to integrate optical signal transduction functions directly into proteins by incorporating environmentally sensitive fluorescent groups that are allosterically linked to a ligand binding site, using a structure-based rational design approach. The location of potential fluorescent allosteric signal transduction (FAST) sites were predicted in the periplasmic Glucose Binding Protein of *Escherichia coli*. Single cysteine mutations were constructed at these locations, which allowed site-specific, covalent coupling of environmentally sensitive fluorophores. The fluorescence of these conjugates was shown to be hyperbolically dependent upon glucose concentration, indicating that the behavior of the fluorophore is allosterically coupled to the degree of occupancy at the glucose binding site, as predicted. Glucose Binding Proteins with engineered FAST functions may allow the development of a new class of optical glucose sensors.

Introduction

Biosensors couple highly specific biomolecular ligand binding events to changes in physical signals, thereby providing analytical tools that can measure the presence of single molecular species in complex mixtures.[1] Most biosensors use naturally occurring macromolecules, such as enzymes or antibodies, which provide the desired analyte specificity, but often are not well suited to simple signal transduction mechanisms.[2] One solution to this problem is to use protein engineering techniques to integrate signal transduction functions directly into proteins, adapting them to straightforward detection technologies, rather than developing instrumentation specific to the properties of a particular protein.[3−15] A simple approach to building such integrated signal transducers is to exploit optical detection strategies based on changes in fluorescent reporter groups which respond to ligand binding.[16,17] Fluorophores can be site-specifically introduced into a protein by using total synthesis,[9,15] semisynthesis,[3,6,8,10,11,14] or gene fusions.[12] In this way pairs of fluorophores can be arranged for detection of binding by fluorescence energy transfer,[3,9,12,14] or a single, environmentally sensitive fluorophore can be positioned to respond to conformational changes accompanying binding events.[6,8,10,11,13,15]

Ideally, the structural relationship between ligand binding site and reporter group is such that each can be manipulated independently, allowing a modular approach to the optimization of the properties of the binding site or the fluorophore.[10,18−21] One way to achieve such modularity is to spatially separate the two sites to minimize steric interference between them. Spatial separation of the reporter group and the binding site requires that the behavior of the fluorophore remain coupled to the degree of occupancy of the ligand binding site via an allosteric linkage mechanism. Recently we have shown that it is possible to engineer such integrated fluorescent allosteric signal transducer (FAST) functions in the Maltose Binding Protein (MBP) of *E. coli* by taking advantage of the large conformational changes that occur upon ligand binding in this protein, using a structure-based rational design approach.[10] Here we present how these results can be used to design FAST sites into the Glucose/

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Galactose Binding Protein (GBP), which is structurally related to MBP.[22,23] This engineered GBP has the potential of forming the basis for the development of a new class of fluorescent glucose sensors with potential applications in the food industry[24] or clinical chemistry.[25−27]

Materials and Methods

Mutagenesis. The gene for the cytoplasmic form of GBP[28] (i.e., lacking the leader sequence peptide) was amplified from *E. coli* genomic DNA using the Polymerase Chain Reaction (PCR) with flanking primers designed to introduce an *Eco*RI restriction site 5′ to the start codon N-Terminus (5′ CCG GAA TTC GGA GAT ACC ATG GCT GAT ACT CGC ATT GGT GTA ACA ATC TAT 3′; restriction site and start codon are underlined), and a *Bam*HI site just before the stop codon (5′ AAG CTT TCA TTA GGA TCC TTT CTT GCT GAA CTC AGC CAG GTT GCT TTT 3′). The resulting fragment was cloned into the pKK223-3 expression vector (Pharmacia). An oligonucleotide cassette coding for a His_5 oligopeptide was subsequently cloned into the *Bam*HI site to allow single-step purification by Immobilized Metal Affinity Chromatography[29] (IMAC). Individual cysteine mutants were made by overlapping PCR fragment mutagenesis.

Protein Expression and Purification. A colony of *E. coli* XL1-blue cells (Stratagene) freshly transformed with a plasmid expressing a mutant GBP protein was grown at 37 °C overnight in 20 mL of 2XYT medium containing 100 μg/mL ampicillin. 2XYT medium (1 L) supplemented with 0.2% (w/v) glucose was inoculated with 10 mL of the overnight culture and grown with vigorous shaking at 37 °C until $OD_{600} = 0.5$. Protein expression was induced with 1 mM IPTG and grown for a further 4 h. Cells were harvested by centrifugation at 3000*g*, resuspended in 40 mL of high salt buffer (1 M NaCl, 50 mM phosphate, pH 7.0; HSB), and stored frozen at −80 °C. The cells were thawed and lysed in a chilled French press at 1200 psi. Cellular debris was removed by centrifugation at 6000*g* for 10 min. DNA was precipitated by addition of polyenimine (pH8) to 5% (w/v) and removed by centrifugation at 6000 g for 30 min. GBP was purified with a single-step IMAC[29] procedure. Cleared lysate was diluted to 100 mL with HSB onto a 30 mL iminodiacetate/zinc column (Pharmacia) and extensively washed with HSB to remove unbound proteins (and bound glucose), followed by elution of mutant GBP with use of a 200 mL, 0−100 mM imidazole gradient in HSB. GBP was located in a single late peak that revealed only one protein band on an overloaded SDS/polyacrylamide gel stained with Coomassie Blue. Yields were typically 5 mg/L growth.

Fluorophore Coupling. Fluorophores were conjugated to the mutant GBP proteins via the single free cysteine. Acrylodan[30] and (((2-(iodoacetoxy)ethyl)methyl)amino)-7-nitrobenz-2-oxa-1,3-diazole[31] (IANBD) were purchased from Molecular Probes and used without further purification. The fluorophores were dissolved in acetonitrile and reacted with freshly purified cysteine mutants (5:1 molar ratio) of GBP (∼1 mg) in 1 M NaCl, 50 mM phosphate buffer (pH 7.0), for 3−5 h at room temperature. Unreacted fluorophore was separated from protein by gel filtration. The extent of coupling was measured both by determining the remaining free thiol concentration with use of Ellman's reagent[32] and by using the ratio of the absorbances of the major protein and fluorophore chromophores (ϵ_{280}(GBP) = 37 mM^{-1} cm^{-1} (this study); ϵ_{469}(IANBD) = 23 mM^{-1} cm^{-1}; ϵ_{392}(acrylodan) = 20 mM^{-1} cm^{-1}). Coupling was always found to be greater than 95%. The conjugates were stable at 4 °C for a period of months, as determined by glucose binding assays.

Measurement of Glucose and Galactose Binding. Sugar binding was determined by measuring changes in fluorescence of the conjugated fluorophores on a SLM-Aminco-Bowman series-2 fluorimeter at 25 ± 1 °C. Glucose or galactose (Sigma) was titrated into a 50 nM conjugated protein solution in 0.1 M NaCl, 50 mM phosphate (pH 7.0), which was continuously mixed with a magnetic stirrer. For titrations, the excitation and emission slit widths were set to 4 and 16 nm, respectively (IANBD: λ_{ex} = 469 nm, λ_{em} = 540 nm; acrylodan: λ_{ex} = 392 nm, λ_{em} = 520 nm). Under these conditions the instrument noise was <1% of the fluorescence signal observed in saturating solutions of glucose. Experimentally observed binding curves were fit to a binding isotherm:

$$\Delta F = \Delta F_{max}\left(1 + \frac{K_d}{S}\right)^{-1} \quad (1)$$

where ΔF is the change in fluorescence, ΔF_{max} the fluorescence change at saturating concentrations of ligand, K_d the binding constant, and S the concentration of ligand.

Results

Identification of Allosterically Linked Reporter Sites. GBP and MBP are both members of a group of periplasmic proteins involved in the active transport and chemotaxis of a variety of ligands.[33] Although this group shares little sequence identity and is diverse in molecular weight, they have similar overall structures,[34] consisting of a single chain that folds into two domains connected together by a hinge region. Ligands bind in the interface between the two domains. In a number of these proteins it has been shown that the domains can adopt at least two different, stable conformations: an "open" form in which the domains are relatively far apart, and a "closed" form which envelops the bound ligand. These interconvert via a bending and twisting motion around the hinge region.

In any protein it is possible to establish allosteric linkage between two sites if they are located within an interface between rigid units that undergo a rearrangement relative to each other, such as the two domains in the periplasmic binding proteins.[35] Using this structural principle, we analyzed the conformational differences of the high-resolution X-ray structures of the open[36] and closed[37] forms of MBP, and identified regions that were predicted to be allosterically linked to the binding site.[10] Site-specific attachment of environmentally sensitive fluorophores demonstrated that these regions are allosterically coupled to maltose binding. GBP has been crystallized only in the closed conformation, precluding direct calculation of potentially allosteric site (PAS) locations. Instead we used the results obtained on MBP, and relied on the rough structural similarity between MBP and GBP to predict the location of analogous PASs in the latter, even though the two proteins share little

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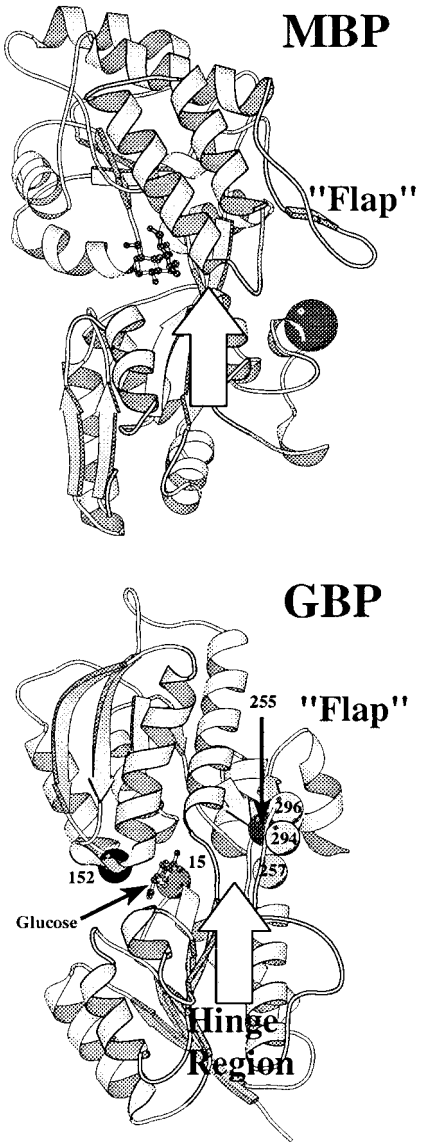

Figure 1. Comparison of the closed forms of MBP[36] (top; Protein Data Bank ref 2MBP) and GBP[22,23,40] (bottom; PDB ref 1GLG) complexed with their respective substrates, showing position. Attachment sites of conjugated fluorophores are indicated by spheres. The sphere located in the "flap" region of MBP indicates the position of the fluorophore that was found to give the best allosteric response[10] (attached to Asp95Cys). On the basis of this result, four sites in the analogous region of GBP (255, 257, 294, 296) are predicted to be potentially allosterically linked to the glucose binding pocket. Sites 15 and 152, located in the glucose binding pocket, are positions for potential nonallosteric reporter groups. The ribbon diagrams were produced with Molscript.[49]

sequence homology and are of different molecular weight and somewhat different secondary structure topology.[38]

The site that gave the most pronounced allosteric signaling in MBP is located in a region that forms a mobile "flap" covering the actual hinge. This flap is formed by two unconnected halves, each confined to one of the domains. Their relative movement changes the environment of an attached fluorophore which is completely separated from the binding pocket by the hinge β-sheet. The equivalent flap region in GBP is much smaller, with only one-half truly retained, which limits the attachment positions in GBP to the hinge itself (Figure 1).

Table 1. Binding Properties of Fluorescent Conjugates of the Mutant Proteins

	IANBD			acrylodan		
mutant	R	K_d(Glc) μM	K_d(Gal) μM	R	K_d(Glc) μM	K_d(Gal) μM
N15C	0.8	0.13	0.1	0.7	0.17	0.15
H152C	4.0	20	160	1.0	nd	nd
L255C	0.8	0.32	0.49	0.5	0.43	0.62
D257C	1.6	0.80	1.5	0.8	0.5	0.5
P294C	1.0	nd	nd	0.9	1	1
V296C	0.7	0.1	0.3	1.0	nd	nd

[a] R: ratio of fluorescence of fully saturated GBP (10 mM glucose) to apoprotein ($R = 1.0$ indicates no change; $R < 1.0$ indicates a decrease upon glucose binding; $R > 1.0$ indicates an increase). K_d(Glc), K_d(Gal): binding constant (μM) for glucose and galactose, respectively (nd: not done; binding constants were determined for all cases where $R \neq 1.0$). Wild-type has a K_d(Glc) = 0.2 μM, and K_d(Gal) = 0.4 μM.[48] N15C and H152C are the positions for nonallosteric reporter groups. the other four mutants are located in the hinge region of the allosteric flap.

Faced with the limited amount of structural information available for GBP, we were restricted to this region in our search for PAS locations. Since it is impossible to predict which of the residues in the flap region is likely to give the most pronounced allosteric response to ligand binding, we chose to scan the β-sheet portion of the flap and identified four sites for reporter group attachment (L255, D257, P294, V296). These positions are all located in one side of the hinge β-sheet, forming a surface onto which the flap α-helix is packed. Their microenvironment is therefore predicted to change if the flap region rearranges upon ligand binding.

Nonallosteric Reporter Sites. In addition to the PAS mutations, we identified two sites for attachment of reporter groups in the binding site itself (N15, H152). Fluorophores placed in these positions are predicted to respond to changes in their microenvironment by direct interaction with the ligand, by protein conformational changes as the "jaws" of the binding site close around the ligand, or by changes in solvation. This strategy has been used successfully to introduce nonallosteric signal transducing fluorescent reporter groups in MBP[8] and Phosphate Binding Protein[6] (PBP), another member of the periplasmic binding protein family, which binds to inorganic phosphate. In both cases the ligand binding constant of the conjugated protein is significantly increased relative to wild-type, indicating a significant degree of steric interference between the ligand and the fluorophore, which may also account for the change in the microenvironment of the fluorophore.[39] This strategy therefore loses the steric independence between reporter group and binding site inherent in the allosteric approach.

Signal Transduction Properties of the Mutants. The six GBP variants with single cysteines introduced for site-specific covalent attachment of fluorophores were constructed by a PCR mutagenesis strategy. Table 1 shows the results of the acrylodan[30] or (((2-(iodoacetoxy)ethyl)methyl)amino)-7-nitrobenz-2-oxa-1,3-diazole[31] (IANBD) conjugates of these mutant proteins. These two fluorophores have been selected because of their known sensitivity to the polarity of their microenvironments.

All four mutants in the hinge region showed a change in fluorescence of their acrylodan or IANBD conjugates upon ligand binding. The acrylodan conjugate at position 255 gives the largest change (2-fold decrease; Figure 2A). In all cases, a single-site hyperbolic binding curve could be constructed by

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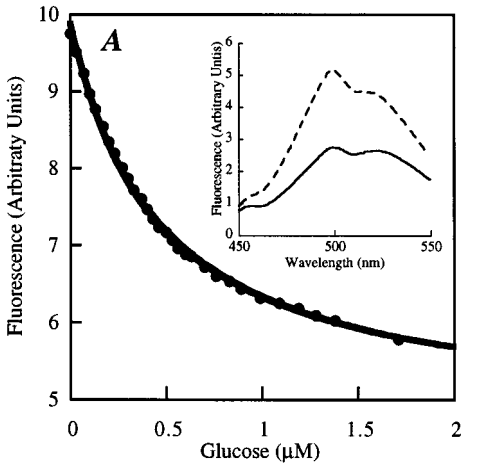

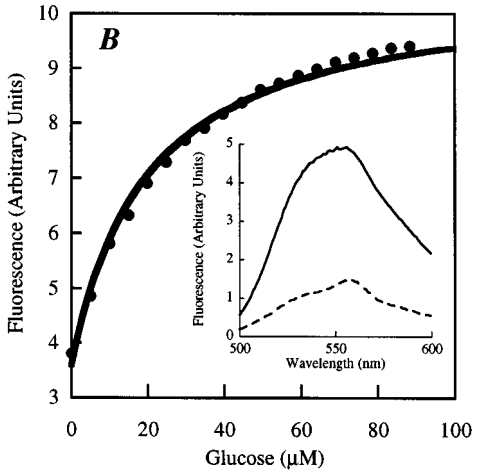

Figure 2. Binding of glucose to the L255C-acrylodan (A) and H152C-IANBD (B) conjugates. The binding curve is the average of three separate titrations (error bars are smaller than the circles shown). Inset shows changes in the emission spectra upon addition of saturating glucose: no glucose (dashed line), 10 mM glucose (solid line).

measuring the change in fluorescence as a function of glucose or galactose concentration, from which we conclude that the fluorophores attached to the hinge region are allosterically linked to the sugar binding pocket, as predicted. Furthermore, the sugar binding constants are affected by no more than a factor of 4, indicating that the FAST and ligand binding sites are sterically separated, as intended.

Two cysteine mutations were also constructed in the binding pocket itself (Figure 1). H152C interacts directly with the sugar, since it replaces His152 which forms a hydrogen bond with the O6 oxygen of both galactose and glucose.[40] The largest change in fluorescence of all the variants explored in this study (4-fold increase) was observed with IANBD attached at this position. However, this conjugate shows a large increase in the binding constants for glucose (~100-fold) and galactose (~500-fold) as would be expected both from the loss of the hydrogen bond to the O6 oxygen and from direct steric interference with the bound sugar.

Fluorophores attached to N15C are intended to respond to changes in the interdomain distance, rather than by direct interaction with the sugar, since Asn15 points away from the sugar binding pocket. Both the acrylodan and the IANBD conjugates show a change upon sugar binding, though not as large as IANBD at the 152 position. However, the conjugates at the 15 position do not greatly perturb the sugar binding constants, indicating that there is no direct interaction with the sugar.

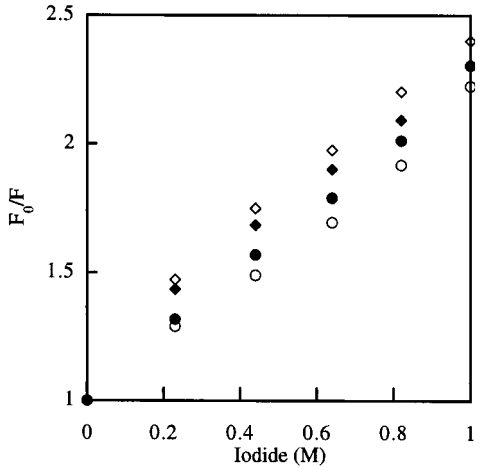

Figure 3. Solute quenching by iodide of the L255C-acrylodan conjugate. F_0/F fractional change in fluorescence emission at 498 nm upon addition of iodide. Quenching was determined for apoprotein (circles) and in the presence of 10 mM glucose (diamonds), and is plotted for both emission maxima at 498 nm (closed symbols) and 520 nm (open symbols).

Microenvironment of the Fluorophore Conjugates. Both acrylodan and IANBD are sensitive to changes in the polarity of their microenvironment,[31,41,42] which may result from changes in solvent accessibility, probe mobility, and changes in the steric interactions with the surrounding protein (or ligand, in the case of the H152C-NBD conjugate). Such microenvironmental changes may manifest themselves as differences in emission intensity as well as shifts in the wavelengths of their maxima. The emission maxima of acrylodan are known to be particularly dependent on the polarity of the environment, showing a significant blue shift in nonpolar relative to aqueous environments.[41,42]

The responses of the different sites vary widely. In several cases only one of the two conjugates coupled at a particular site responds to binding of the sugar (see Table 1). Furthermore, none of the conjugates show an appreciable shift in emission maxima upon ligand binding. The behavior of the best allosteric signal transducer in the hinge region, L255C−acrylodan (Figure 3), was examined in more detail. The emission spectrum of this conjugate has two maxima (498 and 520 nm; Figure 2A), suggesting that the attached acrylodan is present in two distinct environments differing in their polarities which are intermediate between water and ethanol based on their blue shifts relative to water.[30] Both peaks are present in the apo and sugar-bound forms, although their relative intensity changes somewhat upon ligand binding, suggesting a slight redistribution between the two states. To examine the potential contribution of differences in the solvent accessibility of the attachment site to changes in dipolar relaxation of the fluorophore that occurs upon ligand binding, we determined the effect of iodide on the fluorescence in the presence and absence of glucose. Iodide selectively quenches solvent-exposed fluorophores. The degree of quenching follows the Stern−Volmer equation describing steady-state collisional quenching:[43]

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$$F_0/F = 1 + K[I^-] \quad (2)$$

where F_0/F is the fractional decrease in fluorescence, and K the Stern−Volmer quenching constant ($K > 1.0$ for a solvent-exposed flurophore) which is related to the degree of solvent accessibility. It was found that the quenching constants measured for both emission maxima of the acrylodan are approximately the same, do not change upon glucose addition, and are >1 (Figure 3), indicating that both acrylodan conformations are partially solvent exposed, and the change in the microenvironment is unlikely to involve a change in solvent accessibility. Similar observations were made on other conjugates (data not shown).

These results suggest that the mechanism by which the conformational changes affect the dipolar relaxation of the attached fluorophore does not involve change in the local solvent-accessibility of the attachment position, but is dependent on the detailed interaction of the fluorophore with its microenvironment, as was also observed for IANBD attached in the binding site of MBP.[39] Further studies will need to be done to characterize the detailed mechanism of the change in dipolar relaxation of the attached fluorophores as a consequence of protein conformational change in these constructs.

Discussion

Here we show how the transmission of conformational changes in a protein can be exploited to construct integrated signal transduction functions that convert a ligand binding event into a change in fluorescence via an allosteric coupling mechanism. We have also demonstrated that fluorescent allosteric signal transduction (FAST) functions interfere minimally with the intrinsic binding properties of the sugar binding pocket in GBP. These results also provide evidence that GBP undergoes a domain closure upon ligand binding, similar to other periplasmic binding proteins. Furthermore, design of the FAST site was based on exploiting structural similarities between the hinge regions of MBP and GBP, indicating that the same approach can be extended to other periplasmic binding proteins which have similar structural features, widening the repertoire of analytes that can be detected in this manner.

Glucose sensing is important both in the food industry[24] and in clinical chemistry.[25−27,44,45] For instance, tight regulation of glucose levels within the physiological range is necessary to control the long-term complications associated with diabetes,[46] and requires close monitoring of blood glucose. Nearly all glucose sensors which use a biological element for signal transduction rely on the electrochemical detection of glucose oxidase activity,[25−27] which has a number of associated difficulties such as the presence of inhibitors in the blood, control of activity due to variation in oxygen levels, and electrode fouling.[47] The GBP with an engineered FAST function may allow the development of a new class of optical glucose sensor devices, based on the construction of a fluorescent flow cell containing immobilized GBP-FAST conjugates. This requires development of a suitable immobilization strategy which does not interfere with the conformational changes in GBP, and the "tuning" of the GBP binding constant for glucose to be compatible with the physiological concentration of blood glucose, which fluctuates around a mean value of 10 mM. We have demonstrated in MBP that it is possible to manipulate the intrinsic binding constant for the sugar by mutations in the ligand binding site without destroying linkage to the FAST function, thereby creating a collection of proteins capable of measuring sugar concentrations over a 0.1 μM to 15 mM concentration range. It should be possible to manipulate GBP in a similar manner to create a protein optimized for glucose sensing in operational concentration ranges.

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