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Transcriptional regulatory proteins as biosensing tools†

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Abstract

We have developed sensing systems employing different classes of transcriptional regulatory proteins genetically and chemically modified to incorporate a fluorescent reporter molecule for detection of arsenic, hydroxylated polychlorinated biphenyls (OH-PCBs), and cyclic AMP (cAMP). These are the first examples of optical sensing systems based on transcriptional regulatory proteins.

Regulatory proteins in bacteria are fundamental since they control many aspects of cell function including metabolism and reproduction, and are part of resistance machineries organized in the form of plasmid or chromosomal encoded resistance operons.^{1–4} Binding to their ligands triggers expression of the remaining genes of the operon. These natural sensory proteins have been exploited in development of whole-cell sensing systems, which have found applications in biomedical and environmental analysis.⁵ By employing a reporter gene that is co-expressed along with the operon genes and controlled by the operator/promoter region of the operon, we have previously developed, among others, biosensors for arsenic/antimony,^{6–10} copper,¹¹ cadmium/lead,¹² and OH-PCBs.¹³ Whole-cell biosensors have excellent sensitivity and selectivity, and provide cellular bioavailability of the analyte since the sensor is only turned “on” when the analyte is inside the cell.

Use of entire microorganisms in biosensing offers advantages, however, interference from cellular molecules and nutrients may result in a marked loss of detection limits. Also, expression of a reporter protein within an organism results in longer assay times as compared to biosensors that use isolated proteins as sensing elements. To that end, herein we describe a strategy whereby the exquisite molecular recognition of isolated natural regulatory proteins forms the basis for development of protein-based biosensors.

Regulatory proteins and periplasmic binding proteins undergo a similar conformational change upon binding to their ligands.^{14–16} Periplasmic-binding proteins modified with a signal-transduction element have been extensively employed in sensing systems.^{17–24} Employing similar strategies, herein, we have fused a signal-transduction element to

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representative regulatory proteins and developed sensing systems for detection of environmentally-relevant pollutants (arsenic and OH-PCBs) and the signal transducer, cAMP. The regulatory proteins ArsR, HbpR-A family of proteins, and cyclic AMP receptor protein (CRP) have been shown to undergo conformational changes upon binding their respective ligands.^{25–28}

The ability of regulatory proteins to change conformation upon binding to a ligand has not been exploited in optical protein-based biosensors. To that end, we employed two different approaches (a) genetically fuse a regulatory protein to a reporter protein and (b) label regulatory proteins with environmentally sensitive fluorophores. Protein–ligand binding can be determined by addressing the signal from the fluorophore/reporter protein attached to the regulatory protein. Altered signal reflects ligand-induced conformational changes, which are directly proportional to the ligand analyte concentration in a sample (Scheme 1). This mechanism constitutes the basis of our biosensing systems, which to our knowledge, are the first examples of directly labeled transcriptional regulatory protein-based sensing systems employing optical detection.

Biosensors based on transcriptional regulatory proteins expand the range of analytes detectable by periplasmic binding proteins. While us and others have demonstrated the utility of whole cell sensing systems based on transcriptionally regulatory operons, the use of isolated proteins as the biorecognition element should improve the response and assay times, detection limits, specificity, and selectivity of whole cell sensing. Moreover, isolated proteins are more amenable for adaptation to varied sensing platforms. Genetic operons controlled by transcriptional regulatory proteins are ubiquitous in nature, and, therefore, elucidation of the proteome will identify many more of regulatory proteins. Thus, our approach should provide with opportunities for development of new biosensors for target molecules that previously were not available.

In our first example, we engineered a fusion protein of ArsR and EGFP. ArsR is a *trans*-acting regulatory protein responsible for basal expression of the *ars* operon, which consists of genes that regulate survival of cells in arsenic and antimony polluted environments.²⁹ ArsR is a 13 kDa homodimer with a metal binding domain for induction,³⁰ a putative DNA binding domain for repression, and a dimerization domain.³¹ The ArsR–EGFP fusion protein was engineered so that the C-terminus of ArsR was fused to the N-terminus of EGFP with a Pro–Pro–Ala–Thr linker present in the multiple cloning site of EGFP vector. Excitation at 485 nm of the EGFP results in a 510 nm emission of fluorescence. Presence of arsenite or antimonite causes a change in the intensity of the fluorescence emitted by EGFP, which forms the basis for arsenite biosensing. Addition of sodium-*m*-arsenite to the ArsR–EGFP protein caused an immediate increase in fluorescence intensity (altered fluorescence reflects ligand-induced conformational changes, which is directly proportional to the ligand analyte concentration in the sample) that was identical to that obtained with longer incubation times, thus, demonstrating rapid detection of arsenite by the biosensor. Fig. 1 shows a sigmoidal dose–response curve for sodium-*m*-arsenite with a dynamic detection range that extends over 3 orders of magnitude. The detection limit is 5×10^{-7} M or 37.46 ppb allowing for monitoring of arsenic below the limits established by EPA of 10 ppb. When compared to a GFP based whole-cell sensing system that employs the *ars* operon, our

protein-based biosensor has a faster response time (immediate response vs. 30 min), lower detection limits (5×10^{-7} M vs. 8×10^{-6} M), and a wider dynamic range (3 orders of magnitude vs. 1 order of magnitude)³² and needs no pretreatment of the sample prior to analysis as compared to whole-cell sensing systems. The selectivity of the biosensor based on ArsR-EGFP toward anions similar in size, charge, and structure to arsenite and antimonite indicate that structurally similar anions to arsenite/antimonite do not bind ArsR and thus, fluorescence measurements are not significantly affected by their presence (see Table 1).

To explore potential use of other regulatory proteins in biosensing, we chose HbpR, a protein known to bind hydroxylated polychlorinated biphenyls, OH-PCBs.^{28,33} HbpR has four distinct domains. The A-domain interacts directly with an effector molecule causing a drastic conformational change relayed to the rest of the protein through a helix linker region in the B-domain, which is translated to the C-domain, whose ATPase activity is then triggered recruiting σ 54 RNA polymerase. Simultaneously, the DNA-binding D-domain undergoes a conformational change, triggering its release from the DNA's operator region, resulting in gene under control of this promoter.³³ In the subclass of proteins XylR, DmpR, and HbpR, the effector molecule is aromatic, and the A-domain binds to its aromatic effector molecule.³⁴ This class of proteins are difficult to express, purify, and stabilize in solution, and, as a result, there is sparse structural information.^{28,35} Homology studies have been performed,^{28,35} but still there is a lack of specific information on the overall protein structure. This makes our prior rational design strategy of these protein biosensors a nearly impossible proposition.

As an alternative, we have taken a shot-gun approach by attaching a fluorophore simultaneously to cysteines present in the isolated, analyte-binding A-domain of HbpR, HbpR-A. For that, we first truncated HbpR to isolate HbpR-A and then fused HbpR-A to maltose binding protein (MBP) to aid in the expression of soluble, active HbpR-A. MBP is exceptionally soluble and often confers this property to its protein fusion partner. Thus, fusions with MBP are used to increase the solubility of insoluble proteins. We previously developed a whole cell sensing system for PCB-OHs based on the use of an *E. coli* strain harboring pHYBP109, a plasmid encoding for HbpR. Selectivity studies performed with the HbpR whole cell system, showed greatest response to its native effector, 2-hydroxybiphenyl, but also to other structurally related compounds such as 2,2'-dihydroxybiphenyl, 2-aminobiphenyl, 2-hydroxydiphenylmethane and many OH-PCBs.^{13,29} However, since these results were afforded by a whole cell-based sensing, it could not be ruled out that the selectivity was not only provided by the HbpR protein but also, perhaps, by a combination of systems within the cell machinery. It should be noted that due to HbpR solubility problems, the isolated protein had never been directly used to investigate binding to any of these molecules. To that end, we labeled the purified HbpR-A-MBP fusion protein with the environmentally-sensitive fluorophore, IAEDANS and evaluated its response to a family of PCB-OH compounds. HbpR-A-MBP-IAEDANS responded in a dose-dependent manner to 2-hydroxy-3',4'-dichlorobiphenyl (Fig. 2).

We also investigated the recognition of the biosensing system, to detect the family of OH-PCBs listed in Table 2. Most of the OH-PCBs tested, with the exception of 2-hydroxy-3',

5,5'-trichlorobiphenyl and 2,3-dihydroxy-3',4'-dichlorobiphenyl were able to elicit a response. Since HbpR-A is expressed as a fusion to MBP, which contains one cysteine that could be modified with IAEDANS, we also examined the interaction between MBP-IAEDANS and 2-hydroxybiphenyl to rule out any potential non-specific binding and response from MBP alone (data not shown). This ensured that the signal generated was due to selective binding of OH-PCBs to HbpR-A, and not to binding to MBP. The system afforded detection limits of 1×10^{-8} M (Table 2) compared to 1×10^{-8} M using whole cell-based bioluminescence assay¹³ with both systems showing a dynamic range over three orders of magnitude. Intra-assay reproducibility had a % relative standard deviation (%RSD) that ranged from 0.01 to 10.8% calculated by determining the %RSD for each data point for all compounds. Inter-assay reproducibility performed by generating curves at different times, was found to range from 1.1 to 2.3%RSD. These data suggest that the performance of the biosensor is highly reproducible.

To demonstrate that use of transcriptional regulatory proteins spans to different types of molecular recognition systems and mechanisms, we developed a sensing system for cAMP by site-specific labeling of cAMP receptor protein, CRP. CRP is a transcriptional regulator of a large number of genes in *E. coli*. CRP is a 23 kDa homodimer of identical subunits.^{36,37} The N-terminal domain binds to cAMP and the C-terminal domain binds to DNA; the domains are held together by a flexible hinge.³⁸ Upon binding to cAMP, CRP undergoes a number of conformational changes that alter its structure to allow binding to DNA and interact with RNA polymerase, thus regulating gene expression.

Herein, we examined the ability of CRP as a potential biosensing element for cAMP detection. For that, we prepared a series of CRP conjugates with environmentally sensitive fluorophores and evaluated changes in fluorescence of CRP-fluorophore conjugates in response to changes in cAMP concentration. Native CRP contains three cysteines at positions 18, 92, and 178. Previous studies demonstrated the ability to modify cysteine 178 increases with increasing cAMP concentration, indicating that the environment of cysteine 178 changes upon addition of cAMP.³⁹ In our work, a CRP mutant (C178) containing a unique cysteine at position 178 was constructed by site-directed mutagenesis. The mutant and native protein were then conjugated with two environmentally-sensitive, thiol-reactive fluorophores, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) and acrylodan through the cysteine residues in the protein structure. The response of the labeled protein to cAMP was evaluated as shown in Table 1. Our biosensing system exhibited changes in fluorescence at low concentrations of cAMP, thus demonstrating that this approach leads to a sensitive (Table 3) and selective (Table 4, details in ESI†) sensing system for this critically important biomolecule. When compared to a molecular switch-based system that employs CRP,⁴⁰ our protein-based biosensor has a lower detection limits (1×10^{-13} M vs. 1×10^{-8} M) and a faster response time (30 min vs. 18 h).

In summary, we have described a series of strategies demonstrating use of transcriptional regulatory proteins in protein-based biosensors by coupling the molecular recognition properties of three regulatory proteins to three different optical reporting systems. Specifically, we have engineered the metalloregulatory protein ArsR and genetically fused it to EGFP; engineered the truncated protein HbpR-A, and fused it to the environment-

sensitive fluorophore IAEDANS; engineered the cAMP receptor protein and fused it to two different environment-sensitive fluorophores CPM and acrylodan. These proteins are representative examples of a broad class of transcriptional regulatory proteins that can serve as models for the design of biosensors based on the ever-expanding library of newly discovered transcriptional regulators. Additional strategies such as split-protein, molecular switches, and circular permutations could be attempted using the transcriptional regulatory proteins to further improve the analytical parameters of the biosensing systems described here. Protein-based biosensing systems that employ regulatory proteins should not only find applications in analysis, but in the understanding of natural processes, diagnostics, and drug screening, among others. These systems should be amenable to miniaturization and integration into a wide array of sensing platforms.

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Fig. 1.
Response of ArsR-EGFP to sodium-*m*-arsenite.

Fig. 2.
Typical dose–response curve of HbpR-A-IAEDANS to an OH-PCB (2-hydroxy-2',4',5,6'-tetrachlorobiphenyl).

Scheme 1.

Schematic of a transcriptional regulatory protein-based sensor. Binding to the analyte (brown) triggers a conformational-change in the regulatory protein (green), resulting in a change in emission of the attached signal transduction molecule (blue).

Table 1

Selectivity of ArsR–EGFP to other ions

Ion (1×10^{-5} M)	Normalized fluorescence ^a (%)	RSD (%)
Arsenite	100	0.05
Ferricyanide	25	0.32
Dichromate	0	0.62
Sulfate	25	0.26
Arsenate	25	0.13
Protein blank ^b	0	0.53

^aValues represent average ($n = 3$). Normalized fluorescence = $\{[(\text{FL}_{\text{ion}})/\text{FL}_{\text{blank}}]/[(\text{FL}_{\text{arsenite}})/\text{FL}_{\text{blank}})]\} \times 100$. FL – fluorescence.

^bThe blank corresponds to 100 μL of buffer incubated with 100 μL of the protein.

Table 2

Selectivity of HbpR-A-IAEDANS to various OH-PCBs

Compound	Detection limit ^a (M)	RSD ^b (%)
2-Hydroxy-2',3',4',5,5'-pentachlorobiphenyl	1.0×10^{-6}	3.5
2-Hydroxy-2',3,4',5',6-pentachlorobiphenyl	1.0×10^{-6}	3.7
2-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	1.0×10^{-7}	3.9
2-Hydroxy-2',3',5',6'-tetrachlorobiphenyl	1.0×10^{-8}	0.96
2-Hydroxy-2',4',5',6'-tetrachlorobiphenyl	1.0×10^{-7}	0.45
2-Hydroxy-2',4',6'-trichlorobiphenyl	3.2×10^{-7}	3.5
2-Hydroxy-3',4'-dichlorobiphenyl	1.0×10^{-7}	0.56
4-Hydroxy-2',3',4',5'-tetrachlorobiphenyl	1.0×10^{-6}	3.5
4-Hydroxy-2',3',5'-trichlorobiphenyl	1.0×10^{-7}	4.0
4-Hydroxy-2',4',6'-trichlorobiphenyl	1.0×10^{-7}	3.8
4-Hydroxy-3,3',4',5-tetrachlorobiphenyl	1.0×10^{-7}	4.4
4-Hydroxy-3,3',5'-trichlorobiphenyl	1.0×10^{-8}	0.56
4-Hydroxy-3,5-dichlorobiphenyl	1.0×10^{-8}	1.4

^aValues represent the minimum tested concentration of analyte that is greater than three times the standard deviation added to the average of the blank sample.

^bValues represent the average of the relative standard deviation for all the concentrations of a given compound.

Table 3

Response of fluorophore labeled wild-type and mutant CRP

CRP	Fluorophore	Detection limit (M)	Fluorophores/CRP molecule
Wild type	Acrylodan	1×10^{-13}	1.5
Wild type	CPM	1×10^{-6}	1.8
C178	Acrylodan	1×10^{-15}	1.1
C178	CPM	1×10^{-10}	1.0

Table 4

Response of CRPC178-acrylodan to cyclic nucleotides

Nucleotide	Response ^a (% enhancement)	Maximum response ^b (M)
cAMP	9.1	14.0 (10 ⁻¹⁵)
cCMP	4.6	7.1 (10 ⁻¹¹)
cGMP	-1.0	4.8 (10 ⁻¹⁰)
cIMP	5.3	8.1 (10 ⁻⁷)
cUMP	-0.6	4.3 (10 ⁻¹⁰)
2'3'-cAMP	8.3	8.3 (10 ⁻¹²)

% Enhancement = (fluorescence (with nucleotide) - fluorescence (without nucleotide)) × 100%.

^aResponse at 1 × 10⁻¹² M.

^bGiven as % enhancement. In parentheses are the concentrations at which the maxima occur.