Binding and signaling of surface-immobilized reagentless fluorescent biosensors derived from periplasmic binding proteins

ROBERT M. DE LORIMIER, YAJI TIAN, AND HOMME W. HELLINGA

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA

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Abstract

Development of biosensor devices typically requires incorporation of the molecular recognition element into a solid surface for interfacing with a signal detector. One approach is to immobilize the signal transducing protein directly on a solid surface. Here we compare the effects of two direct immobilization methods on ligand binding, kinetics, and signal transduction of reagentless fluorescent biosensors based on engineered periplasmic binding proteins. We used thermostable ribose and glucose binding proteins cloned from *Thermoanaerobacter tengcongensis* and *Thermotoga maritima*, respectively. To test the behavior of these proteins in semispecifically oriented layers, we covalently modified lysine residues with biotin or sulfhydryl functions, and attached the conjugates to plastic surfaces derivatized with streptavidin or maleimide, respectively. The immobilized proteins retained ligand binding and signal transduction but with adversely affected affinities and signal amplitudes for the thiolated, but not the biotinylated, proteins. We also immobilized these proteins in a more specifically oriented layer to maleimide-derivatized plates using a His₂Cys₂ zinc finger domain fused at either their N or C termini. Proteins immobilized this way either retained, or displayed enhanced, ligand affinity and signal amplitude. In all cases tested ligand binding by immobilized proteins is reversible, as demonstrated by several iterations of ligand loading and elution. The kinetics of ligand exchange with the immobilized proteins are on the order of seconds.

Keywords: biosensor; surface immobilization; periplasmic binding protein; fluorescence; zinc finger; biotin

Solute binding members of the periplasmic binding protein (PBP) superfamily have been intensively studied as receptors for sensor applications (Hellinga and Marvin 1998). These proteins exhibit high specificity and affinity for their natural cognate ligands and can be designed to bind nonnatural ligands (Marvin and Hellinga 2001; Looger et al. 2003; Allert et al. 2004). Ligand binding is accompanied by conformational changes in the protein, which can be linked to changes affecting site-specifically-attached fluorophores, thereby transducing binding into

a fluorescent signal (Gilardi et al. 1994; Marvin et al. 1997; de Lorimier et al. 2002). This engineered reagentless sensing mechanism is potentially well suited for real-time sensing applications.

Development of sensor devices requires incorporation of sensing proteins into a detector element by encapsulation or surface immobilization on a suitable material for interfacing with detectors. Here we describe studies for the immobilization of engineered fluorescent signal transducing PBPs. Reagentless sensing systems may have the signal transducing protein separated from the fluid sample by a diffusion barrier. For example, the protein may be entrapped in a porous material through which small molecules diffuse to reach equilibrium with the immobilized receptor (Topoglidis et al. 1998; Alarcon et al.

Reprint requests to: Homme W. Hellinga, Department of Biochemistry, Duke University Medical Center, Box 3711, Durham, NC 27710, USA; e-mail: hwh@biochem.duke.edu; fax: (919) 684-8885.

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2005). Alternatively, the receptor protein is directly linked to a surface and exposed to the fluid sample without an intervening barrier. This arrangement potentially reduces the time for an analyte molecule to reach equilibrium with the receptor, which is desirable for monitoring real-time analyte concentration fluctuations. PBPs linked directly to surfaces exhibit signal transduction by surface plasmon resonance when covalently attached to carboxymethyl dextran chips (Hsieh et al. 2004) or directly to gold (Luck et al. 2003). We are unaware of reports describing signal transduction by fluorophore-labeled PBPs linked directly to surfaces. The present studies were undertaken to explore such schemes. We examined whether the proteins could be attached to surfaces and, if so, whether ligand-specific fluorescence signal transduction is retained. The degrees of signal transduction and ligand affinity for different immobilization methods were also examined. Other critical questions that were pursued concern the reversibility of the response to ligand binding, and the useful lifetime of the surface as measured by the maximum number of consecutive cycles of binding and elution.

The proteins used in this study were thermostable sugar binding proteins cloned from the genomes of two thermophilic bacteria: a ribose binding protein from *Thermotoga tengcongensis* (TteRBP) and a glucose binding protein from *Thermotoga maritima* (TmGBP). These proteins have been characterized with regard to thermostability, ligand binding, crystal structure, and fluorescence signal transduction in solution (S. Rizk, Y. Tian, J. Qiu, and H.W. Hellinga, unpubl.; Y. Tian, A. Changela, M.J. Cuneo, B. Höcker, L.S. Beese, and H.W. Hellinga, unpubl.). Both proteins exhibit sequence and structural homology with other periplasmic monosaccharide binding proteins, facilitating the selection of residues for attachment of fluorophores based on observations from studies on other monosaccharide binding proteins (Marvin and Hellinga 1998; de Lorimier et al. 2002) and other classes of PBP (Marvin et al. 1997; Marvin and Hellinga 2001; de Lorimier et al. 2002). Because of their thermostability, these and other such proteins offer potential advantages for use in harsh environments or as scaffolds for designing novel binding sites.

Results

We examined surface immobilization and signal transduction using 96-well microtiter plates preactivated with various linking functions, to which fluorescent signal-transducing periplasmic binding protein was attached and from which fluorescence emission was quantified using a microplate reader. This system allows rapid measurements of samples under multiple treatments.

Two general modes of protein attachment to microplate wells were examined. In the semispecific immobilization

mode, amine groups on the protein were covalently conjugated with biotin or thiol groups. (We use the term semispecific rather than nonspecific immobilization because the latter implies physisorption through indeterminate interactions.) This conjugate was immobilized by reacting with either streptavidin-coated microplate wells (for biotin conjugation) or maleimide-derivatized wells (for thiol conjugation). Conjugation reactions were controlled to limit the extent of biotin or thiol addition to protein (averaging between two and three adducts per protein), but because of the large number of primary amines in TteRBP (29 lysines and the N terminus), there are many possible permutations of the conjugation pattern.

In the specific immobilization mode of protein attachment, the signal transduction protein was expressed as a fusion to a zinc finger domain at either its N or C terminus. After labeling the unprotected thiol (TteRBP Cys168 or TmGBP Cys13) with a thiol-reactive fluorophore, the fusion protein was prepared for covalent attachment to microplate wells by removing the coordinated zinc from the Cys₂His₂ zinc finger and reducing the resulting zinc finger disulfide to thiols, which then reacted with maleimide groups in the wells of the plate.

Semispecific immobilization via biotin

TteRBP-D168C labeled with Cy3 or Cy5 and conjugated with PEO₄-biotin was attached to streptavidin-coated wells in microplates. The amount of protein bound per well was estimated by fluorescence intensity, calibrated with a serial dilution of the unconjugated labeled protein in solution. Typically 2–3 pmol of biotin-conjugated protein was bound per well, quantified by absolute fluorescence intensity. Two possible sources of error in this estimate may derive from (1) a difference in the efficiency with which the instrument optics collects fluorescence from the bottom of wells compared to the full volume of the wells, and (2) a difference in quantum yield of the fluorophore attached to protein in solution compared with that attached to immobilized protein. For comparison, the maximum biotin binding capacity of the wells as stated by the suppliers is 125 (Pierce) or 300 (Sigma) pmol, but these figures are for small standards such as biotin-fluorescein and are not expected to be achieved for larger molecules such as biotin-conjugated proteins. The observed 2–3 pmol of protein per well is consistent with the expected maximum amount of 14 pmol, assuming a completely packed monolayer of PBP having a molecular diameter of 30 Å, a diameter of 4 mm for the microtiter well, and 4 mm for the height of the active surface on the wall of a well.

Biotin-conjugated TteRBP-D168C labeled with Cy3 or Cy5 was immobilized on streptavidin-coated wells and titrated with ribose. Fitting of the titration curve to a simple two-state hyperbolic binding isotherm produced a parameter

Figure 1. Ribose titration of biotin-conjugated TteRBP-168C-Cy5. The protein was titrated in solution (squares/dashed line, right axis) or immobilized on streptavidin-coated microtiter wells (circles/solid line, left axis). For each titration, the displayed curve is the best fit to a two-state binding model (Marvin et al. 1997). We observe an increased affinity for ribose on the surface ($K_d = 85$ nM), compared with the protein in solution ($K_d = 360$ nM). Data are presented as the average of at least three measurements.

for ligand binding affinity (K_d) and a parameter for signal transduction ($\Delta F_{\max}$), as illustrated in Figure 1. These parameters were derived for the protein titrated in solution and for unconjugated TteRBP-D168C, and compared to reveal the effects of conjugation and surface immobilization on ligand affinity and signal transduction, as summarized in Table 1. We observed that conjugation with PEO₄-biotin had little effect on K_d or $\Delta F_{\max}$ for either the Cy3- or Cy5-labeled protein. Upon immobilization of biotin-conjugated TteRBP, K_d and $\Delta F_{\max}$ decrease about twofold for the Cy5-labeled protein but change only slightly for the Cy3-labeled protein. We conclude that semispecific surface immobilization by the biotin-streptavidin interaction generally preserves the ability of TteRBP to bind ligand and transduce a fluorescent signal.

Semispecific immobilization via thiols

TteRBP-D168C labeled with Cy3 or Cy5 and conjugated with 2-iminothiolane was titrated both in solution and immobilized on maleimide-activated plates and compared with unconjugated protein (Table 1). Conjugation had slight effect on K_d and $\Delta F_{\max}$ in solution, but immobilization resulted in greatly diminished $\Delta F_{\max}$ for both fluorophores studied (Table 1).

Specific immobilization

A defined single attachment point from a protein to a surface can be achieved through the use of particular peptide sequences fused to a terminus of the protein. Here we use a zinc finger domain containing two cysteine thiols coordinated to a zinc ion. Previously we have demonstrated that these two cysteines can be functionalized independently of other cysteine residues in the fusion partner by removing the zinc ion, reducing the resulting disulfide, and reacting with a thiol-reactive moiety (Smith et al. 2005).

Genes encoding zinc finger fusions at the N or C terminus of TteRBP-D168C and TmGBP-Y13C were constructed and expressed in *Escherichia coli*. TteRBP-CZif and TteRBP-NZif labeled with Cy5 at Cys168 were immobilized to maleimide-activated microtiter wells to quantify the efficiency of binding by fluorescence intensity, calibrated with a serial dilution of the nonimmobilized protein in solution. Typically 1.5–2.5 pmol of bound protein per well was estimated by this method. For comparison, the stated binding capacity of the wells is 100–150 pmol, using sulfhydryl-containing peptides as the standard. As for the biotin-immobilized proteins described above, the observed 1.5–2.5 pmol of protein bound per well is consistent with the estimated maximum capacity for a protein of this size.

Table 1. Signal transduction and ligand affinity in fluorophore-labeled TteRBP

		Solution		Immobilized	
Fluorophore	Immobilization mode	$\Delta F_{\max}$ (%) ^a	K_d (nM) ^a	$\Delta F_{\max}$ (%) ^a	K_d (nM) ^a
Cy5	in solution	–15 (5)	260 (38)	N.A.	N.A.
Cy5	biotin	–24 (4)	300 (87)	–13 (3)	130 (57)
Cy5	2-iminothiolane	–15 (0.4)	190 (6)	–6 (3)	110 (26)
Cy5	N-Zif	–17 (2)	300 (70)	–70 (11)	60 (11)
Cy5	C-Zif	–25 (2)	240 (21)	–54 (1)	37 (17)
Cy3	in solution	–25 (a)	65 (a)	N.A.	N.A.
Cy3	biotin	–24 (a)	68 (a)	–27 (1)	58 (6)
Cy3	2-iminothiolane	–20 (5)	53 (11)	–3 (0.6)	65 (50)
Cy3	N-Zif	–38 (2)	38 (4)	–42 (4)	29 (4)
Cy3	C-Zif	–25 (0.2)	40 (7)	–18 (1)	50 (19)

N.A., not applicable; (a), determined from a single experiment.
^a Values have standard deviation in parentheses.

TteRBP-CZif and -NZif labeled with Cy3 or Cy5 were titrated in solution with ribose to determine the effect of the zinc finger domain on ligand affinity and signal transduction (Table 1). For both fluorophores, little difference in K_d or $\Delta F_{\max}$ between the nonfusion and the zinc finger fusion was observed (Table 1). When immobilized to maleimide-activated plates, Cy5-labeled TteRBP-CZif and TteRBP-NZif showed striking and reproducible differences with the nonimmobilized proteins, in both K_d and $\Delta F_{\max}$ (Fig. 2). For both proteins, the K_d improved five- to sixfold, while $\Delta F_{\max}$ increased two- to fourfold. In contrast to Cy5-labeled protein, Cy3-labeled TteRBP-CZif showed minor differences in ligand affinity and signal transduction between the immobilized protein and the protein in solution (Table 1). Hence, depending on the fluorophore, specific immobilization preserves or enhances ligand affinity and signal transduction.

For all derivatives of TteRBP the signal transduction responses were specific to D(-) ribose. For example, titration with D-(+) glucose over a similar range of concentrations failed to elicit a change in fluorescence emission for immobilized TteRBP-CZif labeled with Cy3 (Fig. 3).

Specific immobilization was also tested with TmGBP-CZif and TmGBP-NZif, labeled with Cy5 at Cys13. This combination of fluorophore and amino acid residue was studied because it causes the affinity of TmGBP for glucose ($K_d = 10$ mM) to be in a range close to that of the concentration of glucose (5 mM) in human blood (Burtis and Ashwood 1994), a necessary parameter for use as a medical glucose sensor. TmGBP-CZif and -NZif were titrated in solution with glucose to determine the effect of the zinc finger domain on ligand affinity and signal transduction. For both zinc finger fusion proteins,

Figure 2. Ribose titration of TteRBP-D168C-Cy5 fused to a zinc finger QNK domain and immobilized on maleimide-derivatized microtiter wells. (Circles/dashed line) N-terminal fusion (TteRBP-NZif); (squares/solid line) C-terminal fusion (TteRBP-CZif). The K_d values of the N- and C-terminal fusions are 51 and 81 nM, respectively. Values of $\Delta F_{\max}$ are -79% and -41%, respectively. Data are presented as the average of at least three measurements.

Figure 3. Immobilized TteRBP-CZif-Cy3 binds ribose specifically. Protein immobilized in maleimide-activated wells was titrated with D-(-) ribose (circles/dashed line) or D-(+) glucose (squares/solid line). Data are presented as the average of at least three measurements.

no large differences in $\Delta F_{\max}$ were observed compared with the nonfusion proteins. The zinc finger fusion had little effect on the affinity of TmGBP-NZif for glucose, but in the case of TmGBP-CZif, it was correlated with an approximately twofold decrease in affinity (Table 2).

Both zinc finger fusion derivatives of TmGBP couple to maleimide-activated microtiter wells to approximately the same degree (~ 2 pmol/well) as did the TteRBP fusions. Compared with solution titration, specifically immobilized TmGBP-CZif and -NZif both exhibit about a twofold decrease in $\Delta F_{\max}$ and an increase in glucose affinity of two- or threefold (Table 2).

Reversibility of ligand binding to immobilized protein

The reversibility of ligand binding was examined for both semispecifically (biotin) and specifically (zinc finger) immobilized TteRBP-D168C-Cy5 and for specifically immobilized TmGBP-Y13C-Cy5. Arrays of wells containing immobilized protein were titrated with ligand, as described above, and then repeatedly rinsed with buffer to remove ligand, as monitored by recording fluorescence emission after each rinse. Elution of ligand was assumed to reach completion when the fluorescence from each well showed no significant change between rinses. The cycle of titration and rinsing was repeated up to six times.

Comparison of titration curves reveals that immobilized receptor maintains function over multiple cycles of binding and elution. Figure 4A shows titration data for the first and the fifth such cycle for maleimide-immobilized TmGBP-CZif. Among five titrations, values for K_d were 6.8 ± 1.7 mM for TmGBP-CZif and 9.3 ± 1.7 mM for TmGBP-NZif. No correlation was observed between K_d and titration number, suggesting that ligand affinity is unaffected by multiple titration treatments. Values for $\Delta F_{\max}$ were $-25 \pm 1\%$ for TmGBP-CZif, and $-24 \pm 2\%$

Table 2. Signal transduction and ligand affinity in fluorophore-labeled TmGBP

		Solution		Immobilized	
Fluorophore	Immobilization mode	$\Delta F_{\max}$ (%) ^a	K_d (mM) ^a	$\Delta F_{\max}$ (%) ^a	K_d (mM) ^a
Cy5	in solution	−47 (2)	15 (1)	N.A.	N.A.
Cy5	N-Zif	−47 (2)	16 (1)	−24 (2)	9.3 (1.7)
Cy5	C-Zif	−42 (3)	27 (2)	−25 (1)	6.8 (1.7)

N.A., not applicable.
^aValues have standard deviation in parentheses.

for TmGBP-NZif, also with no correlation to titration number. Thus TmGBP specifically immobilized on a maleimide-derivatized surface exhibits reproducible ligand affinity and signal transduction over several cycles of titration and elution. Similar results were observed for

maleimide-immobilized TteRBP-CZif-Cy3 over six cycles of titration (Fig. 4B) and for biotin-conjugated TteRBP-D168C-Cy5 immobilized to streptavidin-coated microtiter wells, over two cycles (data not shown). We conclude that both methods of immobilization retain reversible ligand binding.

Figure 4. Successive cycles of titration and washing of zinc finger immobilized sugar binding proteins. (A) Cy5-labeled TmGBP-Y13C-CZif was cycled five times with glucose. The first (circles/solid line) and last (open squares/dashed line) titration curves are shown, having K_d values of 4.6 and 8.0 mM, respectively. The average K_d for all five titrations was 6.8 ± 1.7 mM. (B) Cy3-labeled TteRBP-D168C-CZif was cycled six times with ribose; the first (circles/solid line) and last (open squares/dashed line) titration curves have K_d values of 15 and 42 nM, respectively. The average K_d for all six titrations was 34 ± 19 nM, and the average $\Delta F_{\max}$ was $-17 \pm 1\%$. Data are presented as the average of at least three measurements.

Ligand-exchange kinetics

The on- and off-rates for glucose with respect to immobilized TmGBP were within the mixing time for adding or eluting glucose (<30 sec), as judged by a constant fluorescence intensity between the first and subsequent readings of a plate after adding glucose or rinsing with buffer. The on-rate for ribose with respect to immobilized TteRBP is also within these limits. However, wells containing immobilized TteRBP-Cy5 had to be rinsed several times over many minutes or hours to reach fluorescence levels for the apoprotein. We estimated the off-rate of ribose from immobilized TteRBP-CZif-Cy5 by measuring the time dependence of fluorescence after removing ribose solution from wells containing protein. The data were fit to a first-order exponential rate model, from which a pseudo-first-order rate constant was derived. Figure 5 shows typical data and a fit for an experiment in which the plate was rinsed four times over 2 min, and then fluorescence was recorded every 60 sec for a duration of 30 min, shaking for 45 sec between each reading. The derived rate constant is $3.5 \times 10^{-3} \text{ sec}^{-1}$, about 0.2% of the off-rates for arabinose and glucose binding proteins in solution (Miller et al. 1983). Extrapolation of fluorescence to the time of the initial rinse gives 54%, an estimate of the fraction of the fluorescence that recovers within a few seconds of rinsing. Therefore ~46% of the protein population exhibits a slow change in fluorescence. An estimate of the expected off-rate for ribose from immobilized TteRBP-CZif-Cy5 was made using the apparent K_d of 37 nM (Table 1) and a typical on-rate of $2 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ found for other mono-saccharide binding proteins (Miller et al. 1983). Assuming the relation $K_d = k_{\text{off}}/k_{\text{on}}$, the expected off-rate for ribose from immobilized TteRBP-CZif-Cy5 is 0.7 sec^{-1} , about 200-fold more rapid than that estimated for the slowly recovering population.

Figure 5. Time-dependence of the recovery of fluorescence from immobilized TteRBP-CZif-Cy5. The fluorescence ratio prior to the ribose elution step was 1.0. At $t = 0$ the immobilized protein was rinsed to elute ribose, and fluorescence was first recorded at $t = 2.25$ min. The curve is the best fit with a first-order exponential model to the slow phase, yielding the fit parameters of $k_{\text{off}} = 3.5 \times 10^{-3} \text{ sec}^{-1}$ and fluorescence ratios of 1.40 at $t = 0$ min and 1.76 at $t = \alpha$. The ratio of 1.40 is 54% of the range from 1.0 to 1.76, representing the fast phase of exchange that is complete within a few seconds of rinsing.

Discussion

The choice of a method for confining a signal transducing receptor molecule to a surface or a volume element for applications in sensing depends in part on the sampling method and on limitations of the signal transduction mechanism. Topoglidis et al. (1998) observed that fluorophore-labeled maltose binding protein displayed attenuated signal transduction (about fourfold less), but relatively constant affinity, when confined in TiO_2 gel. However, Alarcon et al. (2005) found enhanced signal transduction (two- to threefold higher) for fluorophore-labeled glucose binding protein when entrapped in a glycerol modified silicate condensate sol-gel. We have examined schemes for attaching receptors directly to solid surfaces to construct chemo-responsive surfaces that are in rapid equilibrium with solutes, which may be appropriate for applications that require observing at closely spaced time intervals.

We observed that surface-immobilized fluorophore-tagged PBPs retain their response to ligand binding. We found that structural details of the immobilization mechanism can significantly affect ligand affinity and signal transduction. Immobilization of semispecifically labeled biotin conjugates to a streptavidin-coated surface perturbed ligand affinity and signal transduction by less than twofold. However, semispecific derivatization with 2-iminothiolane and attachment to a maleimide-activated surface causes a three- to sixfold decrease in signal transduction. Orientation-specific immobilization using zinc finger fusions either retained or enhanced ligand affinity (two- to sixfold) and signal amplitude (two- to fourfold), and is therefore the most reliable and robust method.

We examined the ligand binding reversibility in the semispecifically biotinylated and specifically immobilized proteins and found that ligand affinity and signal transduction are reproducible over at least five or six cycles of ligand loading and elution. This is a critical requirement for continuous or repeated use of a sensor.

The rate at which the ligand binding equilibrium is established is also an important factor for real-time sensing: For use in a continuous flow biosensor, ligand-exchange kinetics must be matched to the timescale of fluctuations in ligand concentration. Ligand exchange observed for immobilized PBPs in this study was complete within 135 sec. The exception is immobilized TteRBP, where the ligand off-rate is characterized by two populations, one ($\sim 54\%$) having a rapid off-rate ($k_{\text{off}} > 0.1 \text{ sec}$) and the other ($\sim 46\%$) with an estimated k_{off} of $3.5 \times 10^{-3} \text{ sec}^{-1}$. The off-rate for other monosaccharides, such as arabinose and glucose, from their respective binding proteins in solution is $\sim 1.5 \text{ sec}^{-1}$ (Miller et al. 1983).

Taken together, our results demonstrate that thermostable PBPs that have been engineered to function as reagentless fluorescent biosensors retain their ability to signal when immobilized on surfaces and have reasonably rapid ligand-exchange kinetics. The resulting chemo-responsive surface potentially could be used to construct optical-based sensors for the monitoring of analyte concentrations that fluctuate over a period of seconds. Further development of surface-immobilized signal-transducing proteins for incorporation into sensor devices will require an assessment of the heterogeneity of the protein population with respect to ligand-exchange kinetics. Also, the upper limits of iterative cycling will need to be explored. Thermostable proteins such as used in this study may offer advantages for robustness of ligand affinity and signal transduction with respect to multiple cycles of use. They may also be useful as scaffolds for designing novel binding functions, as structural stability may be better retained than for designed proteins derived from scaffolds from mesophilic organisms.

Materials and methods

Gene construction and expression

The gene encoding a ribose binding protein from the thermophilic bacterium TteRBP was cloned from the genome (Bao et al. 2002) into the plasmid vector pET21a (S. Rizk, Y. Tian, J. Qiu, and H.W. Hellinga, unpubl.). The gene has been modified from the wild-type sequence in three ways: (1) 20 residues comprising the presumptive signal peptide at the N terminus have been removed and replaced with a Met codon for translation initiation; (2) Asp168 has been replaced with Cys for fluorophore attachment; and (3) a six-His tag preceded by a GlySer linker has been fused to the C terminus for protein purification using immobilized metal affinity chromatography.

The gene encoding a glucose binding protein from the thermophilic bacterium TmGBP was cloned from the genome (Nelson et al. 1999) into the plasmid vector pET21a (Y. Tian, A. Changela, M.J. Cuneo, B. Höcker, L.S. Beese, and H.W. Hellenga, unpubl.). The coding sequence has been modified in three ways: (1) replacing the first 31 residues (signal peptide) with a Met codon for translation initiation, (2) mutating Tyr13 to Cys for fluorophore attachment, and (3) fusing to the C-terminal Phe304 residue a GlySer linker followed by a six-His tag for protein purification using immobilized metal affinity chromatography.

Genes encoding TteRBP-D168C or TmGBP-Y13C fused to a 33-residue His₂Cys₂ zinc finger domain (ZifQNK) at either the N or C termini of the former two proteins were constructed as described (Smith et al. 2005). The gene containing ZifQNK fused to the N terminus of TteRBP-D168C (TteRBP-NZif) has a two-residue linker of GlySer between the two domains and has the same C-terminal His tag as above. For fusion to the N terminus of TteRBP-D168C, a 109-bp PCR product containing ZifQNK was constructed using the following two partially complementary oligonucleotide primers: 1 (sense), CACCATG ACAGGTGAGAAACCGTACAAGTGCCCGGAGTGTGGCAA ATCATTTCTCTCGATCGGACCATCTATCCCGTCACCAACGGA CATATCAGAACAAAGAAGGGTTCT; 2 (antisense), AGAACCC TTCTTGTCTGATGTGTCGTTGGTGACGGGATAGATGGTC CGATCGAGAGAATGATTT. The 5' end of the sense primer contains the sequence CACC for cloning into the vector pET101/D-TOPO (Invitrogen) according to the instructions of the supplier. This 109-bp fragment was fused to TteRBP using an oligonucleotide primer having overlapping homology with the C terminus of ZifQNK and the N terminus of TteRBP. The resulting 1-kb fragment was cloned into pET101/D-TOPO. The gene containing ZifQNK fused to the C terminus of TteRBP-D168C (TteRBP-CZif) had a GlyGlySer linker between the C-terminal residue (Gln278) of TteRBP and Thr2 of ZifQNK; Met1 of the latter being deleted. The C terminus of this ZifQNK domain was linked by GlySer to a six-His tag. For fusion to the C terminus of TteRBP-D168C, a 124-bp PCR product containing ZifQNK was constructed using the following four partially complementary oligonucleotide primers: 1 (sense), ACAGGTGAGAAACCGTACAAGTGCCCG GAGTGTGGCAAATCATTC; 2 (sense), ATCGGACCATCTATC CCGTCACCAACGGACATCAGAACAAAGAAGGGTT; 3 (antisense), GACGGGATAGATGGTCCGATCGAGAGAATGATTTGC CACTCCGGG; and 4 (antisense), GTTAATGGTGGTGGTGA TGATGAGAACCCTTCTTGTCTGATGTGTCC. Primer 4 encodes a six-His tag linked to the C terminus of ZifQNK by GlySer. The resulting 124-bp fragment was fused to TteRBP using an oligonucleotide primer having overlapping homology with the N terminus of ZifQNK and the C terminus of TteRBP. The resulting 1-kb fragment was cloned into pET21a.

The gene containing ZifQNK was fused to the N terminus of TmGBP-Y13C with similar method mentioned above, with a two-residue linker of GlySer between the two domains, and has the same C-terminal His tag as above. As done with TteRBP, the gene containing ZifQNK was also fused to the C terminus of TmGBP-Y13C with a GlyGlySer linker between the C-terminal residue (Phe304) of TmGBP and Thr2 of ZifQNK, with Met1 of the latter being deleted. The C terminus of this ZifQNK domain is linked by GlySer to a six-His tag. The expression vector for both TmGBP-CZif and TmGBP-NZif is pET21a.

Plasmids were transformed into the *E. coli* strain Rosetta-gami (DE3; Novagen), and transformants were grown in Hyper Broth medium (Athena Enzyme Systems) at 37°C. Protein expression was induced by adding isopropyl- β -D-thiogalactoside to the cultures when OD₆₀₀ reached ~0.6 units. After shaking

overnight, the culture was centrifuged, and the cell pellet was suspended in a solution of 500 mM NaCl, 10 mM imidazole, and 20 mM MOPS (pH 7.8) and stored frozen at -80°C. The cell suspension was thawed, chilled on ice, and disrupted by sonication with a Sonifier 250 (Branson) and narrow-tip probe, using six cycles of 30 bursts each at 50% duty cycle and an Output Control setting of 5. Five minutes of sample cooling followed each cycle. The cell lysate was centrifuged for 30 min in the cold at 30,000 rcf, and the supernatant was collected and heated in a water bath for 15 min at 65°C. The resulting precipitate was pelleted by centrifugation, and the cleared supernatant, containing the thermostable PBP, was loaded on a 3-mL column of Chelating Sepharose Fast Flow (Amersham Biosciences) preloaded with Ni²⁺. The column was washed with 40 mL of 500 mM NaCl, 10 mM imidazole, and 20 mM MOPS (pH 7.8). Subsequent 20 mL washes contained the same buffer with increasing concentrations of imidazole: 25, 50, 75, 100, 200, and 400 mM. Fractions were collected for each increment of imidazole concentration and analyzed by SDS-PAGE. Suitable fractions were concentrated to 10 mL and further fractionated by gel filtration on a column of Superdex 75 HiLoad (Amersham Biosciences). Protein-containing fractions were concentrated and dialyzed into MOPS-buffered saline (MBS; 100 mM NaCl, 20 mM MOPS at pH 7.0). Stock solutions of ZifQNK-fusion proteins also contained ZnCl₂ at 100 μ M.

Fluorophore labeling

Proteins were prepared for labeling by first adding Tris[2-carboxyethyl] phosphine to 300 μ M and incubating at room temperature for 10–30 min to reduce any disulfide bonds. This treatment does not affect the zinc-coordinated ZifQNK domain. Next, the thiol-reactive dyes Cy3- or Cy5-maleimide (Amersham Biosciences) were added in molar excess according to the manufacturer's protocol. After 4 h of reaction at room temperature in the dark, the solution was fractionated by gel filtration to separate unincorporated dye from labeled protein. The relative amounts of labeled and unlabeled protein were estimated by MALDI-TOF mass spectrometry; all preparations had at least 75% labeling efficiency. No species were detected in ZifQNK fusions that would correspond to additional labeling of the zinc finger Cys residues.

Protein immobilization

TteRBP-D168C labeled with Cy3 or Cy5 was conjugated to biotin using the reagent NHS-PEO₄-biotin (Pierce), following the manufacturer's protocol. The mass distribution of PEO₄-biotin moieties on the protein was assessed by MALDI-TOF mass spectrometry. The mean number of biotins per protein ranged from two to three. A typical distribution of biotin adducts per protein, having an average of 2.0, is as follows (adducts, fraction of protein): (0, 0.15), (1, 0.25), (2, 0.25), (3, 0.19), (4, 0.11), (5, 0.05). Biotin-conjugated protein was diluted to 1.5 μ M and added in 100 μ L to each well of a 96-well streptavidin-coated microplate. Two sources of plate yielded similar results: Reacti-Bind streptavidin-coated high binding capacity plates (Pierce) and SigmaScreen streptavidin HC coated plates. Plates were shaken for 4 h in the dark at room temperature, after which the wells were rinsed four times with MBS.

Fluorophore-labeled TteRBP-D168C was decorated with sulfhydryl groups at lysine residues by conjugation with the reagent 2-iminothiolane (Traut's reagent) following the manufacturer's protocol (Pierce). The mass distribution of adduct on

the protein was assessed by MALDI-TOF mass spectrometry and found to average 2.4–2.7 adducts per protein molecule. The distribution of 2-iminothiolane adducts per protein molecule was not quantified because the smaller mass of the 2-iminothiolane adduct (137 amu) led to poor resolution of adduct peaks compared with PEO₄-biotin adducts (474 amu).

Fluorophore-labeled zinc finger fusion proteins were prepared for immobilization according to the method of Smith et al. (2005). First, Zn²⁺ was removed from the protein by incubating overnight in a solution of MBS containing 5 mM Na₂EDTA and 2 mM 1,10-phenanthroline. Next, chelators were separated from protein by gel filtration (BioRad 10DG column) in MBS. The reductant Tris[2-carboxylethyl] phosphine (Molecular Probes) was added to 300 μM and incubated for 10 min to reduce the zinc finger disulfide to thiols. Reduced protein was diluted to 1.5 μM in MBS and pipetted at a volume of 100 μL into each well of a 96-well Reacti-Bind maleimide-activated clear strip plate (Pierce) that had been previously rinsed twice with 200 μL of MBS. The plate was shaken at room temperature in the dark for 4 h, after which the wells were rinsed four times with 200 μL MBS.

Titration with ligand

Fluorophore-labeled proteins that were titrated in solution were diluted to 10–30 nM in MBS in a fluorescence cuvette with constant stirring. To this solution was added incrementally increasing amounts of D-(–) ribose or D-(+) glucose (Sigma) dissolved in MBS. After each addition of ribose, the fluorescence at 666 nm (Cy5) or 570 nm (Cy3) was recorded using a Tau3 Fluorolog spectrofluorometer (Horiba Jobin Yvon). A titration curve (see Results) was obtained by fitting the data (fluorescence as a function of ribose concentration) to a hyperbolic binding isotherm for a two-state model (Marvin et al. 1997):

$$F = \frac{K_d F_F + [S] F_B}{K_d + [S]}$$

where F is the fluorescence at ligand concentration $[S]$, K_d is the dissociation constant, and F_F and F_B are the fluorescence intensities of the ligand-free and ligand-saturated states, respectively. The signal transduction parameter $\Delta F_{\max}$ is defined as the fractional change $(F_B/F_F) - 1$.

Proteins immobilized to microtiter plates were titrated in an array of wells consisting of two to three rows of eight to 12 wells each. Fluorescence emission from each well in the array was recorded with a SpectraMax GeminiXS microplate reader (Molecular Devices). Instrument settings were as follows: PMT voltage, high; reads per well, 30; and autocalibration, on. The temperature was regulated at 25°C. Optical parameters for Cy5 were 640 nm excitation, 665 nm cutoff, and 680 nm emission. For Cy3 these parameters were 530 nm excitation, 550 nm cutoff, and 570 nm emission. After loading with protein, the wells were rinsed, usually four times, until the fluorescence emission remained constant. The wells were filled with 200 μL of MBS and fluorescence emission recorded two or three times. In the case of TteRBP, 2 μL of ribose solution was added to each well. Replicate wells in the array received aliquots of ribose stock, which ranged from 10 nM to 3 mM in decade intervals along the rows of the array. In the case of TmGBP, the initial buffer was decanted from the wells and replaced with 200 μL of glucose solution. The sugar solutions were mixed, and fluores-

cence was recorded. Two or three recordings of fluorescence were made after addition of ligand, and the average reading per well was used for further data processing. For each well, the ratio of fluorescence in the presence of ligand to fluorescence without ligand was computed. The average of this ratio was computed for replicate wells. This average ratio, as a function of sugar concentration, was fit to the equation above, but fluorescence intensity was replaced with fluorescence ratio.

Kinetics of fluorescence recovery

Cy5-labeled TteRBP-CZif, TmGBP-CZif, or TmGBP-NZif was immobilized to 16 wells (two strips) of maleimide-activated wells as described above. For TteRBP-CZif, ribose in MBS was added to all wells at a concentration of 30 μM. For TmGBP-CZif and TmGBP-NZif, glucose in MBS was added to all wells to 1 M. Fluorescence emission from the wells was recorded, and the wells were rinsed four times with MBS to dilute residual sugar solution to <1 nM. Next 200 μL of MBS was added to eight wells (one strip), and 200 μL of the original sugar concentration was added to the remaining eight wells. Within 135 sec of rinsing, the plate was placed in the microplate reader and the emission at 680 nm was recorded at equal time intervals, with shaking during the interval to mix the contents of the wells. For each time point, the fluorescence from the eight wells containing MBS and from the eight wells containing sugar was separately averaged. Next, the ratio of fluorescence from MBS wells to fluorescence from wells containing saturating sugar concentration was computed for each time point. This ratio was plotted as a function of time and fit to a single exponential function to derive a pseudo-first-order rate constant (see Results).

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