Analysis of ligand binding to a ribose biosensor using site-directed mutagenesis and fluorescence spectroscopy

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Abstract

Computational design of proteins with altered ligand specificity is an emerging method for the creation of new biosensing systems. In this work, we investigated the outcome of site-directed mutagenesis on the *Escherichia coli* ribose binding protein (RBP), which is frequently used as a design scaffold for computational searches. A ribose biosensor was first constructed whereby an environmentally sensitive fluorescent probe was covalently attached to RBP at position S265C. This protein conjugate displayed a 54% decrease in emission intensity upon the addition of saturating ribose concentrations and exhibited an apparent dissociation constant (K_d) of 3.4 μM. Site-directed mutants within the RBP binding pocket were created and examined for ribose binding ability and overall structural stability. Because as many as 12 mutations are needed to alter ligand specificity in RBP, we measured the effect of single and multiple alanine mutations on stability and signal transduction potential of the ribose biosensor. Single alanine mutations had significant impact on both stability and signaling. Mutations of N190A and F214A each produced melting temperatures >8°C below those observed for the wild-type protein. Residue Q235, located in the hinge region of RBP, appeared to be a hot spot for global protein stability as well. Additional single alanine mutations demonstrated as much as 200-fold increase in apparent K_d but retained overall protein stability. The data collected from this study may be incorporated into design algorithms to help create more stable biosensors and optimize signal transduction properties for a variety of important analytes.

Keywords: biosensor; periplasmic binding proteins; ribose binding protein; fluorescence

Protein engineering is an emerging field that utilizes natural or designed polypeptide scaffolds for molecular recognition processes (Goodchild et al. 2006; Hosse et al. 2006). Although nature has provided biomolecules that are able to bind a number of potential analytical targets, there are many compounds of interest for which a binding molecule does not exist. Synthetic chemical warfare agents may be one of the most notable categories for which this is true (Igbal et al. 2000). To meet this need, stochastic and deterministic computational algorithms have been written to alter or to redesign the specificity of naturally occurring polypetides for the interaction with non-natural ligands for the purpose of chemical detection (DeGrado et al. 1999; Looger and Hellinga 2001; Rohl et al. 2004). Computational design of scaffolds and binders for many different chemical agents is an area of highly active research (Lee et al. 2000; Looger et al. 2003; Allert et al. 2004; Medintz et al. 2005).

The *Escherichia coli* periplasmic binding proteins (PBPs) are one family of scaffolds used for the design of fluorescent protein biosensors (Higgins 1992; Dwyer and Hellinga 2004). This family of structurally similar

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Abbreviations: PBP, bacterial periplasmic binding protein; RBP, *Escherichia coli* ribose binding protein; ABD-F, [4-Fluoro-7-aminosulfonylbenzofurazan]; CD, circular dichroism.

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proteins is characterized by a single polypeptide chain folded into two domains connected by a hinge region that forms a cleft for substrate binding (Higgins 1992). In the presence of ligand, these proteins undergo a hinge-bending motion almost completely occluding the ligand from solvent (Quiocho and Ledvina 1996). Using X-ray crystallographic data for the open and closed forms, cysteine codons are engineered into the genetic code at specific locations that allow for the covalent modification of environmentally sensitive fluorophores (Marvin et al. 1997; Dattelbaum and Lakowicz 2001; deLorimier et al. 2002; Salins et al. 2004; Rizk et al. 2006). The ability to make quantitative measurements using other techniques (Tolosa et al. 2003; Wells 2006), including FRET (Deuschle et al. 2005; Medintz and Deschamps 2006) and electrochemistry (Benson et al. 2001), have also been explored using PBPs.

Although the main goal of protein design is to build a biomolecule with high specificity for the desired target, the overall stability of the newly designed scaffolds merits additional consideration during their construction. The wild-type PBPs are highly stable, making them attractive targets for protein engineering. However, the production of practical biosensors using this family necessitates that their stability be investigated following the high number of point mutations needed for building new analytical specificity. This work begins to analyze the contribution of individual amino acids toward the overall stability of PBP scaffolds. As a model system, we chose the *Escherichia coli* ribose binding protein (RBP) to investigate the effect of such large-scale site-directed mutation on protein stability. When using RBP as a design scaffold, there are at least 12 amino acids commonly mutated within the binding pocket of RBP to transform this protein from binding the sugar ribose to some other analyte (Looger et al. 2003; Allert et al. 2004; Dwyer et al. 2004). We have created mutations at each of these sites, singly and in tandem, to determine the effect on both RBP thermal stability and on the fluorescent allosteric signaling mechanism used to report ligand binding. The incorporation of these data into computational models may aid in the design of additional field-ready biosensors specific for many analytical targets.

Results

Construction of a ribose sensing fluorescent protein

The periplasmic space of gram-negative bacteria is host to a variety of soluble binding proteins which help bacteria obtain nutrients from the environment. These periplasmic binding proteins (PBP) typically undergo large conformational changes upon interaction with a specific ligand (Fig. 1). The ability to detect ligand binding to a specific PBP is commonly performed using environmentally sensitive fluorophores covalently attached at specific sites on the protein. Many groups have published work describing the rational placement of fluorophores using both inductive and computational methods (Gilardi et al. 1994; Brune et al. 1998; Marvin and Hellinga 1998; Dattelbaum and Lakowicz 2001; deLorimier et al. 2002; Shrestha et al. 2002; Thomas et al. 2006). Choosing the site of fluorophore attachment as well as the identity of the fluorophore is consistently an empirical exercise for each binding protein under study. In order to measure the interaction between RBP and ribose, we constructed a single cysteine mutant of RBP as described previously (deLorimier et al. 2002). Wild-type RBP does not contain any Cys residues; hence, introduction of an L265C modification allows for the covalent and site-specific modification of RBP with a thiol-reactive fluorophore. This mutant was constructed as a translational fusion with an N-terminal His-Tag for affinity purification.

A number of commercially available fluorescent probes were investigated for their ability to signal ribose binding to RBP, including fluorescein iodoacetamide, acrylodan, ABD-F, IANBD ester, and IANBD amide. Also used for this purpose were JPW4095 and JPW4044 (Millard et al. 2004), provided as a kind gift from Leslie Lowe and colleagues (University of Connecticut). Most of these probes yielded little or no change in fluorescence upon ribose binding (data not shown). However, covalent modification of RBP L265C with ABD-F displayed a 54% decrease in fluorescence emission upon titration of the conjugated protein with ribose (Fig. 2). The peak of fluorescence emission recorded for each measurement was then used to calculate a dissociation constant of 3.4 μM for ribose interaction with ABD-labeled RBP

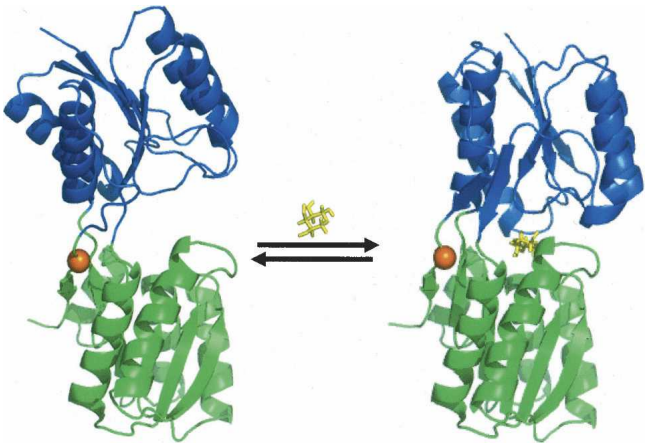

Figure 1. Conformational change observed for the *E. coli* ribose binding protein in the absence (1URP) and presence (2DRI) of ribose. The alpha carbon of residue L265C, which serves as the site of covalent attachment for ABD-F, is marked by a sphere in the hinge region. Molecular rendering performed using PyMOL (DeLano 2002).

(Fig. 2, insert). This number is consistent with that previously reported using other environmentally sensitive probes for signal transduction of ribose binding (deLorimier et al. 2002).

Single alanine mutations affect RBP stability

In order to examine the contribution to overall stability of individual amino acids in the ribose binding pocket, we constructed 13 individual alanine mutations in RBP L265C (Fig. 3). These particular residues were chosen based on their proximity to ribose in the X-ray crystal structure of RBP as well as from previous computational work using RBP as a design scaffold. To confirm proper folding of each mutant, wavelength circular dichroism (CD) spectra were measured. All mutant CD spectra were nearly identical to those observed for the wild-type protein, which is consistent with little or no change to overall RBP structure, as expected (Fig. 4). While global RBP structure appeared unchanged, the temperature of protein unfolding showed great variations, even with single alanine mutations. The data summarized in Table 1 include thermal unfolding measurements for WT RBP (RBP with the L265C mutation) in the presence and absence of ribose. CD data for WT RBP indicate an increase in melting temperature from 57.5°C to 63.7°C upon the addition of ribose. In contrast, only four of the single alanine mutants (S9A, N13A, S103A, and I132A) displayed a significant increase in stability in the presence of ribose similar to the wild-type protein with melting temperatures above 60°C. Many of the single alanine mutations resulted in a greater than expected decrease in overall protein stability as analyzed by melting temperature. In particular, F214A and N190A produced the most significant destabilization of the global protein structure. Mutation at these two sites resulted in a decrease of melting temperature to 49°C and 48°C, respectively, demonstrating almost no change upon the addition of ribose. These two residues are located in the lower RBP domain in close proximity to each other with an inter alpha-carbon distance of ~4 Å (Fig. 3).

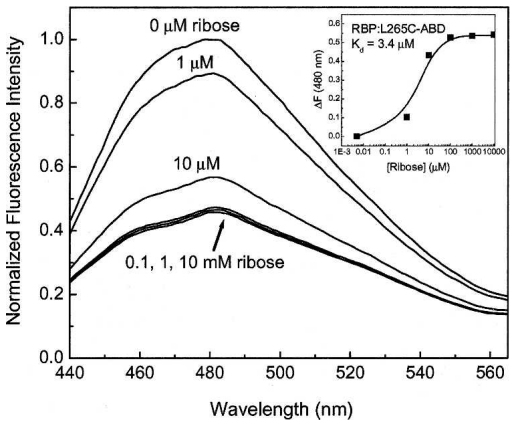

Figure 2. Normalized fluorescence emission spectra for RBP:L265C-ABD in the presence of increasing amounts of ribose. Measurements were performed in 5 mM phosphate buffer (pH 7.0) at room temperature.

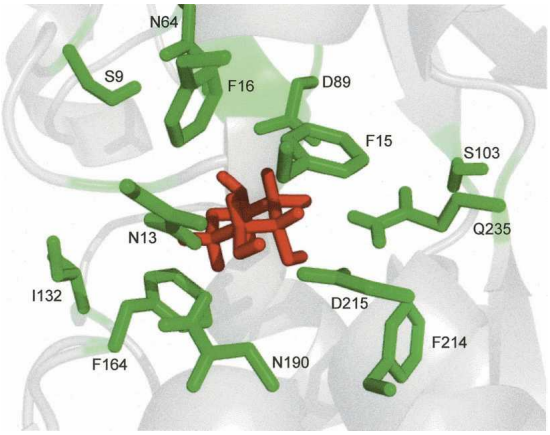

Figure 3. Amino acids involved in RBP binding to D-ribose and mutated to alanine (shown in green; ribose ligand is shown in red). Figure generated with PyMOL (DeLano 2002).

Two separate peptide segments comprise the hinge region that connects the upper and lower domains of RBP (Fig. 1). S103A is found on one of these segments and Q235A on the other. While S103A produced a melting temperature similar to the wild type, Q325A demonstrated no change in melting temperature upon interaction with ribose. This mutation may have locked the RBP into a stable yet open structure even after binding to ribose. Examples of ligand binding to the open confirmation of PBPs have been seen for the structurally similar maltose binding protein as well (Duan et al. 2001; Duan and Quiocho 2002).

Ribose sensing is significantly altered with binding pocket mutations

While mutations to amino acids located in the RBP binding pocket were expected to alter K_d, extreme changes in ΔF were not expected for most single alanine mutations. Figure 5 shows the apparent dissociation constants obtained for the wild-type RBP and two single alanine mutants. Although the actual change in fluorescence intensity does not vary greatly (Table 1), there was a 250-fold variation in binding constant among these mutants. To determine if specificity for ribose binding was altered, RBP N64A, which displayed an increased K_d value, was screened for cross reactivity with structurally similar sugars. There were no observable changes in fluorescence

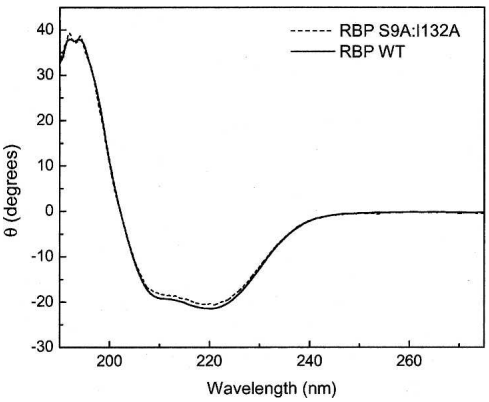

Figure 4. Representative circular dichroism wavelength scans for WT RBP and RBP S9A. Measurements were performed in 5 mM phosphate buffer (pH 7.0) at room temperature.

emission upon the addition of 10 mM fructose, glucose, or potassium gluconate to either the wild-type RBP or RBP N64A construct (data not shown). These data demonstrate that simple mutations within the ligand binding pocket may be useful for altering the dynamic range for analyte sensing while retaining sugar specificity.

We hypothesized that single alanine mutations would not disrupt the signal transducing properties for ribose sensing. However, we found that the majority of single alanine mutations caused significant decreases in ΔF (Table 1). Eight of the 13 mutants demonstrated less than 15% change in fluorescence intensity (down from 54% in the wild type) upon binding ribose. Five of these mutations (F16A, D89A, F164A, F214A, and D215A) produced almost no measurable change in intensity upon the addition of ribose. While it is not surprising that these results suggest that ligand binding is a complex process governed by many intermolecular forces, subtle alterations of these forces may have significant impact on not only ribose binding ability but also on signal transducing potential. This latter realization has implications in the sensitivity one can expect when building biosensors using this protein as a scaffold.

Multiple alanine mutations may be tolerated by the RBP scaffold

While single alanine mutations may yield information about specific locations within the ribose binding pocket, construction of computationally designed proteins typically involves as many as 13 simultaneous mutations. As a result, we examined the tolerance of RBP to multiple alanine mutations within the ribose binding pocket (Table 2). We started with amino acids on the outside of the binding pocket and added additional mutations reaching toward the hinge at the back of the binding pocket, resulting in a quintuple alanine mutant. The ability to bind ribose quickly diminishes as the number of mutations is increased. Changes in fluorescence intensity in response to ribose were observed for the first two mutations, but no change was observed with additional alanine insertions (Fig. 6). The decrease in ribose-dependent fluorescence was accompanied by an increase in K_d (Table 2). Additionally, an increase in destabilization was observed as alanine residues were added further into the binding pocket. Interestingly, mutation Q235A inserted into a quadruple alanine background showed an ~5°C increase in protein stability. These data indicate that, while multiple

Table 1. *Single amino acid substitution in RBP binding site*

Mutant[a]	T_m no ribose	T_m + 10 mM ribose	ΔF[b] (% decrease)	K_d (μM)	$\Delta\Delta G$[c] (kcal/mol)
WT RBP	57.5	63.7	54 ± 4	3.4 ± 0.8	
S9A	56.0	63.8	46 ± 2	30 ± 4	1.3
N13A	58.2	60.1	21 ± 1	20 ± 3	1.0
F15A	52.5	54.2	45 ± 2	667 ± 20	3.1
F16A	58.9	57.4	NB[d]	NB	
N64A	52.9	53.9	41 ± 1	741 ± 88	3.2
D89A	58.2	57.2	NB	NB	
S103A	57.8	60.7	12 ± 1	3.9 ± 1.2	0.08
I132A	57.3	61.2	24 ± 1	9.0 ± 1.9	0.57
F164A	51.7	53.3	<2.0	NB	
N190A	49.4	51.5	14 ± 1	103.1 ± 2.4	2.0
F214A	48.0	46.0	<3.0	NB	
D215A	51.2	51.3	NB	NB	
Q235A	58.2	58.9	14 ± 1	55 ± 10	0.83

[a] All mutants constructed into RBP-L265C background and labeled with ABD-F.
[b] $\Delta F = 1 - (I_{sat}/I_{apo})$.
[c] $\Delta\Delta G = -RT\ ln(K_d^{WT}/K_d^{mutant})$.
[d] (NB) No binding or no reproducible change in fluorescence.

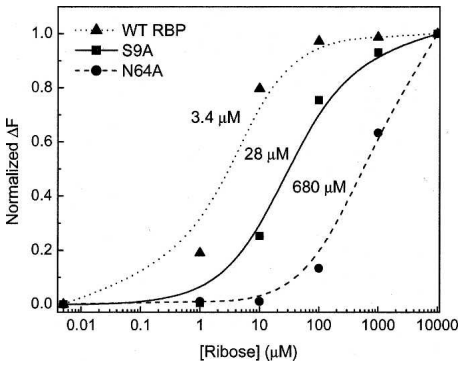

Figure 5. Relative binding constants for WT RBP and for two single alanine mutants. Data from normalized fluorescence emission were replotted and were analyzed using the nonlinear curve fitting algorithm in Origin 6.0.

mutations are in general detrimental to overall protein stability, there may be some sites that can aid in designing more stable scaffolds for the design process.

Discussion

Many successful examples of using RBP and other periplasmic binding proteins as scaffolds for the design of new ligand binders exist in the literature (Looger et al. 2003; Allert et al. 2004; Dwyer et al. 2004). While these proteins are known to be quite stable, the large number of localized mutations necessary to alter ligand specificity may cause significant destabilization and reduction in signal transducing ability. In order to design highly specific binding molecules, one does not necessarily want to give up sensitivity. The limits on the interplay between specificity and sensitivity in biosensor development are a focus of this project.

Using site-directed mutagenesis, we investigated the possibility of incorporating experimentally determined constraints back into design algorithms in order to produce more stable biosensing systems. The constructed fluorescent protein biosensors show a large decrease in fluorescence intensity in response to ribose. Alteration of specific residues to alanine within the RBP binding pocket results in the ability to detect ribose over several orders of magnitude (Fig. 5). To examine the effective shelf life of the RBP biosensors, fluorescence response was monitored periodically over the past 5 mo for wild-type RBP and for RBP Q235A stored at 4°C. Both of these systems retained consistent ribose-dependent fluorescence changes over this time period. In previous work mutating a few of the amino acids in the RBP binding pocket using a genetically encoded ribose biosensor, Lager et al. (2003) observed similar changes in binding constant. The energetic contribution of the alanine mutations to the binding constant is seen using $\Delta\Delta G$ (Table 1). For example, mutation of N64A is found to contribute over 3 kcal/mol to the binding of ribose by RBP. This significant change in binding free energy shows that careful consideration is needed for introducing site-specific mutations into an RBP scaffold.

The amino acid mutations described here result in loss of signaling, loss of stability, both, or neither. Peristeric residues (e.g., I132A and S9A) that are along the binding cleft but not directly involved in ligand binding produce almost no change to either signaling or thermal stability. Mutation of endosteric residues that directly contact the ribose ligand and are located further into the binding cleft appear to have a greater impact on stability and signaling but not necessarily both (e.g., D89). Residues N190 and F214 seem to act as loci for both stability and signaling. Thermal unfolding data for residue Q235, which is located in the hinge region, indicate the presence of a hot spot for global protein stability (Table 1). Indeed, a mutation of Q235V retained ribose binding ability and resulted in an increase in overall RBP stability to 68°C in the presence of ribose (data not shown), which is higher than that found for the wild-type protein. Taken together, these data further support the important role residue 235 may have in overall protein stability. Because of the increase in stability for this mutant, the design process could be altered so that

Table 2. *Multiple alanine substitutions within the ribose binding pocket*

Mutant[a]	T_m no ribose	T_m + 10 mM ribose	ΔF[b] (% decrease)	K_d (μM)	$\Delta\Delta G$[c] (kcal/mol)
WT	57.5	63.7	54 ± 4	3.4 ± 0.8	
S9A	56.0	63.8	44 ± 1	30 ± 4	1.4
S9A, I132A	56.8	66.1	33 ± 1	52 ± 7	1.6
Double + S103A	53.4	53.7	NB[d]		
Triple + N13A	54.1	54.7	NB		
Quad + Q235A	58.9	59.0	NB		

[a] All mutants constructed into RBP-L265C background and labeled with ABD-F.
[b] $\Delta F = 1 - (I_{sat}/I_{apo})$.
[c] $\Delta\Delta G = -RT\ ln(K_d^{WT}/K_d^{mutant})$.
[d] (NB) No binding or no reproducible change in fluorescence.

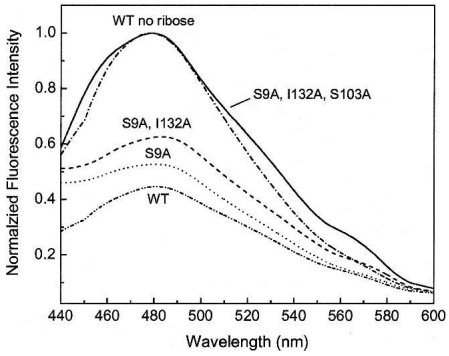

Figure 6. Normalized fluorescence emission spectra for RBP containing multiple alanine mutations within the ribose binding pocket. Emission spectra for WT and all mutants are shown in the presence of ribose. The spectra for the triple-, quad-, and quintuple-alanine RBP mutants in the presence of ribose overlap the WT no ribose spectra. Measurements were performed in 5 mM phosphate buffer (pH 7.0) at room temperature.

this mutation is fixed during the search algorithm. While this may limit the number of possible ligand poses used during the computational search, a significant benefit to signal transduction and stability may be realized.

While a substantial gain in stability is observed following ligand binding and hinge bending conformational changes, RBP is optimized for its role in *E. coli* nutrient acquisition and not necessarily for biosensor design. We show that RBP is able to absorb at least five alanine mutations simultaneously within the binding pocket without too much loss in stability (Table 2). Increasing the stability of the starting scaffold is one way to create more stable biosensing systems. In addition to point mutation described here, homologous proteins from thermophilic organisms offer attractive alternatives (Cuneo et al. 2006; deLorimier et al. 2006). However, because ligand binding is measured as a function of global conformational changes, these proteins may require higher incubation temperatures to function properly. Because of the known structural homology between RBP and other such binding proteins, these results may serve as a model to help influence the computational design of other members of this structural superfamily.

Materials and methods

Preparation of RBP mutants

Primers for the *E. coli rbsB* gene, encoding the ribose binding protein, and a His_{10}-C-terminal tag were used to clone into the NdeI–EcoRI site of pET21a (Novagen, Inc.). For insertion of point mutations, overlapping PCR site-directed mutagenesis was performed on this construct as previously described (Ho et al. 1989). All constructs were confirmed by DNA sequencing (VCU Nucleic Acid Research Facility). RBP mutants were expressed from *E. coli* BL21(DE3) pLysS and purified using Ni-NTA affinity chromatography (GE Biosciences) according to manufacturer-recommended protocols. Protein purity was checked using a Bruker Omniflex MALDI-TOF as well as SDS-PAGE. Purified RBP mutants were applied to an Econo-Pac 10 DG gel filtration column (Bio-Rad) to equilibrate the samples in 5 mM phosphate buffer (pH 7.0) for storage at 4°C.

Preparation of protein conjugates

In a typical labeling procedure, 10–50 μM protein in 5 mM phosphate buffer (pH 7.0) was incubated with a threefold molar excess of thiol-reactive fluorophore in the dark, at 4°C overnight. After an initial screening of the available environmentally sensitive probes (see list below) using RBP:L265C, ABD-F was used to covalently modify all RBP mutants. Unreacted fluorophore was removed from labeled protein conjugates using Econo-Pac 10 DG columns pre-equilibrated with 5 mM phosphate buffer (pH 7.0). For simplicity, RBP:L265C will be designated WT RBP. 60

Fluorescein-iodoacetamide, acrylodan, NBD ester, and NBD amide were purchased from Invitrogen/Molecular Probes. ABD-F was purchased from AnaSpec, Inc. JPW4095 and JPW4044 were kind gifts of Leslie Lowe at the University of Connecticut. All dyes tested were dissolved in dry DMSO and used without further purification.

Steady-state fluorescence

Fluorescence measurements were performed using a Varian Cary Eclipse spectrofluorometer equipped with a plate reader and thin film polarizers set to magic angle (0° excitation and 54.7° emission). Titrations of protein conjugates (1 μM) in 5 mM phosphate buffer (pH 7.0) were performed using a 96-well plate reader while increasing ribose concentrations from 0 to 10 mM ribose and using an excitation wavelength of 380 nm. Fluorescence intensity at the emission maximum as a function of ribose concentration was used to determine binding constants by fitting data to a single binding isotherm as previously described (deLorimier et al. 2002). All data analysis was performed using the nonlinear curve fitting procedures employed in the Origin 6.0 software package. A minimum of three experimental trials were averaged for calculation of binding constants. Unless otherwise stated, all measurements were conducted at room temperature.

Circular dichroism

All CD measurements were performed using a Jasco J-720 spectrometer equipped with a thermal peltier temperature control module, and data were analyzed using the spectra manager software (ver. 5.1.0.0) supplied with the instrument. All measurements were performed using stirred proteins solutions of ~0.5 μM in 5 mM phosphate buffer (pH 7.0) with or without the addition of 10 mM ribose.

Acknowledgments

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