DNA backbone interactions impact the sequence specificity of DNA sulfur-binding domains: revelations from structural analyses

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ABSTRACT

The sulfur atom of phosphorothioated DNA (PT-DNA) is coordinated by a surface cavity in the conserved sulfur-binding domain (SBD) of type IV restriction enzymes. However, some SBDs cannot recognize the sulfur atom in some sequence contexts. To illustrate the structural determinants for sequence specificity, we resolved the structure of SBDSpr, from endonuclease SprMcrA, in complex with DNA of GPSGCC, GPSATC and GPSAAC contexts. Structural and computational analyses explained why it binds the above PT-DNAs with an affinity in a decreasing order. The structural analysis of SBDSpr–GPSGCC and SBDSco–GPSGCC, the latter only recognizes DNA of GPSGCC, revealed that a positively charged loop above the sulfur-coordination cavity electrostatically interacts with the neighboring DNA phosphate linkage. The structural analysis indicated that the DNA–protein hydrogen bonding pattern and weak non-bonded interaction played important roles in sequence specificity of SBD protein. Exchanges of the positively-charged amino acid residues with the negatively-charged residues in the loop would enable SBDSco to extend recognition for more PT-DNA sequences, implying that type IV endonucleases can be engineered to recognize PT-DNA in novel target sequences.

INTRODUCTION

Bacterial DNA phosphorothioate (PT) modification involves the replacement of the R_P non-bridging oxygen of a given phosphodiester bond with sulfur by Dnd proteins (1,2). Natural PT modifications dynamically occur to both DNA strands at the consensus sequences of G_PGCC (P denotes PT link) in Streptomyces lividans 66, G_PS AAC/G_PS TTC in Escherichia coli B7A and Salmonella enterica 87, and G_PS ATC in Bermanella marisrubri RED65, or to a single strand at C_PS CA sites in Vibrio cyclitrophicus FF75 (3,4). dnd gene clusters governing PT modification are present in more than 1,300 bacterial and archaeal species (5,6). PT modification has been implicated in conferring resistance to oxidation to the host bacteria (7,8), influencing the global transcriptional response (9), and participating in restriction-modification systems in bacteria (10).

DNA modifications, primarily base methylation, participate in DNA replication and gene regulation through interactions with different nucleic acid binding proteins, also known as ‘readers’ (11), which transmit methylation information to other systems. For example, 5mCpG, the major eukaryotic methylated dinucleotide, is recognized by the methyl-GpG-binding (MBD) domain and the SET and RING finger-associated (SRA) domain, the prevalent 5mC reader in three life kingdoms (12,13). The SRA domain is often fused to other domain(s) that function in versatile cellular processes related to 5mC metabolism, or fused with a nuclease motif to cleave DNA in a modification-dependent way (14–18). Therefore, studies of the recognition mechanism of DNA modification by these readers are important to understand the flow of epigenetic information.

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We recently identified a new type of reader in the *Streptomyces coelicolor* type IV restriction enzyme ScoMcrA, which specifically recognizes and cleaves PT-modified DNA (19), and this reader is a sulfur-binding domain (SBD) (20,21). PT-dependence is dictated by SBDs, whose carboxyl termini almost exclusively contain HNH nuclease motifs (21).

The complex structure of SBD$_{\text{Sco}}$ bound to the PT-DNA sequence 5'-CCG$_{\text{Ps}}$GCACC-3' was determined [Protein Data Bank (PDB) accession number: 5ZMO] (20). Discrimination of sulfur from oxygen by the SBD was achieved by concurrent interactions with the sulfur atom, the non-bridging oxygen of neighboring phosphates, as well as with the base pairs surrounding the PT linkage, in a manner generally similar to the recognition of 5mC or 5hmC by the base pairs surrounding the PT linkage, in a manner generally similar to the recognition of 5mC or 5hmC by the SRA. SBD$_{\text{Spr}}$ only contacted the PT linkage on one DNA strand even though both strands were phosphorylated.

Three phosphates in the vicinity of the central PT link were wrapped in a cleft edged by two positively-charged patches on the surface of SBD$_{\text{Sco}}$, where the R$_{\text{P}}$ sulfur was firmly coordinated by a hydrophobic concave pointing to the bottom of this cleft (20). SBD$_{\text{Sco}}$ only binds to PT-DNA in the sequence G$_{\text{Ps}}$GCC and not to the other four natural PT modification sequences, in sharp contrast to that the SRA domain has high flexibility in target sequence selection (22).

In the complex structure of SBD$_{\text{Sco}}$–G$_{\text{Ps}}$GCC, four residues on a ‘base-contacting’ loop formed seven hydrogen bonds with the three base pairs across the PT link. Single mutation of individual residues lowered the binding affinity to G$_{\text{Ps}}$GCC by varying amounts, ranging from 20% to 80% (20). By contrast, the SBD of the PT-dependent restriction endonuclease (REase) SprMcrA from *Streptomyces pristinaespiralis* has a relatively relaxed sequence specificity, targeting G$_{\text{Ps}}$GCC, G$_{\text{Ps}}$AAC and G$_{\text{Ps}}$ATC, but not G$_{\text{Ps}}$TTC or C$_{\text{Ps}}$CA (21).

To understand the reasons underlying the differences in sequence specificity among SBDs, we crystallized SBD$_{\text{Spr}}$ with G$_{\text{Ps}}$GCC, G$_{\text{Ps}}$AAC and G$_{\text{Ps}}$ATC. Comparative structural analysis revealed that a surface patch on two SBDs possesses a reverse charge, which exerts repelling and attracting strength on DNA by SBD$_{\text{Sco}}$ and SBD$_{\text{Spr}}$, respectively. Mutation of E156R/D157R in this patch from SBD$_{\text{Sco}}$ conferred the mutant domain with the ability to bind G$_{\text{Ps}}$AAC and G$_{\text{Ps}}$ATC. Additionally, we provide evidence for why both SBDs showed a higher affinity for G$_{\text{Ps}}$GCC than for the other DNA sequences. This study reports that variation in DNA binding affinity constitutes a key determinant of the sequence specificity for SBDs and provides new insights into approaches for engineering the specificity of modification-dependent REases by altering their contacts with DNA phosphates other than the nucleotide bases.

**MATERIALS AND METHODS**

**Construction of protein expression vector and site-directed mutagenesis**

DNA fragments encoding wild-type SBD$_{\text{Spr}}$ and SBD$_{\text{Sco}}$ were cloned into the pET28a vector (Novagen), with N-terminal 6xHis tags. His-tagged SBD$_{\text{Spr}}$ and SBD$_{\text{Sco}}$ mutant variants were constructed by the whole-plasmid PCR and DpnI digestion method (23). The *Escherichia coli* strain DH10b was used as a transformation host. The mutations were confirmed by DNA sequencing of the entire gene. Primers used for plasmid construction were listed in Supplementary Table S1.

**Preparation and purification of stereospecific PT-DNA**

The PT-DNA oligonucleotides were chemically synthesized and PAGE-purified. The concentration of oligonucleotides was determined by spectrophotometric measurement on a NanoDrop 2000 spectrophotometer (Thermo), and double-stranded DNA was prepared by mixing equimolar concentrations of complementary PT-modified oligonucleotides, followed by heating to 95°C for 2 min and gradual cooling. The R$_{\text{P}}$ and S$_{\text{P}}$ stereoisomers of double-stranded PT-DNA were separated by anion exchange HPLC with a DNA-Pac PA-100 analytical column (Thermo) on an Agilent 1260 Infinity Series system at a flow rate of 1 ml/min with the following parameters (column at room temperature; solvent A, 10 mM Tris–HCl, pH 8.0; solvent B, 10 mM Tris–HCl, pH 8.0, 1 M NaCl; gradient, 10% B to 70% B over 40 min; detection by UV absorbance at 260 nm). The eluent was desalted with a Copure C18 column (Biocomma), dried on an RVC 2–25 rotational vacuum concentrator (Martin Christ), and dissolved with distilled deionized water.

**Protein expression and purification**

Proteins were expressed in the *Escherichia coli* strain BL21(DE3) at 16°C; a 10-ml culture grown overnight from a single colony was inoculated into 1 l of Luria Broth medium supplied with 50 ug/ml kanamycin. The culture was incubated at 37°C to an OD$_{600}$ of 0.6–0.8 and induced by the addition of 0.2 mM isopropyl-D-1-thiogalactopyranoside (IPTG) for another 20 h at 16°C. The cells were harvested and resuspended in 20 ml binding buffer (20 mM MES, pH 6.8, 20 mM imidazole, and 300 mM NaCl) and lysed by sonication in an ice bath. After centrifugation at 16 000 g for 60 min at 4°C, the supernatant was applied to 2 ml Ni-NTA column (GE Healthcare), and a Superdex 200 10/300 GL gel filtration chromatography column (GE Healthcare) equilibrated with binding buffer. The Ni-NTA column was eluted with 10 ml of elution buffer (20 mM MES, pH 6.8, 300 mM imidazole and 300 mM NaCl), and lysozyme was lyzed by sonication in an ice bath. After centrifugation at 16 000 g for 60 min at 4°C, the supernatant was applied to 2 ml Ni-NTA column (GE Healthcare), and a Superdex 200 10/300 GL gel filtration chromatography column (GE Healthcare) equilibrated with 10 mM Tris–HCl (pH 8.0), 100 mM NaCl and 1 mM DTT, using an AKTA FPLC system (GE Healthcare). The peak fractions were combined and concentrated to 10 mg/ml. Purified proteins were visualized by Coomassie-stained 15% SDS-PAGE analysis, and protein concentration was determined using a Bradford Protein Assay Kit (Bio-Rad).

**Crystallization, data collection and structure determination**

Crystals for SBD$_{\text{Spr}}$ in complex with the R$_{\text{P}}$ form of the 8-bp hemi-PT DNA oligonucleotide 5'-GGCG$_{\text{Ps}}$GCC-3' were grown at 14°C using the sitting-drop vapor-diffusion method (23). The crystals were transferred to a cryo-protectant solution consisting of the same buffer supplemented with 25% polyethylene glycol 4000 (PEG 4000). The complex structure of SBD$_{\text{Spr}}$ bound to the PT-DNA sequence 5'-CCG$_{\text{Ps}}$GCACC-3' was determined [Protein Data Bank (PDB) accession number: 5ZMO] (20). Discrimination of sulfur from oxygen by the SBD was achieved by concurrent interactions with the sulfur atom, the non-bridging oxygen of neighboring phosphates, as well as with the base pairs surrounding the PT linkage, in a manner generally similar to the recognition of 5mC or 5hmC by the SRA. SBD$_{\text{Spr}}$ only contacted the PT linkage on one DNA strand even though both strands were phosphorylated.

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method in 48-well plates (24). Typically, 1 μl of reservoir solution was mixed with 1 μl of protein–DNA solution and equilibrated against 80 μl of reservoir solution. After optimization and macroseeding efforts, diffraction crystals of SBD_{Spr}–G_{PS}GCC were obtained from a buffer of 0.01 M magnesium acetate tetrahydrate, 0.05 M sodium cacodylate trihydrate pH 6.5, and 1.3 M lithium sulfate monohydrate. Crystal diffraction datasets at a resolution of 2.06 Å for the SBD_{Spr}–G_{PS}GCC complex were collected at the BL19U1 beamline at the National Center for Protein Science Shanghai and processed using HKL2000 (25). The crystal belonged to space group P6_{1}2_{1}2_{1}, and contained three molecules of SBD_{Spr}, in complex with three molecules of PT-DNA in each asymmetric unit. The crystal structure was determined by the molecular replacement method with the Phaser program (26), using the structure of SBD_{Spr}–G_{PS}AAC as the searching model. The structure of the SBD_{Spr}–G_{PS}AAC complex was refined and rebuilt using Coot (27) and Refmac (28).

The co-crystal of SBD_{Spr} with 8-bp oligos with G_{PS}AAC sequence was not successfully obtained. Crystals for SBD_{Spr} in complex with the R_{P} form of the 10-bp hemi-PT DNA oligonucleotide 5′-GGCG_{PS}AACGTTG-3′ were grown and obtained at 14°C with the reservoir solution containing 0.1 M Bis-Tris pH 5.5, 0.15 M ammonium acetate, and 25% PEG 3350. The SBD_{Spr}–G_{PS}AAC complex crystals belonged to the P1 space group, with two molecules of SBD_{Spr} and two molecules of G_{PS}AAC–DNA; the structure of the complex was determined to 2.42 Å by the molecular replacement method with the phenix.rosetta refinement program (29), using the SBD domain of the ScoMcrA structure (PDB code: 5ZMO) as the searching model. The structure of the SBD_{Spr}–G_{PS}AAC complex was refined and rebuilt using Coot and Phenix.refine.

Crystals for SBD_{Spr} in complex with the R_{P} form of the 8-bp hemi-PT DNA oligonucleotide 5′-GATG_{PS}ATCC-3′ were grown and obtained at 14°C with the reservoir solution containing 0.1 M Tris–HCl pH 8.5 and 4.5% PEG 8000. The SBD_{Spr}–G_{PS}ATC complex crystals belonged to the C222_{1} space group, with two molecules of SBD_{Spr} and two molecules of G_{PS}ATC–DNA in the asymmetric unit; the structure of this complex was determined to 3.3 Å by the molecular replacement method with the Phaser program (30), using the structure of SBD_{Spr}–G_{PS}AAC as the searching model. The structure of the SBD_{Spr}–G_{PS}ATC complex was refined and rebuilt using Coot, Refmac and Phenix.refine.

The data collection statistics and the refinement statistics for the SBD_{Spr}–G_{PS}GCC, SBD_{Spr}–G_{PS}AAC and SBD_{Spr}–G_{PS}ATC complexes are summarized in Supplementary Table S2.

**Electrophoretic mobility shift assay (EMSA)**

Each EMSA reaction contained 6 pmol DNA and protein at a concentration 4-fold higher than the DNA concentration (molar ratio) in 10 μl binding buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl and 5% glycerol). After incubation at room temperature for 5 minutes, the reaction mixtures were loaded onto 12% non-denaturing polyacrylamide gels (acrylamide:bisacrylamide ratio of 79:1, w/w) and electrophoresed in 0.5× TBE buffer at 15 mA for 30 min. Ten bp-oligonucleotides used for EMSA assay were listed in Supplementary Table S1.

**Fluorescence polarization assay for analysis of DNA binding**

5′-FAM-labeled hemi-PT-DNA, labeled on one strand only, was synthesized and purified (Supplementary Table S1). Protein solutions were diluted serially using 2-fold dilutions (5 μM starting concentration, 16–20 dilutions) and mixed with a 5 nM final concentration of DNA probe in a Corning 3575 plate, using binding buffer of 20 mM Tris–HCl pH 8.0, 5% glycerol, 50 mM NaCl and 1 mM DTT. The mixture was incubated for 10 min at room temperature, and fluorescence polarization was measured at room temperature on a SpectraMax i3x (Molecular Devices) using 485/20 nm and 528/20 nm filters for emission and excitation, respectively. The dissociation constants (K_D) were calculated by fitting the experimental data (from two experimental replicates) to the following equation using GraphPad Prism software (version 6.0): [mP] = [maximum mP][C] / (K_D + [C]) + [baseline mP], and then the curve was replotted using percent saturation calculated as ([mP] – [baseline mP])/[maximum mP] – [baseline mP]), where mP is millipolarization and [C] is protein concentration. The binding experiments were performed under the same laboratory conditions.

**Transformation efficiency assay**

The pACYCDuet™-1 vector (PT+) and its derivative (PT−) carrying the _ndd_ gene cluster from _Salmonella enterica_ serovar _Cerro_ 87 were introduced to _E. coli_ BL21(DE3), and competent cells of the resulting strains were prepared using the standard calcium chloride protocol. Transformation frequency was determined by introducing 100 ng pET28a derivatives carrying _scoMcrA_ or its mutant variants to the competent cells. The number of _E. coli_ colonies in each experiment was determined by serial dilutions. Each experiment was repeated three times and the mean value of the transformation frequency was reported.

**All-atom molecular dynamics simulation**

The co-crystal structure of the SBD domain of ScoMcrA and the natural PT-DNA fragments (G_{PS}GCC) was used as a starting model to build up the nucleotide-mutant models of G_{PS}ATC and G_{PS}AAC, and the protein-mutant models of E156R and E156R/D157R, with the modeling software package of Molecular Operating Environment v2018 (31). All the molecular dynamics simulations (MDs) were performed with the AMBER 16 software (30). For protein and DNA parts, Amber ff14SB and OL15 force field were used, respectively (32,33). The phosphorothioate force field employed these parameters developed by Mukherjee and Bhattacharyya _et al._ (34). The PROPKA algorithm determined the protonation of the SBD–DNA complex on the PDB2PQR web server (35). The protein-DNA complexes were then solvated within a cubic box and the TIP3P water model (36), in which the minimum distances between any protein atom and edges of the water box was set to
be 12 Å. The systems were neutralized by adding appropriate numbers of Na⁺ and Cl⁻ ions. Long-range electrostatic interactions were calculated with the Particle-Mesh-Ewald (PME) method (37), and van der Waals interactions were truncated within 12 Å. The time interval was set as 2 fs, and the SHAKE (38) algorithm was used to constrain the bonds-connecting hydrogen atoms. The entire system was first minimized and heated up to 298 K before the production process. The CPPTRAJ tool implemented in the AMBER 16 software package was used for trajectory analyses, such as the popular root-mean square deviation (RMSD) and cluster analysis. Solvent accessible surface area (SASA) is a parameter that measures the fraction of the protein surface interacting with the solvent molecules. The term corresponding to the SASA was calculated through BIOVIA Discovery Studio (39).

**Binding Free Energy Calculation**

The binding free energy between PT-DNA and SBD was calculated by the MM/GBSA approach (40), using the following equations,

\[
\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{PT-DNA}} + G_{\text{protein}}),
\]

\[
\Delta G_{\text{bind}} = \Delta H - T \cdot \Delta S \approx \Delta E_{\text{MM}} + \Delta G_{\text{solv}} - T \Delta S,
\]

\[
\Delta E_{\text{MM}} = \Delta E_{\text{int}} + \Delta E_{\text{vdW}} + \Delta E_{\text{ele}},
\]

\[
\Delta G_{\text{solv}} = \Delta G_{\text{GB}} + \Delta G_{\text{SA}},
\]

where \( \Delta E_{\text{int}} \) is negligible with the single-trajectory strategy. The nonpolar part of the solvation free energy (\( \Delta G_{\text{SA}} \)) was calculated with the solvent-accessible surface area (SASA) through the LCPO algorithm (41), by using \( \Delta G_{\text{SA}} = \gamma \cdot \text{SASA} + \beta \) (the surface tension constants \( \gamma \) and \( \beta \) were set to 0.0072 and 0, respectively). The polar part of the solvation energy (\( \Delta G_{\text{GB}} \)) was estimated using the Generalized Born (GB) model proposed by Onufriev et al. (GB(\( \Delta \text{g}=2 \)) (42). The \( \Delta E_{\text{vdW}}, \Delta E_{\text{ele}}, \Delta G_{\text{GB}} \) and \( \Delta G_{\text{SA}} \) terms were computed based on the 500 snapshots extracted from the last 20 ns MD trajectories. Each trajectory was calculated individually, and then all energies were analyzed statistically.

**Non-covalent interaction (NCI) analysis**

The independent gradient model (IGM) (43) and reduced density gradient (RDG) (44) analyses were carried out using Multiwfn 3.6 program (45). Molecular plots were visualized with the VMD 1.9.3 program (46).

The IGM analysis depends on the topological characteristics of the electron density, \( \rho \). The IGM descriptor \( \delta_{\text{inter}} \) is calculated as the difference between the first derivatives of electron density of the whole system and the fragments:

\[
\delta_{\text{inter}} = |\nabla \rho|_{\text{IGM,inter}} - |\nabla \rho|
\]

\( \delta_{\text{inter}} > 0 \) indicates the presence of weak interactions and its magnitude denotes the interacting intensity.

The non-covalent interaction RDG method is an alternative method to reveal weak interlayer interactions (44), with a dimensionless form of electron density gradient norm function:

\[
\text{RDG}(r) = \frac{1}{2(3\pi)^{1/3}} \frac{|\nabla \rho(r)|}{\rho(r)^{4/3}}.
\]

The sign of the second eigenvalue of the electron density Hessian matrix, sign(\( \lambda_2 \)), was used in the RDG analyses to judge the attractive and repulsive interaction, that is, corresponding to negative and positive values of sign(\( \lambda_2 \)) \( \rho \), respectively.

**RESULTS**

**Affinity of SBD_{Spr} for PT-DNA of varied sequence contexts**

The sulfur modification-dependent REases SprMcrA and ScoMcrA use an SBD to recognize the DNA backbone phosphorothioate link of the \( R_P \) stereoisomer, which is adopted by the naturally occurring PT modifications in five DNA core sequence contexts (Table 1) in prokaryotes. ScoMcrA only recognizes G\(_{\text{Pr}}\)GCC, whereas SprMcrA binds and shifts DNA of the sequences G\(_{\text{Pr}}\)GCC, G\(_{\text{Pr}}\)ATC and G\(_{\text{Pr}}\)AAC in EMSAs (21). To compare the affinity of SBD_{Spr} (aa 1–165 of SprMcrA) for PT-DNA of the five natural PT sequence contexts, a set of hemi-modified DNA duplexes, which differed from each other in the core sequence bearing the PT link in either the \( P_T \) or \( R_P \) configuration (Table 1) were assayed (Supplementary Figure S1). In agreement with the reported EMSA results (21), no \( S_P \) PT-DNA nor \( R_P \) PT-DNA in sequences of G\(_{\text{Pr}}\)TTC or C\(_{\text{Pr}}\)CA could be recognized by SBD_{Spr}, indicating that recognition of the PT link is coupled with interactions with surrounding nucleobase or phosphate groups. SBD_{Spr} showed the highest binding affinity for G\(_{\text{Pr}}\)GCC, with a dissociation constant value (\( K_D \)) of 5.55 nM, followed by a \( K_D \) of 38.33 nM for G\(_{\text{Pr}}\)ATC and 94.67 nM for G\(_{\text{Pr}}\)AAC (Table 1). By comparison, SBD_{Sco} showed a \( K_D \) of 102 nM for G\(_{\text{Pr}}\)GCC (Table 2), 18.5-fold weaker binding than SBD_{Spr} had to the same DNA duplex.

**Structure of SBD_{Spr} complexes**

To determine why SBD_{Spr} showed varying affinity for G\(_{\text{Pr}}\)GCC, G\(_{\text{Pr}}\)ATC and G\(_{\text{Pr}}\)AAC, as well as a much higher
Table 2. $K_D$ (nM) value of SBD$_{Sc}$ and SBD$_{Sc}$ mutants for PT-DNA of different core sequences

| Hemi PT-DNA duplex | G$_{Ps}$GCC | G$_{Ps}$ATC | G$_{Ps}$AAC |
|--------------------|-------------|-------------|-------------|
| SBD$_{Sc}$ (wild type) | $102 \pm 1.2^*$ | $1091 \pm 58$ | $567 \pm 37$ |
| E156D | $112 \pm 12$ | $1322 \pm 99$ | $846 \pm 74$ |
| E156Q | $137 \pm 18$ | $1015 \pm 49$ | $618 \pm 43$ |
| E156L | $110 \pm 12$ | $791 \pm 42$ | $499 \pm 37$ |
| E156K | $130 \pm 11$ | $370 \pm 14$ | $297 \pm 19$ |
| E156R | $133 \pm 21$ | $346 \pm 17$ | $267 \pm 15$ |
| E156R/D157R | $123 \pm 8$ | $192 \pm 12$ | $183 \pm 9$ |

* $K_D$ (dissociation constant, nM).

Comparison of the structure of SBD$_{Spr}$ and SBD$_{Sc}$ complexed with G$_{Ps}$GCC

Generally, the overall structure of the SBD$_{Spr}$ monomer, including the sulfur-binding cavity, was similar to SBD$_{Sc}$ except for three flexible loops that swayed differently (Supplementary Figure S5). Among them, loop 12 made no contact with DNA substrates in either structure. Loop 34 was three amino acids longer in SBD$_{Spr}$ than in SBD$_{Sc}$ and made contacts with the DNA phosphate backbone. The location of loop A5 in SBD$_{Spr}$ was shifted by four amino acids from the equivalent loop S6 in SBD$_{Sc}$ (Figure 1C); however, both of these loops were inserted into the major groove of the DNA substrates and contacted the DNA bases (Figure 1A, B). The sequence and spatial arrangement of these residues involved in sulfur coordination were well aligned with the equivalent residues of SBD$_{Sc}$, but differed slightly by the presence of an additional electrostatic bond with the guanine base G117 in SBD$_{Sc}$ (Supplementary Figure S6). The $S$ oxygen symmetric to sulfur was stabilized by two hydrogen bonds with Q32 and A22 in SBD$_{Spr}$ whereas it only bonded to the amino group of R117 in SBD$_{Sc}$. The Q32A mutation in SBD$_{Spr}$ caused an ~50-fold decrease in binding affinity, but the equivalent mutation of R117A or R117G in SBD$_{Sc}$ caused a ~50-fold decrease in binding affinity, but the equivalent mutation of R117A or R117G in SBD$_{Sc}$, completely abolished the affinity for PT-DNA, possibly due to the loss of three bonds between R117 and the sulfur and oxygen atoms.

For the electrostatic interaction between the phosphate group of G4 and R85, the remaining four bonds lack equivalents in the structure of the SBD$_{Sc}$ complex (20). Therefore, the striking structural difference between the two complexes with respect to the interaction with the phosphate backbone lies in the lack of any interaction with the phosphate group of C8, immediately downstream of the phosphorothioate in SBD$_{Sc}$. The contrary, in SBD$_{Spr}$, Y31 also makes a hydrogen bond to the fifth DNA phosphate in addition to coordination with the sulfur atom (Figure 2C).

Base contact by SBD$_{Spr}$ determines the variation in binding affinity

As mentioned above, SBD$_{Spr}$ displayed varied affinity to PT-DNA of different core sequences (Table 1). In three SBD$_{Spr}$ co-crystal structures, the H102–G103–D104 motif all inserted into the DNA major groove to make contacts with bases, but the numbers of H-bonds were slightly different (Figure 3). In all structures, the ND1 atom of H102 formed H-bond with O6 atom of G4, and the OD2 atom of D104 bonded to the N4 atom of C7. It’s worth noting that the ND1 atom of H102 also formed H-bond with N7 atom of G4 in G$_{Ps}$GCC and G$_{Ps}$AAC sequences, while this H-bond was not existing in SBD$_{Spr}$-G$_{Ps}$ATC complex. When the central SG5C6 are changed to SA5A6 or SA5T6, the H-bonds patterns formed by the central bases showed some differences in three complexes. The N atom in the main chain of G103 bonded to N7 atom of SG3 from G$_{Ps}$GCC sequence and N7 atom of SA3 from G$_{Ps}$ATC and G$_{Ps}$AAC sequences. The carbonyl O atom of G103 formed an additional H-bond with N4 atom of base C6 in SBD$_{Spr}$.
GPSGCC complex. SA5 showed a significant deflection in GPSATC sequence, compared with GPSGCC and GPSAAC sequences, leading to formation of another H-bond between the ND1 atom of H102 with the N6 atom of SA5. In conclusion, HGD motif formed five H-bonds in SBDSpr–GPSGCC complex while four H-bonds in SBDSpr–GPSATC and SBDSpr–GPSAAC complexes, which explained why SBDSpr showed a highest affinity for GPSGCC.

The base recognition pattern by HGD motif in complexes of SBDSpr–GPSAAC and SBDSpr–GPSATC is different (Figure 3). What’s more, close comparison of two structures revealed that the binding of the GPSAAC released the methyl group of T6′ on the complementary strand from the binding site, and converts the weak non-bonded interaction to unfavorable thymine methyl-solvent accessibility (Supplementary Figure S7). In the case of GPSATC, the percent solvent accessibility of T6 methyl group was calculated to be only 10–15, corresponding to fully buried with the SBD-DNA interface. The multiple C–H...O contacts between the methyl and Y78 were believed to be attractive for GPSATC (47).
Figure 2. Details of the SBD$_{Spr}$–DNA interactions. (A) The SBD$_{Spr}$ binds specifically to G$_{PS}$GCC. Residues that interact with DNA are colored as follows: Tyr31, Gln32, Tyr78, Pro79 and Ala82, which form the sulfur atom binding pocket, are in yellow; His102, Gly103 and Asp104, which recognize DNA bases, are in purple; and Arg29, Arg73 and Arg85, which interact with phosphates through electrostatic interactions, are in blue. (B) Sulfur atom-binding pocket on SBD$_{Spr}$ formed by Tyr31, Gln32, Tyr78, Pro79 and Ala82. (C) Schematic summary of the interactions between SBD$_{Spr}$ and PT-DNA, mC represented the main chain of amino acid.

Figure 3. Comparison of HGD motif of SBD$_{Spr}$ interaction with three DNA core sequences of (A) G$_{PS}$GCC, (B) G$_{PS}$ATC and (C) G$_{PS}$AAC. SG5 and SA5 represents the nucleoside G5 and A5 with PT modification. The base contact motif HGD of SBD$_{Spr}$ in the three complexes was shown in cyan, purple and green, respectively.
In the case of GPSAAC, the T′6 methyl group was fully exposed to solvent and lack of specific interaction with SBD protein. Difference in the weak non-bonded interaction between GPSATC and GPSAAC results in a lower KD values for GPSATC than GPSAAC bound by SBD_Spr.

To evaluate the contribution of these interactions with the bases to the DNA binding affinity, the three aa residues HGD were independently mutated, and the binding affinity of the resulting mutated proteins to semi-PT-DNA of 5′-GGCCGPSGCCC-3′ was measured by fluorescence polarity (Supplementary Table S3). The H102A and D104A mutants showed a 370-fold and 25-fold decrease, respectively, in binding affinity compared with wild-type protein, demonstrating that base contact constitutes an important component of the total affinity for PT-DNA by ensuring the formation of a stable DNA/protein complex. Unexpectedly, the G103A mutation almost abolished the affinity for PT-DNA as evidenced by the strikingly increased KD value of >9000 nM (Supplementary Table S3). The G103A mutation introduced an additional C-C side chain, which increased the main chain rigidity and affecting the hydrogen bonding network of base G5 and C6, leading to drastic decrease in binding affinity.

Opposite interactions with PT-DNA by loop 34 of SBD_Spr and SBD_Sco

When the structures of SBD_Spr–GPSGCC and SBD_Sco–GPSGCC were compared, a striking DNA strand distortion at the two phosphodiester bonds proximal to the 3′ terminus of the PT-DNA strand was observed in the SBD_Sco–GPSGCC complex. Phosphorus atoms of the seventh and eighth bases in the PT-modified strand were extruded by 3.5 and 5.0 Å relative to those in the SBD_Spr–GPSGCC structure. (Figure 4A). Compared with SBD_Sco, SBD_Spr possesses a longer loop 34, containing the three positively charged residues R69, R73, and R75, which constitute a local positive interface with DNA wherein R73 bonds to the phosphate group of C6 (Figure 4B, D). By contrast, the corresponding interface of the SBD_Sco loop34 features two tandem acidic residues, E156 and D157, and a spatially adjacent D160 (Figure 4C, E). These residues form a negatively charged surface area, which is repulsive towards the DNA phosphate backbone and may account for the distortion of the DNA double helix structure in the SBD_Sco–GPSGCC complex.

Ability of SBD_Sco–E156R/D157R to bind PT-DNA of GPSAAC and GPSATC

Given that SBD_Sco can only bind to GPSGCC, and that SBD_Spr has maximum affinity for GPSGCC, we hypothesized that the repulsive force exerted by the negative interface of SBD_Sco weakened its overall affinity for PT-DNA, leading to the failure to recognize GPSAAC or GPSATC, although this repulsive force was not sufficient to disrupt the most stable complex formed with GPSGCC.

To test this hypothesis, E156 of SBD_Sco, structurally equivalent to R73 of SBD_Spr, was mutated into basic (R and K), neutral (L and Q), and acidic (D) residues. Affinity quantification of each E156 mutant by fluorescence polarization assay showed that the mutants containing the R, K or L substitutions all showed an increased affinity for GPSAAC and GPSATC relative to the wild-type protein. In particular, the E156R mutant displayed the most significant increases in DNA binding affinity for GPSATC and GPSAAC by; respectively, ~3-fold and ~2-fold (Table 2, Figure 4F, Supplementary Figure S8). The double-mutation protein, E156R/D157R, showed further increases in DNA binding affinity for GPSATC and GPSAAC in EMSA (Figure 5A), which were quantified to be ~5.7-fold and ~3-fold increases for GPSATC and GPSAAC, respectively (Table 2). Unfortunately, we were unable to purify the triple-mutation protein, E156R/D157R/D160R, probably because the significant decrease in protein expression. However, mutations of the three acidic residues were constructed in the full-length ScoMcrA, and the in vivo nuclease activities of the mutants were analyzed by comparing the transformation efficiency of their coding DNA into a PT and non-PT E. coli host (Figure 5B). In agreement with the EMSA results for the SBD mutants, the uptake efficiency of scoMcrA_E156R by the PT host was 2.5-fold less than by the non-PT host, while that of other two single-mutant genes had no significant difference in the PT and non-PT hosts. In parallel, the transformation efficiency of scoMcrA_E156R/D157R was 500-fold less with PT E. coli than with non-PT E. coli (Figure 5B), implying that the double-mutation protein acquired restriction activity for GPSAAC DNA, but kept the ability to discriminate the unmodified DNA. However, the triple-mutant gene did not show distinctive transformation efficiency between the PT and non-PT hosts. In addition, the transformation efficiency of scoMcrA_E156R/D157R/D160R was 500-fold less with PT E. coli than with non-PT E. coli (Figure 5B), implying that the double-mutation protein acquired restriction activity for GPSATC and GPSAAC, which were built and mutated through Molecular Operating Environment. The most populated conformations sampled during our simulations that contain the interaction regions were chosen for RDG analysis. The calculated RDG isosurfaces with BGR color scales representing sign(λ3) ρ values are given in Supplementary Figure S9 for E156R and E156R/D157R in SBD_Sco–GPSATC and SBD_Sco–GPSAAC. In E156R, two hydrogen bonds, N7G7⋯NH1R156 and O6G7⋯NH2R156, were strong. When we introduced a double mutation (E156R/D157R), two newly formed hydrogen bonds (HH12R157⋯O6G7 and HH22R156⋯O2P6) were observed, similar to the case of E156R. Additionally, van der Waals interactions were also observed between the adjacent HH22R157 and O6G7 and N7G7, indicated by the green color of the RDG surfaces. The gain of GPSATC and GPSAAC interactions with both R156 and R157 might be crucial to triggering the changes leading to the acquisition of the enhancement of non-covalent interaction. Replacement of E156 and D157 with arginine introduced hydrogen bond interactions as well as van der Waals interactions, which significantly strengthened the binding, resulting in a concerted interplay of interactions between the SBD and PT-DNA.
Figure 4. Comparison of SBD<sub>Spr</sub> and SBD<sub>Sco</sub> interactions with PT-DNA. (A) Superimposition of SBD<sub>Spr</sub> loop34 (cyan) with DNA (orange) and SBD<sub>Sco</sub> loop34 (grey) with DNA (yellow). Phosphorus atoms of the seventh and eighth bases in the PT-modified strand of SBD<sub>Sco</sub>-GPSGCC were extruded by 3.5 and 5.0 Å relative to those in the SBD<sub>Spr</sub>-GPSGCC. (B) Arg69, Arg73 and Arg75 of SBD<sub>Spr</sub> loop 34 form a positive interface with the DNA. (C) Glu156, Asp157 and Asp160 of SBD<sub>Sco</sub> loop 34 and helix 3 form a negatively charged surface area to DNA. (D, E) The surface charge of (D) SBD<sub>Spr</sub> and (E) SBD<sub>Sco</sub>. The surface charge distribution at neutral pH is displayed with blue for positive, red for negative, and white for neutral. (F) Influence of mutations in E156 on the ability of SBD<sub>Sco</sub> to bind GPSGCC, GPSATC and GPSAAC in EMSAs. N, no protein added to the EMSA.

Figure 5. Sequence specificity of ScoMcrA mutants. (A) Ability of SBD<sub>Sco</sub> mutants to bind GPSGCC, GPSATC and GPSAAC in EMSAs. N, no protein added to the EMSA; WT, the wild-type SBD<sub>Sco</sub>; E156R, SBD<sub>Sco</sub>-E156R mutant; E156R/D157R, SBD<sub>Sco</sub>-E156R/D157R mutant. (B) Uptake efficiency of sco<sup>−</sup>McrA and its mutants by the PT<sup>−</sup> host and PT<sup>+</sup> host. The PT<sup>+</sup> host contains the expression vector with the <i>ndl</i> gene cluster from <i>Salmonella enterica</i>, which encodes the 'writer' proteins for phosphorothioation of GPSAAC / GPSGTTC. Transformation efficiency obtained with the <i>ndl</i> host (PT<sup>+</sup>) and the negative control host (PT<sup>−</sup>) is indicated by black bars and white bars, respectively.
Recognition of PT-DNA by the SBD of the type IV REase ScoMcrA is not only phosphorothioate-dependent but also DNA sequence-specific as this enzyme only recognizes PT-DNA of the GPSGCC core in vivo and in vitro, whereas it does not bind to the PT-DNA of other four core sequences found in prokaryotes. However, five SBDs Sco homologs, including SprMcrA, generally display a more relaxed sequence specificity in target DNA selection. Although both SBD Spr and SBD Sco clearly shifted the GPSGCC DNA duplex in EMSAs, affinity quantification showed that the former had an 18.2-fold higher affinity than the latter did, with SBD Spr also showing a 28.7-fold and 6-fold higher affinity for GPSATC and GPSAAC, respectively (Tables 1 and 2). These differences in binding affinity between the two SBD domains lead to a distinctive presence or absence of in vivo restriction activity. For example, ScoMcrA can restrict the uptake of ddN dideoxy nucleotides, generating the GPSGCC modification (20) but not those generating the GAAC/GTTC modifications (Figure 5B). In contrast, SprMcrA can block the establishment of PT modifications at GPSAAC/GPSATC and GPSGCC sites as it showed an overall higher affinity for PT-DNA compared to ScoMcrA. Through comparative analysis, we attributed this difference in binding affinity to the reverse charge of loop 34 in both structures, which functions like a switch by changing between positive and negative electric charges in the different structures. Mutations of negatively charged amino acids into positively charged ones on loop 34 of SBDs significantly enhanced the binding affinity for PT-DNA. For example, the E156R/D157R mutation conferred ScoMcrA with the ability to bind to GPSAAC and GPSATC, and thus conferred SprMcrA with in vivo restriction activity for ddN encoding GPSAAC/GPSATC (Figure 5B). This structural switch offers us an opportunity to engineer a flexible or stringent sequence specificity for a given SBD.

In our study, the SBD Sco–E156R and SBD Sco–D157R mutants had significant increases in binding affinity for GPSATC and GPSAAC when compared with the wild-type SBD Sco. The MD simulations showed that R156 and R157 participate in van der Waals interactions and hydrogen-bond interactions with C6 and G7 of PT-DNA with GPSATC and GPSAAC core sequences, resulting in the higher binding affinity compared to the wild-type SBD Sco. However, the binding affinity of these two mutants for GPSGCC showed a slight decrease in comparison to the wild-type protein, in contrast to the increased affinities for GPSATC and GPSAAC. The superposition of the mutants and wild type structures after MD simulations gave a root mean square deviation (RMSD) value of 0.636 Å by using backbone atoms (Ca), indicating the mutations do not lead to vast structural changes in the MD simulations (Supplementary Figure S10). Next, the binding affinity of GPSGCC and SBD Sco were carefully examined to understand the geometrical disturbance of E156R/D157R mutation with the MM/GBSA method (40). As shown in Supplementary Table S4, the ΔΔGb value for PT-DNA binding SBD Sco–E156R/D157R was positive (0.9 kcal/mol), suggesting that the mutation slightly weakened the binding affinity, consistent with the experimental observation (Table 2). It is noticed that deformability of the DNA structure may contribute to the sequence specificity (48). As the conclusions of MD simulations, R156 and R157 are not directly involved in influencing binding interaction with C6 and G7 in GPSGCC sequence, however, they may affect the orientation of other residues that are involved in direct interaction with PT-DNA. We speculate that will lead to the twisting of DNA, which then results in an imperfect match of the hydrophobic pocket with the Rp sulfur atom, thus reducing the affinity. These interactions ultimately lead to an overall decrease in the binding affinity of mutant SBD Sco–E156R/D157R to GPSGCC. Interestingly, the SBD Sco–E156R/D157R mutant gained the ability to bind with the Sα stereoisomers of GPSGCC (Supplementary Figure S11) probably because the twisting of the GPSGCC strand by the E156R/D157R mutation positioned the sulfur of Sα within the sulfur-coordination cavity.

SBD homologs are widely represented in at least 1059 sequenced species from 14 phyla of bacteria (20). In addition to SBD Sco, four SBD homologs, including SBD Spr, displayed flexibility in the selection of substrate PT-DNA with different core sequences (20). It is notable that loop 34 is rich in basic amino acids in four of the SBDs (Supplementary Figure S12). With its acidic amino acids, loop 34 of SBD Sco is unique among SBD homologs, which may be related to the unique domain composition of SBD-SRA-HNH for ScoMcrA. Multiple DNA recognition domains of ScoMcrA result in reduced ability to distinguish between modified and non-modified DNA substrates, in turn resulting in nonspecific cleavage activity. In order to maintain the specificity of cleavage activity and low toxicity, the distribution of positive charges on the surface may have become reduced in ScoMcrA during evolution. Consequently, ScoMcrA can only recognize and restrict GPSGCC, the most common core sequence of PT-DNA in Streptomyces, with flexibility lost in the selection of substrate PT-DNA with different core sequences. Overall, our study illustrates structural features that impact the recognition of PT-DNA by SBDs of type IV restriction enzymes.

DATA AVAILABILITY

Atomic coordinates and structure factor for the sulfur-binding domain (SBD) from full-length Streptomyces pristinaespiralis endonuclease SprMcrA in complex with 5′-GGCGPSGCCC-3′, 5′-GATGPSATCC-3′ and 5′-GGCGPSAACGTG-3′ have been deposited with the Protein Data Bank under accession numbers 7CC9, 7CCJ and 7CCD, respectively.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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