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An Engineered SS Bridge Blocks the Conformational Change Required for the Nuclease Activity of BfiI

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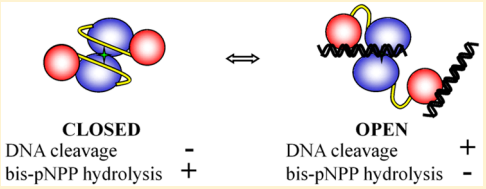
Supporting Information

ABSTRACT: The type IIS restriction endonuclease BfiI is a homodimer, and each monomer is composed of the N-terminal catalytic and C-terminal DNA recognition domains connected by a 28-residue linker segment. In the crystal in the absence of cognate DNA, BfiI exists in a "closed" conformation, in which an interdomain linker occludes a putative DNA binding surface at the catalytic domain and sterically hinders access to the active site. Cognate DNA binding presumably triggers a conformational change from the inactive "closed" state to the catalytically competent "open" state. Here we show that the disulfide SS bridge engineered at the domain interface locks the enzyme in the "closed" state. In the "closed" SS-linked state, BfiI binds cognate DNA with the same affinity as the wild-type enzyme but does not cut it, indicating that cross-linking introduces a restraint on the conformational transition, which couples DNA recognition and cleavage. Disruption of the interdomain SS bridge by the reducing agent restores the DNA cleavage ability of BfiI.



The type II restriction endonucleases (REases) recognize short nucleotide sequences and cleave phosphodiester bonds in DNA within or close to their target sites. Being a component of restriction−modification (RM) systems that are widespread in bacteria and archaea, REases cut invading foreign DNA, providing a defense barrier against bacteriophage or plasmid infection.[1] Host DNA is refractory to REase cleavage because of the target site methylation by an accompanying methyltransferase of the RM system. Faithful recognition and cleavage of the target sequence by REase is crucial for host survival: cleavage at off-target sites that are not protected by methylation would be lethal for the host cell. Not surprisingly, REases evolved various mechanisms that tightly couple recognition and catalysis to secure DNA cleavage at the target sites.

Orthodox type IIP REases like EcoRV are dimers that recognize palindromic target sites. Each EcoRV subunit interacts with one half of the symmetrical site and contains a single active site. Intersubunit communication is essential for catalysis to occur: if one of the EcoRV subunits does not make all specific contacts with its half-site, DNA cleavage does not occur at either active site.[2] Thus, residues involved in base-specific contacts in one subunit cross-talk with the catalytic centers of both subunits. Similar "cross-talk rings" at the DNA recognition/catalytic interface of EcoRI and MunI restriction enzymes[3,4] ensure tight coupling between DNA recognition and cleavage at both half-sites of the target sequence.[5]

A different mechanism is employed by type IIE restriction enzymes. NaeI REase dimer is comprised of two domains and requires binding of an effector DNA molecule to execute cleavage of another DNA copy.[6−8] Allosteric cross-talk between NaeI domains contributes to the coupling of DNA recognition and catalysis. Binding of the effector DNA at the C-terminal domain dimer triggers structural rearrangements at the dimer interface that promote binding and cleavage of the substrate DNA at the catalytic N-terminal domain dimer. In EcoRII, effector DNA binding domains sterically block the DNA binding interface at the C-terminal catalytic domain dimer. Cognate DNA binding at the N-terminal domain triggers the conformational change that opens the DNA binding cleft at the C-terminal domain and promotes phosphodiester bond cleavage.[9−12]

Yet another mechanism is used by a type IIS REase FokI that is fully active on DNA containing two recognition sequences.[13] First, the FokI monomer binds DNA at its recognition site and dislodges the C-terminal cleavage domain back-packed on the N-terminal recognition domain.[14−16] Then, cleavage domains of two DNA-bound FokI monomers dimerize on DNA and cut both DNA strands at one of the target sites.[16−20]

The type IIS restriction endonuclease BfiI acts at an asymmetric 5′-ACTGGG-3′ sequence and cleaves top and bottom DNA strands five and four nucleotides downstream of the recognition sequence.[21] X-ray studies[22,23] and biochemical analysis[24] showed that BfiI consists of the N-terminal catalytic and C-terminal DNA recognition domains (Figure 1A).[22−24] The N-terminal phospholipase D (PLD)-like domains form a dimer that has a single active site located in the intersubunit cleft. The C-terminal domain is capable of binding DNA containing a recognition sequence.[23,24] BfiI requires two copies of the recognition site for efficient DNA cleavage.[25] Isolated N-terminal domain dimer possesses a nonspecific nuclease activity;[24] therefore, BfiI evolved a mechanism to limit double-strand breaks at the specific recognition sequence.

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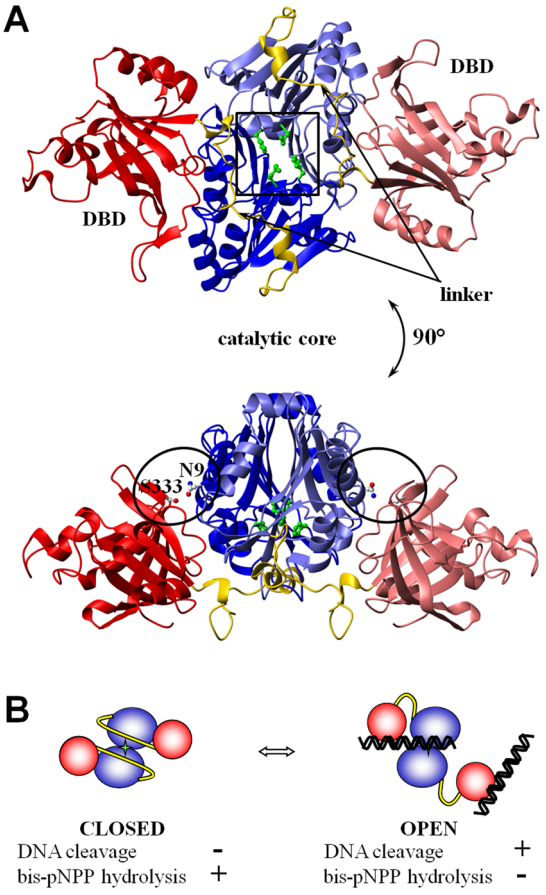

Figure 1. (A) Overall BfiI structure. BfiI (shown in two different orientations) is a homodimer. Each monomer is comprised of the N-terminal PLD-like domain and the C-terminal DNA binding domain (DBD) of the B3 family. Two N-terminal domains (light and dark blue) make a dimeric catalytic core flanked by two C-terminal DBDs (light and dark red). The linker connecting the N- and C-terminal domains is colored yellow. The active site residues (framed in the top structure) are shown as balls and sticks and colored green. The N9 and S333 residues (circled in the bottom structure) shown as balls and sticks were replaced with Cys to generate a double N9C/S333C mutant that allows introduction of a SS cross-link between the N- and C-terminal domains. (B) Cartoon representation of BfiI. The BfiI enzyme in the absence of DNA is in a "closed" conformation. Linkers connecting the N- and C-terminal domains run across the putative DNA binding interface at the catalytic domain and hinder the access of DNA to the active site (star) but do not interfere with small bis(*p*-nitrophenyl) phosphate (bis-pNPP) diester hydrolysis. Binding of cognate DNA to the C-terminal domains presumably triggers a conformational rearrangement that opens the active site at the catalytic domain and allows DNA cleavage at a specific site.

Taking together structural and biochemical data, we proposed the following model for BfiI catalytic activity control (Figure 1B). The interdomain linker connecting N- and C-terminal domains sterically occludes the putative DNA binding surface at the catalytic domain and prevents the entry of DNA into the active site but does not interfere with a bis(*p*-nitrophenyl) phosphate (bis-pNPP) diester hydrolysis (Figure 1A).[22] Therefore, in the absence of cognate DNA, BfiI is in the "closed" state that is not capable of DNA cleavage (Figure 1B). Cognate DNA binding at the C-terminal domain presumably triggers a conformational change to the "open" state of the enzyme. The dislodged interdomain linker opens an entrance to the active site cleft at the N-terminal domains (Figure 1B), resulting in site-specific DNA cleavage. We aimed to test the proposed model of BfiI activity regulation by engineering a disulfide bridge at the interface between the N- and C-terminal domains.

MATERIALS AND METHODS

Plasmids and Strains. *Escherichia coli* strain ER2566 and plasmid pBAD24-*bfi*IR5.7 (M. Zaremba, unpublished observations) were used for the cloning and expression of the wt and mutant BfiI protein carrying His tags on their N-termini. This plasmid was made by cloning an ~1.1 kb NcoI−PstI fragment containing the gene for the BfiI endonuclease, *bfi*IR, fused with an N-terminal His_6 tag sequence into vector pBAD24.[26] A compatible plasmid p*Bfi*IM9.1 carrying genes for the BfiI methyltransferases was co-expressed together with the pBAD24-*bfi*IR5.7 plasmid to protect the *E. coli* genomic DNA from cleavage.[25]

Oligonucleotides. The specific 16 bp duplex containing the recognition sequence of BfiI was obtained by annealing two oligodeoxyribonucleotides of 16 nucleotides, 5′-AGCGTAGC-ACTGGGCT-3′ and 5′-AGCCCAGTGCTACGCT-3′ (the BfiI site is underlined). The nonspecific duplex that lacks the recognition site for BfiI was obtained by annealing two oligodeoxyribonucleotides of 16 nucleotides, 5′-AGCGTAGC-CCGGGGCT-3′ and 5′-AGCCCCGGGCTACGCT-3′. A 30 bp duplex containing the recognition sequence and the cleavage site for BfiI was obtained by annealing two oligodeoxyribonucleotides of 30 nucleotides: 5′-AGCGTAGCACTGGGCTGC-TGAACTGTGCTG-3′ and 5′-CAGCACAGTTCAGCAGCC-CAGTGCTACGCT-3′ (the BfiI site is underlined). The BfiI cleavage resistant 30 bp oligoduplex was assembled by annealing a 5′-AGCGTAGCACTGGGCTGCTGAACTGTG-CTG-3′ with 5′-CAGCACAGTTCA(s)GCAGCCCAGTGC-TACGCT-3′ [where (s) designates the phosphorothioate linkage].

Mutagenesis. The N9C/S333C double mutant was produced by QuikChange site-directed mutagenesis.[27] Sequencing of the entire gene confirmed that only the designed mutations had been introduced.

Protein Purification. The bacterial cells of *E. coli* strain ER2566 carrying compatible plasmids pBAD24-*bfi*IR5.7 and p*Bfi*IM9.1 were grown at 37 °C to late logarithmic phase in Luria broth medium[28] containing 50 mg/L ampicillin and 30 mg/L chloramphenicol. After being induced with L-arabinose (0.2%, 4 h, at 37 °C), the cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl (pH 8.0 at 25 °C) and 500 mM NaCl. Crude cell extract was obtained by sonication, and cell debris was separated by centrifugation. The resulting supernatant was loaded onto the Ni^{2+}-charged HiTrap Chelating HP column (GE Healthcare) and the His-tagged BfiI eluted using a linear gradient in imidazole (final concentration of 500 mM). The fractions containing BfiI were pooled, dialyzed against 20 mM Tris-HCl (pH 8.0 at 25 °C), 200 mM NaCl, and 20% (v/v) glycerol, and loaded onto the HiTrap Heparin HP column (GE Healthcare). BfiI proteins were eluted using a linear gradient in NaCl (final concentration of 1 M). Final fractions containing purified enzymes were pooled and dialyzed against storage buffer [10 mM HEPES (pH 8.0 at 25 °C), 200 mM KCl, and 50% (v/v) glycerol] with air bubbling (0.5 L/h, 18 h, at 8 °C) through it to promote the formation of disulfide bonds in the N9C/S333C double mutant and stored at −20 °C.

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The homogeneity (>90%) of the protein preparations was estimated by SDS−PAGE. Concentrations of the wt and mutant protein were determined by absorbance at 280 nm using extinction coefficients of 95300 M^{-1} cm^{-1} (wt protein) and 95460 M^{-1} cm^{-1} (N9C/S333C mutant) for the dimer. Enzyme concentrations are expressed in terms of dimer.

SDS−PAGE Analysis. SDS−PAGE analysis of the proteins was performed as described previously[28] using 4% stacking and 12% separating gels. The non-cross-linked and cross-linked samples of the wt and N9C/S333C mutant proteins were prepared in storage buffer (see above) in the absence and presence of DTT, respectively. The non-cross-linked samples were incubated with 1 mM DTT for 20 min at room temperature to remove disulfide bonds. All protein samples were mixed in a 1:1 (v/v) ratio with a saturated NEM solution (100 mM) in buffer [100 mM Tris-HCl (pH 6.8 at room temperature) and 4% SDS (w/v)] to prevent disulfide bond formation between free thiols under denaturing conditions. Then samples were placed for 3 min into a bath with 95 °C water before being loaded. Electrophoresis was conducted at room temperature for 1−1.5 h at ∼30 V/cm. Gels were stained with Coomassie Blue and scanned.

DNA Binding Studies. DNA binding by the wt BfiI and cross-linked N9C/S333C mutant was analyzed by an EMSA using the 16 bp specific or nonspecific oligonucleotide duplexes (see above). The individual DNA strands of oligonucleotide duplexes were 5′-labeled with [γ-^{33}P]ATP (GE Healthcare) and PNK (UAB Fermentas) prior to being annealed with unlabeled strands. The duplexes (1 nM) were incubated for 10 min at room temperature with different amounts of the wt or mutant enzyme in 20 μL of binding buffer [40 mM Tris-acetate (pH 8.3 at 25 °C), 0.1 mM EDTA, 0.1 mg/mL BSA, and 10% (v/v) glycerol]. Samples were loaded onto 8% acrylamide gels [29:1 acrylamide/bis(acrylamide)] and run in 40 mM Tris-acetate (pH 8.3 at 25 °C) and 0.1 mM EDTA for 2 h at ∼6 V/cm. DNA binding under reducing conditions was analyzed in the presence of 2 mM DTT in binding and loading buffers. After electrophoresis, the gels were analyzed using a Cyclone Storage Phosphor System with OptiQuant Image Analysis Software, version 3.00 (Packard Instrument Co). Apparent K_d values were determined as described previously.[12]

DNA Cleavage. The specific catalytic activity of the wt BfiI and N9C/S333C mutant was determined in the absence and presence of DTT (2 mM) using phage λ DNA. Varied protein amounts (from 23 pM to 115 nM) were incubated with 1 μg of λ DNA in 50 μL of 33 mM Tris-acetate (pH 7.9 at 37 °C), 66 mM KOAc, 10 mM magnesium acetate, and 0.1 mg/mL BSA for 1 h at 37 °C. Phage λ DNA cleavage by the wt BfiI and N9C/S333C mutant at 55 °C was performed in the absence and presence of DTT (2 mM) using 1 μg of λ DNA and 115 nM proteins in 50 μL of 15 mM Tris-acetate (pH 8.0 at 25 °C), 55 mM KOAc, and 0.1 mg/mL BSA for 1 h under a mineral oil layer. Reactions were terminated by the addition of 20 μL of "STOP" solution [75 mM EDTA (pH 9.0), 0.3% SDS, 0.01% bromophenol blue, and 50% (v/v) glycerol] and heating at 65 °C for 20 min. The DNA was analyzed by electrophoresis through agarose.

Reactions on a plasmid substrate under reducing conditions were performed using pUCBFI-2 plasmid DNA (bears two recognition sites of BfiI)[25] at 25 °C in a buffer containing 30 mM Tris-acetate (pH 8.0 at 25 °C), 110 mM KOAc, 2 mM DTT, and 0.1 mg/mL BSA. Reaction mixtures contained 2.3 nM plasmid substrate and 0.5−10 nM wt BfiI or N9C/S333C mutant. The reactions were initiated by adding a non-cross-linked enzyme solution with DTT (2 mM) to the mixture of the other components. In the activation experiment with the cross-linked N9C/S333C mutant, the reaction was initiated in the absence of the reducing agent, and then DTT was added to a final concentration of 2 mM 8 min after the beginning of the reaction. Aliquots were removed at timed intervals and quenched with phenol and chloroform. The aqueous phase was mixed with a loading dye solution [0.01% (w/v) bromophenol blue, 75 mM EDTA, and 20 mM DTT in 50% (v/v) glycerol] and analyzed by electrophoresis through agarose. Digital images of the gels were taken with the Biometra BioDocAnalyze gel documenting system. The amounts of supercoiled, open-circular, and linear forms of the plasmid were quantified with 1-D Main software (Advanced American Biotechnology).

Bis-pNPP Hydrolysis. The hydrolysis of bis-pNPP by the wt BfiI and N9C/S333C mutant was measured in the absence and presence of DTT (2 mM) by incubating 250 nM enzymes with 2 mM bis-pNPP in 30 mM MES-KOH (pH 6.5 at 25 °C) and 110 mM KOAc at 37 °C. Reaction mixtures with specific DNA contained 500 nM 30 bp oligonucleotide duplex with the BfiI recognition site (see above). The reactions were initiated by adding the bis-pNPP solution to the mixture of the other components. At timed intervals, samples were withdrawn from the total reaction volume and reactions quenched by mixing 1:2 (v/v) with 0.5 M Na_2CO_3, and the absorbance of *p*-nitrophenolate at 405 nm was measured.

Data Analysis. Nonlinear regression analysis used the KyPlot version 2.0.[29]

RESULTS

Engineering of the SS Cross-Link at the Domain Interface of BfiI. The BfiI conformation observed in the crystal is incompatible with DNA cleavage because the interdomain linker occludes the catalytic site at the N-terminal domain interface. Because BfiI does not cleave DNA that lacks the recognition sequence, we assumed that BfiI conformation in solution may be similar to that observed in the crystal. Guided by the crystal structure of the "closed" conformation, we aimed to engineer a disulfide bridge at the interface between the N- and C-terminal domains that would lock the enzyme in the "closed" conformation.[22] The transition from the "closed" to the catalytically active "open" BfiI conformation requires a large rearrangement at the interdomain interface to displace a linker. Although the crystal structure of the "open" BfiI conformation is unknown, it is unlikely that the engineered SS bridge designed for the "closed" conformation will be compatible with the "open" BfiI conformation. Analysis of the BfiI apo form structure revealed that the side chain oxygen atom of N9 and the hydroxyl group of the S333 residue are engaged in H-bond interaction across the domain−domain interface (O···O distance of 3.1 Å) (Figure 1A). The N9C/S333C double mutation should position SH groups of engineered Cys residues at a distance favorable for the formation of a disulfide bridge under oxidizing conditions.[30] Double-Cys mutant N9C/S333C of BfiI was obtained by site-directed mutagenesis, expressed in *E. coli*, and purified under oxidizing conditions, which promote disulfide bridge formation (see Materials and Methods).

First, the N9C/S333C mutant was analyzed by SDS−PAGE under reducing and oxidizing conditions. In the presence of DTT, the N9C/S333C mutant shows the same electrophoretic

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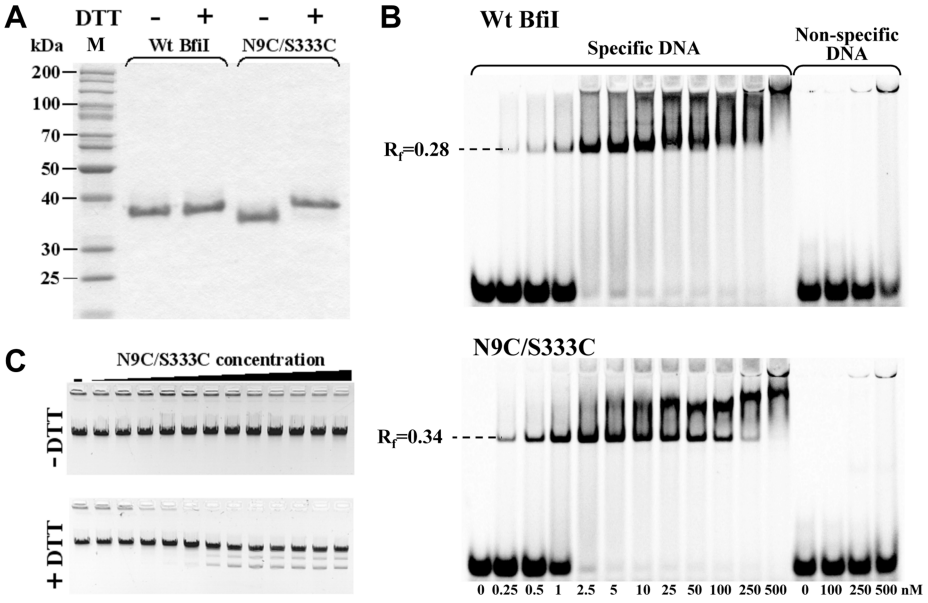

Figure 2. (A) SDS−PAGE analysis of the wt BfiI and the cross-linked N9C/S333C mutant. Protein samples of the wt BfiI and N9C/S333C mutant in the absence and presence of DTT were subjected to SDS−PAGE and visualized by Coomassie brilliant blue staining (for details, see Materials and Methods). Lane M contained molecular mass markers. (B) EMSA analysis of the wt BfiI and cross-linked N9C/S333C mutant. Samples (20 μL) of 16 bp ^{33}P-labeled specific and nonspecific oligoduplexes (1 nM) were incubated with varying protein concentrations (indicated below) and samples analyzed on the polyacrylamide gel (see Materials and Methods). The electrophoretic mobility index (R_f) for cognate DNA complexes of the wt BfiI and the cross-linked N9C/S333C variant was calculated as the migration distance ratio of the specific protein−DNA complex and free DNA. The calculated K_d values for the wt BfiI and the cross-linked N9C/S333C variant are 0.71 ± 0.42 and 0.61 ± 0.15 nM, respectively. (C) Phage λ DNA cleavage assay. Phage λ DNA (1 μg/50 μL) samples were incubated with increasing N9C/S333C protein concentrations (from 23 pM to 115 nM) for 1 h at 37 °C in the absence and presence of 2 mM DTT. The reactions were quenched and analyzed by electrophoresis through agarose as described in Materials and Methods.

mobility on a gel as the wt enzyme (Figure 2A) monomer, while under oxidizing conditions, the mutant moves slightly faster than the wt BfiI monomer (Figure 2A). The difference in the electrophoretic mobility of the BfiI and the cross-linked mutant in the SDS gel suggests that the oxidized mutant has a more compact conformation because of the intramonomer SS cross-link in the SDS-unfolded state and therefore migrates faster through the gel than the reduced form.

DNA Binding by the Cross-Linked Variant. To determine whether the cross-linked N9C/S333C mutant is capable of binding DNA, we performed the electrophoretic mobility shift analysis (EMSA) experiments in the absence of DTT using 16 bp ^{33}P-labeled specific and nonspecific oligoduplexes with and without the recognition sequence, respectively (Figure 2B). Binding patterns of the wt BfiI and the cross-linked mutant are nearly identical: (i) both proteins show a clear preference for cognate DNA binding, and (ii) the cross-linked mutant binds cognate DNA with the same affinity as the wt enzyme (K_d values of 0.61 ± 0.15 nM for the mutant and 0.71 ± 0.42 nM for the wt). The only difference is that the relative mobility index values slightly differ for the specific BfiI−DNA (R_f = 0.28) and cross-linked mutant−DNA (R_f = 0.34) complexes, suggesting a more compact conformation of the cross-linked variant in the DNA-bound form. The cross-linked mutant and the wt BfiI possess similar affinities (Supplementary Figure 1B) for a longer 30 bp oligoduplex containing both the recognition and the cleavage sites, indicating that under our experimental conditions the N-terminal nucleolytic domain does not significantly contribute to the overall binding affinity. In the case of the 16 bp specific oligoduplex, addition of DTT did not change the mobility of the wt BfiI−DNA complex but yielded two bands on a gel for the N9C/S333C mutant (Supplementary Figure 1A). R_f values suggest that incomplete DTT reduction of the disulfide bonds in the mutant variant results in a mixture of "closed" and "open" complexes (Supplementary Figure 1A). Taken together, these data indicate that locking BfiI in the "closed" conformation does not compromise cognate DNA binding.

DNA Cleavage by the Cross-Linked Variant. The phage λ DNA cleavage activity of the wt BfiI and N9C/S333C mutant was monitored in the absence and presence of the DTT. The wt BfiI shows the same specific activity under both reducing and oxidizing conditions (Supplementary Figure 2). In the absence of DTT, the SS-cross-linked N9C/S333C variant shows no DNA cleavage activity (Figure 2C). However, addition of DTT restores the catalytic activity of the N9C/S333C mutant to that of the wt BfiI (Figure 2C and Supplementary Figure 2). Thus, locking of BfiI in the "closed" conformation by the SS bridge results in an inactive enzyme, whereas reduction of the disulfide bond fully restores its catalytic activity.

Next, the catalytic properties of the N9C/S333C mutant were examined under reducing conditions (in the presence of DTT) using a two-site plasmid DNA substrate, which is an optimal substrate for BfiI.[25] The plasmid DNA cleavage was performed under single-turnover conditions with enzyme in excess of the DNA and multiple-turnover conditions with DNA in excess over the enzyme (Supplementary Figures 3 and 4). The cleavage rate constants for the N9C/S333C mutant are nearly identical to that of the wt enzyme under both sets of reaction conditions (Supplementary Figures 3 and 4). Thus, the non-cross-linked mutant possesses catalytic properties similar to those of the wt BfiI.

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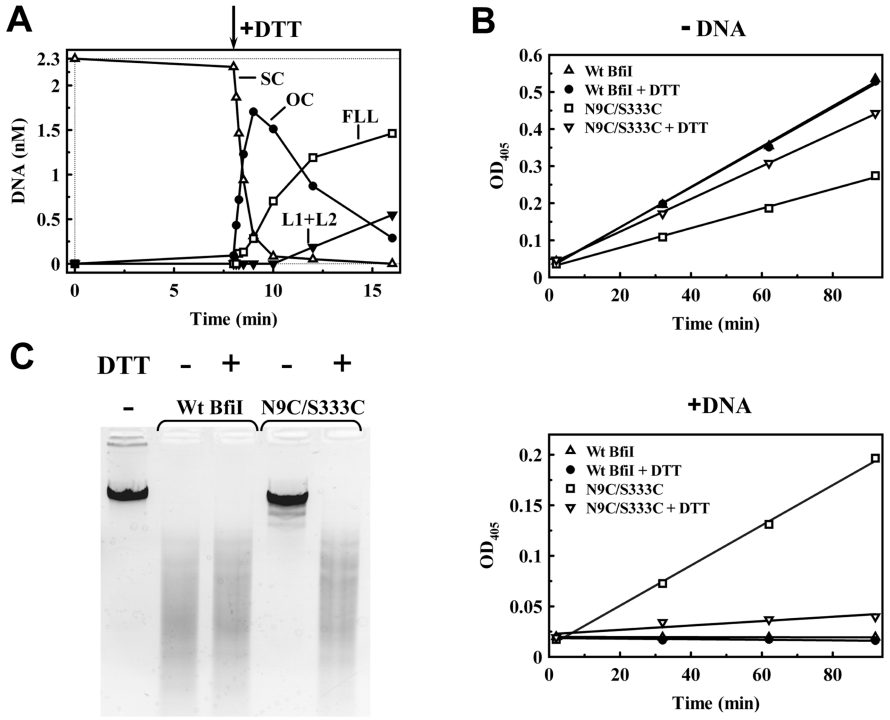

Figure 3. (A) DTT activation of plasmid DNA cleavage by the cross-linked N9C/S333C variant. The reaction was initiated in the absence of DTT by mixing of 2.3 nM two-site plasmid DNA and 10 nM cross-linked N9C/S333C mutant. After incubation for 8 min, DTT was added to a final concentration of 2 mM and the mixture incubated for an additional 8 min. Samples were removed from the reactions at timed intervals, reactions quenched with phenol and chloroform, and mixtures analyzed as described in Materials and Methods to determine the amounts of the following forms of the plasmid DNA: supercoiled DNA SC (△), open-circular DNA OC (●), linear DNA cut at one BfiI site FLL (□), and linear DNA cut at both BfiI sites L1 and L2 (▼). (B) Bis-pNPP hydrolysis by the wt BfiI and N9C/S333C mutant. The reaction mixtures contained 2 mM bis-pNPP and 250 nM wt BfiI or N9C/S333C mutant, and formation of the *p*-nitrophenolate in the absence of DNA (top) or presence (bottom) of the 30 bp cognate oligoduplex (500 nM) was monitored spectrophotometrically at 405 nm. Reactions under reducing conditions were performed in the presence of 2 mM DTT (see Materials and Methods). Samples were removed from the reaction mixtures at timed intervals and reactions quenched, and the absorbance of *p*-nitrophenolate was quantified as described in Materials and Methods. (C) DNA cleavage at 55 °C. Phage λ DNA cleavage by the wt BfiI and N9C/S333C mutant at 55 °C was monitored under reducing (+DTT) and oxidizing (−DTT) conditions. Phage λ DNA (1 μg/50 μL) was incubated with the wt BfiI or N9C/S333C mutant (115 nM) for 1 h at 55 °C in the absence or presence of DTT (2 mM). The reactions were quenched and analyzed by electrophoresis through agarose as described in Materials and Methods. In contrast to the clear DNA fragmentation at 37 °C resulting from the site-specific DNA cleavage (Figure 2C), smears are observed at 55 °C because of a nonspecific DNA cleavage by the nuclease domain.

In the absence of DTT, plasmid DNA, like phage λ DNA, is not cleaved by the cross-linked N9C/S333C mutant. To determine if DTT addition can trigger plasmid DNA cleavage by the mutant, we first incubated plasmid DNA with an excess of the N9C/S333C variant in the absence of DTT for 8 min (Figure 3A) and then added DTT. In the absence of DTT, only slow DNA nicking occurs presumably because of the presence of traces of the non-cross-linked variant. DTT addition triggers the plasmid DNA cleavage that proceeds with a rate nearly identical to that of the non-cross-linked mutant (Figure 3A and Supplementary Figure 3B). Taken together, plasmid DNA cleavage data indicate that the catalytically impaired cross-linked form of BfiI fully regains its cleavage activity upon addition of DTT and displays catalytic properties characteristic of the wt enzyme.

Hydrolysis of a Bis(*p*-nitrophenyl) Phosphate. Similar to other PLD family enzymes, BfiI hydrolyzes the small artificial phosphodiester substrate bis(*p*-nitrophenyl) phosphate (bis-pNPP).[31,32] The N9C/S333C mutant like BfiI hydrolyzes bis-pNPP in the absence and presence of DTT (Figure 3B). The capability of the cross-linked mutant to hydrolyze bis-pNPP indicates that in the "closed" conformation BfiI possesses a preorganized active site, which is accessible for a small artificial substrate but not for DNA.

BfiI cuts phoshodiester bonds in DNA and bis-pNPP at the same active site; therefore, bis-pNPP hydrolysis is inhibited by BfiI preincubation with the 30 bp oligoduplex containing the recognition and cleavage site of BfiI.[32] We performed similar competition experiments with the cross-linked mutant under reducing and oxidizing conditions (see Materials and Methods). Unlike the wt enzyme, the cross-linked N9C/S333C mutant hydrolyzes bis-pNPP even in the presence of the specific DNA substrate (Figure 3B). DTT addition results in the inhibition of bis-pNPP hydrolysis by the N9C/S333C mutant in a manner similar to that of the wt enzyme (Figure 3B). These results indicate that the SS bridge at the domain interface of the cross-linked N9C/S333C mutant restricts the domain reorientation upon specific DNA binding and prevents the entrance of DNA into the active site; however, it does not interfere with bis-pNPP hydrolysis. Removal of the SS cross-link by DTT treatment allows the conformational transition of the enzyme from the "closed" to the "open" state, promoting DNA binding at the catalytic N-terminal domain and inhibition of bis-pNPP hydrolysis.

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Temperature-Induced Unfolding of the DNA Binding Domain. BfiI possesses a modular structure with DNA recognition and cleavage functions located on separate domains. The N-terminal domain dimer forms a catalytic core, which can act as a stand-alone, nonspecific nuclease capable of degrading both phage λ DNA and synthetic oligonucleotide duplexes.[24] Two C-terminal domains flank the nuclease unit and provide cognate DNA binding.[22−25] In the wt BfiI dimer, one of the C-terminal domains presumably binds one copy of the recognition sequence, while the other C-terminal domain binds the second site. The C-terminal domains in the absence of DNA are less stable than the N-terminal domain dimer. As a consequence, at a low GdmCl concentration (2.5 M), the C-terminal DNA binding domains are unfolded, while the N-terminal domain maintains its dimeric structure and retains its nonspecific nuclease activity that causes DNA degradation.[24]

We have found that at elevated temperatures wt BfiI also displays a nonspecific nuclease activity (Figure 3C) that presumably results from the temperature-induced unfolding of the C-terminal cognate DNA binding domains and elimination of steric constraints introduced by the interdomain linker. In contrast to the wt enzyme, the cross-linked N9C/S333C mutant shows no nonspecific nuclease activity at elevated temperatures under oxidizing conditions (Figure 3C). However, addition of DTT triggers nonspecific DNA degradation activity similar to that of the wt BfiI (Figure 3C). Taken together, these results suggest that because of the interdomain SS bridge the active site of the cross-linked mutant is inaccessible for DNA at an elevated temperature, preventing nonspecific DNA degradation observed in the case of the wt BfiI.

DISCUSSION

Here we aimed to establish the mechanism that allows coupling between DNA recognition and catalysis and activity regulation in BfiI. It has been proposed that to perform DNA cleavage at the target site BfiI has to undergo a conformational transition from the inactive "closed" state observed in the crystal to the "open" catalytically active state (Figure 1B). In this paper, we demonstrate that the SS bridge engineered at the dimer interface creates a physical constraint that locks the enzyme in the "closed" conformation and limits the conformational transition to an "open" state. The selection of amino acid positions for the Cys replacement and the SS bridge engineering was guided by the BfiI crystal structure. The successful engineering of the SS bridge at the domain interface indicates that in solution BfiI exists in the "closed" conformation observed in the crystal structure (Figure 1A). The N9C/S333C double mutant in the presence of a reducing agent (DTT) shows DNA binding and cleavage properties that are nearly indistinguishable from those of the wt BfiI (Supplementary Figures 1, 3, and 4). In the SS-cross-linked form, the N9C/S333C mutant is unable to cleave DNA, although it binds it with the same affinity as wt BfiI. In contrast to the case in BfiI, the SS bridge engineered in the flexible loops of the PI-SceI homing endonuclease locked the enzyme in a nonproductive conformation that showed a compromised DNA binding ability.[33] The cross-linking of BfiI restricts the conformational transition that couples DNA recognition and cleavage (Figure 4). Because of the constraints imposed by the SS bridge in the cross-linked form, the cognate DNA binding at the C-terminal domains is unable to promote the conformational change into the "open" catalytically active state. In the cross-linked "closed" state, a small model phosphodiester bis-pNPP is still hydrolyzed, indicating that the active site is fully assembled and predisposed for cleavage (Figure 3B). Addition of the reducing agent (DTT) efficiently destroys the SS bridge at the domain interface and allows a conformational transition from the "closed" to the "open" catalytically competent state (Figure 3A). Therefore, the SS bridge at the domain interface acts as a redox switch that regulates the catalytic activity of the BfiI restriction enzyme.

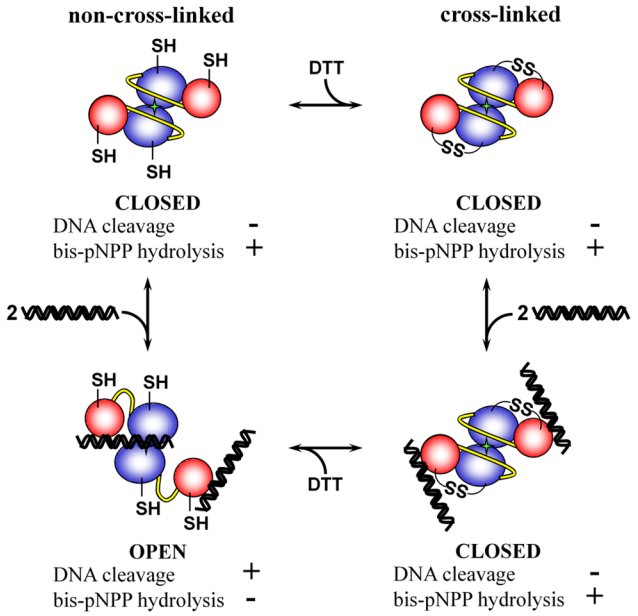

Figure 4. Schematic model for catalytic activity control of the N9C/S333C mutant. In the absence of cognate DNA, both non-cross-linked and cross-linked forms of the N9C/S333C mutant are in the "closed" conformation, where the interdomain linker (yellow) connecting N-terminal (blue) and C-terminal domains (red) crosses over the putative DNA binding surface at the catalytic domain and sterically blocks the access of DNA to the active site (green star). Nevertheless, in the "closed" conformation, both forms are still able to hydrolyze the small artificial phosphodiester substrate bis-pNPP, indicating that the active site is functional. Binding of the cognate cognate DNA (black double helices) by the non-cross-linked form switches BfiI from the "closed" to the "open" conformation, where the active site at the N-terminal domain becomes engaged in DNA cleavage that inhibits bis-pNPP hydrolysis. The cross-linked form bound to DNA remains in the "closed" conformation; therefore, no DNA cleavage occurs, but bis-pNPP is hydrolyzed.

The catalytic activity control in the BfiI restriction enzyme is achieved through the intrasteric/active site-directed inhibition. Such a mechanism has been demonstrated before for protein kinases, protein phosphatases, proteinases, metabolic enzymes, transport receptors, and targeting domains, allowing control of various cellular processes.[34] In general, protein activity control is achieved by masking of the active site by an intrasteric autoregulatory sequence (IARS). Allosteric activation by an activatory ligand or protein results in the release of the IARS from the active site. In the case of the BfiI restriction enzyme, the interdomain linker connecting the N- and C-terminal domains acts as an IARS. In the "closed" BfiI conformation, the interdomain linker sterically blocks the DNA binding cleft at the N-terminal domain interface. Cognate DNA acts as an allosteric activator: DNA binding at the C-terminal domain triggers domain reconfiguration and repositioning of the linker

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Biochemistry 2015, 54, 5340−5347

promoting DNA cleavage at the catalytic N-terminal domain (Figure 4). The conformational transition from the "closed" to "open" state couples DNA sequence recognition and catalysis. Considering the fact that the isolated N-terminal domain of BfiI is a nonspecific nuclease, such tight activity control ensures DNA cleavage only at BfiI recognition sequences and prevents nonspecific cleavage by the nuclease domain that would be lethal for the host cell. It remains to be established whether a similar mechanism of the catalytic activity control is employed in the ATP-dependent CglI and NgoAVII restriction endonucleases[35,36] that are structurally similar to the BfiI restriction enzyme.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00437.

DNA binding and cleavage by the wt BfiI and the N9C/S333C mutant under reducing and oxidizing conditions (Supplementary Figures 1–4) (PDF)

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Notes

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■ ABBREVIATIONS

DBD, DNA binding domain; bis-pNPP, bis(*p*-nitrophenyl) phosphate; BSA, bovine serum albumin; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; FLL, full-length linear; IARS, intrasteric autoregulatory sequence; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NEM, *N*-ethylmaleimide; OC, open-circular; RM, restriction–modification; SC, supercoiled; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; wt, wild-type.

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