ISSN 0026-8933, Molecular Biology, 2020, Vol. 54, No. 4, pp. 599–610. © Pleiades Publishing, Inc., 2020.
Russian Text © The Author(s), 2020, published in Molekulyarnaya Biologiya, 2020, Vol. 54, No. 4, pp. 667–679.

PROTEOMICS

UDC 577.151.45;577.113.4

The Role of Cysteine Residues in the Interaction of Nicking Endonuclease BspD6I with DNA

L. A. Abrosimova[a], *, A. R. Samsonova[b], T. A. Perevyazova[c], A. K. Yunusova[c], R. I. Artyukh[c], E. A. Romanova[d], L. A. Zheleznaya[c], †, T. S. Oretskaya[d], and E. A. Kubareva[d]

[a] *Chemistry Department, Moscow State University, Moscow, 119991 Russia*
[b] *SABNP, University of Evry, INSERM U1204, Université Paris-Saclay, Evry 91025 France*
[c] *Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*
[d] *Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119991 Russia*
**e-mail: abrludmila@gmail.com*

Received March 5, 2020; revised March 5, 2020; accepted March 16, 2020

Abstract—Nicking endonucleases (NEs) are a small, poorly studied family of restriction endonucleases. The enzymes recognize a target sequence in DNA, but catalyze the hydrolysis of only one strand. The mechanism of their action is important to study because NEs with new specificities are necessary to design to solve the practical tasks of biotechnology. One of the modern approaches for investigation of protein-nucleic acid interactions is fluorescence spectroscopy, which involves the introduction of fluorophores into proteins, mainly through Cys residues due to the high reactivity of their thiol group. To implement this approach, it is necessary to clarify the role of Cys residues in the functioning of the native protein and the possible consequences of their modification. Crosslinking was used to study whether Cys residues are close to DNA in the complex with NE BspD6I. Reactions were carried out using the wild-type enzyme, its mutant form NE BspD6I(C11S/C160S), and modified DNA duplexes containing the 2-pyridyldisulfide group at the C2' atom of the sugar-phosphate moiety in different positions of the oligonucleotide strand. The Cys residues of NE BspD6I were for the first time shown to be in close proximity to DNA during the binding process, including the step of a nonspecific complex formation. The substitutions C11S and C160S in the N-terminal domain of the enzyme slightly decreased the efficiency of substrate hydrolysis. Construction of a cysteine-free NE BspD6I variant and examination of its properties will provide additional information about the functional significance of the Cys residues for this unique enzyme.

Keywords: nicking endonuclease, BspD6I, mutant forms, DNA duplex, 2-pyridyldithio group, crosslinking, *Bacillus species* D6I

DOI: 10.1134/S0026893320040020

INTRODUCTION

Site-specific endonucleases that cleave only one strand in double-stranded DNA (dsDNA) at a certain position are in increasing demand due to a rapid development of molecular biological and biotechnological methods [1]. The enzymes are known as nicking endonucleases (NEs). NEs are thought to have a high potential for gene therapy and are used to construct chimeric proteins with a preset specificity [2]. Such chimeric proteins may stimulate homologous recombination to eliminate DNA lesions [3]. However, the mechanism of action is poorly understood for NEs, thus limiting their applications.

We studied the NE BspD6I from thermophilic *Bacillus* species [4]. The enzyme is a large subunit of heterodimeric restriction endonuclease (RE) BspD6I [5, 6]. NE BspD6I recognizes the 5'-GAGTC-3'/ 5'-GACTC-3' sequence in dsDNA. The sequence is often found in the promoters of phage genes, e.g., those of the T7 bacteriophage. NE BspD6I hydrolyzes the 'top' DNA strand after the fourth nucleotide to 3'-end of the recognition site: 5'-GAGTCNNNN↓-3'/ 5'-NNNNGACTC-3'. According to REBASE (http://rebase.neb.com/cgi-bin/isoget?Nt.BspD6I), five isoschizomers hydrolyzing the 5'-GAGTC-3'/ 5'-GACTC-3' sequence are known for NE BspD6I and two other isoschizomers have been predicted.

Kachalova et al. [7] solved the crystal structure of NE BspD6I (PDB ID 2EWF) in the absence of DNA

Abbreviations: dsDNA, double-stranded DNA; CD, circular dichroism; NE, nicking endonuclease; PAGE, polyacrylamide gel electrophoresis; RE, restriction endonuclease; FAM, carboxyfluorescein; PEG-Mal, polyethylene glycol maleimide; SPDP, succinimidyl 3-(2-pyridyldithio)propionate.

† Deceased.

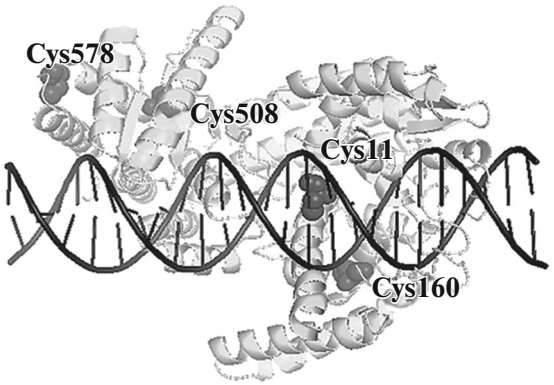

Fig. 1. Positions of Cys residues in the complex of NE BspD6I with DNA according to the model proposed earlier [12].

substrate. NE BspD6I has three domains. One is a putative DNA-binding domain and consists of two structural subdomains, D1 (residues 1–116 and 280–300) and D2 (117–279). The two others are a linker domain (residues 301–381) and a catalytic domain (382–604). The domains presumably responsible for the recognition and catalysis are spatially separate, and NE BspD6I therefore provides a convenient enzyme to introduce substitutions in the DNA-binding (recognizing) domain in order to obtain enzymes with new specificities.

We have previously studied the phenomenon of NE BspD6I oligomerization at higher protein concentrations [8], developed the approaches to regulating activity of the enzyme [9, 10], determined the thermodynamic and kinetic parameters of its interaction with the substrate, and identified the groups of atoms that are potentially in contact with the enzyme in the recognition site and the DNA sequence adjacent to internucleotide cleavage site [6, 10]. However, the available data are still insufficient for understanding the roles that particular amino acid residues play in the function of the enzyme because the structure of its complex with DNA is still unsolved.

The C-terminal domain of the NE harbors a catalytic motif. The motif includes the amino acid residues Pro455, Asp456, Glu469, Val470, and Glu482. The properties of NE BspD6I mutant forms with the single substitutions Asp456Ala and Glu418Ala have confirmed that the respective amino acid residues play a substantial role in the catalysis [11]. A model of the NE–DNA complex has been constructed on the basis of the crystal structure of NE BspD6I [12]. There are four Cys residues in NE BspD6I: two are in positions 11 and 160 of the N-terminal domain and two are in positions 508 and 578 of the C-terminal domain. The Cys residues of the N-terminal domain are close to DNA according to the model of the protein–nucleic acid complex [12] (Fig. 1). Cys508 is in the vicinity of the putative NE catalytic center, while Cys578 is distant and seems to exert no effect on the interaction with DNA. It is possible to use crosslinking of a protein with a DNA duplex containing a pyridyldithio group to estimate how close a particular Cys residue is to DNA. The interaction is based on the thiol–disulfide exchange reaction [13–21]. This approach still provides a relatively inexpensive, reliable, and reversible means to conjugate DNA, RNA, and their analogs with each other and with other biomolecules, providing a convenient tool to study their structures and functions [22].

It should be noted that Cys residues are usually essential for maintaining the protein structure because they are capable of disulfide bonding and coordinating metal ions. On the one hand, the high reactivity of Cys residues determines their key role in the redox potential and active centers of enzymes [23, 24]. Along with Arg and Lys residues, Cys residues may interact with the DNA sugar-phosphate backbone in protein–nucleic acid complexes [25, 26]. A classic example is provided by Cys62 located in the DNA-binding center of the p50 subunit of the human NF-κB transcription factor. According to X-ray crystallography and crosslinking data, Cys62 is close to the 3'-phosphate groups of nucleotides 6 and 7 in the κB site of the enhancer of the immunoglobulin light chain genes [27–30]. On the other hand, Cys residues are rare in protein sequences and their thiol group specifically interacts with compounds carrying the haloacetyl, disulfide, or maleimide groups. Cys residues are consequently attractive as targets for introducing fluorophores in protein molecules [31]. Fluorescence labeling of proteins at Cys residues have become widespread in recent years because methods based on fluorescence resonance energy transfer and fluorescence microscopy of single molecules were employed in studying the mechanisms underlying the function of biolomolecules and their complexes [20, 32–34]. To use such a method, it is important to understand what role Cys residues play in the function of the protein of interest and what sequels are possible to expect from their modification in terms of its enzymatic activity.

Our study was the first to demonstrate the role of the Cys residues in the NE BspD6I interaction with DNA. We used the crosslinking technique and the DNA duplexes that contained the 2-pyridyldisulfide group at the C2' atom of the sugar-phosphate moiety in certain positions of the oligonucleotide strand. The Cys residues of the N-terminal DNA-binding and C-terminal catalytic domains of NE BspD6I were found to be close to DNA during the formation of an enzyme–substrate complex. However, a substitution of Ser for the Cys residues of the N-terminal domain did not substantially affect the protein function.

EXPERIMENTAL

Oligodeoxyribonucleotides, reagents, and buffer solutions. Oligodeoxyribonucleotides carrying the

2'-amino group were synthesized by the team headed by T.S. Zatsepin (Skolkovo Institute of Science and Technologies, Moscow). Nonmodified and fluorescently labeled oligodeoxyribonucleotides were purchased from Syntol (Russia) and Evrogen (Russia).

We used N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), 5-kDa polyethylene glycol maleimide (PEG-Mal), kanamycin (Km), chloramphenicol (Cam), and isopropyl-β-D-1-thiogalactopyranoside (IPTG) from Sigma-Aldrich (United States); sodium dodecyl sulfate (SDS) from Amresco (United States); threo-2,3-dihydroxy-1,4-dimercaptobutane (1,4-dithiothreitol, DTT) from AppliChem (Germany); PageRuler protein molecular weight markers (10–200 kDa) from Thermo Fisher Scientific (United States); Bromophenol Blue (BPB) from Reanal (Hungary); Ni-NTA agarose from Novagen (Germany); NAP-5 gel filtration mini columns from GE HealthCare (United States); tryptone from VWR Life Science (United States); and a yeast extract from Helicon (Russia).

The following buffer solutions were used in experiments: buffer A (10 mM Tris-HCl, pH 7.8, 150 mM KCl, 10 mM $CaCl_2$), buffer B (10 mM Tris-HCl, pH 7.8, 150 mM KCl, 10 mM $MgCl_2$), buffer C (20 mM HEPES, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 50% (m/V) glycerol), buffer D (50 mM Tris-HCl, pH 8.3, 50 mM boric acid , 200 mM EDTA, 50% (m/V) glycerol), buffer E (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 125 mM KCl, 1 mM ADP), buffer TAE (40 mM Tris-CH_3COOH, pH 7.5, 1 mM EDTA), buffer TBE (50 mM Tris-HCl, pH 8.3, 50 mM boric acid, 1 mM EDTA), buffer TG (25 mM Tris-HCl, pH 8.3, 250 mM glycine, 0.1% (m/V) SDS), buffer LAP (50 mM Tris-HCl, pH 6.8, 2.5% (m/V) SDS, 10% (m/V) glycerol, 0.01% (m/V) BPB, and buffer CD (10 mM K-phosphate, pH 7.6, 150 mM KCl, 0.1 mM EDTA). The LB culture medium contained 10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract.

Construction of the NE BspD6I(C11S/C160S) gene. The plasmid pET28b carrying the gene for the wild-type NE BspD6I with a hexahistidine tag (His_6-tag) at the C-terminus [35] was used to construct the plasmids carrying a mutant gene that coded the enzyme with substitutions of two Cys residues with Ser in the N-terminal domain. A gene for the protein with the Cys11Ser substitution was initially constructed and then mutated to introduce the Cys160Ser substitution into the protein.

To introduce the Cys11Ser substitution, side-directed mutagenesis of the wild-type NE BspD6I gene was carried out using the following primers:

11F (forward), 5'-GGTATGTTTCTTCTTCACCTAGAAGTCCAG-3'), and

11R (reverse), 5'-CTGGACTTCTAGGTGAAGAAGAAACATACC-3').

Mutagenesis was performed using a QuikChange kit (Agilent Technologies, United States) according to the manufacturer's protocol. DNA was amplified using Pfu Turbo DNA polymerase (Agilent Technologies). Amplification included 1 cycle of 95°C for 30 s and 16 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 7 min. To construct the NE BspD6I (C11S/C160S) gene, the plasmid with the gene coding for the NE BspD6I(C11S) was mutated using the primers 160F (5'-CTTTAACAGCAACAAATAATAATCAGGTGGA-3') and 160R (5'-AGAACATAGCAATATCTAATTTCTGAGGCCGCCT-3') and VELOCITY DNA polymerase (Bioline, United Kingdom) according to the manufacturer's protocol. Amplification included 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 4 min; and 1 cycle of 72°C for 10 min. In either case, the reaction mixture was supplemented with 10 units of RE DpnI after PCR to hydrolyze the template (methylated) plasmid and incubated at 37°C for 1 h. The resulting plasmid was used to transform *Escherichia coli* NovaBlue(DE3) cells, which additionally carried the pRARE plasmid with the gene for SscL1I methyltransferase to protect DNA from NE BspD6I hydrolysis [35]. Cells were grown on the LB agar medium supplemented with antibiotics (40 μg/mL Km and 10 μg/mL Cam). Colonies were tested for pET28b coding for the NE BspD6I mutants by PCR with primers directed to the NE BspD6I gene: Nick1 (5'-GCGCCATGGCTAAAAAAGTTAATTG-3') and Nick2 (5'-GCGGTCGACAAACCTTACCTCCTTC-3'). Colonies carrying the plasmid with the NE BspD6I(C11S/C160S) gene were grown in 5 mL of the LB medium supplemented with the antibiotics (40 μg/mL Km and 10 μg/mL Cam). The plasmid was isolated using a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific). A plasmid fragment carrying the mutations was PCR amplified with primers Nick3 (5'-AAAGTCGACAAATGAAAACTGTGGTAAAC-3') and T7 (5'-TAATACGACTCACTATAGGG-3'), separated from by-products by electrophoresis in 0.8% agarose gel in buffer TAE at 10 V/cm, and isolated from gel with a Gel extraction kit (Qiagen, United States). To verify the presence of the nucleotide substitutions of interest, the PCR product was sequenced on an ABI Prism310 genetic analyzer (Applied Biosystems) at the Institute of Protein Research (Russian Academy of Sciences).

Protein isolation. *Escherichia coli* NovaBlue(DE3) cells carrying pRARE with the SscL1I methyltransferase gene and pET28b with the gene for NE BspD6I or its mutant form were grown at 37°C until A_{590} = 0.6 optical units. The LB medium contained 40 μg/mL Km and 10 μg/mL Cam. The cell culture was supplemented with 0.7 M IPTG to induce NE expression and incubated with intense aeration at 20°C for 12 h to reach the late logarithmic growth phase. The further isolation and purification procedures were the same for the wild-type BspD6I and its mutant form. Biomass obtained from a bacterial cell suspension was diluted with a cell lysis buffer (0.02 M K-phosphate,

pH 7.5, 7 mM β-mercaptoethanol, 1 mM EDTA, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride). The preparation was stirred to obtain a homogeneous suspension and sonicated. Cell debris was removed by centrifugation (18000 *g*, 4°C, 30 min). NE BspD6I and NE BspD6I(C11S/C160S) were isolated via two steps of low-pressure column chromatography on a BioLogic LP system (Bio-Rad Laboratories). The cell lysate was first applied onto a 5-mL Whatman p11 phosphocellulose column (GE HealthCare), which was equilibrated with column chromatography (CC) buffer (0.02 M K-phosphate, pH 7.5, 7 mM β-mercaptoethanol) supplemented with 10 mM KCl. Elution was performed using a linear gradient of increasing KCl concentration (0.1–1.0 M in 50 mL of buffer CC) at a rate of 30 mL/h; 0.5-mL fractions were collected.

The fractions were tested for the protein of interest by Laemmli electrophoresis [36]. Electrophoresis was carried out in gel slabs (10 × 8 cm) in electrode buffer TG at 18 V/cm, using a Mini-PROTEAN TetraSystem camera (Bio-Rad Laboratories). The separating gel contained 12% acrylamide, 0.4–0.5% *N*,*N*'-methylenebisacrylamide, 0.1% SDS, 375 mM Tris-HCl (pH 8.8). The stacking gel contained 4% acrylamide, 0.1–0.2% *N*,*N*'-methylenebisacrylamide, 0.1% SDS, 130 mM Tris-HCl (pH 6.5). Samples (10 μL) were prepared in buffer LAP without DTT. Prior to application, the samples were heated at 95°C for 5 min and chilled in ice. To visualize the proteins, gels were stained with PageBlue (Thermo Fisher Scientific), which is based on Coomassie Brilliant Blue G-250.

The fractions that contained the target protein were dialyzed against buffer CC containing 10 mM KCl and applied onto a 1-mL column with Ni-NTA agarose (Qiagen). Elution was carried out using a stepwise gradient of imidazole concentration in buffer CC at 30 mL/h; 30 mM imidazole was used to remove impurities, and 150 mM imidazole was used to elute the target protein. Purified preparations of NE BspD6I and NE BspD6I(C11S/C160S) were dialyzed against a storage buffer (10 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol (v/v)) and then used in experiments or stored at −20°C.

The concentrations of NE BspD6I and NE BspD6I(C11S/C160S) were estimated spectrophotometrically, using the extinction coefficient ε_{280} = 77240 M^{-1} cm^{-1}. The concentrations were 6.15 mg/mL wild-type NE BspD6I and 1.5 mg/mL NE BspD6I(C11S/C160S).

A MutS preparation was kindly provided by M.V. Monakhova (Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University).

Circular dichroism (CD) spectra of NE BspD6I and NE BspD6I(C11S/C160S) were recorded using a Chirascan spectrometer (Applied Photophysics, United Kingdom) at 20°C. NE samples in buffer CD were tested in a cylindrical cuvette with an optical path length of 0.05 cm.

Reactions of NE BspD6I and NE BspD6I(C11S/C160S) with PEG-Mal were carried out in buffer A without DTT at 37°C for 15 min. The proteins were used at 10 μM; PEG-Mal was used in a 200-fold excess. Conjugation was monitored by Laemmli electrophoresis [36], which was carried out as above.

Binding of NE BspD6I and NE BspD6I(C11S/C160S) with DNA duplex I (Fig. 2) was carried out in 10 μL of buffer A supplemented with 1 mM DTT, 15% glycerol, and 0.1 mg/mL BSA at 37°C for 30 min. To select the conditions for crosslinking of the proteins with DNA duplex I, the reaction mixtures did not contain DTT and BSA, DNA duplex I was used at 0.5 μM, and the NE BspD6I or NE BspD6I(C11S/C160S) concentration was varied from 0.1 to 5 μM. To estimate the apparent dissociation constant (K_d) for the complex of NE BspD6I(C11S/C160S) with DNA duplex I (10 nM), the protein was used at increasing concentrations in a range of 10–100 nM. The formation of DNA–protein complexes was monitored by PAGE in gel slabs (200 × 200 × 1.5 mm) containing 7% acrylamide and *N*,*N*'-bisacrylamide (acrylamide : *N*,*N*'-bisacrylamide = 29 : 1). Electrophoresis was carried out in buffer TBE at 15 V/cm. Fluorescently labeled DNA was detected in gel with a Typhoon FLA 9500 instrument (GE Healthcare). A quantitative analysis of fluorescent bands was performed using ImageQuant software (GE Healthcare). The extent of DNA binding with the enzyme was calculated as a ratio of the fluorescence intensity of the band corresponding to the complex to the total fluorescence intensity of all bands in a lane. K_d of the complex of NE BspD6I(C11S/C160S) with DNA duplex I was obtained as the protein concentration at which 50% of the carboxyfluorescein (FAM)-labeled DNA substrate is in complex with the enzyme. Using the Origin program, the standard error was estimated as $SE = s/n^{0.5}$, where SE is the standard error, s is the standard deviation, and n is the number of independent binding experiments ($n = 3$).

Crosslinking of the proteins with modified DNA duplexes. At the first step, oligonucleotides containing the amino group in the 2'-amino-2'-deoxyuridine moiety were modified using SPDP. The reaction mixture contained 800 pmol of an oligonucleotide and 2.4 mmol of SPDP (a 3000-fold excess) in 50 μL of a Na-borate buffer (pH 8.5). Mixtures were incubated at 37°C for 24 h. Modified oligonucleotides were separated from the excess of reagents on an Illustra NAP-5 column (GE Healthcare) according to the manufacturer's protocol and precipitated with acetone in the presence of 0.4 M $LiClO_4$. To obtain DNA duplexes at the next step, the complementary strand was added to the oligonucleotide samples, and the samples were heated at 95°C for 5 min and slowly chilled at room temperature. The modified DNA duplexes (0.5 μM)

I
5'–CGTGGTCTC**GAGTC**TTCTCAAGGTAC–*FAM*–3'
3'–GCACCAGAG**CTCAG**AAGAGTTCCATG–5'

II
5'–CGTGGTCTC**GAGUC**TTCTCAAGGTAC–*FAM*–3'
3'–GCACCAGAG**CTCAG**AAGAGTTCCATG–5'

III
5'–CGTGGTCTC**GAGTC**TTC**U**CAAGGTAC–*FAM*–3'
3'–GCACCAGAG**CTCAG**AAGAGTTCCATG–5'

IV
5'–CGTGGTCTCTCA**U**CTTCAAGGTACTG–*FAM*–3'
3'–GCACCAGAGAGTAGAAGTTCCATGAC–5'

Fig. 2. DNA duplexes labeled with FAM at the 3' end of the upper strand. The NE BspD6I recognition site is in bold. **U** is the 2'-deoxy-2'-[3-(2-pyridylthio)propionamido]uridine residue.

were crosslinked with NE BspD6I or NE BspD6I(C11S/C160S) (1 μM) in 20 μL of buffer A at 37°C for 30 min. The crosslinking reaction of modified DNA duplex IV (0.5 μM) with MutS (2.1 μM), which is a component of the *E. coli* mismatch repair system, was carried out in 20 μL of buffer E in the same incubation conditions. The crosslinking products were examined by PAGE, which was carried out in parallel in denaturing (see Protein isolation) and nondenaturing (7% gel, see Binding of NE BspD6I and NE BspD6I(C11S/C160S) with DNA duplex I) conditions. A modified SDS-PAGE variant was used to retain the oligonucleotides in gel because the oligonucleodites were far lower in molecular mass than the proteins. A trapping gel was added to the bottom of the separating gel and contained 0.375 M Tris-HCl (pH 8.8), 20% acrylamide, 1% *N*,*N*'-methylenebisacrylamide, and 0.1% SDS [37].

Endonuclease activity assays of NE BspD6I and NE BspD6I(C11S/C160S) were carried out at 37°C for 30 min. The reaction mixture contained 10 μL of buffer B supplemented with 1 mM DTT, 0.1% BSA, and 15% glycerol. The enzymes were used at 1, 10, 20, and 100 nM; and the DNA duplex, at 10 nM. Prior to loading to the gel, the reaction mixture was combined with 2 μL of buffer D and heated at 95°C for 3 min. The cleavage products were separated by PAGE in 20% gel (200 × 200 × 1 mm, acrylamide : *N*,*N*'-bisacrylamide = 19 : 1) with 7 M urea in buffer TBE at 30 V/cm. Fluorescence of DNA-containing bands was detected using a Typhoon FLA 9500 instrument and analyzed using the ImageQuant program. The extent of DNA substrate cleavage by the enzyme was calculated as a ratio of the fluorescence intensities of the bands corresponding to the hydrolysis products to the total fluorescence intensity of all bands (the initial DNA and the reaction products).

RESULTS AND DISCUSSION

Covalent Binding of the Cys Residues of NE BspD6I with Pyridyldisulfide DNA Derivatives

To check whether the Cys residues of NE BspD6I are close to DNA, we used crosslinking of the wild-type and mutant NEs with modified DNA duplexes, which contained the 2-pyridyldithio group in the 2' position of a certain nucleotide of DNA strand. The same DNA modification has previously been used to obtain a conjugate with the p50 subunit of the human NF-κB transcription factor [16]. The 2-pyridyldithio group is known to selectively interact with Cys residues of a protein via the thiol–disulfide exchange reaction in an aqueous buffer solution at pH 7–8 (Fig. 3). Metelev et al. [16] have shown that an attack on the sulfur atom that belongs to the thiol group of a Cys residue and is close to the 2'-propionamide group is the only way for the reaction to proceed.

26-bp duplexes (Fig. 2) were used as analogs of the NE BspD6I substrate. DNA binding and hydrolysis by the enzyme are efficient only when the flanking regions are at least 4 bp in length upstream (from the left side) of the recognition site (from the 5' end of the 'top' strand) and at least 3 bp in length downstream of the cleavage site [6]. The reactive group was introduced in the 'top' strand of the NE BspD6I recognition site in place of T (duplex II) or immediately at the 5'-side of the internucleotide bond that is hydrolyzed by the enzyme (duplex III). As a control, we used duplex IV, which had the 2-pyridyldithio group and lacked the NE recognition site, and duplex I, which lacked the reactive group.

To obtain duplexes II–IV containing the 2-pyridyldithio group, we used the 26-mer precursor oligonucleotides that had a single 2'-amino-2'-deoxyuridine residue in a preset position [16] and the FAM fluorescent label at the 3' end. The 2'-amino-2'-deoxyuridine oligonucleotide derivatives and complementary oligonucle-

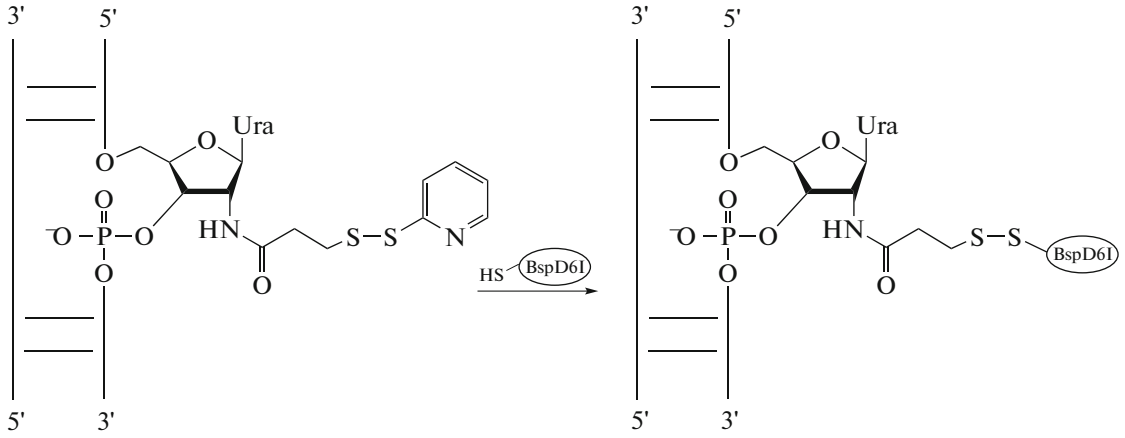

Fig. 3. Scheme of interaction of the DNA contained 2-pyridyldithio group with the SH group of a Cys residue of NE BspD6I.

otides were used to produce the duplexes containing the NE BspD6I recognition site. The duplexes were hydrolyzed by the enzyme with an efficiency of 80–97% as compared with nonmodified duplex I (data not shown). Thus, the modification did not interfere with enzymatic activity when introduced at the 2' position of the sugar moiety within the NE BspD6I recognition site or close to the hydrolyzable internucleotide bond.

The 2'-amino group of the oligonucleotides was modified with SPDP. One end of the modifying reagent had the succinimide group, which is capable to interact with the NH_2 group at pH 8.3. The other end had the 2-pyridyldithio group, which potentially interacts with the SH groups of Cys residues in a protein at pH 7.5 (Fig. 3). SPDP is the shortest of all commercially available crosslinking agent with the 2-pyridyldithio group. Its length is 6.8 Å. The reactive group is less conformationally mobile because of the short length, and the conjugate yield might be lower because of this circumstance. However, the short length makes it possible to identify the amino acid residue of a protein and the nucleotide that are brought close together during the formation of the DNA–protein complex. Reactive DNA duplexes II–IV were obtained by hybridizing the modified oligonucleotides with the respective complementary strands.

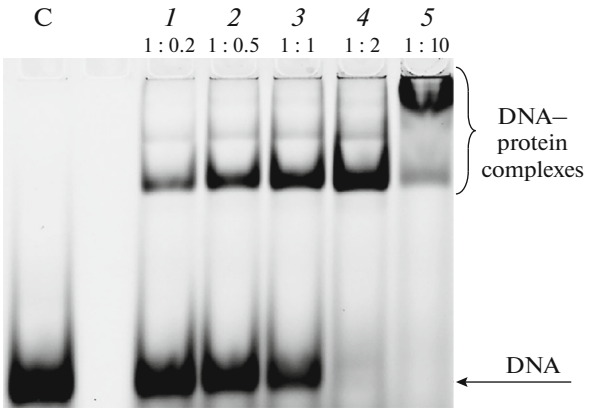

Fig. 4. Analysis of the complex formation between DNA duplex I (0.5 μM), which contained a FAM-labeled 'top' strand, and NE BspD6I. PAGE in 7% gel was carried out in nondenaturing conditions. Lanes: C, initial DNA; (*1*–*5*) reaction mixtures containing 0.1, 0.25, 0.5, 1.0, and 5.0 μM NE BspD6I, respectively. The DNA duplex–NE BspD6I concentration ratio is shown at the top for each lane. The reaction was carried out at 37°C for 30 min.

The crosslinking conditions were selected so that NE BspD6I efficiently bound with DNA. The optimal temperature for the NE BspD6I function is 55°C [4], but the properties of the enzyme were studied at 37°C to allow comparisons with the majority of other restriction endonucleases. Magnesium ions serve as a natural RE cofactor and are necessary for the catalysis of DNA hydrolysis. Calcium ions facilitate the formation of an enzyme–substrate complex, but the resulting complex is unproductive [38]. Taking advantage of this circumstance, the efficiency of RE–DNA binding is conventionally assayed in the presence of Ca^{2+} to prevent substrate hydrolysis. NE BspD6I was incubated with DNA duplex I (0.5 μM) in buffer A for 30 min. The NE concentration was varied within a range of 0.5–5.0 μM (Fig. 4).

A twofold excess of NE BspD6I was already sufficient for its efficient binding with substrate I. The thiol–disulfide exchange reaction between the enzyme and DNA duplexes II–IV was carried out at 37°C for 30 min; the reaction buffer did not contain DTT (Fig. 5). The DNA concentration was 0.5 μM; the enzyme was used at 1 μM. The reaction products were resolved by SDS-PAGE (see Crosslinking of the proteins with modified DNA duplexes in Experimental) and detected by fluorescence of the FAM label introduced in DNA. As expected, conjugate bands were detected upper than the band of a 70-kDa marker

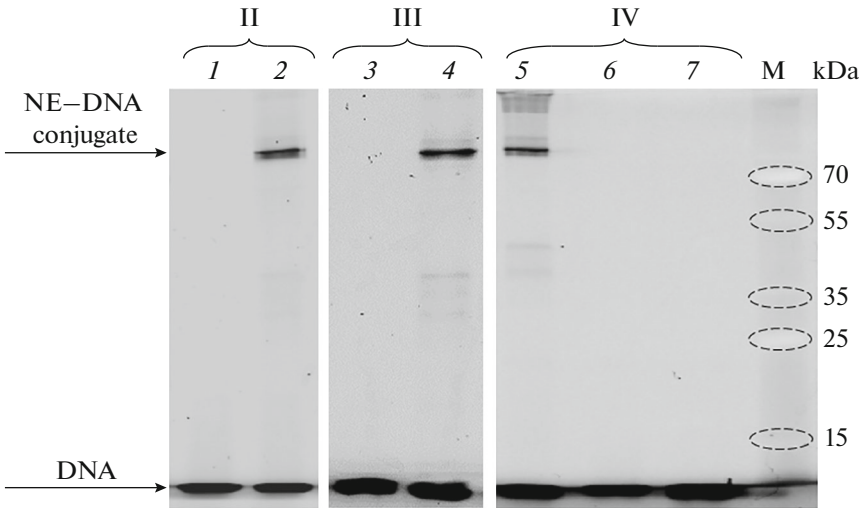

Fig. 5. Analysis of conjugate formation of NE BspD6I with DNA duplexes II–IV (as specified at the top of the lanes) and MutS with DNA duplex IV. The products were resolved by SDS-PAGE in 12% gel. DNA-containing bands were detected by FAM fluorescence. Lanes: (*1, 3*, and *7*) the reaction mixtures contained only the initial DNA; (*2*, *4*, and *5*) DNA duplex II, III, or IV (0.5 μM), respectively, was incubated with NE BspD6I (1 μM); (*6*) MutS (2.1 μM) was incubated with DNA duplex IV (0.5 μM). M, protein molecular mass markers (kDa). The reaction was carried out at 37°C for 30 min.

protein (Fig. 5). NE BspD6I is 70.8 kDa, and the crosslinked DNA strand is 7.9 kDa in molecular mass.

Products of crosslinking with NE were detected with all reactive duplexes, that is, duplexes II–IV (Fig. 5, Table 1). Thus, at least one of the four Cys residues is brought close to DNA during the formation of the protein–nucleic acid complex. A DNA–protein conjugate band was observed even in the case of duplex IV, which lacked the NE BspD6I recognition site. NE BspD6I has high affinity for nonspecific DNA ligands, as has been demonstrated by gel retardation assays performed in the binding conditions [8]. It is known that RE initially form a primary contact with DNA and then slide along DNA to find a recognition site [38]. A certain level of background nonspecific binding is therefore always observed, especially when the DNA substrate is sufficiently long and has regions similar in sequence to the RE recognition site. Note that the p50 subunit of NF-κB also has high affinity for nonspecific DNA and similarly interacted with both a duplex containing 2-pyridyldisulfide group in the κB site and a reactive DNA duplex of a random sequence [16].

The *E. coli* MutS protein was used as a control. MutS is a homodimeric repair protein, binds with DNA regardless of its sequence, and has seven Cys residues per monomer. However, a conjugate was not formed when MutS was incubated with DNA duplex IV (Fig. 5). The finding indicates, first, that the Cys residues of MutS do not come into contact with DNA and, second, that the reaction is possible to employ in probing such interactions in the process of protein functioning.

The above data do not make it possible to identify the particular Cys residue that interacts with the reactive groups of duplexes II–IV. We can only assume that the Cys residues of the N-terminal domain (positions 11 and/or 160), which is responsible for NE BspD6I binding with DNA, react with the modified DNA fragments. Crosslinking with duplex III, which carries the modification in the vicinity of the target internucleotide bond, suggests a possible role in complex formation with DNA for the Cys residues of the C-terminal domain, which is responsible for the catalysis. Cys508 is close to the catalytic center of the enzyme and may also contribute to the crosslinking.

To further study the role that the Cys residues play in NE BspD6I interactions with DNA, we used the mutant NE BspD6I(C11S/C160S), which contained Ser in place of the two Cys residues that are in the N-terminal domain and are presumably close to the recognition site in the protein–nucleic acid complex [12].

Table 1. Conjugate yields after the interaction of NE BspD6I and NE BspD6I(C11S/C160S) with DNA duplexes II–IV

DNA duplex	Wild-type NE BspD6I, %	NE BspD6I(C11S/C160S), %
II	16	6
III	18	5
IV	10	5

Results were averaged over three experiments; the measurement error did not exceed 10% of the respective average yield. The conjugate yield was calculated as a ratio of the fluorescence intensity of the band corresponding to the conjugate to the total fluorescence intensity of all bands in the lane.

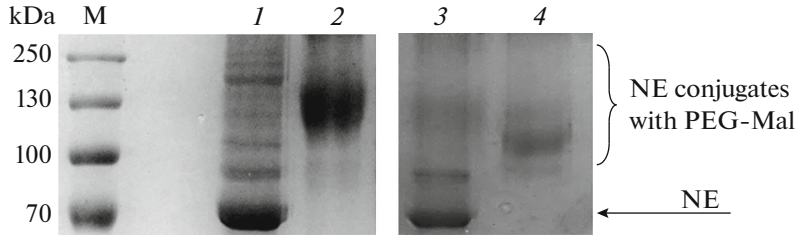

Fig. 6. Results of the interaction of PEG-Mal (5 kDa, 200-fold excess) with the wild-type NE BspD6I (10 μM) or NE BspD6I(C11S/C160S) variant (10 μM). The products were analysed by PAGE in 12% gel, and the gel was stained with Coomassie G-250. M, molecular mass markers (kDa). The wild-type NE BspD6I and NE BspD6I(C11S/C160S) variant were examined in the absence (lanes *1* and *3*, respectively) or presence (lanes *2* and *4*, respectively) of PEG-Mal.

The relative yields of the DNA–protein conjugates are possible to estimate with due regard to nonspecific binding, and the following assumptions can be made on the basis of the results. If the Cys residues of the N-terminal DNA-binding domain are the only Cys residues involved in the interaction with DNA, then NE BspD6I(C11S/C160S) would not crosslink with reactive duplexes II–IV. If NE BspD6I(C11S/C160S) covalently binds with the DNA duplexes, then Cys508 and/or Cys578 of the C-terminal catalytic domain are in close proximity of the duplex.

Structural and Functional Characterization of the NE BspD6I(C11S/C160S)

The presence of Cys residues in a protein and their accessibility to chemical reagents are possible to verify in a qualitative reaction with PEG-Mal (5 kDa). An increase in molecular mass due to conjugation with the reagent was detected by Laemmli PAGE. The reaction was performed using a 200-fold excess of PEG-Mal relative to the protein at 37°C for 15 min. As is seen from Fig. 6, conjugates of different molecular mass formed with the wild-type NE BspD6I and with the NE BspD6I(C11S/C160S) variant, demonstrating that the Cys residues are fewer in the mutant protein.

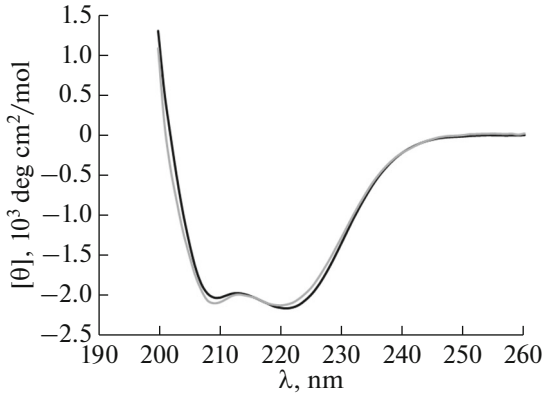

Fig. 7. CD spectra of the wild-type NE BspD6I (a black line) and the NE BspD6I(C11S/C160S) variant (a gray line).

When the reaction mixture was supplemented with DTT after a 15-min incubation, the thioester bond produced by the PEG-Mal interaction with the thio group of Cys was broken and the initial protein released (data not shown).

When mutant proteins are constructed, Ser is often used as a substitute for Cys because the two amino acid residues are similar in properties and the protein secondary structure is consequently preserved in the majority of cases after the substitution. We used CD to evaluate the effect of the amino acid substitutions on the structure of NE BspD6I(C11S/C160S). CD spectra of the mutant and wild-type proteins were recorded in a wavelength range of 200–260 nm in buffer CD at 20°C (Fig. 7). As is seen, the character of the CD spectrum did not substantially change after the substitution of Ser for Cys11 and Cys160, indicating that the secondary structure of NE BspD6I(C11S/C160S) was much the same as that of the original protein.

Functional activity of NE BspD6I(C11S/C160S) was evaluated by its efficiency in introducing a single-strand break in 26-bp duplex I (Fig. 8). The reaction was carried out at 37°C for 30 min. A FAM-labeled 8-nt oligonucleotide was one of the reaction products and was detected by PAGE in 20% gel in the presence of 7 M urea. The enzyme concentration was varied to be 1, 10, 20, and 100 nM, while the DNA duplex was constant, 10 nM, in these experiments. The extent of hydrolysis was averaged over three independent experiments (Fig. 9). The results showed that the NE BspD6I(C11S/C160S) was quite efficient in hydrolyzing the substrate, although its efficiency was 1.5 times lower than that of the wild-type enzyme.

The model assumed for the complex of NE BspD6I with DNA [12] predicts that Cys11 interacts with DNA in the vicinity of the adenosine residue of the 'bottom' strand of the recognition site (5'-GAGTC-3'/ 3'-CTCAG-5'). A lower hydrolytic activity of NE BspD6I(C11S/C160S) is possibly explained by the involvement of Cys11 in DNA substrate binding. The dissociation constant of the complex of NE BspD6I(C11S/C160S) with duplex I was estimated by the gel retardation assay (see Experimental, Binding of NE BspD6I and NE BspD6I(C11S/C160S) with

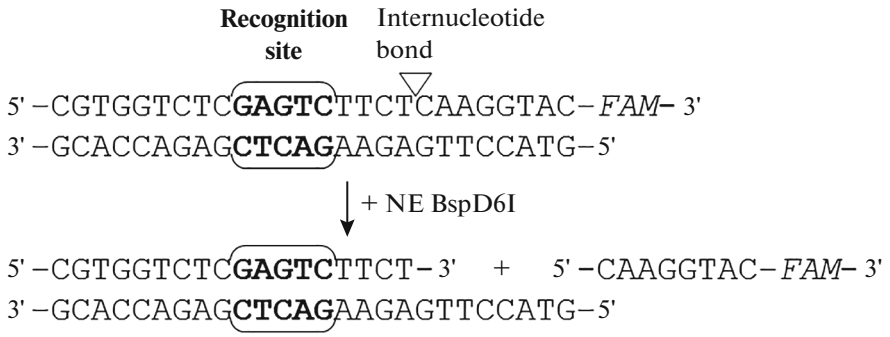

Fig. 8. Scheme of hydrolysis of DNA duplex I by NE BspD6I.

DNA duplex I). The apparent dissociation constant K_d was calculated as the enzyme concentration that ensured a 50% binding of the substrate. The binding reaction was carried out in buffer A; the DNA concentration was 10 nM; the concentration of NE BspD6I(C11S/C160S) was varied in a range of 10–100 nM (Fig. 10). K_d was estimated at 35 ± 9 nM. Previously, K_d has similarly been estimated for the complex of the wild-type NE BspD6I with duplex I and found to be 8 ± 2 nM [10]. The results demonstrate that the substitutions for Cys11 and Cys160 caused an approximately fourfold decrease in enzyme binding with DNA, implicating the residues in enzyme interactions with DNA.

Interaction of NE BspD6(C11S/C160S) with Pyridyldisulfide DNA Derivatives

Crosslinking reactions of the NE BspD6I(C11S/C160S) with DNA were carried out in the same conditions as in the case of the wild-type enzyme. Figure 11 shows an example electrophoretic pattern of the crosslinking products of the two enzymes with DNA duplex II, which contained the NE BspD6I recognition site. Conjugates with duplexes I–IV were produced by the NE BspD6I(C11S/C160S), which lacked Cys residues in the N-terminal domain (Fig. 11, Table 1). Therefore, Cys508 and/or Cys578 of the C-terminal catalytic domain are close to DNA at the step of complex formation. Note that we did not expect a priory that the C-terminal catalytic domain would contribute to substrate DNA binding because RE FokI, which shows the greatest structural similarity to NE BspD6I, contacts DNA with its catalytic domain only at the stage of substrate hydrolysis [39].

NE BspD6I(C11S/C160S) produced conjugates with duplex IV, which lacked the enzyme recognition site. Therefore, one or both of the Cys residues of the catalytic domain are close to DNA as early as the nonspecific binding stage. Similar yields were obtained for the NE BspD6I(C11S/C160S) with duplexes II and III, which differ in the position of the reactive group. The result supports the hypothesis that the Cys resi-

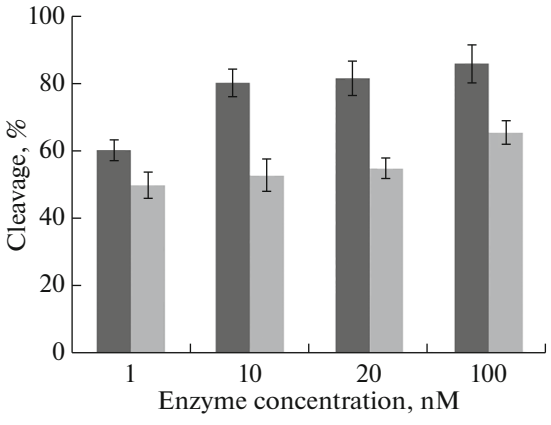

Fig. 9. The results of hydrolysis of DNA duplex I by the wild-type NE BspD6I (dark gray columns) and NE BspD6I(C11S/C160S) (light gray columns). The extent of DNA hydrolysis was averaged over three experiments. The reaction was carried out at 37°C for 30 min.

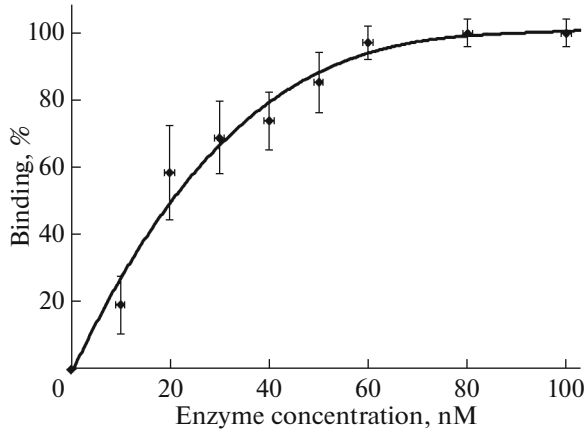

Fig. 10. Binding of DNA duplex I (10 nM) with the NE BspD6I(C11S/C160S) as dependence on the enzyme concentration. The extent of DNA binding was averaged over three experiments. The reaction was carried out at 37°C for 30 min.

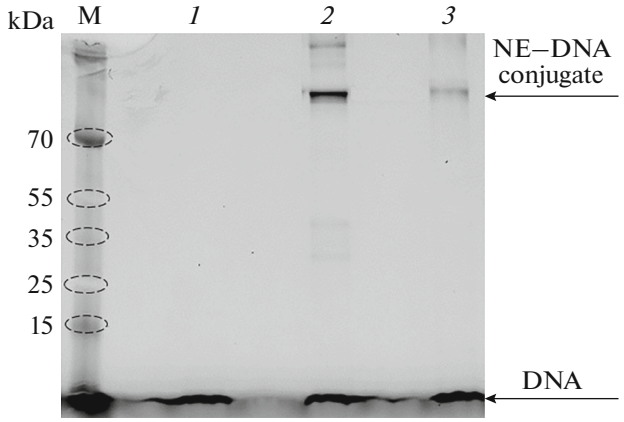

Fig. 11. Analysis of conjugate formation of the wild-type NE BspD6I (lane *2*) and NE BspD6I(C11S/C160S) (lane *3*) with DNA duplex II. The products were resolved by SDS-PAGE in 12% gel. DNA-containing bands were detected by FAM fluorescence. M, protein molecular mass markers (kDa). Lane *1*, initial DNA. The DNA duplex was used at 0.5 μM; the protein concentration was 1 μM. The reaction was carried out at 37°C for 30 min.

dues of the C-terminal domain of NE are involved in initial DNA scanning.

Note that the conjugate yield with the wild-type NE was reproducibly higher than with the mutant enzyme (Table 1). The difference might arise because the mutant NE BspD6I(C11S/C160S) has lower affinity for DNA (see above). However, we cannot exclude that Cys11 and/or Cys160 are directly involved in DNA cleavage by the enzyme. The product yield of the reaction with the wild-type enzyme would inevitably be higher than with the NE BspD6I(C11S/C160S) in this case. In general, our findings agree with the model of NE BspD6I interactions with DNA [12], although the model has been built disregarding the more recent finding that DNA is bent by 66 ± 4 degrees in complex with the enzyme [6].

To summarize, we were the first to demonstrate using the crosslinking technique that the Cys residues of NE BspD6I are in the immediate vicinity of DNA as early as the formation of a nonspecific protein–nucleic acid complex. In particular, the Cys residues of the C-terminal catalytic domain of NE BspD6I were found to be close to DNA. Functional properties were characterized for the NE BspD6I variant that lacked the Cys residues of the N-terminal domain. The substitutions Cys11Ser and Cys160Ser were shown to cause a fourfold decrease in the efficiency of NE binding with DNA, while hydrolytic activity of the enzyme decreased only by a factor of 1.5.

Understanding the protein–protein and protein–nucleic acid interactions in intricate complexes of biomolecules is one of the main problems of functional proteomics. An efficient strategy is to construct the protein mutant forms that contain only one Cys residue in a preset position of the polypeptide chain. Subsequent modification of the single-Cys variants with a commercial fluorophore or a crosslinking agent carrying the maleimide group provides an opportunity to probe the interactions of the respective proteins with their partner biomolecules. An important condition for the strategy to be suitable is that functional activity is preserved in the protein after all of its Cys residues have been substituted; the protein is then used as a basic tool to construct single-Cys mutant forms [20, 40]. Construction of the NE BspD6I variant that lacks Cys residues and investigation of its properties will help to understand whether the strategy is suitable for studying the protein–protein interactions of the heterodimeric RE BspD6I.

ACKNOWLEDGMENTS

We are grateful to T.S. Zatsepin (Skolkovo Institute of Science and Technologies, Moscow) for the oligodeoxyribonucleotides carrying the 2'-amino group and M.V. Monakhova (Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University) for the MutS preparation.

FUNDING

This work was supported by the Russian Science Foundation (project no. 18-74-00049).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

This work does not contain any studies involving animals or human subjects performed by any of the authors.

REFERENCES

1. Abrosimova L.A., Kisil O.V., Romanova E.A., Oretskaya T.S., Kubareva E.A. 2019. Nicking endonucleases as unique tools for biotechnology and gene engineering. *Russ. J. Bioorg. Chem*. **45** (5), 303–320.
2. Gabsalilow L., Schierling B., Friedhoff P., Pingoud A., Wende W. 2013. Site-and strand-specific nicking of DNA by fusion proteins derived from MutH and I-SceI or TALE repeats. *Nucleic Acids Res.* **41**, e83.
3. Van Nierop G.P., de Vries A.A.F., Holkers M., Vrijsen K.R., Gonçalves M.A. 2009. Stimulation of homology-directed gene targeting at an endogenous human locus by a nicking endonuclease. *Nucleic Acids Res.* **37**, 5725–5736.
4. Zheleznaya L.A., Perevyazova T.A., Alzhanova D.V., Matvienko N.I. 2001. Site-specific nicjase from *Bacillus* species strain D6. *Biochemistry* (Moscow). **66** (9), 989–993.
5. Yunusova A.K., Rogulin E.A., Artyukh R.I., Zheleznaya L.A., Matvienko N.I. 2006. Nickase and a protein encoded by an open reading frame downstream from the nickase BspD6I gene form a restriction endo-

nuclease complex. *Biochemistry* (Moscow). **71** (7), 815–820.

6. Abrosimova L.A., Kubareva E.A., Migur A.Y., Gavshina A.V., Ryazanova A.Y., Norkin M.V., Perevyazova T.A., Wende W., Hianik T., Zheleznaya L.A., Oretskaya T.S. 2016. Peculiarities of the interaction of the restriction endonuclease BspD6I with DNA containing its recognition site. *Biochim. Biophys. Acta—Proteins Proteom.* **1864**, 1072–1082.
7. Kachalova G.S., Rogulin E.A., Artyukh R.I., Perevyazova T.A., Zheleznaya L.A., Matvienko N.I., Bartunik H.D. 2005. Crystallization and preliminary crystallographic analysis of the site-specific DNA nickase Nb.BspD6I. *Acta Crystallogr. F: Struct. Biol. Cryst. Commun.* **61**, 332–334.
8. Sekerina S.A., Grishin A.V., Ryazanova A.Yu., Artyukh R.I., Rogulin E.A., Yunusova A.K., Oretskaya T.S., Zheleznaya L.A., Kubareva E.A. 2012. Oligomerization of site-specific nicking endonuclease BspD6I at high protein concentrations. *Russ. J. Bioorg. Chem.* **38** (4), 376–382.
9. Abrosimova L.A., Migur A.Yu., Kubareva E.A., Vende V., Zheleznaya L.A., Oretskaya T.S. 2015. Development of inhibitors for nicking endonuclease BspD6I based on synthetic DNA fragments. *Izv. Vyssh. Uchebn. Zaved., Prikl. Khim. Biotekhnol.* **2**, 48–59.
10. Abrosimova L.A., Migur A.Y., Kubareva E.A., Zatsepin T.S., Gavshina A.V., Yunusova A.K., Perevyazova T.A., Pingoud A., Oretskaya T.S. 2018. A study on endonuclease BspD6I and its stimulus-responsive switching by modified oligonucleotides. *PLoS One.* **13**, e0207302.
11. Yunusova A.K., Artyukh R.I., Perevyazova T.A., Abrosimova L.A., Kachalova G.S., Zheleznaya L.A. 2017. Restriction endonuclease R.BspD6I is active only in the presence of catalytically active large subunit. *MEDLINE.RU.* **18**, 200–208.
12. Kachalova G.S., Rogulin E.A., Yunusova A.K., Artyukh R.I., Perevyazova T.A., Matvienko N.I., Zheleznaya L.A., Bartunik H.D. 2008. Structural analysis of the heterodimeric type IIS restriction endonuclease R.BspD6I acting as a complex between a monomeric site-specific nickase and a catalytic subunit. *J. Mol. Biol.* **384**, 489–502.
13. Verdine G.L., Norman D.P.G. 2003. Covalent trapping of protein–DNA complexes. *Annu. Rev. Biochem.* **72**, 337–366.
14. Metelev V.G., Kubareva E.A., Vorob'eva O.V., Romanenkov A.S., Oretskaya T.S. 2003. Specific conjugation of DNA binding proteins to DNA templates through thiol-disulfide exchange. *FEBS Lett.* **538**, 48–52.
15. Vorob'eva O.V., Romanenkov A.S., Metelev V.G., Karyagina A.S., Lavrova N.V., Oretskaya T.S., Kubareva E.A. 2003. Crosslinking of Cys142 of methyltransferase SsoII with DNA duplexes containing a single internucleotide phosphoryldisulfide link. *Mol. Biol.* (Moscow). **37** (5), 772–779.
16. Metelev V., Romanenkov A., Kubareva E., Zubin E., Polouchine N., Zatsepin T., Molochkov N., Oretskaya T. 2006. Structure-based cross-linking of NF-κB p50 homodimer and decoy bearing a novel 2'-disulfide trapping site. *IUBMB Life.* **58**, 654–658.
17. Romanenkov A.S., Kisil O.V., Zatsepin T.S., Yamskova O.V., Karyagina A.S., Metelev V.G., Oretskaya T.S., Kubareva E.A. 2006. DNA-methyltransferase SsoII as a bifunctional protein: Features of the Interaction with the promoter region of SsoII restriction–modification genes. *Biochemistry* (Moscow). **71** (12), 1341–1349.
18. Heinze R.J., Sekerina S., Winkler I., Biertümpfel C., Oretskaya T.S., Kubareva E., Friedhoff P. 2012. Covalently trapping MutS on DNA to study DNA mismatch recognition and signaling. *Mol. Biosyst.* **8**, 1861–1864.
19. Monakhova M., Ryazanova A., Hentschel A., Viryasov M., Oretskaya T., Friedhoff P., Kubareva E. 2015. Chromatographic isolation of the functionally active MutS protein covalently linked to deoxyribonucleic acid. *J. Chromatogr. A.* **1389**, 19–27.
20. Monakhova M., Ryazanova A., Kunetsky V., Li P., Shilkin E., Kisil O., Rao D.N., Oretskaya T., Friedhoff P., Kubareva E. 2020. Probing the DNA-binding center of the MutL protein from the *Escherichia coli* mismatch repair system via crosslinking and Förster resonance energy transfer. *Biochimie.* **171**, 43–54.
21. Stasińska A.R., Putaj P., Chmielewski M.K. 2020. Disulfide bridge as a linker in nucleic acids' bioconjugation: 2. A summary of practical applications. *Bioorg. Chem.* **95**, 103518.
22. Stasińska A.R., Putaj P., Chmielewski M.K. 2019. Disulfide bridge as a linker in nucleic acids' bioconjugation: 1. An overview of synthetic strategies. *Bioorg. Chem.* **92**, 103223.
23. Leichert L.I., Jakob U. 2006. Global methods to monitor the thiol-disulfide state of proteins in vivo. *Antioxid. Redox Signal.* **8**, 763–772.
24. Holliday G.L., Mitchell J.B., Thornton J.M. 2009. Understanding the functional roles of amino acid residues in enzyme catalysis. *J. Mol. Biol.* **390**, 560–577.
25. Luscombe N.M., Laskowski R.A., Thornton J.M. 2001. Amino acid-base interactions: A three-dimensional analysis of protein-DNA interactions at atomic level. *Nucleic Acids Res.* **29**, 2860–2874.
26. Luscombe N.M., Thornton J.M. 2002. Protein–DNA interactions: amino acid conservation and the effects of mutations on binding specificity. *J. Mol. Biol.* **320**, 991–1009.
27. Ghosh G., Duyne G.V., Ghosh S., Sigler P.B. 1995. Structure of NF-κB p50 homodimer bound to a κB site. *Nature.* **373**, 303–310.
28. Chen Y.-Q., Ghosh S., Ghosh G. 1998. A novel DNA recognition mode by NF-κB p65 homodimer. *Nat. Struct. Biol.* **5**, 67–73.
29. Escalante C.R., Shen L., Thana D., Aggaraiwal A.K. 2002. Structure of the NF-κB p50/p65 heterodimer bound to the PRDII DNA element from the interferon-β promoter. *Structure.* **10**, 383–391.
30. Romanenkov A.S., Ustyugov A.A., Zatsepin T.S., Nikulova A.A., Kolesnikov I.V., Metelev V.G., Oretskaya T.S., Kubareva E.A. 2005. Analysis of DNA–protein interactions in complexes of transcription factor NF-κB with DNA. *Biochemistry* (Moscow). **70** (11), 1212–1222.
31. Kim Y., Ho S.O., Gassman N.R., Korlann Y., Landorf E.V., Collart F.R., Weiss S. 2008. Efficient

site-specific labeling of proteins via cysteines. *Bioconjug. Chem.* **19**, 786–791.

32. Kapanidis A.N., Weiss S. 2002. Fluorescent probes and bioconjugation chemistries for single-molecule fluorescence analysis of biomolecules. *J. Chem. Phys.* **117**, 10953–10964.
33. Reck-Peterson S.L., Derr N.D., Stuurman N. 2010. Imaging single molecules using total internal reflection fluorescence microscopy (TIRFM). *Cold Spring Harb. Protoc.* **3**, pdb.top73.
34. Liu J., Hanne J., Britton B.M., Bennett J., Kim D., Lee J.B., Fishel R. 2016. Cascading MutS and MutL sliding clamps control DNA diffusion to activate mismatch repair. *Nature*. **539**, 583–587.
35. Rogulin E.A., Perevyazova T.A., Zheleznaya L.A., Matvienko N.I. 2004. Plasmid pRARE as a vector for cloning to construct a superproducer of the site-specific nickase N.BspD6I. *Biochemistry* (Moscow). **69** (10), 1123–1127.
36. Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature.* **227**, 680–685.
37. Monakhova M.V., Kubareva E.A., Romanova E.A., Semkina A.S., Naberezhnov D.S., Rao D.N., Zatsepin T.S., Oretskaya T.S. 2019. Synthesis of β-diketone DNA derivatives for affinity modification of proteins. *Russ. J. Bioorg. Chem.* **45** (2), 144–154.
38. Pingoud A., Wilson G.G., Wende W. 2014. Type II restriction endonucleases: A historical perspective and more. *Nucleic Acids Res.* **42**, 7489–527.
39. Bitinaite J., Wah D.A., Aggarwal A.K., Schildkraut I. 1998. FokI dimerization is required for DNA cleavage. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 10570–10575.
40. Friedhoff P., Manelyte L., Giron-Monzon L., Winkler I., Groothuizen F.S., Sixma T.K. 2017. Use of single-cysteine variants for trapping transient states in DNA mismatch repair. *Methods Enzymol.* **592**, 77–101.

Translated by T. Tkacheva