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Peculiarities of the interaction of the restriction endonuclease BspD6I with DNA containing its recognition site*



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ABSTRACT

Background: Nicking endonucleases are enzymes that recognize specific sites in double-stranded DNA and cleave only one strand at a predetermined position. These enzymes are involved in DNA replication and repair; they can also function as subunits of bacterial heterodimeric restriction endonucleases. One example of such a proteins is the restriction endonuclease BspD6I (R.BspD6I) from *Bacillus species* strain D6, which consists of the large subunit — nicking endonuclease BspD6I (Nt.BspD6I), and the small subunit (ss.BspD6I). Nt.BspD6I can function independently. Similar enzymes are now widely used in numerous biotechnological applications. The aim of this study was to investigate the fundamental properties of two subunits of R.BspD6I and their interdependence in the course of R.BspD6I activity.

Methods: The binding and hydrolysis of DNA duplexes by R.BspD6I are primary analyzed by gel electrophoresis. To elucidate the difference between Nt.BspD6I interaction with the substrate and product of hydrolysis, the thickness shear mode acoustic method is used.

Results and conclusions: The thermodynamic and kinetic parameters of the Nt.BspD6I interaction with DNA are determined. For the first time we demonstrated that Nt.BspD6I bends the DNA during complex formation. Nt.BspD6I is able to form complexes with the product nicked in the top strand and ss.BspD6I cleaves the bottom strand of the DNA consecutively. Furthermore, the influence of dA methylation in the R.BspD6I recognition site on ss.BspD6I activity is analyzed.

General significance: The obtained results provide evidence that Nt.BspD6l coordinates the activity of R.BspD6l by strictly coupling of the bottom strand cleavage by ss.BspD6l to the top strand cleavage.

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1. Introduction

The widely used type II restriction endonucleases (REs) are a class of enzymes that recognize a short (4–8 bp) specific sequence in doublestranded DNA and cleave both DNA strands inside or near the recognition site. However, these enzymes differ substantially by the composition of the recognition site, organization of their catalytic centers and quaternary structure. The palindromic DNA sequences are usually cleaved by homodimeric REs, containing one catalytic center in each subunit. To introduce a double-strand break into an asymmetric sequence, several strategies are adopted by REs [1]. Two catalytic centers can be localized (i) in one monomeric molecule (e.g. R.Sap, R.Mva1269I [2,3]), (ii) in two identical (e.g. R.Fok [4,5]) or (iii) different subunits (e.g. R.BbvCI [6]). There is a

Abbreviations: AFM, atomic-force microscopy; Bt, biotin residue; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; IC₅₀, half-maximal inhibitory concentration; *K*_{d(app)}, apparent dissociation constant; MTase, M., methyltransferase; m⁶A, 6-methyl-2'-deoxyadenosine; NE, Nt., nicking endonuclease; PAG, polyacrylamide gel; RE, R., restriction endonuclease; R-M, restriction-modification; SD, standard deviation; SDS, sodium dodecyl sulfate; SE, standard error of the mean; ss., small subunit; TBE, Tris-borate-EDTA; TSM, thickness shear mode acoustic method.

 $[\]star$ The paper is dedicated to the memory of Prof. Alfred M. Pingoud, an outstanding scientist in the field of restriction endonuclease investigations.

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Fig. 1. The positions of DNA cleavage by large and small subunits of R.BspD6I. The recognition site is shown in gray, the arrows indicate the DNA cleavage positions. The large subunit of R.BspD6I alone cleaves only one DNA strand, thus, it is nicking endonuclease – Nt.BspD6I. The small subunit (ss.BspD6I) possesses a catalytic site that is only functional when complexed with the large-nicking subunit.

subset of heterodimeric REs with one subunit which can act as individual enzyme, so-called nicking endonucleases (NEs) cleaving specifically only one strand of DNA [7]. These heterodimeric REs represent rather special group of enzymes and the mechanism of their action has not been thoroughly studied yet. NEs themselves nowadays are widely used, e.g. for DNA amplification [8], in sequencing procedures [9], for DNA [10] or

Table 1

DNA duplexes used in experiments.

RNA detection and quantification [11]. To improve the existing methods and to develop new approaches, it is necessary to acquire information about the mechanism of NE action. Moreover, natural NEs have separate DNA-binding and DNA-cleaving domains that make them ideal targets for engineering enzymes with the new specificities [1].

The object of the present study is the heterodimeric restriction endonuclease BspD6I (R.BspD6I) which consists of the large subunit the nicking endonuclease BspD6I (Nt.BspD6I), and the small subunit (ss.BspD6I). Nt.BspD6I from *Bacillus* sp. strain D6 recognizes the pseudosymmetric 5-bp sequence (5'-GAGTC-3'/5'-GACTC-3') and cleaves only one DNA strand containing the sequence GAGTC (top strand) at the distance of four nucleotides from the 3'-end of the recognition site [12] (Fig. 1).

The protein encoded by the open reading frame (ORF) adjacent to the bspD6I gene was found to be the small subunit (ss) of R.BspD6I [13]. It was also shown that ss.BspD6I apart from Nt.BspD6I cannot bind DNA and does not display any nuclease activity. In the presence of Nt.BspD6I, ss.BspD6I cleaves the bottom strand of the DNA duplex preferentially six nucleotides to the 5'-end from the recognition site, while approximately 10% of DNA molecules are cleaved five nucleotides apart (Fig. 1).

DNA duplex $(5' \rightarrow 3'/3' \rightarrow 5')$	Length, bp	Abbreviation
TC GAGTC TTCT↓CAA AG CTCAG AAGA-GTT	14	I-A
TC GAGTC TTCT↓CAAGG AG CTCAG AAGA-GTTCC	16	I-B
TCTC GAGTC TTCT↓CAAGGT AGAG CTCAG AAGA-GTTCCA	19	I-C
TC GAGTC TTCT1CAAGGTACCTG AG CTCAG AAGA-GTTCCATGGAC	22	I-D
CGTGGTCTC GAGTC TTCT↓C-A-AGGTAC GCACCAGAG CTCAG AAGA-G↑T↑TCCATG	26	I-E
GCGTGGTCTC GAGTC TTCT↓C - A-AGGTACCTG CGCACCAGAG CTCAG AAGA-G↑T↑TCCATGGAC	30	I-F
GCGTGGTCTC CAGGT CTTCTCAAGGTACCTG CGCACCAGAG CTCAG AAGAGTTCCATGGAC(T) ₁₀ - Bt	30	I-F'
GCGTGGTCTC GAGTC TTCT↓ pC-A-AGGTACCTG CGCACCAGAG CTCAG AAGAG↑T↑TCCATGGAC	30	I-G
GCGTGGTCTC GAGTC TTCT pCAAGGTACCTG CGCACCAGAG CTCAG AAGAGTTCCATGGAC(T) ₁₀ - <u>Bt</u>	30	I-G'
NNNNN GAGTC NNNNN NNNNN CTCAG NNNNN	120	II-A – II-H*
GTATGAAGCTAGAGCCAGGTTGGCAGCATC CATACTTCGATCTCGGTCCAACCGTCGTAG	30	III
GATGCTGCCAA GAGTC CTCT↓AGCTTCATAC-TAMRA CTACGACGGTT CTCAG GAGA-TCGAAGTATG	30	IV
CGTGGTCTC GAGTC TTCT↓C-C-TGG GCACCAGAG CTCAG AAGA-G↑G↑ACC	23	V-A
CGTGGTCTC GmAGTC TTCT↓C−C−TGG GCACCAGAG C−TCAG AAGA−G↑G↑ACC	23	V-B
CGTGGTCTC GAG-TC TTCT↓C−C−TGG GCACCAGAG CTC<u>mA</u>G AAGA-G↑G↑ACC	23	V-C
CGTGGTCTC GmAG-TC TTCT↓C-C-TGG GCACCAGAG C-TCmAG AAGA-G↑G↑ACC	23	V-D

The recognition site of R.BspD6I is indicated by bold; <u>Bt</u> – biotin residue; \downarrow – the position of Nt.BspD6I cleavage, \uparrow – of ss.BspD6I cleavage; <u>mA</u> – 2'-deoxy-6-methyladenosine (m⁶A). *120-bp duplexes **II-A** – **II-H** contain R.BspD6I recognition site in variable positions (see section "Determination of the DNA bending angle induced by Nt.BspD6I").

In contrast to R.BsrDI and R.Btsl [7], the reconstituted heterodimers consisting of a cleavage-deficient, but DNA binding proficient large subunit (Nt.BspD6I(E418A)) and the wild-type small subunit (ss.BspD6I), result in an inactive enzyme [Yunusova, personal communication]. The crystal structures of Nt.BspD6I and ss.BspD6I without DNA substrate were obtained with high resolution (PDB ID: 2ewf and 2p14, respectively). Nt.BspD6I consists of three domains: DNA recognition and catalytic domains are connected by the linker domain. The structure of ss.BspD6I represents only a catalytic domain and has no DNA recognition domain. Ss.BspD6I shares high sequence and structure similarity to the catalytic domain of the large nicking subunit [14]. It is still unknown how ss.BspD6I interacts with Nt.BspD6I and with DNA. All the attempts to detect such complexes of ss.BspD6I were unsuccessful. Thus, this interaction is supposed to be transient and very weak so the protein-protein complex is very unstable [13].

We are interested to understand the interrelation of the R.BspD6I subunits in DNA cleavage. In this study, we analyzed enzyme activity using modified and non-modified DNA substrates. Moreover, we characterized the nicking endonuclease BspD6I interaction with DNA using short synthetic substrates (14–30 bp) and investigated the influence of the flanking regions on the enzyme activity.

2. Materials and methods

2.1. DNA fragments

Oligonucleotides composing duplexes **I-F**' and **I-G**' (Table 1) were purchased from Thermo Fisher Scientific (USA). 23-mer DNA fragments containing 6-methyl-2'-deoxyadenosine (m⁶A) (Table 1) were purchased from Metabion (Germany). Other oligonucleotides were assembled by the standard phosphoramidite method using an automatic DNA ASM-800 synthesizer (Biosset, Russia) and commercial reagents and solvents (Glen Research, USA).

Labeling the 5'-ends of oligonucleotides with radioactive phosphate was performed by addition of $[\gamma$ -³²P]ATP (1000 Ci/mol, Institute of Molecular Biology, Russia) and T4 polynucleotide kinase (Thermo Fisher Scientific) during 30 min at 37 °C in the buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT. The labeled oligonucleotides were purified on MicroSpin G-50 columns (GE Healthcare, USA). The quantification of the radioactivity was performed by the Cherenkov method with a Tracor Analytic Delta 300 instrument (ThermoQuest/CE Instruments, USA).

DNA duplexes were formed by mixing the complementary oligonucleotides in equimolar amounts, heating to 90 °C and further gradual cooling to room temperature. ³²P-labeled duplexes were composed from labeled and unlabeled oligonucleotides to ensure a uniform radioactivity and equal amounts of the complementary oligonucleotides.

2.2. Protein purification

The gene encoding Nt.BspD6I was expressed in Nova Blue (DE3) Escherichia coli strain (Novagen, USA). Cells were transformed by electroporation first with the pRARE/M.SscL11 plasmid, which contained the gene of methyltransferase SscL11 (M.SscL11) and the genes of tRNA for rare E. coli codons. Subsequently, the cells were transformed by pET28b/ Nick plasmid coding Nt.BspD6I with an additional hexahistidine tag at the C-terminus of the protein [15]. The cells were grown in LB medium containing kanamycin (35 µg/ml, Sigma-Aldrich, USA) and chloramphenicol (10 μ g/ml, Sigma-Aldrich), at 37 °C under the conditions of intensive aeration, until an optical density A_{600} of 0.6 to 0.8. The expression of the recombinant protein was induced by IPTG (Thermo Fisher Scientific) with a final concentration of 0.5 mM. The cultures were grown for additional 3 h and then pelleted by centrifugation (4000 g, 30 min, 4 °C) and frozen. The cells were resuspended in 200 ml of lysis buffer (20 mM K₂HPO₄/KH₂PO₄ (pH 7.5), 1 M KCl, 0.1 mM EDTA, 7 mM βmercaptoethanol) containing the inhibitor of proteases - PMSF (2 mM, AppliChem, Germany). Cell suspensions were sonicated on ice 4×60 s with the interval of 60 s between the impulses. The cellular debris was pelleted by centrifugation during 30 min at 4 °C and 15,000 g. The supernatant was then mixed with imidazole solution to its final concentration of 10 mM and applied to a column filled with 5 ml of Ni-NTA agarose (QIAGEN, Germany) previously equilibrated by buffer A (50 mM K₂HPO₄/KH₂PO₄ (pH 7.5), 1 M KCl, 10 mM imidazole). Nt.BspD6I purification on Ni-NTA agarose was performed according to the protocol QIAexpressionist (QIAGEN). After application of the supernatant, the sorbent was washed with 10 column volumes of the buffer A. Following the first wash step, the column was washed by buffer A containing different concentrations of imidazole: 30 mM for elimination of non-specifically bound proteins and 200 mM for elution of Nt.BspD6I. All procedures of Nt.BspD6I purification were carried out at 4 °C. The purity of Nt.BspD6I preparation was analyzed by SDS-PAGE. After purification Nt.BspD6I preparation was dialyzed against the storage buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 50% glycerol). The concentration of Nt.BspD6I was assessed according to the Beer-Lambert law at 280 nm using the molar extinction coefficient (77,240 M^{-1} cm⁻¹). The active concentration of Nt.BspD6I estimated by Scatchard analysis was about 40% of total protein concentration. Ss.BspD6I was expressed and purified according to the protocol described earlier [13].

2.3. EMSA experiments

Nt.BspD6I–DNA complexes have been formed by incubation of the reaction mixture for 30 min at 37 °C in buffer B (10 mM Tris-HCl (pH 7.5), 150 mM KCl, 1 mM DTT) contained 10 mM CaCl₂ and 0.1 mg/ml BSA. Gel electrophoresis was performed using 7% nondenaturing PAG in TBE buffer (89 mM Tris–borate (pH 8.3), 2 mM EDTA) at 15 mA. A Typhoon FLA 9500 was used to obtain the autoradiographs of the gels. These were analyzed using Quantity One software from Bio-Rad Laboratories (USA). The extent of DNA–protein complex formation (binding, %) was determined as the ratio of radioactivity of the band corresponding to DNA–protein complex to the total radioactivity of the bands corresponding to free DNA and DNA-protein complex. Each experiment was performed at least three times; the values of standard error (SE) did not exceed 8–10%.

The apparent dissociation constants ($K_{d(app)}$) based on the results of EMSA experiments were determined graphically as the point where the fraction of bound DNA equals 0.5. The standard error of the mean was calculated in Origin (OriginLab, USA) according to the formula SE = $s/n^{0.5}$, where SE – standard error, s – standard deviation, and n – number of independent experiments.

2.4. Hydrolysis of DNA duplexes

Hydrolysis of DNA duplexes was performed by incubation of the reaction mixture during 30 min at 37 °C in the buffer B containing 10 mM MgCl₂ and 0.1 mg/ml BSA. The concentrations of DNA duplexes, Nt.BspD6I, and ss.BspD6I are indicated in the text. Gel electrophoresis was performed using 20% PAG with 7 M urea in TBE buffer at 30 mA. The amount of DNA hydrolysis (% cleavage) was determined separately for each strand (radioactively or fluorescently labeled) as the ratio of the product to the total DNA. Each measurement was performed at least three times; the SE did not exceed 8–10%.

2.5. Determination of the DNA bending angle induced by Nt.BspD6I

Circular permutation analysis [16] was used for the estimation of the DNA bending angle during Nt.BspD6l binding. First, 120-bp duplexes **II-A** – **II-H** containing Nt.BspD6l recognition site at different positions within the linear DNA fragment were generated. The plasmid pBEND2 (kindly gift of Dr. S. Adhya), bearing the tandem recognition sites of several REs (Fig. 2(a)), was used for this purpose. This plasmid was hydrolyzed by REs from Thermo Fisher Scientific: BglII, NheI, SpeI, XhoI,



Fig. 2. Analysis of DNA bending by Nt.BspD6I. (a) Fragment of pBEND2 plasmid containing tandem recognition sites of R.BgIII, R.Nhel, R.Spel, R.Xhol, R.EcoRV, R.Smal, R.Stul, R.BamHI and 120-bp duplexes **II-A** – **II-H** with Nt.BspD6I recognition site (gray rectangle) at different positions, which were obtained by digestion of the plasmid by appropriate REs. (b) Complex formation of Nt.BspD6I with DNA fragments **II-A** – **II-H** (indicated above gel lines on photography). A 7% non-denaturing PAG after staining with SYBR Gold. (c) Fitting the obtained data to parabolic function *y* = 0.85*x*²–0.83*x* + 0.63 (see the text in paragraph 2.5.).

EcoRV, Smal, Stul or BamHI at 37 °C for 30 min in buffer supplied with the enzyme. The resulting 120-bp fragments **II-A** – **II-H** were purified by PCR clean-up and gel extraction kit (Macherey-Nagel, Germany) after electrophoresis in a 2% agarose gel.

Fragments **II-A** – **II-H** were incubated with Nt.BspD6I in the buffer B at 37 °C for 30 min. Each reaction mixture (10 μ l) contained 100 nM DNA and 50 nM Nt.BspD6I. DNA–protein complexes and unbound substrates were separated by electrophoresis in 7% non-denaturing PAG as described above. The gels were finally stained with SYBR Gold (Invitrogen, USA) for 20 min and documented with a Typhoon FLA 9500.

The DNA bend angle was determined using Ferrari model [16]. Data were fitted to the parabolic function $y = ax^2 - bx + c$, where *x* stands for the ratio of the length from the middle of the binding site to the end of the fragment and total fragment length, *y* stands for the ratio of the mobility of the DNA–protein complex and the mobility of free DNA, and the coefficients *a*, *b*, and *c* determine the angle value as

$$a = -b = 2c \times (1 - \cos \alpha) \tag{1}$$

The experiment was repeated 4 times, the SE for the angle value did not exceed 4° .

2.6. Immobilization of DNA at the gold surface of thickness shear mode transducer

In the binding studies of Nt.BspD6I at surface we used AT-cut quartz crystal transducer with a fundamental frequency of 8 MHz (CH Instruments, USA). The transducer was covered on both sides by thin gold layers that served as electrodes (working area 0.2 cm^2). After the careful cleaning of the crystal (see [17] for details) it has been mounted between two silicon O-rings in the flow-through cell. The working volume of the cell was $100 \,\mu$ [18]. The analyte was introduced into the cell using a Genie Plus syringe pump (Kent Scientific, USA) with a flow rate of $50 \,\mu$ /min. Every solution was applied until the resonance frequency stabilized. The transducer surface was first washed with deionized water, and then 0.2 mg/ml NeutrAvidin (Pierce Biotechnology, USA) solution in water was applied. Approx. 15 min were necessary for signal stabilization in a constant flow rate of the NeutrAvidin. It was followed by water in order to wash out all non-chemisorbed NeutrAvidin molecules.

30-bp DNA duplexes **I-F'** and **I-G'** containing biotin moiety at 5'end of the bottom strand were used in the experiments. 1 μ M DNA duplex **I-F'** or **I-G'** dissolved in a binding buffer B, containing 10 mM CaCl₂, was applied and subsequently the crystal was rinsed with the same buffer again. This buffer was suitable for the study of Nt.BspD6I–DNA binding. Then Nt.BspD6I was applied in concentrations of 0.03, 0.06, 0.1, 0.3 and 1.0 μ M in the buffer B* that represented the buffer B containing 10 mM CaCl₂ and 2.9% of glycerol. After every addition of enzyme the surface of the sensor was washed with the buffer B* and subsequently Nt.BspD6I with a higher concentration was added.

2.7. Detection of Nt.BspD6I–DNA interaction by thickness shear mode method

The thickness shear mode (TSM) acoustic method is based on application of a high frequency AC voltage to an AT-cut quartz crystal. The acoustic shear wave is generated due to the piezoelectric effect and propagates through the sensing layer into the liquid. This acoustic wave can be characterized by storage and dissipation processes. The



Fig. 3. Cleavage (a) and binding (b) of different DNA duplexes by Nt.BspD6I analyzed by gel electrophoresis. All duplexes contain ³²P-lable at the 5'-end of the top strand. Autoradiographs of a 20% PAG with 7 M urea (a) and 7% non-denaturing PAG gel (b). Numbers and lengths of the duplexes are indicated above gel lines on autoradiographs. Concentration of DNA was 10 nM, concentration of Nt.BspD6I in DNA cleavage experiments was 10 nM, in binding assays – 25 nM. The SE did not exceed 10% of the indicated extent of DNA cleavage or binding by Nt.BspD6I.

measured electrical impedance of the sensor is related to the complex acoustic impedance. The imaginary part of the impedance represents the energy storage and is proportional to the change in the series resonance frequency, Δf_s , while the real part is connected with the energy dissipation and is related to the motional resistance, R_m . The viscous forces result in a friction between the surrounding liquid and the layer immobilized on the surface of the crystal [18] being reflected in an increase of R_m and a decrease of f_s . A network analyzer 8712ES (Agilent Technologies, USA) was used to measure the impedance properties of the sensor and for determination of f_s and R_m . See Snejdarkova et al. [19] for the details of the experimental setup. Please note that TSM experiments were performed at 25 °C. This is due to the fact that at this temperature the AT-cut quartz crystals provide most stable oscillations. At the higher temperatures, above 35 °C the oscillations depend on the temperature, therefore certain errors can be expected.

3. Results and discussion

3.1. Characterization of nicking endonuclease BspD6I interaction with DNA

3.1.1. Screening of DNA substrate of optimal length for efficient enzyme binding and hydrolysis

The specific interaction of RE with DNA takes place only in the presence of its recognition site. However, the sequences flanking the recognition site also influence DNA hydrolysis. Especially, the length of these flanking regions can be an essential factor during formation of the enzyme–substrate complex [20]. Nt.BspD6I was used for screening the optimal substrate of R.BspD6I since the DNA binding region of R.BspD6I is supposed to be located only in Nt.BspD6I [14]. In the previous studies the catalytic activity of Nt.BspD6I and its isoschizomers from different organisms [21–25] were tested using substrates of different length (plasmids, phage T7 DNA). However, the interaction of these enzymes with short synthetic DNA fragments was not investigated at all. These data are essential for some application of the enzyme (see above).

To analyze the influence of the regions flanking the recognition site of Nt.BspD6I on its activity, the substrates with different length were constructed (Table 1, duplexes I-A, I-B, I-C, I-D, I-E, I-F). The length of these duplexes varied from 14 to 30 bp. Duplexes I-A, I-B and I-D have short sequence (2 bp) flanking the recognition site at the 5'-end, whereas the sequences after the position of hydrolysis vary from 3 to 11 bp. Duplex I-C contains 4 bp in the front of the recognition site and 6 bp after the position of hydrolysis. The regions flanking the binding and cleavage sites are extended in substrates I-E and I-F, their length exceeds 7 bp.

Nt.BspD6I is a thermophilic enzyme with optimal temperature for DNA cleavage at 55 °C. However, it can bind and hydrolyze the substrate at lower temperature as well [10]. To avoid the dissociation of the short DNA duplexes and weak protein–DNA complexes we performed the experiments at 37 °C. All DNA duplexes contained ³²P-label at the 5'-end of the top strand.

Nt.BspD6I was shown to hydrolyze all duplexes in the presence of Mg^{2+} as cofactor at 37 °C in 30 min with high efficacy (>80%, Fig. 3(a)). The DNA binding assay was performed in the presence of Ca^{2+} instead of Mg^{2+} to prevent DNA cleavage. Under these conditions, we could not detect stable complexes of Nt.BspD6I with all these duplexes. Only in the case of duplexes **I-E** and **I-F** around 80% of enzyme molecules were bound to DNA, for the other duplexes the extent of binding was between 3 and 50% (Fig. 3(b)).

We can conclude that Nt.BspD6I can hydrolyze short duplexes containing 2 bp at the 5'-end of the recognition sequence and 3 bp downstream the 3'-end of the cleavage site (7 bp from 3'-end of the recognition site). It was known that sequences flanking the recognition site in general modulate the thermodynamic and kinetic parameters of the interaction between restriction endonucleases and their targets [20]. Duplexes **I-E** and **I-F** (with the most extended flanking sequences among the DNA fragments studied in this work) were chosen as the



Fig. 4. Hydrolysis of ³²P-labeled bottom strand of duplex **I-E** (10 nM) by different amount of ss.BspD6I in mixture with Nt.BspD6I. Autoradiograph of a 20% PAG with 7 M urea. Lane 1: initial DNA, lane 2: control reaction mixture containing only duplex and ss.BspD6I (180 nM), lanes 3–6: DNA cleavage by ss.BspD6I (30, 60, 120, 180 nM, respectively) in the presence of Nt.BspD6I (10 nM). The reaction mixtures were incubated at 37 °C for 30 min. DNA size markers – 6-mer (5'-GTACCT-3') and 7-mer (5'-GTACCTT-3') are presented in lines 7 and 8, respectively. The SE did not exceed 8% of the indicated extent of DNA cleavage by ss.BspD6I in the presence of Nt.BspD6I.

optimal substrates for further investigation of Nt.BspD6l activity to minimize the impact of flanking sequences on the interaction of Nt.BspD6l with DNA. The apparent dissociation constants ($K_{d(app)}$) determined by EMSA for Nt.BspD6l complexes with 26 bp duplex **I-E** (7 \pm 1 nM) and 30 bp duplex **I-F** (8 \pm 1 nM) indicate the high affinity of enzyme to DNA (data not shown).

3.1.2. Does small subunit of BspD6I restriction endonuclease have an influence on the DNA hydrolysis by nicking endonuclease BspD6I?

It was previously shown that Nt.BspD6I is essential for DNA hydrolysis by ss.BspD6I [13]. Does ss.BspD6I on the other hand have an influence on Nt.BspD6I functionality? To answer this question we studied the hydrolysis of the top strand of the 26-bp DNA substrate **I-E** by Nt.BspD6I alone and in the presence of ss.BspD6I.

The initial step of investigation was the selection of the concentrations of Nt.BspD6I and ss.BspD6I for the formation of heterodimeric R.BspD6I, which could efficiently hydrolyze both strands of a DNA substrate. For this purpose, the ratio of R.BspD6I subunits resulting in the highest activity was determined experimentally. The 26-bp duplex **I-E**



Fig. 5. Time course of ³²P-labeled top strand of duplex **I-E** cleavage performed by Nt.BspD6I alone (black curve) or in the presence of ss.BspD6I (gray curve).

was chosen as a substrate. The hydrolysis of each DNA strand was investigated in two separate experiments. Duplex **I-E** with a 5'-³²P-labeled top or bottom strand was used for the study of the hydrolysis by Nt.BspD6I or by small subunit (ss.BspD6I) of R.BspD6I, respectively. Concentrations of 10 nM for Nt.BspD6I and 10 nM for the substrate were chosen for the efficient hydrolysis of the top strand during 30 min, at 37 °C, in the presence of 10 mM MgCl₂. Under such conditions Nt.BspD6I cleaves > 80% of 5'-³²P-labeled top strand of substrate I-E (Fig. 3(a)). For the investigation of ss.BspD6I activity, the substrate (10 nM), containing ³²P-label at the 5'-end of the bottom strand, and Nt.BspD6I (10 nM) were mixed with small subunit of R.BspD6I. The ratio of Nt.BspD6I to ss.BspD6I was 1:3, 1:6, 1:12 and 1:18. Ss.BspD6I hydrolyzed the substrate efficiently at a 12- or 18-fold excess relative to Nt.BspD6I, the extent of hydrolysis was 63 \pm 5% or 72 \pm 6% respectively (Fig. 4). We detected a prevalence for the 6-mer product (cleavage at 6th position to the 5'-end from the recognition site, Fig. 1) over the 7-mer (cleavage at 5th position to the 5'-end from the recognition site, Fig. 1) in the case of bottom-strand hydrolysis by ss.BspD6I of 26 bp duplex I-E that is consistent with the previous studies [13,26].

Next, we analyzed the steady state kinetics of the hydrolysis of **I-E** duplex (10 nM) containing the radioactively labeled top strand by Nt.BspD6I alone (10 nM) and in the presence of 12-fold excess of ss.BspD6I. It was shown that the initial hydrolysis rate by Nt.BspD6I was 2 times higher in the presence of small subunit (Fig. 5). Probably Nt.BspD6I dissociates from the duplex cleaved in the both strands faster than from the nicked duplex, and therefore can perform catalysis on other DNA molecules. The plateau of the kinetic curves corresponded to 80% cleavage of the substrate, so Nt.BspD6I was inhibited in the course of the steady-state cleavage reaction.

According to these data, not only Nt.BspD6I is essential for the activity of ss.BspD6I, but also ss.BspD6I facilitates efficient hydrolysis by Nt.BspD6I.

3.1.3. Estimation of DNA bending angle induced by Nt.BspD6I

The process of DNA bending is essential for some restriction enzymes when forming the catalytically active complex (e.g. R.EcoRV). This angle can be determined by direct measurements (e.g. X-ray analvsis, AFM), which require sophisticated instrumentation and have some limitations. There is no experimental information about DNA bending induced by R.BspD6I. Previous attempts to crystallize the complexes of R.BspD6I–DNA, ss.BspD6I–DNA or Nt.BspD6I–DNA have failed. Since the latter complex is detected by the gel shift assay, the DNA bending angle induced by Nt.BspD6I was estimated by so-called circular permutation analysis described by Ferrari et al. [16]. The method is based on the fact that the electrophoretic mobility of the bent DNA depends on the position of the bend. In case of DNA bending induced by protein, mobility of DNA fragments depends on the position of the protein recognition site. To perform the experiment it is necessary to obtain DNA fragments of the equal length, but with different position of protein recognition site. To generate such DNAs we used special bending vector pBend2. Plasmid pBend2 was originally constructed by Kim et al. [27]. It contains tandem repeats of restriction endonucleases sites and the Nt.BspD6I recognition site at its center (Fig. 2(a)). Sequential digestion with different restriction endonucleases produces DNA fragments of the equal length but with different position of Nt.BspD6I recognition site.

First of all, the obtained DNA fragments (100 nM) were incubated with Nt.BspD6I in different concentrations (5–60 nM). Corresponding DNA–protein complexes were analyzed via EMSA. The predominant formation and the best yield of a complex between an Nt.BspD6I monomer and each duplex (100 nM) was found at protein concentration of 50 nM (see, e.g., Fig. S1 in supplement). The results of 120-bp DNA fragments **II-A** – **II-H** interactions with Nt.BspD6I are presented in Fig. 2(b). In our experiments coefficients *a* and –*b* in Eq. (1) in Ferrari method were very similar and indicated a DNA bending angle of $66 \pm 4^{\circ}$ for



Fig. 6. Complex formation of Nt.BspD6l, ss.BspD6l, and R.BspD6l with duplexes **I-F** and **I-G** (the top strands are ³²P-labeled). Autoradiograph of a 7% non-denaturing PAG. Numbers of the duplexes are indicated above gel lanes. Lanes 1, 5: initial DNA fragments; lanes 2, 6: complex formation of 25 nM Nt.BspD6l with 10 nM duplex **I-F** or **I-G**; lanes 3, 7: complex formation of 300 nM ss.BspD6l with 10 nM duplex **I-F** or **I-G**; lanes 4, 8: – complex formation of R.BspD6l (25 nM Nt.BspD6l + 300 nM ss.BspD6l) with 10 nM duplex **I-F** or **I-G**. The reaction mixtures were incubated at 37 °C, 30 min. The SE did not exceed 8% of the indicated extent of DNA binding by protein.

DNA in the complex with Nt.BspD6I (Fig. 2(c)). Our result is contradictional to the proposed model of R.BspD6I complexed with DNA [14]; where the DNA is in a straight conformation. It seems that this model requires some correction considering the obtained data, particularly as also Machulin et al. [28] observed a DNA bending by Nt.BspD6I using AFM.

There is no common rule for the DNA bending among type II restriction endonucleases. In some cases, no significant deviations from the canonical DNA form can be observed in complex with RE (for R.BamHI, R.BsoBI), but in the specific DNA–R.EcoRV complex, the DNA is bent by about 50° [20]. As mentioned earlier, the formation of a DNA– ss.BspD6I complex has not been detected. Since the small subunit lacks DNA-binding ability, it cannot induce DNA bending. Furthermore, the presence of ss.BspD6I did not affect the DNA bending by Nt.BspD6I (data not shown). Therefore, the small subunit of the heterodimeric



Fig. 7. The plot of the changes in the series resonant frequency Δf_S as a function of the protein (Nt.BspD6I or human serum albumin (HSA)) concentration for the acoustic biosensor based on DNA duplexes of different structures. In addition, the data of two control experiments are shown: Nt.BspD6I application to the surface without DNA and HSA application to the surface modified with duplex I-F'.

R.BspD6I does not contribute to DNA bending by Nt.BspD6I. However, we suggest that the DNA bending by Nt.BspD6I is a prerequisite for Nt.BspD6I cleavage activity as well as for ss.BspD6I binding to the Nt.BspD6I-DNA complex.

3.1.4. Nicking endonuclease BspD6I binding with DNA fragment that is identical to the product of its own hydrolysis

DNA duplex **I-G** consists of the three oligonucleotides, one of them in the top strand carries a phosphate group at the 5'-terminus. Thus, DNA duplex **I-G** mimics the product of **I-F** hydrolysis by Nt.BspD6I (Table 1).

We assessed the affinity of Nt.BspD6I to the product of its hydrolysis – the duplex **I-G** with a single-strand break in the top strand. The EMSA demonstrated the ability of Nt.BspD6I to bind duplex **I-G** almost with the same efficacy as the initial substrate **I-F** (Fig. 6). Both duplexes contained ³²P-label at the 5'-end of the top strand. However, complexes detected by gel shift assays reflect the equilibrium state of protein–DNA interaction and do not allow to study the kinetics of this process. The kinetic properties of Nt.BspD6I binding to the DNA have been studied by the TSM acoustic method. This method is very effective for analyzing on-line kinetics of binding processes at surfaces [29] and has been successfully applied for studying (cytosine-5)-DNA methyltransferase SsoII interaction with different DNA substrates [30].

The advantage of TSM method is that it does not require any labeling of the interacting molecules like in the conventional gel shift assays. On the other hand, it is necessary to immobilize DNA on the surface of a sensor, therefore we used duplexes I-F' and I-G' that contained a biotin residue covalently attached to the 5'-end of the bottom strand (Table 1). Biotin containing duplexes I-F' and I-G' have been immobilized at the NeutrAvidin layer chemisorbed at the gold surface of quartz crystal transducer. The interaction of Nt.BspD6I with DNA has been analyzed at 25 °C by measurement of the changes of series resonant frequency of crystal oscillation, Δf_s , and by motional resistance, ΔR_m . The changes in resonance frequency are attributed to the changes of thickness or mass of the DNA layer following binding of Nt.BspD6I, while the changes in motional resistance reflect the viscosity contribution that arises from friction between the DNA layer and the surrounding water solution. The addition of the I-F' duplex onto the surface of NeutrAvidin layer in a concentration of 1 µM resulted in decrease of the resonant frequency by 20 Hz. Considering the molecular weight of I-F' duplex (21.7 kDa), the surface concentration can be estimated using Eq. (2)as 6 pmol cm $^{-2}$:

$$\Delta f_{S} = -2.26 \times 10^{-6} f_{0}^{2} \Delta m / A \tag{2}$$

where *A* is the area (0.2 cm² in our case) and f_0 is the fundamental frequency of the crystal (8 MHz). Similar surface concentration (8 pmol cm⁻²) has been obtained also for **I-G**' duplex.

The addition of Nt.BspD6l onto the surface of TSM transducer with immobilized **I-F**' or **I-G**' duplexes resulted in decrease of resonant frequency, f_s . The plot of the changes of resonant frequency vs. Nt.BspD6l concentration obtained for duplexes **I-F**' and **I-G**' had, however, a different maximum frequency change: almost 200 Hz decrease for duplex **I-F**' and 140 Hz for duplex **I-G**' (Fig. 7) observed after applying 1 μ M of Nt.BspD6l. It is previously shown, that Nt.BspD6l is able to oligomerize at concentrations higher than 0.5 μ M [31]. Using the frequency change of 200 Hz and molecular weight of Nt.BspD6l (70.8 kDa) the surface

 Table 2

 Kinetic parameters of Nt.BspD6I binding to different DNA duplexes immobilized on the TSM transducer.

Parameter	Duplex I-F'	Duplex I-G'
k _a , M ⁻¹ s ⁻¹ k _d , s ⁻¹ K _d , nM	$\begin{array}{c} 14,300\pm150\\ 0.0007\pm0.00005\\ 46\pm4\end{array}$	$\begin{array}{c} 10,700 \pm 600 \\ 0.0009 \pm 0.00005 \\ 88 \pm 11 \end{array}$

concentration of NE can be estimated according to Eq. (2) as 19.5 pmol cm⁻²:

Comparison of this surface concentration with that of DNA suggests that at saturation about three molecules of Nt.BspD6I are adsorbed on one DNA duplex. Thus, the possible stoichiometry of Nt.BspD6I-I-F' complex at high Nt.BspD6I concentration is 3:1. However, this is rather rough estimation. Taking into account the increase of motional resistance, $R_{\rm m}$, following addition of Nt.BspD6I, one can expect that the changes of the resonant frequency can be particularly due to the increased friction between the DNA-protein layer and the buffer. In any case, the larger frequency changes for Nt.BspD6I complexes with duplex I-F' suggest that more molecules of Nt.BspD6I are able to bind this duplex compared to the duplex I-G'. Certainly, in latter case the surface concentration of Nt.BspD6I estimated according to Eq. (2) and frequency changes of 140 Hz is 13.7 pmol cm⁻². This corresponds approximately to a 2:1 stoichiometry of Nt.BspD6I on the I-G' duplex. In order to analyze quantitatively the kinetics of Nt.BspD6I association with the DNA, we used an approach based on the principles similarly to those applied by Thompson and Tassew [18]. According to this method the frequency changes can be expressed as

$$-\Delta f_{S}(t) = -(\Delta f_{S})_{max} \times [1 - exp(-kt)]$$
(3)

where $k = k_a[P] + k_d$ (k_a and k_d are the association and dissociation rate constants, respectively; [P] is the concentration of Nt.BspD6I). The dependence of k on Nt.BspD6I concentration should be a straight line [32]. The intercept of the line with the *y* axis gives the k_d value, while the $k_{\rm a}$ is equal to the line's slope. The equilibrium dissociation constant (K_d) could be determined from the k_a and k_d values: $K_d = k_d/k_a$. These calculations allowed us to determine the k_a and k_d values (Table 2). The example of the kinetic changes of the resonant frequency, f_s, following addition of various concentration of Nt.BspD6I to a surface of TSM transducer with immobilized **I-F**' duplex and corresponding plot of k on Nt.BspD6I concentration are shown in supplements (Fig. S2 and S3). As it can be seen from Table 2, the complex of Nt.BspD6I with duplex I-F' is more stable compared to the complex with duplex I-G'. According to the obtained data Nt.BspD6I is able to bind the product of hydrolysis. Binding the product of hydrolysis and hindered dissociation of the enzyme from this complex can be the rate-limiting steps of enzymatic reaction that results in further inhibition of substrate hydrolysis as we demonstrated above.

The formation of Nt.BspD6I complexes with duplexes I-F and I-G was also confirmed by EMSA and is shown in Fig. 6. The K_{app} values



Fig. 8. Inhibition of cleavage of 3'-TAMRA-labeled substrate **IV** (10 nM) by Nt.BspD6I (10 nM) in the presence of duplexes **I-F**, **I-G**, and non-specific duplex **III.** The concentrations of duplexes-competitors varied from 0 to 2 µM. Excess of competitors is indicated on the x-axis in logarithmic scale. Reaction conditions were 25 °C, 30 min.

for Nt.BspD6I complex with duplex I-F determined by TSM method are significantly higher than the ones estimated by EMSA. This can be explained by several reasons. A gel-shift assay allows to study DNAprotein binding in solution and requires the incorporation of fluorescent or radioactive labels into protein or DNA. The peculiarities of TSM method is that it studies protein-DNA interactions at the surfaces and does not require any labeling of the interacting molecules like in the conventional assays. Therefore, rather high concentrations of interacting molecules are needed to get the significant effect using TSM method. Indeed, the maximum Nt.BspD6I concentration applied to the sensing surface with immobilized DNA during our experiments was 1 µM that is considerably higher than the maximum concentration used for EMSA (30 nM). The higher protein concentrations and therefore different stoichiometry of DNA-protein complexes are the other reasons underlying distinction between obtained K_d values. However, the fact of Nt.BspD6I complex formation with product of its DNA hydrolysis was demonstrated by both approaches.

It was previously shown that Nt.BspD6I possesses the ability of nonspecific DNA binding [31]. To assess the extent of such non-specific binding with DNA, we analyzed the hydrolysis of duplex **IV** that contained fluorescent label TAMRA on the 3'-end of its top strand in the presence of different competitors: canonical duplex **I-F**, duplex **I-G** that corresponds to the product of hydrolysis by Nt.BspD6I, and 30-bp non-specific duplex **III** without the Nt.BspD6I recognition site (Fig. 8). The half-maximal inhibitory concentrations (IC₅₀) were determined for these duplex-competitors.

Under the conditions used, duplex **I-F** was converted into duplex **I-G**, therefore DNA duplexes **I-F** and **I-G** almost identically influenced the activity of Nt.BspD6I. The IC_{50} value for these duplexes corresponded to their 3-fold excess in the reaction mixture (30 nM). Non-specific duplex **III** of the same length inhibited the activity of Nt.BspD6I much slower. Only 100-fold excess of this duplex (1 μ M) lowered the enzymatic activity by half. Therefore, the non-specific binding of Nt.BspD6I with DNA is rather low and the obtained kinetic parameters correspond mainly to the specific Nt.BspD6I–DNA interactions.

3.2. Characterization of the DNA cleavage by small subunit of restriction endonuclease BspD6I in the presence of nicking endonuclease BspD6I

To investigate the interrelation between the subunits of R.BspD6I during catalysis at least two issues must be addressed: (i) Is ss.BspD6I able to cleave the bottom strand after Nt.BspD6I has introduced a nick in the top strand of DNA duplex? (ii) Is ss.BspD6I able to hydrolyze the bottom strand if cleavage of the top strand by Nt.BspD6I is blocked?

Two types of R.BspD6I substrate analogues were used for these purposes – DNA duplex with a nick in position of Nt.BspD6I hydrolysis (duplex **I-G**) and DNA duplexes containing 6-methyl-2'-deoxyadenosine ($m^{6}A$) in the recognition site of Nt.BspD6I.

3.2.1. The ability of ss.BspD6I to hydrolyze a substrate after Nt.BspD6I action

Ss.BspD6I does not form complexes with duplexes I-F and I-G either in the presence or in the absence of Nt.BspD6I, it also does not interfere with complex formation of Nt.BspD6I with these duplexes (Fig. 6). We further studied the hydrolysis of duplexes I-F and I-G by ss.BspD6I alone and in the presence of Nt.BspD6I (Fig. 9). Both duplexes contained the bottom strand that was ³²P-labeled at 5'-end. In control experiments in the absence of Nt.BspD6I, ss.BspD6I was not able to hydrolyze duplexes I-F and I-G (Fig. 9(a), lanes 2 and 5). Nt.BspD6I addition to the reaction mixture resulted in the hydrolysis of these duplexes by ss.BspD6I. Two ³²P-labeled DNA products were detected in each case: 10-mer 5'-32P-CAGGTACCTT-3' and dominant 9-mer 5'-32P-CAGGTA CCT-3' (Fig. 9(a), lanes 3 and 6). The initial rate of hydrolysis by ss.BspD6I at 37 °C of I-F duplex was shown to be slightly higher than the one of the **I-G** duplex (Fig. 9(b)). This fact can be explained by the intrinsic higher affinity of Nt.BspD6I to its canonical substrate comparing with the product of cleavage (Table 2) that directly affects ss.BspD6I activity. According to our data, the ability of Nt.BspD6I to stay in the complex with the nicked top strand allows ss.BspD6I to introduce the second break in the bottom strand of the DNA. We assume that the hydrolysis of DNA by Nt.BspD6I and ss.BspD6I is strictly coupled. First Nt.BspD6I binds and bends the DNA substrate. This enables the cleavage domain of Nt.BspD6I to nick the top strand. Ss.BspD6I is capable of binding this binary complex, nicking the bottom strand and thereby to facilitate product release.

3.2.2. Interaction of subunits of restriction endonuclease BspD6I with DNA ligands containing N6-methyl-2'-deoxyadenosine in the recognition site

Next, we analyzed the ability of ss.BspD6I to hydrolyze DNA substrate when the cleavage of Nt.BspD6I is blocked. For this study it was necessary first to choose non-hydrolyzable substrate analogues for Nt.BspD6I. Nt.BspD6I was previously shown to be sensitive to methylation of adenosine in the recognition site in the case of a 23-bp dimethylated substrate [33].



Fig. 9. Analysis of ss.BspD6l activity. (a) Incubation of ³²P-labeled bottom strand of duplexes **I-F** and **I-G** (10 nM) with ss.BspD6l (120 nM) alone and in the presence of Nt.BspD6l (10 nM). Autoradiograph of a 20% PAG with 7 M urea. Numbers of the duplexes are indicated above gel lanes. Lanes 1, 4: initial DNA fragments; lanes 2, 5: duplexes **I-F** and **I-G** mixed with ss.BspD6l; lanes 3, 6: hydrolysis of duplexes **I-F** and **I-G** by ss.BspD6l in the presence of Nt.BspD6l. The reaction mixture was incubated at 37 °C for 30 min. The SE did not exceed 8% of the indicated extent of DNA cleavage by ss.BspD6l in the presence of Nt.BspD6l. (b) Time course of ³²P-labeled bottom strand cleavage of duplexes **I-F** (black curve) and **I-G** (gray curve) performed by ss.BspD6l in the presence of Nt.BspD6l. (b) Time course of ³²P-labeled bottom strand cleavage of duplexes **I-F** (black curve) and **I-G** (gray curve) performed by ss.BspD6l in the presence of Nt.BspD6l. (b) Time course of ³²P-labeled bottom strand cleavage of duplexes **I-F** (black curve) and **I-G** (gray curve) performed by ss.BspD6l in the presence of Nt.BspD6l.



Fig. 10. Nt.BspD6I interaction with the duplexes **V-A** – **V-D** (the top strands are ³²P-labeled) depending on the presence and localization of m⁶A in the recognition site. (a) Complex formation between Nt.BspD6I and duplexes **V-A** – **V-D**. Autoradiograph of a 7% non-denaturing PAG. (b) DNA cleavage by Nt.BspD6I. Autoradiograph of a 20% PAG with 7 M urea. In all the experiments concentrations of DNA duplexes and Nt.BspD6I were 10 nM.

Nt.BspD6I and ss.BspD6I are the part of restriction-modification system (R-M system) [13]. The BspD6I R-M system contains two methyltransferases (MTases). These MTases gene sequences are completely identical with the M1.BstSEI and M2.BstSEI genes found in Bacillus stearothermophilus (T.A. Perevyazova, unpublished data, [34]). Chernukhin et al. [35] showed, that purified recombinant M2.BstSEI alone (in the absence of M1.BstSEI) is capable to modify adenine in both DNA strands of double-stranded 5'-GASTC-3' (S = G or C) sequence. Moreover, the kinetic parameters of DNA methylation by M2.BstSEI (homolog of M2.BspD6I) are similar to those of DNA MTases that recognize the palindromic sites [35]. We expect that one of MTases from R-M systems (like the BspD6I or BstSEI R-M system), containing nicking endonuclease, serves not only for the protection of host DNA from digestion by RE but carries out some other functions, for example, it might take part in DNA repair together with NE. Therefore, it was important to study for the first time Nt.BspD6I interactions with DNA fragments that reflect the DNA modification by cognate MTases. Moreover, the influence of DNA methylation on ss.BspD6I activity has never been studied.

We used duplexes **V-A** – **V-D** (5'-label was at the top strand) to investigate the binding of Nt.BspD6I with mono- (**V-B**, **V-C**) and dimethylated (**V-D**) substrates comparing to non-methylated one (**V-A**) (Fig. 10(a)). The enzyme bound efficiently only the non-methylated duplex **V-A** at equimolar ratio of Nt.BspD6I and DNA. The extents of Nt.BspD6I binding with other DNA duplexes did not exceed 10%.

In the next step we analyzed hydrolysis of these duplexes by Nt.BspD6I. Only non-methylated duplex **V-A** was cleaved (Fig. 10(b)). Probably, the methylation of the dA residue in Nt.BspD6I recognition site interferes with substrate binding and inhibits conformational changes of Nt.BspD6I–substrate complex that are a prerequisite for catalysis.

Further, the influence of the methyl group in the recognition site on the activity of ss.BspD6I (in the presence of Nt.BspD6I) was investigated. Hydrolysis of DNA duplexes (10 nM, 5'-label was in the bottom strand) was performed using 10 nM Nt.BspD6I and 120 nM of ss.BspD6I or 50 nM Nt.BspD6I and 50, 300 or 600 nM of ss.BspD6I. Cleavage of the monomethylated duplexes (**V-B**, **V-C**) by ss.BspD6I was not detected as is shown in supplement (Fig. S4). In other words, the top-strand methylation of dA residue in the recognition site (duplex **V-B**) also blocks the bottom-strand nicking. The same effect was observed when methylated dA residue was presented in the bottom strand (duplex **V-C**) and in both strands (duplex **V-D**). The data obtained confirm the hypothesis that the subunits act in a coordinated manner and show that effective DNA binding and cleavage by Nt.BspD6I are likely to be the essential factors for ss.BspD6I activity.

4. Conclusions

Using the set of DNA duplexes of different length, we demonstrate that Nt.BspD6I can hydrolyze short 14-30 bp duplexes. Only 2 bp at the 5'-end and 7 bp from the 3'-end of the recognition site are sufficient for top-strand nicking. Extension of flanking sequences does not improve the nicking efficiency. DNA fragments of equal length but with different position of Nt.BspD6I recognition site permit us to determine a bending angle of 66 \pm 4° for DNA in complex with Nt.BspD6I. For the first time we demonstrated that the small subunit of R.BspD6I is important for Nt.BspD6I activity increasing 2-fold the initial rate of substrate hydrolysis by nicking endonuclease. Using gel shift assays and the thickness shear mode acoustic method, Nt.BspD6I is shown to be able to form the complex with the product of DNA hydrolysis. Ss.BspD6I effectively hydrolyzes the bottom strand when the top strand of DNA duplex has been cleaved by Nt.BspD6I. Finally, the influence of the methylation of dA residue in R.BspD6I recognition site on ss.BspD6I activity is analyzed. The repression of Nt.BspD6I activity by m⁶A in the recognition site results in the inhibition of bottom strand hydrolysis by ss.BspD6I. The obtained data provide evidence that Nt.BspD6I coordinates the action of ss.BspD6I; two subunits act as the reliable partners that results in effective R.BspD6I DNA cleavage activity.

Author contributions

Conceived and designed the experiments: EAK, LAA, TH, WW. Performed the DNA-protein investigations: LAA, AYuM, AVG, MVN. Purified proteins: AYuR, TAP, LAZ. Provided the DNA synthesis: TSO. Analyzed the data: LAA, AYuM, AVG, TH, EAK. Wrote the paper: EAK, LAA, TH, TSO.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.bbapap.2016.05.008.

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