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DNA-methyltransferase SsoII as a Bifunctional Protein: Features of the Interaction with the Promoter Region of SsoII Restriction-Modification Genes

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Abstract—DNA duplexes bearing an aldehyde group at the 2′-position of the sugar moiety were used for affinity modification of (cytosine-5)-DNA methyltransferase SsoII. It is shown that lysine residues of M.SsoII N-terminal region are located in proximity to DNA sugar-phosphate backbone of a regulatory sequence of promoter region of SsoII restriction-modification enzyme coding genes. The ability of the two M.SsoII subunits to interact with DNA regulatory sequence has been demonstrated by affinity modification using DNA duplexes with two 2′-aldehyde groups. Changes in nucleotide sequence of one half of the regulatory region prevented cross-linking of the second M.SsoII subunit. The results on sequential affinity modification of M.SsoII by two types of modified DNA ligands (i.e. by 2′-aldehyde-containing and phosphoryldisulfide-containing) have demonstrated the possibility of covalent attachment of the protein to two different DNA recognition sites: regulatory sequence and methylation site.

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The subject of this study is DNA methyltransferase SsoII (M.SsoII) isolated from *Shigella sonnei* 47 strain and belonging to the SsoII restriction-modification system [1]. M.SsoII recognizes a pentanucleotide sequence in double-stranded DNA; the recognition site is completely degenerated by a central nucleotide pair 5′-C**C**NGG-3′/3′-GGN**C**C-5′. In the presence of a cofactor, *S*-adenosyl-*L*-methionine (AdoMet), the enzyme methylates the internal dC residue (selected in bold) of the sequence, forming 5-methyl-2′-deoxycytidine [2]. The methyltransferase gene has been cloned in *Escherichia coli* and its amino acid sequence has been determined [3]. Analysis of M.SsoII amino acid sequence demonstrated that the protein region between 72 and 379 amino acid residues (a.a.r.) includes all 10 conservative motifs typical for (cytosine-5)-DNA methyltransferase (MTases) [4, 5]. These motifs are located in the amino acid chain in a strictly determined order (Fig. 1). Motif I is responsible for AdoMet binding, motif IV contains invariant dipeptide ProCys, a part of a catalytic domain responsible for the transfer of methyl group from AdoMet molecule onto methylated cytosine. The only cysteine residue in the M.SsoII molecule, Cys142 in the motif IV,

Abbreviations: AdoHcy) *S*-adenosyl-*L*-homocysteine; AdoMet) *S*-adenosyl-*L*-methionine; a.a.r.) amino acid residue; HTH) helix–turn–helix; MTase) DNA methyltransferase; oU) 2′-*O*-(2-oxoethyl)uridine; PDG(pss)) phosphoryldisulfide group; RM) restriction-modification; N) A, G, T or C; W) A or T. Prefix "d" (deoxy) in the designation of oligodeoxyribonucleotides and DNA duplexes is omitted.

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interacts with the C6 atom in cytosine. In the formed covalent intermediate the C5 atom of the cytosine residue acquires additional negative charge, and, as a consequence, become alkylated by the AdoMet methyl group [4-6]. The variable region located between the motifs VIII and IX determines the specificity of recognition of methylated DNA sequence.

Earlier it was shown that M.SsoII forms a specific and stable complex not only with DNA methylation site, but also with the promoter region of SsoII restriction-modification (RM) genes [7, 8]. Then a decrease in expression of the M.SsoII encoding gene and an increase in expression of the restriction endonuclease gene (R.SsoII) are observed. A 15-base-pair inverted repeat was identified within the promoter region (further referred to as regulatory sequence), enabling, presumably, the maximal number of specific contacts with protein [8]:

5'-**AGGACAAATTGTCCT**-3'

3'-**TCCTGTTTAACAGGA**-5'

It should be noted that regulation of gene expression is extremely important for functioning of RM systems in bacterial cells. The level of methyltransferase expression should always correlate with the level of restriction endonuclease synthesis. It is essential that restriction endonuclease can hydrolyze foreign DNA before it becomes methylated by a corresponding MTase. At the same time, if the methylation level is not sufficiently high, restriction endonuclease will damage DNA of a host cell, thus leading to cell death. Among (cytosine-5)-DNA methyltransferases, the autoregulatory function is also found for restriction-modification enzymes EcoRII and MspI, recognizing similar DNA methylation sequences (M.EcoRII: 5′-C**C**WGG-3′/3′-GGW**C**C-5′; M.MspI: 5′-C**C**GG-3′/3′-GG**C**C-5′) [9, 10]. However, coupled regulation of restriction endonuclease and methyltransferase gene expression was shown only for the SsoII RM system [8].

In M.SsoII structure, the conservative motif I is preceded by a long N-terminal region (a.a.r. 1-71) (Fig. 1), which, as was shown experimentally, is significant for the regulatory role of the protein [7, 8]. Scheme below shows the amino acid sequence of the N-terminal region of M.SsoII. Putative secondary structure elements (α-helices) are marked in gray. Previously it has been predicted with high probability that the second and third α-helices form a helix–turn–helix structural motif (HTH) [8]. It is possible that it is the HTH motif that represents the binding site of the RM gene promoter region.

1-MTDNIAATIKEKRERLHMTQKEFADAL-GLSKYGDRTIRRWERGETKPTGAELKAVIDFPDTP-PYPNNENGR-71

Alignment of the amino acid sequence of the HTH motif identified in the N-terminal region of M.SsoII with HTH motifs of known transcription factors allows assigning M.SsoII to the family of Cro-proteins and repressors [11]. Computer modeling was performed and a putative model of the complex of M.SsoII N-terminal region with DNA regulatory sequence was constructed (Fig. 2) [11]. The modeling was based on X-ray data for the structure of the complex of bacteriophage 434 repressor with DNA [12], as well as on the results of DNA footprinting, which revealed the groups of atoms in the regulatory sequence involved in the interaction with M.SsoII at the stage of specific recognition [13].

The suggested model (Fig. 2a) implies that amino acid residues Arg35, Arg38, and Arg39, located in the third recognizing α-helix, contact with heterocyclic bases of guanosine and thymidine residues in DNA regulatory sequence (Fig. 2b). Since the regulatory sequence is a palindrome and therefore has a second order symmetry axis, it is logical to assume that its binding (similarly to Cro-proteins and repressors) involves two N-terminal regions of two M.SsoII subunits [11]. Each of the subunits forms contacts with one half of the recognition site, where N-terminal regions are located from one side of DNA and are separated by the minor groove (Fig. 2a). It should be mentioned that considering the possibility of DNA bending or twisting, the location of contacts

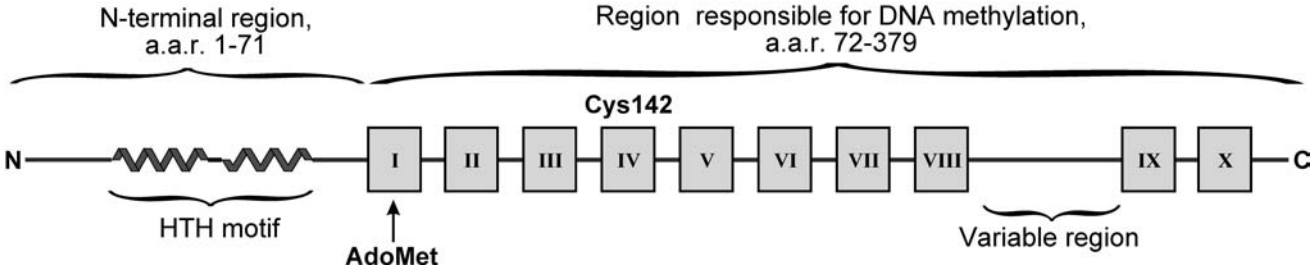

Fig. 1. Structural organization of DNA methyltransferase M.SsoII. The N-terminal protein region (a.a.r. 1-71) putatively contains a helix–turn–helix (HTH) motif responsible for binding with regulatory sequence of the promoter region of SsoII restriction-modification genes. The protein region from a.a.r. 72 to 379 contains motifs I-X, which are conservative for all (cytosine-5)-DNA methyltransferases.

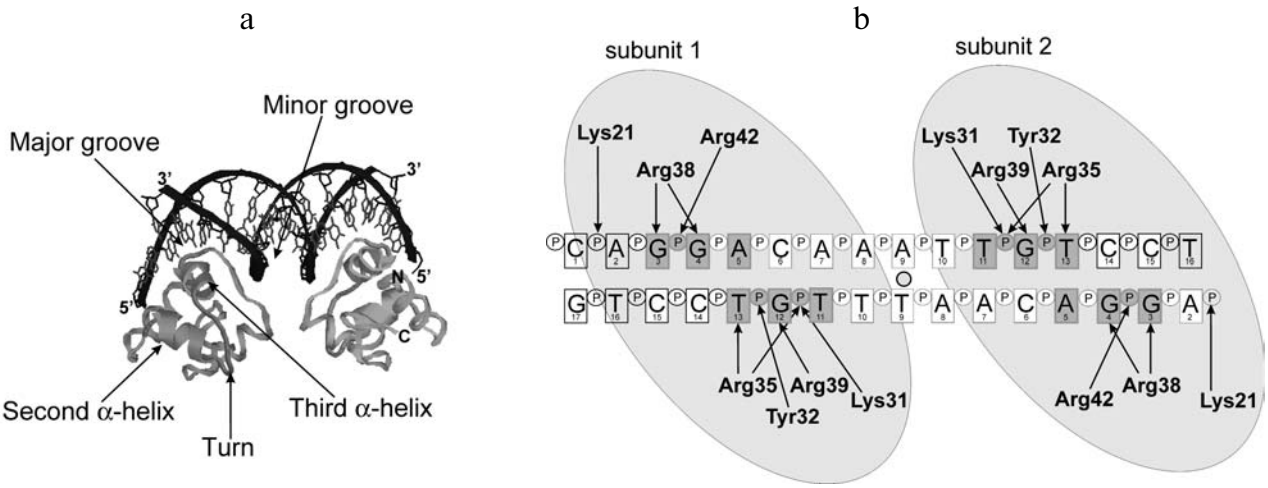

Fig. 2. Dimensional model (a) and scheme of DNA–protein contacts (b) for the complex of M.SsoII N-terminal region with a 15-base-pair DNA regulatory sequence [11].

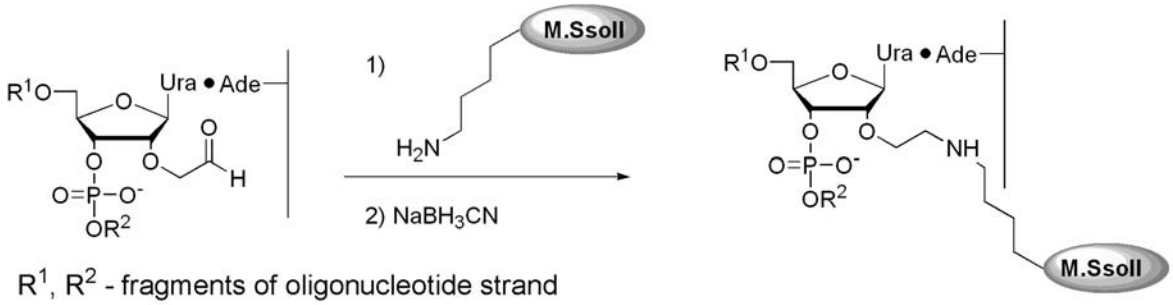

Fig. 3. Scheme of affinity modification of protein lysine residue by DNA duplex containing 2′-*O*-(2-oxoethyl)uridine residue.

between M.SsoII amino acid residues and groups of atoms of the regulatory sequence can be shifted relative to those shown in Fig. 2b.

To confirm the computer modeling data and determine the stoichiometry of DNA–protein complexes formed upon interaction of M.SsoII with the promoter region of SsoII restriction-modification genes, protein affinity modification with reactive DNA analogs was used in this work. Considering that at least two lysine residues, Lys21 and Lys31, of the M.SsoII N-terminal region can interact with DNA regulatory sequence (Fig. 2b), affinity modification was performed by DNA duplexes with one or two 2′-*O*-2-oxoethyl groups, able to selectively interact with spatially close ε-amino groups of lysine residues [14]. The reaction results in the formation of a Schiff base, which, due to its instability under experimental conditions, should be reduced to a secondary amine by sodium cyanoborohydride (Fig. 3). DNA duplexes containing single 2′-*O*-(2-oxoethyl)uridine moieties have been previously used for affinity modification of lysine residues in homodimer of p50 subunit of NF-κB [15, 16] and SsoII restriction endonuclease [17].

MATERIALS AND METHODS

Synthesis of oligodeoxyribonucleotides and DNA duplexes. Oligonucleotides used in this work were obtained by the phosphoroamidite method using an Applied Biosystems 392A synthesizer (USA) and following a standard procedure. Oligonucleotides containing 2′-*O*-(2,3-dihydroxypropyl)uridine residues were synthesized as described in [14], and oligonucleotides containing internucleotide phosphoryldisulfide group (PDG) as reported in [18]. DNA duplexes with 2′-*O*-(2-oxoethyl)uridine residues were obtained using the method described in [16, 17].

Enzymes and proteins. T4 polynucleotide kinase (5 activity units per μl) and terminal transferase (20 units/μl) were commercially available preparations from SibEnzyme

Synthetic DNA duplexes used for studies of interaction of M.SsoII with DNA

	DNA duplex[a] (5′ → 3′)	Yield of DNA–protein conjugate[b], %	
		C1	C2
I	ATCAAAACA**GGACAAATTGTCCT**AAAACCAA TAGTTTTG**TCCTGTTTAACAGGA**TTTTGGTT	—	—
II	GATCCTTTATAATGCTAATTAGTACTGATC CTAGGAAATATTACGATTAATCATGACTAG	—	—
III	ATCAAAACA**GGACAAAToUGTCCT**AAAACCAA TAGTTTTG**TCCTGTTTA-ACAGGA**TTTTGGTT	46 ± 6	—
IV	ATCAAAACA**GGACAAATTGTCCT**-AAAACCAA TAGTTTTG**TCCTGTTTAACAGGA**oUTTTGGTT	42 ± 4	—
V	ATCAAAACA**GGACAAATTGoUCCT**AAAACCAA TAGTTTTG**TCCTGTTTAAC-AGGA**TTTTGGTT	38 ± 5	—
VI	ATCAAAAC-**AGGACAAATTGTCCT**AAAACCAA TAGTTTTG**oUCCTGTTTAACAGGA**TTTTGGTT	35 ± 3	—
VII	ATCA-AAACA**GGACAAATTGTCCT**AAAACCAA TAGToUTTG**TCCTGTTTAACAGGA**TTTTGGTT	34 ± 1	—
VIII	GCATATATATATATAA**TTGTCCT**-AAAACCAA CGTATATATATATATT**AACAGGA**oUTTTGGTT	15 ± 3	—
IX	ACGTTCCoUGGCTATTGACTGC TGCAAGG-ACCGATAACTGACG	2.0 ± 0.5	—
X	ATCAAAACA**GGAC-AAATTGTCCT**-AAAACCAA TAGTTTTG**TCCTGoUTTAACAGGA**oUTTTGGTT	55 ± 3	6 ± 3
XI	ATCAAAAoUA**GGACAAAToUGTCCT**AAAACCAA TAGTTTT-G**TCCTGTTTA-ACAGGA**TTTTGGTT	42 ± 4	5 ± 2
XII	ATCAAAAC-**AGGACAAATTGTCCT**-AAAACCAA TAGTTTTG**oUCCTGTTTAACAGGA**oUTTTGGTT	36 ± 4	11 ± 2
XIII	GCATATATAT-ATATAA**TTGTCCT**-AAAACCAA CGTATATAToUTATATT**AACAGGA**oUTTTGGTT	26 ± 3	—
XIV	ACGTTCC**pss**TGGCTATTGACTGC CTGCAAGG—-ACCGATAACTGACGT	50 ± 5	—

[a] Regulatory sequence or its half is selected in bold; 2′-*O*-(2-oxoethyl)uridine residues are marked in gray, methylation site is underlined.
[b] Average values obtained from at least three independent experiments are shown. "–", DNA duplex does not form conjugate with M.SsoII.

(Russia) and New England Biolabs (USA). The preparations of M.SsoII, M.NlaX, and M.SsoII(C142A) at the concentration of 5 μg/μl were isolated from *E. coli* cell culture following a two-step protocol, including chromatography on heparin-Sepharose and Ni-NTA-agarose.

Study of equilibrium binding of M.SsoII with DNA duplexes containing regulatory sequence (gel retardation assay). Complexing of M.SsoII (20 pmol) with 1 pmol of 5′-^{32}P-labeled DNA duplexes I and II (table) was performed in 20 μl of buffer A (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM dithiothreitol) containing 10% glycerol, in the presence of various concentrations of poly(dI·dC) (0, 5, 10, 12.5, and 25 ng/μl) for 20 min at 37°C. Protein–nucleic acid complex and free DNA duplex were separated using gel electrophoresis in non-denaturing 6% polyacrylamide gel in TBE buffer (50 mM Tris-borate buffer, pH 8.3, 1 mM EDTA) at 4°C. Here and further on the visualization of radioactive bands on the gel and data processing was performed using a Molecular Dynamics PhosphorImager SI (Molecular Dynamics, USA) and computer program ImageQuant version 5.0.

Interaction of M.SsoII with DNA duplexes containing one or two 2′-*O*-2-oxoethyl groups (covalent binding). 5′-^{32}P-labeled modified duplexes III-XIII (1 pmol) (table) were incubated with 20 pmol of M.SsoII in 10 μl of buffer A containing 12.5 ng/μl poly(dI·dC) for 30 min at 37°C. Then 1 μl of 275 mM $NaBH_3CN$ (final concentration in the mixture 25 mM) was added and incubated for 1 h at 37°C. DNA–protein conjugates and unbound DNA and protein were separated by SDS-PAGE in 12% gel with 4% stacking gel. Prior to loading on the gel the samples were heated for 3 min at 95°C in denaturing buffer B (60 mM Tris-HCl, pH 6.8, 1% (v/v) glycerol, 2% (w/v) SDS). Location of 5′-^{32}P-labeled bands was determined by autoradiography. The gel was stained with Coomassie R250 and the molecular weights of radioactive-labeled compounds were determined by correlation with protein markers. The yields of modified oligonucleotide conjugates with one (C1) or two (C2) M.SsoII subunits were defined as ratios between radioactivity of the product and total radioactivity of reaction products and unbound DNA.

Sequential covalent binding of M.SsoII with DNA duplexes containing 2′-*O*-2-oxoethyl and phosphoryldisulfide (PDG) groups. First 80 pmol of PDG-containing DNA duplex XIV, 3′-labeled using terminal transferase and [α-^{32}P]UTP, was incubated with 160 pmol of M.SsoII for 1 h at 37°C in 20 μl of buffer A containing 500 μM *S*-adenosyl-*L*-homocysteine (AdoHcy) and 12.5 ng/μl poly(dI·dC). Then 80 pmol of 2′-aldehyde-containing DNA duplex VI was added, and the reaction mixture was incubated for an additional 30 min at 37°C. In some experiments, modified DNA duplexes were added to the reaction mixture in reverse order. Then 2 μl of 275 mM $NaBH_3CN$ was added and incubated for 1 h at 37°C. The samples were dissolved in buffer B and analyzed by SDS-PAGE in 12% gel. 3′-^{32}P-labeled bands were detected by autoradiography. To visualize protein-containing zones, the gels were stained with Coomassie R250 solution. The data was processed using software supplied with a Sorbfil videodensitometer (Sorbpolymer Ltd., Russia). The yield of covalent binding product formed by M.SsoII and PDG-containing DNA duplex XIV (table) was calculated as the ratio between the intensities of DNA–protein conjugate and total intensity of DNA–protein conjugate and unbound protein.

RESULTS AND DISCUSSION

Interaction of M.SsoII with DNA duplexes containing one or two 2′-*O*-2-oxoethyl groups in the regulatory sequence. To study the interaction of M.SsoII with the promoter region of SsoII restriction-modification genes, 31-mer DNA duplex I containing a 15-base-pair inverted repeat (regulatory sequence) was selected as an initial unmodified substrate (table). The optimal ratio between the concentrations of M.SsoII and DNA ligand I was selected previously [19]. It is 20 : 1 and allows achieving maximal product yield. Studies have demonstrated that M.SsoII forms two complexes with DNA duplex I characterized by different mobility in non-denaturing polyacrylamide gel (Fig. 4, A1 and A2). At the same time, nonspecific DNA duplex II lacking the M.SsoII recognition site (table) forms only complex A1. Considering that the affinity of M.SsoII to nonspecific DNA sequence is just 30 times lower compared to DNA ligand containing the regulatory sequence [8], we have assumed that complex A1 is nonspecific. Experiments on displacement of ^{32}P-labeled duplexes I and II from DNA–protein complexes A1 and A2 were performed. As a competing ligand poly(dI • dC) was used. Its nucleotide sequence does not

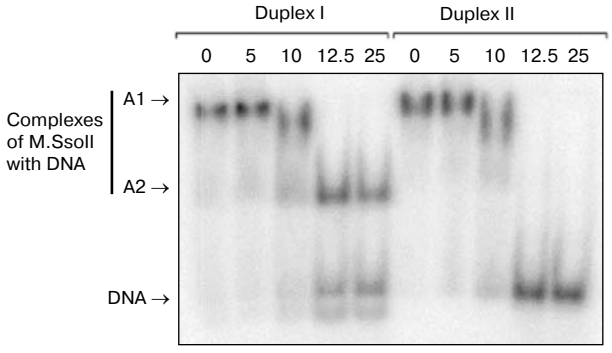

Fig. 4. Analysis of equilibrium binding of M.SsoII with DNA duplexes I and II using the gel retardation assay. 5′-^{32}P-labeled DNA duplexes I or II were incubated with M.SsoII in the absence and presence of poly(dI·dC) (concentration in ng/μl is shown above the lanes). The formed DNA–protein complexes are denoted A1 and A2.

contain the regulatory sequence and has no homology with nucleotide sequence of DNA duplex II. Poly(dI•dC) concentration in the reaction mixture of 12.5 ng/μl results in the displacement of labeled DNA from complex A1; at the same time, it has no effect on the formation of complex A2 (Fig. 4). This shows that complex A2 is specific.

Further experiments on affinity modification of M.SsoII by reactive DNA ligands were performed in the presence of 12.5 ng/μl poly(dI•dC). Under these conditions, the formation of only specific protein–nucleic acid complex with maximal efficiency (~80%) was observed.

To reveal the location of lysine residues in the M.SsoII N-terminal region relative to DNA regulatory sequence, we synthesized a set of modified DNA duplexes III-IX (table). Duplexes III-VI contained 2′-*O*-(2-oxoethyl)uridine residue (oU) either in the positions of putative contacts with Lys21 and Lys31 residues of M.SsoII or in direct proximity to these positions in the DNA strand (Fig. 2b). DNA duplexes VII-IX were used as a control. In DNA duplex VII the 2′-*O*-(2-oxoethyl)uridine residue was located in the flanking region of the regulatory sequence, and DNA duplex VIII preserves only one half of the recognition site. In DNA ligand IX, the reactive group was located at the central position of the methylation site. There was no regulatory sequence in this duplex.

All DNA duplexes were tested for their interaction with M.SsoII under selected conditions for specific binding. It was found that the efficiency of cross-linking of M.SsoII with oligonucleotides comprising DNA duplexes III and IV and resulting in modification of Lys21 and Lys31 residues was approximately 40-45% (table). On the other hand, the yield of products of affinity modification of M.SsoII by duplex IX (which has no regulatory sequence, but contains the methylation site) did not exceed 2%. However, such duplex does form a complex with the enzyme (data not shown). This result indicates the specificity of cross-linking of M.SsoII with the regulatory sequence. The yield of products of affinity modification of M.SsoII by DNA duplex VIII containing only one half of the recognition site was approximately 15% (table). It is evident that the more effective covalent binding of M.SsoII with the promoter region of SsoII RM genes requires both halves of the DNA regulatory sequence. These data are in agreement with the results reported in [8], according to which the affinity of M.SsoII to unmodified duplex I is 13-15 times higher than to duplexes containing only left or right halves of the inverted repeat.

The efficiency of formation of cross-linking products of the enzyme with DNA duplexes V and VI containing modification in the proximity of predicted contacts with Lys21 and Lys31 was up to 35-40%. This value is slightly lower than in the case of affinity modification of M.SsoII by DNA duplexes III and IV, but it is high enough and suggests the proximity of protein lysine residues with modified nucleosides. Similar results were obtained upon the interaction of M.SsoII with DNA duplex VII containing 2′-*O*-(2-oxoethyl)uridine at a random position in oligonucleotide strand outside the regulatory sequence. Discrepancy in the data and results of computer modeling (Fig. 2b) can be explained by the fact that the latter did not take in consideration of a possibility of DNA distortion upon binding to M.SsoII. Moreover, it is quite possible that there is interaction between highly reactive DNA duplexes and lysine residues located on the surface of the protein globule or in the proximity to ligand during the initial stage of recognition [17]. Affinity modification of M.SsoII by other DNA ligands bearing reactive groups at different positions in a flanking region of the regulatory sequence has not been studied. Therefore, high yield of DNA–protein conjugate in the case of duplex VII can be a consequence of a "lucky hit" by a reactive group in the specific position in DNA ligand.

We have performed experiments on covalent binding of modified duplexes III-VII with DNA methyltransferase NlaX, displaying high degree of homology with the amino acid sequence of M.SsoII. The significant difference is the absence of a 70 a.a.r. N-terminal region (that is characteristic for M.SsoII) in the structure of M.NlaX [20]. It was found that M.NlaX did not form conjugates with modified DNA duplexes III-VII. These data indicate that covalent binding of M.SsoII with 2′-aldehyde-containing DNA ligands is mediated by lysine residues in the N-terminal region of the enzyme.

To investigate the possibility of binding of two M.SsoII subunits with the regulatory sequence, DNA duplexes X-XII (containing two 2′-*O*-(2-oxoethyl)uridine residues) were synthesized (table). Both modifications were introduced in the same DNA strand in two half-sites of regulatory sequence in those positions that are presumably in proximity to lysine residues in the N-terminal region of M.SsoII in protein–nucleic acid complex. It was supposed that lysine residues in each of the M.SsoII subunits will interact with a certain modified nucleoside. DNA duplex XIII containing modifications in the same positions as DNA duplex XII but comprising only one half of the inverted repeat was used in control experiments.

Affinity modification of M.SsoII by DNA duplexes X-XIII, ^{32}P-labeled at the 5′-end of the modified strand, was carried out under conditions of specific binding. The efficiency of formation of a conjugate of one M.SsoII subunit with a modified strand of DNA duplexes X-XII (conjugate C1, calculated molecular weight 54.1 kD) was 35-55% (table and Fig. 5). Replacement of one of the halves of the regulatory region with a random sequence resulted in a decrease in efficiency of formation of C1 with DNA duplex XIII by almost two-fold compared to DNA duplexes X-XII. This is in agreement with the data obtained earlier for DNA duplex VIII and indicates that

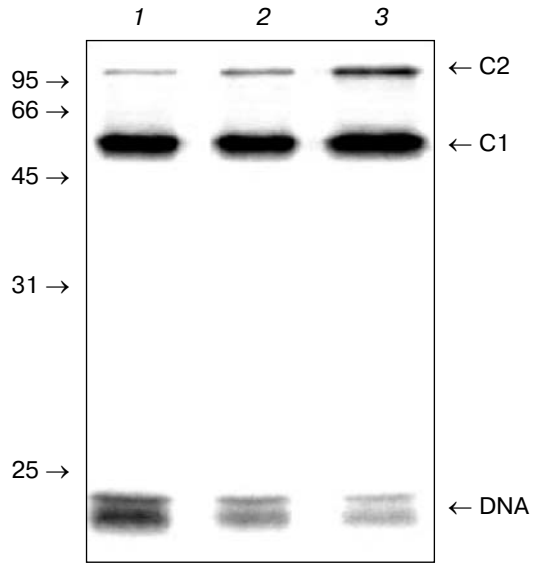

Fig. 5. Analysis of products of covalent binding of M.SsoII with 5′-^{32}P-labeled DNA duplex X (concentration 50 nM). Radioautograph of reaction mixtures after SDS-PAGE in 12% gel. Lanes *1-3*: concentration of M.SsoII in the reaction mixture is 1, 2, and 4 μM, respectively. C1 denotes the conjugate of one M.SsoII subunit; C2 is the conjugate of two M.SsoII subunits with modified strand of DNA duplex X. The arrows on the left show the molecular weight of protein markers, kD.

both half-sites of regulatory sequence are important for M.SsoII binding. As seen from Fig. 5, cross-linking with modified DNA duplexes X-XII leads to the formation of conjugate of two protein subunits with regulatory sequence (C2, calculated molecular weight 98.7 kD), where the yield varies between 5 and 11% (table). Therefore, we have determined that two M.SsoII subunits can interact with the promoter region of RM genes. It is important to note that nucleotide replacement within one of the half-sites of regulatory sequence prevented the formation of the conjugate of two M.SsoII subunits with DNA (duplex XIII). Hence, the presence of two intact halves of inverted repeat is necessary for the formation of conjugate C2.

Therefore, using affinity modification of M.SsoII by DNA ligands containing an aldehyde group at 2′-position of the sugar moiety, it has been demonstrated that binding of DNA regulatory sequence can involve either one or two enzyme molecules. It is worth mentioning that other approaches, such as ultracentrifugation, gel filtration, and cross-linking of enzyme molecules, did not allow determining the stoichiometry of protein–nucleic acid complexes due to the aggregation of M.SsoII upon increase of its concentration in solution.

As follows from the analysis of the interaction of M.SsoII with reactive DNA ligands, the change in nucleotide sequence in one of the half-sites of regulatory sequence interferes with binding of the second enzyme subunit. The yield of the conjugate of two protein subunits with DNA increases with increase in concentration of M.SsoII in the reaction mixture; at the same time the yield of the conjugate of one MTase subunit with DNA does not change (Fig. 5). It can be assumed that the regulatory function of M.SsoII is associated with dimerization of the enzyme upon interaction with the promoter region of SsoII restriction-modification genes, which occurs under the increase in MTase concentration *in vivo*.

Investigation of simultaneous participation of both methylating and N-terminal (regulatory) regions of M.SsoII in interaction with DNA. Of special interest was the question of how two DNA-recognizing functions of M.SsoII are linked between each other, and whether the protein can simultaneously interact with two different recognition sites, methylation site, and regulatory sequence. Previously, a hypothetical model based on computer modeling and demonstrating the possibility of such interaction has been constructed [11]. It includes two full-size M.SsoII molecules, three DNA molecules, and two AdoHcy molecules (Fig. 6). Both M.SsoII subunits are located from one side of the regulatory DNA sequence. The regions responsible for methylation in each M.SsoII molecule are in proximity to substrate and AdoHcy, and enable protein–protein contacts between M.SsoII molecules.

We assumed that the gel retardation assay would allow us to identify DNA–protein complex of M.SsoII with both unmodified DNA duplexes containing regula-

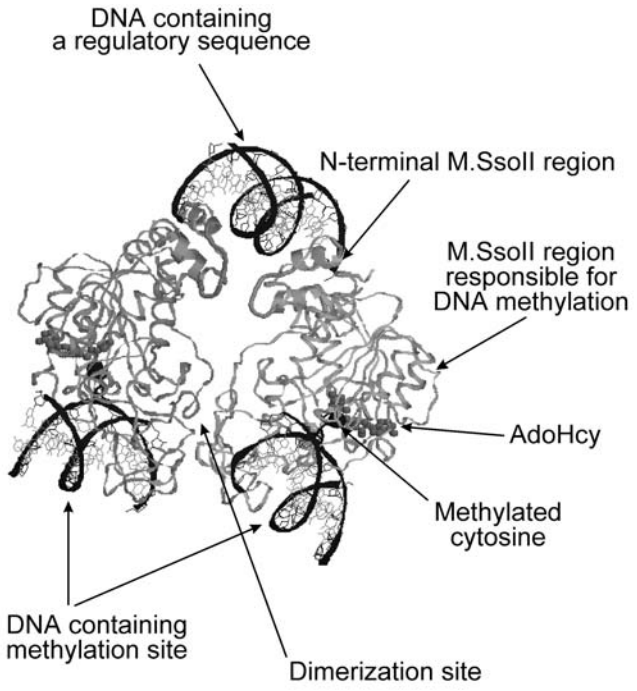

Fig. 6. Model of full-size M.SsoII (dimer) in a complex with DNA regulatory sequence, two DNA molecules containing methylation site, and two AdoHcy molecules [11].

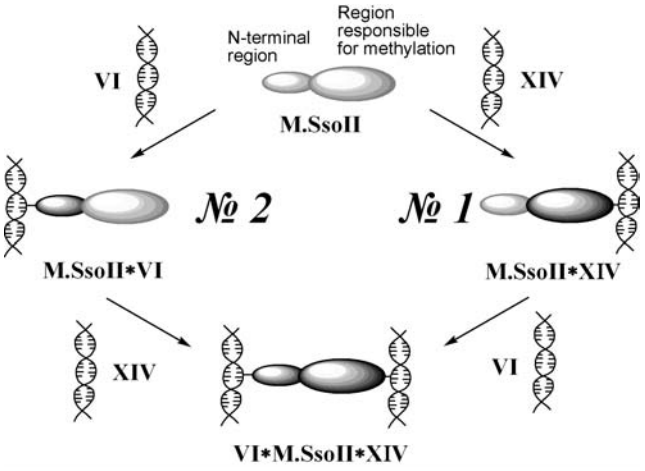

Fig. 7. Scheme of the experiment on sequential affinity modification of M.SsoII by two types of DNA duplexes, one of which contains 2′-*O*-(2-oxoethyl)uridine residue in the regulatory sequence (duplex VI), and the other a phosphoryldisulfide group in the methylation site (duplex XIV). No. 1 and No. 2 describe different order of addition of DNA ligands to M.SsoII.

tory sequence and methylation site, considering the shift in its mobility compared with DNA–protein complex formed by M.SsoII and only one of the duplexes. However, a band that could correspond to M.SsoII complex with two different DNA ligands was not observed on a native gel. For this reason, sequential affinity modification of M.SsoII by two types of modified DNA duplexes (one of which contained 2′-*O*-(2-oxoethyl)uridine residue in the regulatory sequence (duplex VI), while another (duplex XIV) – the phosphoryldisulfide group in the methylation site) were used to verify the results of computer modeling (table). DNA duplex XIV is able to interact with high efficiency with the only Cys142 residue involved in the catalytic center of the C-terminal region of M.SsoII [18].

Different order of addition of 2′-aldehyde-containing and phosphoryldisulfide-containing DNA ligands to M.SsoII was used in this work (Fig. 7). According to the expectations, M.SsoII formed conjugates with each of the modified DNA duplexes VI and XIV (Fig. 8, lanes *2* and *4*). In a separate experiment, it was demonstrated that the presence of sodium cyanoborohydride had no effect on affinity modification of M.SsoII by PDG-containing DNA duplex XIV (Fig. 8, lane *3*). These results on sequential affinity modification of M.SsoII by two types of DNA ligands (3′-^{32}P-labeled XIV and unlabeled VI) confirmed the possibility of formation of a "double" conjugate VI·M.SsoII·XIV, the product of covalent attachment of the protein to reactive groups in both DNA recognition sites (Fig. 8, lane *5*). Initially, the M.SsoII region responsible for methylation of substrate XIV forms the conjugate with the corresponding oligonucleotide, and then the N-terminal enzyme region also can covalently bind to the regulatory DNA sequence (Fig. 7, No. 1). After the affinity modification of M.SsoII in reverse order, when 2′-aldehyde-containing DNA duplex VI was added first, followed by addition of PDG-containing DNA duplex XIV, the efficiency of VI·M.SsoII·XIV conjugate formation was lower (Fig. 8, lane *6*). It is possible that initial covalent binding of the N-terminal M.SsoII region with the regulatory sequence alters the enzyme conformation, thus decreasing the efficiency of its interaction with the methylation site containing PDG (Fig. 7, No. 2).

A set of control experiments has been performed. DNA duplexes XIV and VI were incubated with M.NlaX and a mutant form of M.SsoII, where Cys142 residue was replaced with Ala. As expected, the formation of a double conjugate with duplexes XIV and VI in the case of M.SsoII(C142A) and M.NlaX was not observed (data not shown). The mutant M.SsoII form (C142A) effectively reacted with duplex VI, but not with the duplex bearing phosphoryldisulfide group within the methylation site,

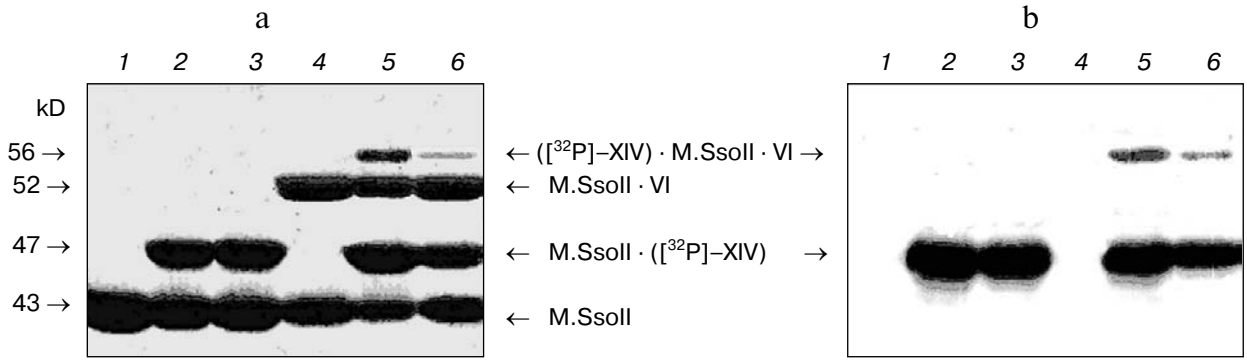

Fig. 8. Electrophoretic analysis of the products of covalent binding of M.SsoII with 2′-aldehyde-containing DNA duplex VI (*4*), 3′-^{32}P-labeled PDG-containing DNA duplex XIV (in the absence (*2*) and presence (*3*) of $NaBH_3CN$), and with two DNA ligands sequentially (*5* – first with XIV, then with VI; *6* – first with VI, then with XIV) in 12% SDS-PAGE. *1*) Initial M.SsoII protein. a) Gel staining with Coomassie R250; b) gel radioautograph. Protein molecular weights are shown on the left.

which is in agreement with the literature [18]. In turn, M.NlaX was involved in a reaction of thioldisulfide exchange with duplex XIV, but, in accordance with earlier results, did not form conjugate with the modified strand of duplex VI, since this protein lacks the N-terminal region responsible for binding with the DNA regulatory sequence.

Therefore, sequential affinity modification of M.SsoII by two types of DNA ligands allowed detecting the formation of a "double" conjugate (regulatory sequence)·M.SsoII·(methylation site). This proves the possibility of formation of multiplex complexes of DNA with M.SsoII-type methyltransferases containing the regulatory domain during their functioning in bacterial cells.

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