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CROSSLINKING OF (CYTOSINE-5)-DNA METHYLTRANSFERASE SsoII AND ITS COMPLEXES WITH SPECIFIC DNA DUPLEXES PROVIDES AN INSIGHT INTO THEIR STRUCTURES

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□ *(Cytosine-5)-DNA methyltransferase SsoII (M.SsoII) functions as a methyltransferase and also as a transcription factor. Chemical and photochemical crosslinking was used for exploring the structure of M.SsoII–DNA complexes and M.SsoII in the absence of DNA. Photocrosslinking with 4-(N-maleimido)benzophenone demonstrated that in the M.SsoII complex with DNA containing the regulatory site, the M.SsoII region responsible for methylation was bound to DNA flanking the regulatory site, which contained no methylation sequence. This required high flexibility of the linker connecting the M.SsoII N-terminal domain and the M.SsoII region responsible for methylation. The flexibility was demonstrated by crosslinking with bis-maleimidoethane and 1,11-bis-maleimidotetraethyleneglycol.*

Keywords DNA methyltransferase SsoII; protein–protein crosslinking; protein–DNA crosslinking; protein–DNA complexes

INTRODUCTION

(Cytosine-5)-DNA methyltransferase SsoII (M.SsoII) from *Shigella sonnei* 47 recognizes the sequence 5′-CCNGG-3′/3′-GGNCC-5′ (N = A, C, G, T) in double-stranded DNA and methylates the C5 atom of the inner cytosine

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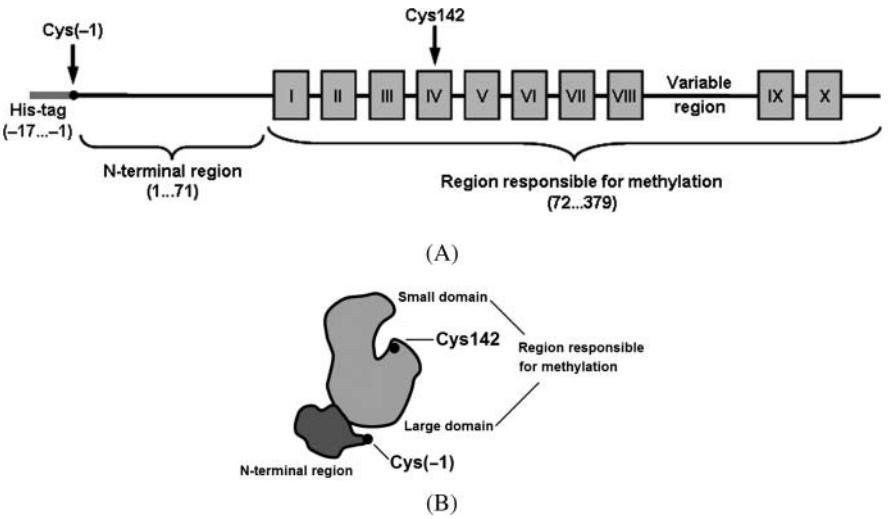


FIGURE 1 Structural organization of M.SsoII molecule: (A) primary and (B) tertiary structure.

residues.[1] Ten conservative amino acid motifs can be distinguished in the primary structure of the (cytosine-5)-DNA methyltransferases.[2] They are arranged in a strictly defined order along the sequence (Figure 1A). In M.SsoII, these conservative motifs are located among residues 72–379—the so-called region responsible for methylation, highly similar to (cytosine-5)-DNA methyltransferase HhaI (M.HhaI) of known 3D structure. The region responsible for methylation consists of a large domain and a small domain separated by a DNA-binding cleft (Figure 1B). Cys142 of the large domain plays a key role in the catalysis of methyl group transfer. This residue is a part of dipeptide Pro-Cys, which is absolutely conservative among (cytosine-5)-DNA methyltransferases due to its functional role.[2]

The first one of the conservative motifs in M.SsoII is preceded by a prolonged N-terminal region (residues 1–71; Figure 1A). The N-terminal region consists of two parts: residues 1–55 are homologous to transcription regulators from the HTH_3 family (PF01381 in Pfam database) whereas residues 56–71 share no similarity with any other protein domain in Pfam.[3] The N-terminal region is responsible for the second type of M.SsoII activity: this protein functions as a transcription factor repressing transcription of its own gene and stimulating transcription of restriction endonuclease *SsoII* gene.[4] This type of activity is mediated via M.SsoII binding to a quasi-palindromic sequence 5′-AGGACAA**A**TTGTCCT-3′/3′-TCCTGTT**T**AACAGGA-5′, which has been identified as the regulatory site in the promoter region of the genes of SsoII restriction–modification system.[4–7] We assume that the N-terminal region can exist as a separated domain in M.SsoII molecule. This assumption is corroborated by the fact that a deletion mutant representing

only the N-terminal region has a pronounced secondary structure and retains the capability to bind DNA that contains the regulatory site.[8] Such proteins that combine enzymatic activity with functioning as transcription factors are interesting research objects, because mechanisms of interrelation between the two activities remain unclear.

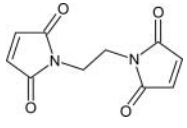
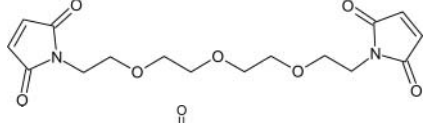
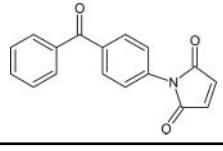
Despite numerous attempts, neither M.SsoII nor any of M.SsoII–DNA complexes were crystallized. A homology-based model was suggested in which two M.SsoII subunits formed a complex with one DNA duplex containing the regulatory site and with two DNA duplexes containing the methylation site.[3] The M.SsoII N-terminal region model was built on the basis of its sequence similarity with transcription repressors of the HTH_3 family, whereas the M.SsoII region responsible for methylation was modeled on the basis of its sequence similarity with (cytosine-5)-DNA methyltransferases HhaI and HaeIII. The M.SsoII–DNA contacts in this model are in agreement with the results of footprinting studies.[6,9] The bottleneck of this model is conformation of the linker between the two regions of the protein: there is no significant sequence similarity between M.SsoII residues 56–71 and any other protein with a determined spatial structure. Thus, the conformation of the residues 56–71 was chosen arbitrarily and requires experimental verification. Moreover, such a linker could be flexible and capable of adopting different conformations.

Recently, it has been shown experimentally that M.SsoII formed only one DNA–protein complex with a stoichiometry of 1:1 when interacting with a 60-bp DNA duplex containing the methylation site (60met).[8] On the other hand, upon M.SsoII interaction with a 60-bp DNA duplex containing the regulatory site (60reg), two DNA–protein complexes were observed. Their stoichiometry was estimated as one M.SsoII molecule per DNA duplex for the complex with a higher electrophoretic mobility and two M.SsoII molecules per DNA duplex for the complex with a lower electrophoretic mobility.[8] Thus, a DNA-mediated dimerization of M.SsoII was proposed upon its interaction with 60reg duplex. However, the arrangement of protein subunits in this complex is unknown, as well as the existence of any protein–protein contacts.

We tested the chemical and photochemical crosslinking approaches for exploring the structure of M.SsoII–DNA complexes and M.SsoII in the absence of DNA. The following reagents were used: *bis*-maleimidoethane (BMOE), 1,11-*bis*-maleimidotetraethyleneglycol (BM[PEO]$_4$), and 4-(*N*-maleimido)benzophenone (BPM; see Table 1). These reagents are commonly used as "molecular rulers" for the distance estimation between the reactive groups in proteins and DNA–protein complexes.[10]

A recombinant form of M.SsoII was used in the present work. It carried an N-terminal His-tag with one cysteine residue, Cys(−1) (Figure 1A). Here, we show that in the absence of DNA, the thiol group of Cys(−1) can get in close

TABLE 1 The crosslinkers used in the work

Crosslinker	Short name	Formula	MM (kDa)	Distance between reacted groups in the product of crosslinking	Target groups
Bis-maleimidoethane	BMOE		220	6.27–10.52 Å[10]; 8.0 Å (Pierce)	–SH
1,11-*bis*-maleimidotetraethyleneglycol	$BM[PEO]_4$		352	3.51–16.56 Å[10]; 17.8 Å (Pierce)	–SH
4-(*N*-maleimido) benzophenone	BPM		277	8.6–11.4 Å[15]	–SH and nonselective

proximity (up to a distance of less than 10.5 Å) to the thiol group of Cys142, which supports the hypothesis about a flexible linker connecting the M.SsoII N-terminal domain to the M.SsoII region responsible for methylation. In the M.SsoII–60reg complex, Cys142 modification by BPM followed by UV illumination yields a protein–DNA crosslink, demonstrating that the active center of M.SsoII is located near nonspecific DNA sequence in this complex. In the light of our new data, the existing model of M.SsoII complex with DNA needs to be revised.[3]

MATERIALS AND METHODS

The crosslinkers BMOE and BM[PEO]$_4$ were obtained from Pierce (Rockford, IL USA), and BPM was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Plasmids used in this work were constructed earlier[11, 12] and were kindly provided by Dr. Karyagina (N.F. Gamaleya Institute of Epidemiology and Microbiology, Russia) and Dr. Solonin (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russia). The proteins were expressed in *Escherichia coli* M15[pRep4] cells. The recombinant proteins carried the N-terminal His-tags: MetArgGlySer(His)$_6$ThrAspPro LeuGluThrCys (M.SsoII) or MetArgGlySer(His)$_6$GlySer [(cytosine-5)-DNA methyltransferase Ecl18kI (M.Ecl18kI)]. The proteins were purified by affinity chromatography using Ni-NTA agarose (Qiagen, Düsseldorf, Germany) followed by heparin sepharose (GE Healthcare, Little Chalfont, USA), then dialyzed into 50 mM Na phosphate with 50% glycerol (pH 7.5), and stored at −20°C.

Oligonucleotides 5′-ACGTTCATAATTGGAATCAAAACAGGACAAATT GTCCTAAAACCAACACTTAATTCTGGT-3′ (60regF), 5′-ACCAGAATTAA GTGTTGGTTTTAGGACAATTTGTCCTGTTTTGATTCCAATTATGAACG T-3′ (60regR), 5′-GACTTACAGTTGATAGTATGAAGCTAGAGCCAGGTTG GCAGCATTCTACTCATGTACTTG-3′ (60metA), and 5′-CAAGTACATGAG TAGAATGCTGCCAACCTGGCTCTAGCTTCATACTATCAACTGTAAGT C-3′ (60metT) were purchased from IBA GmbH (Göttingen, Germany).

Oligonucleotides were ^{32}P-labeled using T4 polynucleotide kinase (10 units; Fermentas, Vilnius, Lithuania) and [γ-^{32}P]ATP in 10 μL of 50 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) for 30 minutes at 37°C. [γ-^{32}P]ATP excess was removed by gel filtration on Illustra MicroSpin G-50 columns (GE Healthcare). Radioactivity of ^{32}P-labeled preparations was determined by the Cherenkov method as counts per minute on a Tracor Analytic Delta 300 counter (ThermoQuest/CE Instruments, Austin, TX, USA).

Solutions of DNA duplexes were prepared by mixing equimolar amounts of the complementary oligonucleotides. ^{32}P-labeled duplexes with known specific radioactivity were obtained by adding corresponding ^{32}P-labeled oligonucleotide to a certain amount of DNA duplex. The mixture was heated to 90°C and slowly cooled to room temperature for annealing.

M.SsoII and M.Ecl18kI (2.4 μM) complex formation with DNA duplexes (1.2 μM) was performed in 20 μL of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5) containing 100 mM NaCl and 5 μM *S*-adenosyl-L-homocysteine (AdoHcy). The reaction mixtures contained M.SsoII in two times molar excess to DNA in order to increase the yield of the complex M.SsoII–60reg with the stoichiometry 2:1. The ratios M.SsoII:60met and M.SsoII:60reg were the same for the purpose of comparison. The presence of the cofactor *S*-adenosyl-L-methionine or its analogue AdoHcy was shown to be necessary for the specific complex formation between M.SsoII and DNA containing the methylation site.[9] AdoHcy was added in two times molar excess to the protein in all the reaction mixtures for the purpose of comparison. Reaction mixtures were kept for 30 minutes at 37°C and then 2 μL of crosslinker solution in dimethyl sulfoxide was added. The final concentration was 5–50 μM for BMOE, 5–50 μM for BM[PEO]$_4$, and 150 μM for BPM. The reaction was stopped by adding 1 μL of 1 M Tris-HCl buffer containing 50 mM β-mercaptoethanol (pH 7.5). In the case of photocrosslinking with BPM, the reaction mixtures were put on Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) in an icebox and irradiated for 20 or 60 minutes at 365 nm with a UV lamp (Bachofer, Reutlingen, Germany).

Every reaction mixture was divided into two parts: 5 μL was analyzed by native polyacrylamide gel electrophoresis (PAGE), and the remaining 17 μL by Laemmli PAGE[13] in 10% sodium dodecyl sulfate gel. Native PAGE was performed in a 100 × 80 × 1 mm gel containing 5.7% acrylamide and 0.3% *N*,*N*′-*bis*-acrylamide, using TBE buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) at 15 mA. PageRuler Unstained Protein Ladder (Fermentas) and O'GeneRuler Ultra Low Range DNA Ladder (Fermentas) were used as markers. Nonradioactive DNA bands were visualized by ethidium bromide staining and photographed using a gel imaging and analysis system BioDocAnalyze (Biometra, Göttingen, Germany). Radioactive DNA bands were detected using an image analyzer FLA-3000 (Fuji-Film, Tokyo, Japan). Protein bands were visualized by colloidal Coomassie staining [0.1% Coomassie Brilliant Blue-250G, 2% (w/v) phosphoric acid, 5% aluminum sulfate, 10% ethanol]. The crosslinking results were compared for M.SsoII–60met complex, M.SsoII–60reg complex, and M.SsoII in the absence of DNA. Bands intensity was assessed using an ImageQuant 6 program.

RESULTS

Protein–DNA Complex Formation

Crosslinking was performed for protein–DNA complexes formed by M.SsoII with 60met, M.SsoII with 60reg, and M.SsoII without DNA (Figures 2 and 3). Despite the protein excess over DNA, only one M.SsoII–60met complex was observed, with the stoichiometry 1:1 (Figure 2A, lane 2 and Figure 3A, lane 1—complex 1). In contrast, two complexes were observed for M.SsoII–60reg (Figure 2A, lane 6 and Figure 3A, lane 4—complexes 1 and 2).

The reaction mixtures were analyzed by native PAGE (Figures 2A and 3A) and Laemmli PAGE (Figures 2B, 2C, 3B, and 3C). Native gels give the opportunity to check the formation of the M.SsoII–DNA complexes with the proper yield under the experimental conditions. M.SsoII demonstrates a high degree of nonspecific binding, i.e., binding to double-stranded DNA of any sequence.[14] M.SsoII has pI at 9.0; therefore, it is positively charged at pH 7.5 and approaches any DNA due to electrostatic interactions. However, nonspecific complexes are much less stable than the specific ones and thus demonstrate a much lower yield according to a gel shift assay.

Protein modification by a crosslinker slightly increases its molecular mass (MM; Table 1). Such a small difference is insufficient to be detected by a gel shift assay. On the other hand, intramolecular crosslink forms a loop inside the protein molecule, resulting in its compactization and therefore a higher electrophoretic mobility in comparison with the noncrosslinked protein. A linkage formation between two protein molecules yields a branched polymer. Electrophoretic mobility of such polymer molecules can differ a lot depending on the position of the linkage.[15, 16] Thus, electrophoretic mobility of crosslinking products does not reflect directly their MM, but can provide information on the position where the linkage was formed.

M.SsoII contains two Cys residues: Cys142 in the active center and Cys(−1) in the His-tag (Figure 1A). Thus, crosslinking through thiol groups can yield only one type of intramolecular crosslink Cys(−1)–Cys142 but three types of intermolecular crosslinks: Cys(−1)–Cys(−1), Cys(−1)–Cys142, and Cys142–Cys142 (Figure 4). In order to distinguish between these products, the same crosslinking reactions were performed with M.Ecl18kI, which shares 99% of the sequence identity with M.SsoII, but carries only one cysteine residue—Cys142 in the active center. Obviously, only one type of intermolecular crosslink, Cys142–Cys142, could occur in the case of M.Ecl18kI and intramolecular crosslink formation was not possible.

Cys–Cys Crosslinking

Thiol modification with maleimide results in a stable thioether bond formation. The reaction is fast and very specific to sulfhydryls at pH 6.5–7.5.[17]

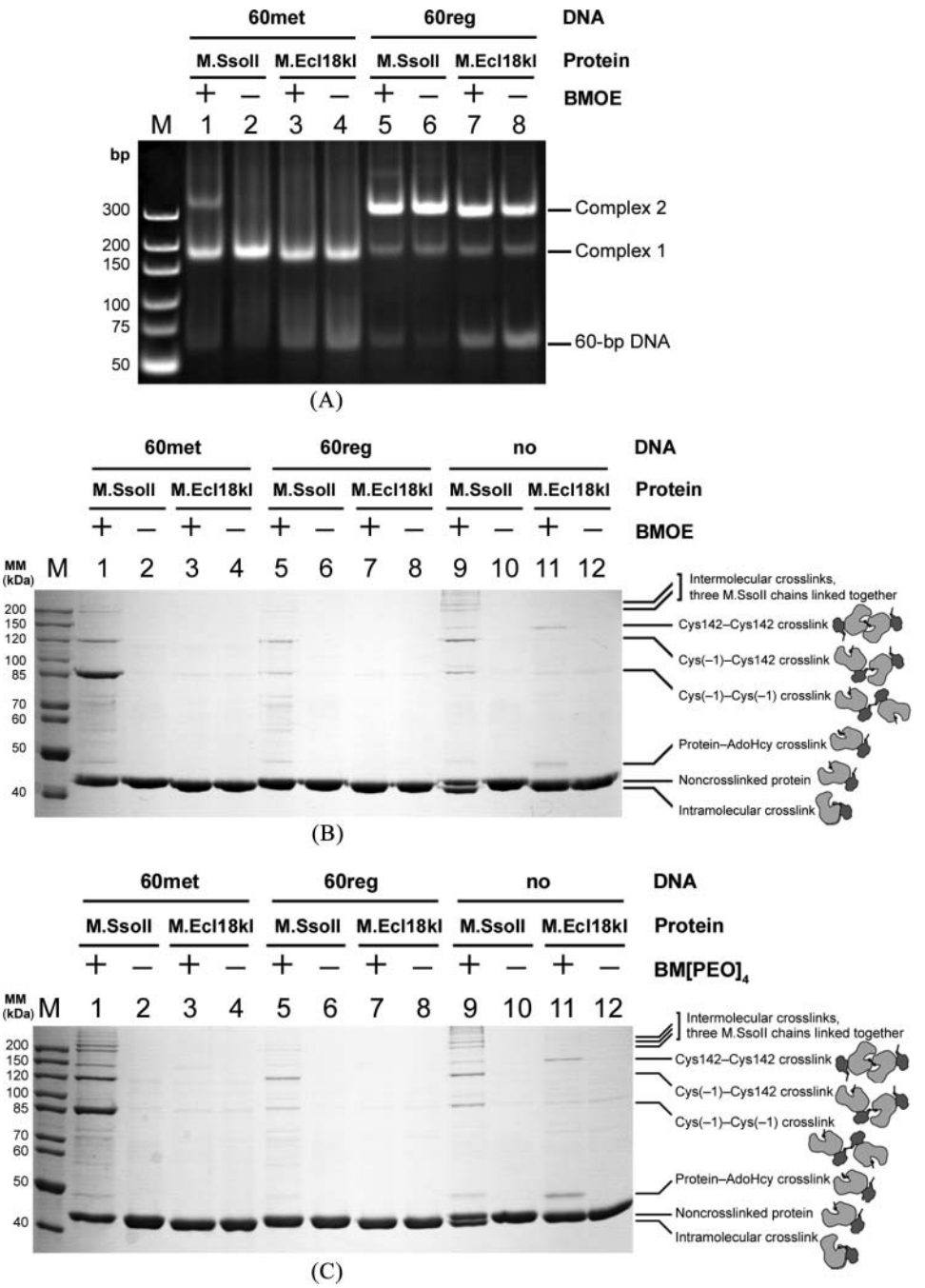


FIGURE 2 Crosslinking of M.SsoII, M.EcI18kI, and their complexes with 60met and 60reg DNA using BMOE or BM[PEO]$_4$. (A) Analysis of BMOE crosslinking products by native PAGE. (B) Analysis of BMOE crosslinking products by Laemmli PAGE. (C) Analysis of BM[PEO]$_4$ crosslinking products by Laemmli PAGE.

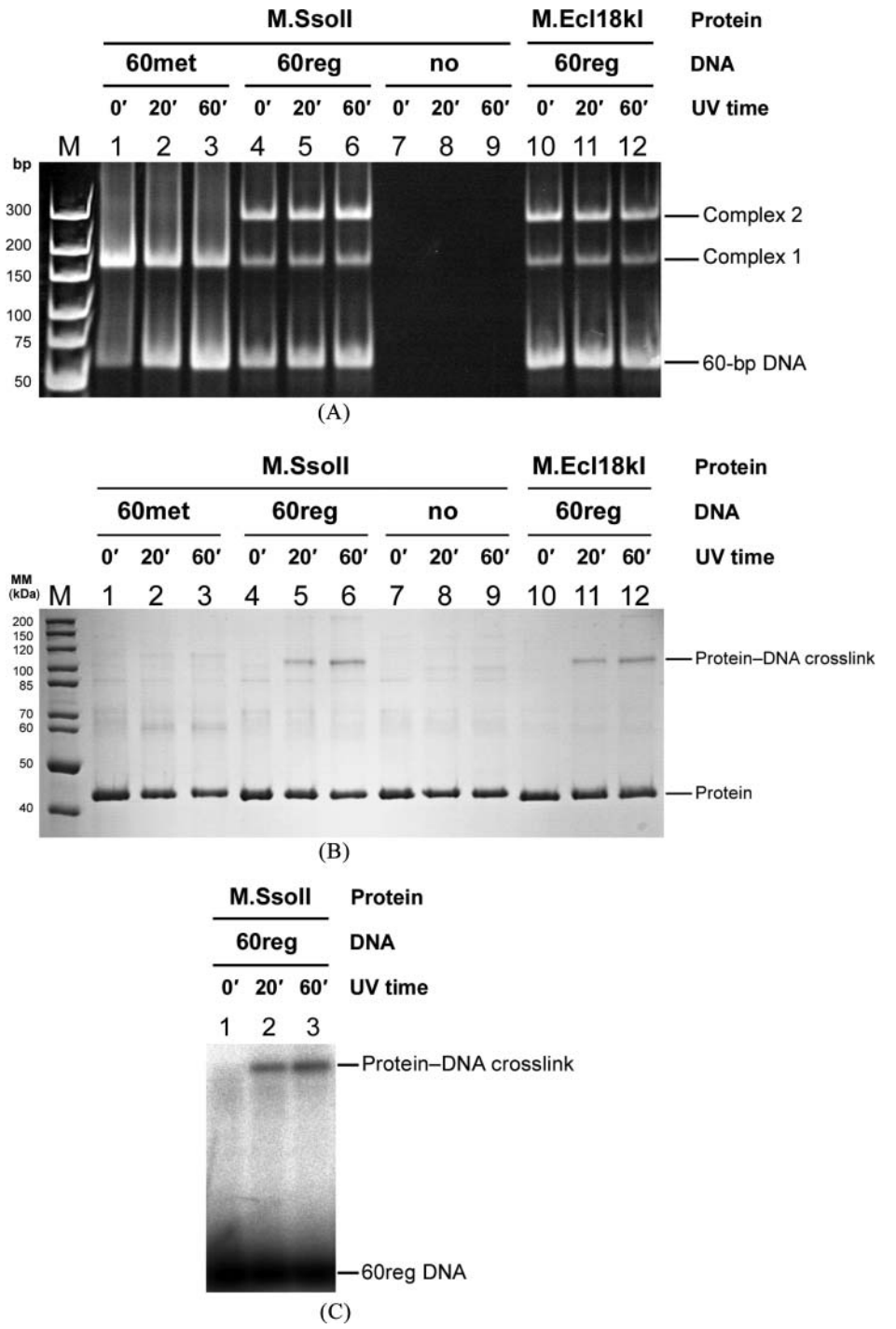


FIGURE 3 Photocrosslinking of M.SsoII, M.Ecl18kI, and their complexes with 60met and 60reg DNA using BPM. The time of UV illumination is shown above the lanes. (A) Analysis of the crosslinking products by native PAGE. (B) Analysis of the crosslinking products by Laemmli PAGE. Protein containing bands are stained with Coomassie. (C) Analysis of the crosslinking products by Laemmli PAGE. ^{32}P-labeled DNA is detected by radioautography. Lanes 1–3 correspond to lanes 4–6 in the Figure (B).

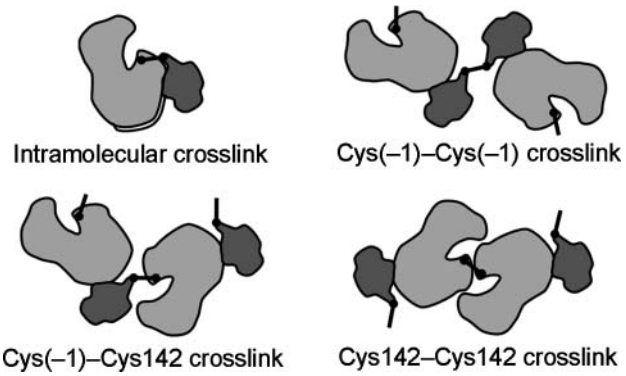


FIGURE 4 Possible variants of Cys–Cys crosslinks in M.SsoII molecules.

To optimize the experimental conditions, we carried out some preliminary experiments using maleimide coupled to a fluorescent dye Alexa Fluor 594. M.SsoII was modified by an excess of this reagent; aliquots of the reaction mixture were taken over time (from 0.5 to 60 minutes) and analyzed by Laemmli PAGE. Fluorescence intensity of the M.SsoII band on the gel was used to estimate the reaction yield. Cysteine modification proved to be completed within 1 minute (data not shown), confirming that the Cys residue was available for the reagent.

The reactions of crosslinking with BMOE and BM[PEO]$_4$ were studied over time (1, 5, and 20 minutes). The total amount of all the crosslinking products was close to its maximum after 1 minute in the case of BM[PEO]$_4$ and after 5 minutes in the case of BMOE (Figure 5A). This testifies to an easier reaction in the case of BM[PEO]$_4$ due to its longer linker between the maleimide groups. In the next experiments, the reaction time for all mixtures was kept fixed at 5 minutes.

Generally, a twofold or threefold molar excess of BMOE or BM[PEO]$_4$ over the amount of Cys-containing protein is recommended by the producer (Pierce). We have compared 2-, 4-, 8-, and 20-fold molar excesses of each crosslinker over the M.SsoII monomer. The highest yield of crosslinking products was obtained for the twofold excess. It corresponds to the twofold excess of maleimide groups over the amount of thiol groups, because a crosslinker molecule contains two maleimide rings and M.SsoII monomer contains two Cys residues. This ratio of crosslinker to protein was maintained in the next experiments where the bands were analyzed quantitatively (Table 2).

Considering the high efficiency of thiol modification with maleimide, we assumed that all protein molecules were modified by the reagent. Because M.SsoII modified by one or two BMOE or BM[PEO]$_4$ molecules could not be separated from unmodified M.SsoII by a gel shift assay, we labeled the corresponding band with an apparent MM of 45 kDa as "noncrosslinked

TABLE 2 Yield of SsoII methyltransferase crosslinking by BMOE and BM[PEO]$_4$. The yield is represented in percentage to the total protein amount. The values for each band are the average of two experiments

Crosslink type		BMOE			BM[PEO]$_4$		
Description	Apparent MM (kDa)	M.SsoII + 60met	M.SsoII + 60reg	M.SsoII	M.SsoII + 60met	M.SsoII + 60reg	M.SsoII
Intramolecular	43	0	0	34	0	0	38
Noncrosslinked protein	45	51	85	43	41	77	36
Cys142–AdoHcy	50	3	2	1	2	2	4
Intermolecular, Cys(−1)–Cys(−1)	85	35	5	5	31	5	5
Intermolecular, Cys(−1)–Cys142	120	8	6	9	14	10	8
Intermolecular, Cys142–Cys142	150	0	0	2	4	2	2
Intermolecular, three M.SsoII	200	3	1	2	4	2	2
chains linked together	220	1	0	3	4	1	3
	240	0	0	0	1	0	1
Intramolecular crosslink		**0**	**0**	**34**	**0**	**0**	**38**
Intermolecular crosslink		**46**	**12**	**22**	**57**	**21**	**22**
Total crosslink		**46**	**12**	**56**	**57**	**21**	**60**
Total protein		**100**	**100**	**100**	**100**	**100**	**100**

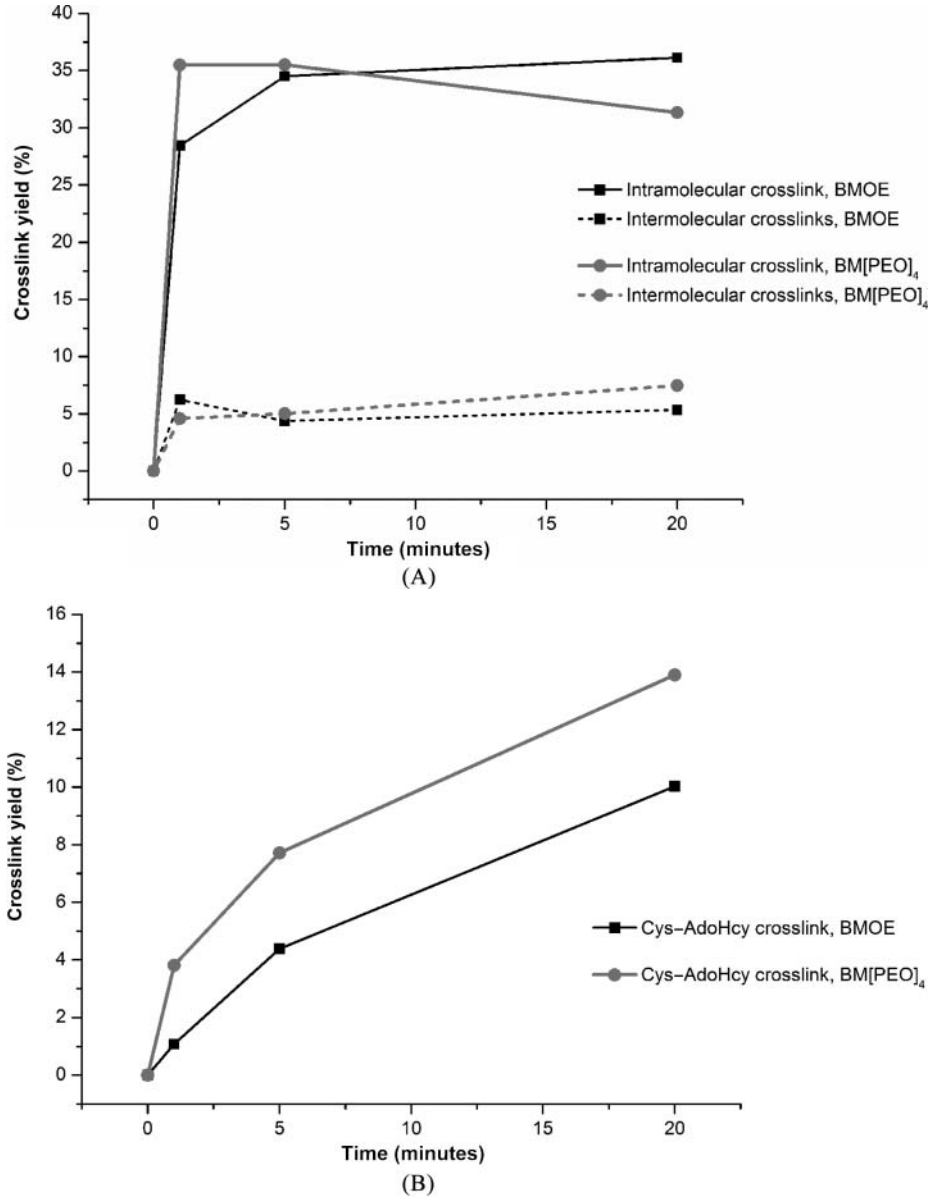


FIGURE 5 Kinetics of M.SsoII crosslinking with BMOE or BM[PEO]$_4$ in the absence of DNA. (A) Formation of intramolecular and intermolecular Cys–Cys crosslinks. (B) Formation of Cys–AdoHcy crosslink.

protein" (Figures 2B, 2C, and 3B; Table 2). The intensity of this band could be used as a measure opposite to the crosslinking efficiency. In the absence of DNA, the intensity was the lowest (i.e., the crosslinking was the most efficient) in the case of M.SsoII treatment by BM[PEO]$_4$ (Table 2).

A product of intramolecular Cys–Cys crosslinking is observed as a result of crosslinking by BMOE or BM[PEO]$_4$ in the absence of DNA (Figures 2B

and 2C, lane 9). It has electrophoretic mobility corresponding to a protein of 43 kDa. With both BMOE and BM[PEO]$_4$, the yield of this intramolecular crosslinking product was equally high (see Table 2), which demonstrates that a Cys(−1) side chain can approach a Cys142 side chain up to the distance less than 10.5 Å without sterical constraints. Such a band was not observed with M.Ecl18kI, which contains only one cysteine residue (Cys142), further supporting the assignment of this band as an intramolecular Cys–Cys crosslink (Figures 2B and 2C, lane 11).

A product with apparent MM of 50 kDa was observed for both M.SsoII and M.Ecl18kI when crosslinked by BMOE or BM[PEO]$_4$ in the absence of DNA (Figures 2B and 2C, lanes 9 and 11, protein–AdoHcy crosslink). This product was not formed in the absence of AdoHcy (data not shown). We can speculate that it is a Cys142–AdoHcy crosslink where one of the maleimide groups is coupled to the amino group of AdoHcy. Although the maleimide group reacts at pH 6.5–7.5 predominantly with free sulfhydryl groups, reactivity toward primary amines can also occur (Pierce). The yield of this product increased over time relatively slowly (Figure 5B), which also suggested a reaction of maleimide with a group different from thiol.

Three types of intermolecular crosslinks can be formed in M.SsoII, as it is mentioned earlier (Figure 4). In the case of M.SsoII crosslinking by BM[PEO]$_4$ in the absence of DNA, we observed three bands on the gel corresponding to these three species, with apparent MM of 85, 120, and 150 kDa (Figure 2C, lane 9). In the control experiment of M.Ecl18kI crosslinking by BM[PEO]$_4$ in the absence of DNA, only one prominent band was observed, with the apparent MM of 150 kDa (Figure 2C, lane 11). Thus, we assigned this band to the Cys142–Cys142 crosslink. The MM of 85 kDa is approximately two times higher than that of M.SsoII (45 kDa). The band of 85 kDa should correspond to Cys(−1)–Cys(−1) crosslink, because the linkage is located close to the N-terminal ends of the polypeptide chains and the geometry of the branched polymer is close to linear. The Cys(−1)–Cys142 crosslink should correspond to 120 kDa as a value between 85 and 150 kDa. In the case of M.SsoII and M.Ecl18kI crosslinking with BMOE in the absence of DNA (Figure 2B, lanes 9 and 11), the bands of intermolecular crosslinks are the same as with BM[PEO]$_4$ (Figure 2C, lanes 9 and 11), only the yield with BMOE is lower. This is explainable by the shorter linker between the maleimide groups in BMOE (Table 2).

Also, several bands with an apparent MM of 200 kDa and more were observed in the case of M.SsoII crosslinking in the absence of DNA by BM[PEO]$_4$ or BMOE (Figures 2B and 2C, lane 9; Table 2). Most likely, they are products of crosslinking between three M.SsoII molecules. Each one of the bands is formed with a very low yield (less than 4%) and therefore can be considered a result of occasional interaction between the reactive groups due to stochastic collision of molecules in solution. Such bands were absent in crosslinking reactions with M.Ecl18kI, as expected.

In the case of M.SsoII–60reg complex crosslinking by BMOE or $BM[PEO]_4$, the intermolecular crosslinks are obtained with a low yield, which is approximately equal to the yield of the same bands in the absence of DNA (compare lanes 5 and 9 in Figures 2B and 2C; Table 2). Therefore, they can be considered a result of occasional reactions due to stochastic collision of molecules in solution. The Cys142–Cys142 crosslink was totally absent with BMOE. This testifies that upon M.SsoII dimerization mediated by its binding to the regulatory site, the distance between the thiol groups of Cys142 residues in different M.SsoII subunits is higher than 10.5 Å. There were no crosslinks in the case of M.Ecl18kI–60reg complex crosslinking with BMOE or $BM[PEO]_4$ as a chemical reagent.

The products of BMOE or $BM[PEO]_4$ crosslinking were formed with the highest yield in the case of M.SsoII–60met complex (Figures 2B and 2C, lane 1). The main species were Cys(−1)–Cys(−1) and Cys(−1)–Cys142 crosslinks. Obviously, these bands were absent in the case of M.Ecl18kI (Figures 2B and 2C, lane 3). The native gels demonstrated the formation of only one M.SsoII–60met complex in the absence of a crosslinker, with stoichiometry 1:1, whereas the crosslinker addition resulted in the appearance of the second M.SsoII–60met complex (Figure 2A, lane 1). It had an electrophoretic mobility similar to that of the M.SsoII–60reg complex with 2:1 stoichiometry, suggesting the same stoichiometry. The fact that the second complex formation was observed only in the presence of the crosslinker points to its nonspecific character. Because M.SsoII is taken in two times molar excess to DNA, a sufficient concentration of unbound M.SsoII molecules remains after M.SsoII–60met specific complex formation. These molecules can bind to the same DNA duplex in the regions that are free from the already bound M.SsoII molecules, i.e., apart from the methylation site. Chemical footprinting revealed that only 8–9 bp took part in forming DNA–M.SsoII contacts in the specific complex with DNA containing the methylation site.[9] A 14-bp duplex was sufficient for obtaining the M.SsoII complex with DNA containing the methylation site and effective DNA methylation.[18] Typically, (cytosine-5)-DNA methyltransferases protect 16–21 bp from DNase I hydrolysis.[19–21] Taken together, these data suggest that only one third or one fourth of the 60-bp DNA duplex is covered by one M.SsoII molecule in the specific M.SsoII–60met complex (Figure 6B). The flanking regions can be bound by free M.SsoII molecules in a nonspecific manner. Considering the high rate of the maleimide reaction with thiol, the crosslinking technique can fix an arrangement of M.SsoII molecules, which has a short lifetime and plays no significant biological role.

Photocrosslinking

Usage of BMOE or $BM[PEO]_4$ is limited by the mutual arrangement of Cys residues in a protein molecule. To examine the local environment

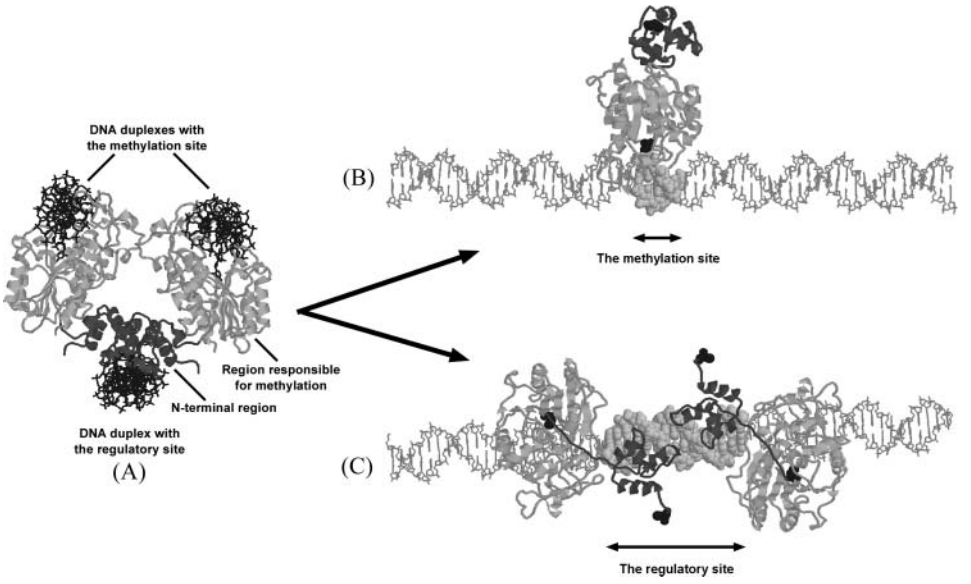


FIGURE 6 Rearranging the structure of M.SsoII complex with DNAs. (A) The previously assumed structure of the complex between two M.SsoII subunits, one DNA duplex containing the regulatory site and two DNA duplexes containing the methylation site according to ref. [3]. Schematic representations of (B) M.SsoII–60met complex and (C) M.SsoII–60reg complex considering the impossibility of M.SsoII simultaneous binding to the regulatory site and the methylation site. The N-terminal domain (residues 1–71) is in dark gray; the region responsible for methylation (residues 72–379) is in light gray. The Cys residues are in black (space-fill representation). Out of the His-tag, only Cys(−1) is shown. DNA is in sticks representation; the methylation site in (B) and the regulatory site in (C) are in space-fill representation.

around a Cys residue, we used a heterobifunctional crosslinker BPM (Table 1). Its maleimide group reacts specifically with the thiol group of cysteine residue, while its benzophenone group can insert into the C–H bond when excited by UV light.[22, 23] The benzophenone group is not decomposed by excitation; it can undergo relaxation and then be excited again. Therefore, prolongation of UV illumination increases the yield of the crosslinked substance.

A product with an apparent MM of 110 kDa was observed upon BPM crosslinking (Figure 3B, lanes 5 and 6). This product was found only in the reaction mixture with 60reg, i.e., it was specific for the M.SsoII–60reg complex. Its yield increased with increasing time of UV illumination and reached 19% after 1 hour. Such a long exposure to UV light did not disrupt the protein–DNA complexes (Figure 3A). The same photocrosslinking product was obtained with M.Ecl18kI with the same yield (Figure 3B, lanes 11 and 12), testifying that Cys142 was involved in this reaction. Experiments with ^{32}P-labeled DNA duplex have shown that this band is indeed a protein–DNA crosslink (Figure 3C, lanes 2 and 3 that correspond to lanes 5 and 6 in Figure 3B).

These results indicate that the Cys142 residue of the M.SsoII active center is in close proximity to DNA without the methylation site in the M.SsoII–60reg complex. This correlates with the low yield of crosslinks upon BMOE or BM[PEO]$_4$ treatment of this complex (Figures 2B and 2C, lane 5). Cys142 seems to be protected from modification due to complex formation with 60reg DNA.

DISCUSSION

A hypothetic model was built earlier[3] that consisted of two M.SsoII subunits in complex with one DNA duplex containing the regulatory site and with two DNA duplexes containing the methylation site (Figure 6A). The model was constructed on the basis of the sequence similarities listed above, based on the results of footprinting and crosslinking analysis of M.SsoII–DNA complexes.[6,9, 24] However, the relative arrangement of the two regions in M.SsoII molecule remained unproven, as well as the relative arrangement of M.SsoII subunits in the complex and the general possibility of such a cell complex formation.

It is worth noting that M.SsoII simultaneous binding to both the DNA ligands (containing the methylation and the regulatory site) was never demonstrated by an electrophoretic mobility shift assay. Such a complex was obtained only by crosslinking of M.SsoII with two types of modified DNA duplexes, where one of the duplexes contained a 2′-*O*-(2-oxoethyl)uridine residue in the regulatory sequence, and the other one carried a phosphoryldisulfide group in the methylation site.[24] However, these conditions are far from native ones and formation of such a complex in the bacterial cell is quite improbable.

In this study, we showed that M.SsoII treatment with BMOE or BM[PEO]$_4$ in the absence of DNA gave a product of an intramolecular crosslink with comparable high yields (Table 2). This testifies that the Cys(−1) thiol group can approach the Cys142 thiol group up to a distance of less than 10.5 Å without sterical constraints. It requires an extremely high mobility of the N-terminal domain relative to the region responsible for methylation and, therefore, strongly supports the hypothesis about the linker flexibility. This does not yet disprove the model proposed earlier,[3] but points to the fact that the linker conformation in the model represents only one of several possible variants. A putative structure of the M.SsoII molecule with the intramolecular crosslink is shown in Figure 7B and demonstrates that the linker has a size that allows an orientation between the two domains such that Cys(−1) is in close proximity to Cys142.

Footprinting studies on DNA containing the regulatory site revealed a very large region protected from DNase I hydrolysis by M.SsoII binding—48/52 bases.[4] Chemical footprinting, on the contrary, revealed a much shorter region of DNA–protein interactions: all the specific

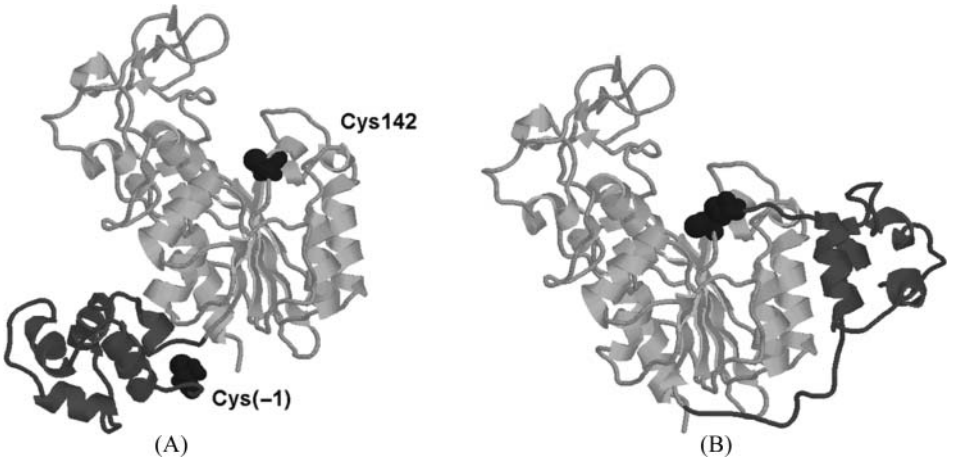


FIGURE 7 (A) The previously assumed location of Cys residues in M.SsoII molecule according to ref. [3]. (B) Schematic representations of M.SsoII with the intramolecular Cys(−1)–Cys142 crosslink. The N-terminal domain (residues 1–71) is in dark gray; the region responsible for methylation (residues 72–379) is in light gray. The Cys residues are in black (space-fill representation). Out of the His-tag, only Cys(−1) is shown.

M.SsoII–DNA contacts were located inside the 15-bp sequence of the regulatory site.[6] The palindromic nature of the regulatory site suggested dimerization of M.SsoII. However, only a complex of 1:1 stoichiometry was obtained upon M.SsoII interaction with a 30-bp DNA containing the regulatory site. Increasing the DNA length up to 60 bp was necessary to demonstrate the DNA-mediated protein dimerization.[8] These data were in agreement with the DNase I footprinting results and proved that the presence of DNA flanking the regulatory site was necessary for the proper M.SsoII–60reg complex formation. Nevertheless, no structural reason was found for it.

The present study gives an explanation for these observations. Photocrosslinking with BPM demonstrates that the Cys142 residue of the M.SsoII active center is located close to the DNA in the M.SsoII–60reg complex, therefore suggesting that the M.SsoII region responsible for methylation binds to the DNA flanking the regulatory site (Figure 6C). Considering that there is no methylation site in 60reg DNA, we assume that M.SsoII binding to the flanking sequences is nonspecific, i.e., based on electrostatic interactions with the sugar-phosphate backbone of the DNA and independent of the DNA sequence. A key structural feature allowing formation of such a complex is the flexibility of the linker connecting the N-terminal region and the region responsible for methylation.

Thus, M.SsoII interaction with the regulatory site implies its nonspecific interaction with DNA flanking the regulatory sequence. Such a structural organization of M.SsoII complex with the regulatory DNA most likely prevents M.SsoII interaction with the methylation site. Therefore, the simultaneous binding of M.SsoII to both the DNA ligands (containing the methylation

and the regulatory site) is hardly possible under native conditions and the model (Figure 6A[3]) needs to be revised in light of the data presented here.

Interaction of the M.SsoII region responsible for methylation with the DNA flanking the regulatory site could play an important biological role. It is likely to increase the stability of the M.SsoII complex with the regulatory site situated in the promoter region of the SsoII restriction–modification system, therefore creating an obstacle for RNA polymerase and preventing its binding to the promoter of *ssoIIM* gene. This leads to transcription repression and thus stabilizes the M.SsoII concentration in the cell.

CONCLUSIONS

The crosslinking with BPM demonstrates that the M.SsoII–60reg complex formation implies nonspecific binding of the M.SsoII region responsible for methylation to the DNA flanking the regulatory site. This requires a high flexibility of the linker connecting the M.SsoII N-terminal domain and the M.SsoII region responsible for methylation, which is demonstrated by the crosslinking with BMOE and $BM[PEO]_4$. Such structural organization provides high stability for the M.SsoII–60reg complex and is necessary for performing the function of a transcription regulator by M.SsoII.

Abbreviations

60met	60-bp DNA duplex containing the methylation site
60reg	60-bp DNA duplex containing the regulatory site
AdoHcy	*S*-adenosyl-L-homocysteine
BMOE	*bis*-maleimidoethane
$BM[PEO]_4$	1,11-*bis*-maleimidotetraethyleneglycol
BPM	4-(*N*-maleimido)benzophenone
MM	molecular mass
PAGE	polyacrylamide gel electrophoresis
M.Ecl18kI	(cytosine-5)-DNA methyltransferase Ecl18kI
M.HhaI	(cytosine-5)-DNA methyltransferase HhaI
M.SsoII	(cytosine-5)-DNA methyltransferase SsoII

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