BioTechniques
The International Journal of Life Science Methods

Chemically-induced affinity star restriction specificity: a novel TspGWI/sinefungin endonuclease with theoretical 3-bp cleavage frequency

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BioTechniques, Vol. 50, No. 6, June 2011, pp. 397–406

Supplementary Material

Supplementary Material (.pdf)

The type IIS/IIC restriction endonuclease *Tsp*GWI recognizes the sequence 5′-ACGGA-3′, cleaving DNA 11/9 nucleotides downstream. Here we show that sinefungin, a cofactor analog of S-adenosyl methionine, induces a unique type of relaxation in DNA recognition specificity. In the presence of sinefungin, *Tsp*GWI recognizes and cleaves at least 12 degenerate variants of the original recognition sequence that vary by single base pair changes from the original 5-bp restriction site with only a single degeneracy per variant appearing to be allowed. In addition, sinefungin was found to have a stimulatory effect on cleavage at these nondegenerate *Tsp*GWI recognition sites, irrespective of their number or the DNA topology. Interestingly, no fixed "core" could be identified among the new recognition sequences. Theoretically, *Tsp*GWI cleaves DNA every 1024 bp, while sinefungin-induced activity cleaves every 78.8 bp, corresponding to a putative 3-bp long recognition site. Thus, the combination of sinefungin and *Tsp*GWI represents a novel frequent cutter, next only to *Cvi*JI/*Cvi*JI*, that should prove useful in DNA cloning methodologies.

Restriction-modification (RM) enzymes are vital systems involved in protecting bacteria and archaea from infection by bacteriophage DNA. Interestingly, a few RM systems with an inverse function have also been found in the viruses infecting eukaryotic, unicellular *Chlorella*. Such systems play a significant role in hosts' genome degradation as well as excluding different types of virus superinfection (1,2). On the basis of subunit composition, cofactor requirements, and the mode of recognition and cleavage, RM systems have been classified into four major types: I, II, III, and IV (3). There are currently 3945 biochemically or genetically characterized restriction enzymes, and of the 3834 type II restriction enzymes, 299 distinct specificities are known. Type II RM systems constitute the largest fraction that has been characterized biochemically (4). Type I and type III restriction endonucleases (REases) are complex multisubunit molecular machines that utilize ATP and perform either DNA modification or cleavage (5).

Orthodox type II REases usually recognize short, palindromic sequences of 4–8 bp in length and cleave phosphodiester bonds in DNA within their recognition sites (6). Most often, type II RM systems contain separate REase and methyltransferases (MTase) enzymes. In contrast to MTases, REase proteins display a high sequence diversity, with only remote similarities detected, such as the catalytic motif PD-(D/E)XnK, and are categorized into 11 overlapping subtypes (3).

Subtype IIS REases that interact with the asymmetric sequence typically cut DNA in a strictly fixed location beyond its recognition site to produce blunt or sticky ends with 3′- or 5′-overhangs (6). Among them, a growing group of atypical type II REases, represented by bifunctional enzymes with REase and MTase activities within a single polypeptide (subtype IIC) is worth mentioning. Examples of this group include *Eco*57I (7,8), *Hae*IV (9), *Alo*I (10), *Bse*MII (11), enzymes from the *Mme*I family (12,13), and enzymes from the *Thermus* sp. family (14-17). Several of them are stimulated by S-adenosyl methionine (AdoMet) or its structural analogs sinefungin (SIN) and S-adenosyl homocysteine (AdoHcy), but otherwise they behave like typical type II enzymes (6,15).

Several type II REases exhibit a "star" activity when used in excess (overdigestion) or under altered reaction buffer conditions. The star (*) activity of bacterial REases is widely described in the literature as modifying reaction conditions such as low ionic strength, high pH, high glycerol concentration, the presence of other organic solvents, high molar ratio of enzyme to DNA, or substituting other divalent cations for magnesium (18,19). These star recognition sequences usually differ from the canonical sequence by a single base pair (20). The star effect is associated with a decrease in the activity of associated water molecules, resulting in altered protein-DNA contacts and affecting recognition and cleavage (21). An unusual type of star activity, caused by an adenine nucleotide, is observed in eukaryotic *Cvi*JI REase (1,2). The new concept of fidelity index (FI), defined as the ratio of the maximum enzyme amount showing no star activity to the minimum amount needed for complete digestion at the cognate recognition site for any particular restriction endonuclease, has recently been proposed. The FI defines the fundamental property by which REases can be characterized (19).

The bifunctional *Tsp*GWI protein is a prototype of the *Thermus* sp. enzyme family. The other members of this family include related thermophilic endonucleases *Taq*II, *Tsp*DTI, and *Tth*111II (14-17). These enzymes belong to the type II REases, but they also have some features of types I and III. The criteria for defining the *Thermus* sp. family were published previously (14,15).

*Tsp*GWI recognizes the 5′-ACGGA-3′ site and cleaves 11/9 nucleotides downstream. The enzyme generates a defined partial fragmentation pattern corresponding to cleavage at the target site (16). Under the variety of conditions tested previously, no complete *Tsp*GWI digestion was obtained. This was probably due to preferential site cleavage in various DNA substrates and the requirement of two recognition sequences for DNA cleavage (14,16). Unlike most of the other bifunctional type IIC REases/MTases, neither exogenous AdoMet nor AdoHcy stimulate *Tsp*GWI REase activity, although AdoMet is required for effective DNA methylation. The restriction activity of *Tsp*GWI is neither inhibited nor stimulated by ATP. Interestingly, in contrast to the *Tsp*GWI enzyme, *Tsp*DTI and *Taq*II of the *Thermus* family of enzymes display marked differences in their response to AdoMet, which effectively stimulates their restriction endonuclease activity (15).

In this paper, we show that SIN, an AdoMet analog that has inverted charge configuration at the $\delta CHN^{+}H3$ center as compared with the $\delta S^{+}CH3$ center of AdoMet, induces a novel type of *Tsp*GWI star activity, converting the 5-bp enzyme to approximately 3-bp, a very frequent cutter. This allows the enzyme to be used for novel molecular biology applications or to improve existing ones. Moreover, SIN stimulates cleavage at canonical *Tsp*GWI recognition sites irrespective of their number, orientation, and DNA topology. The relaxation in the *Tsp*GWI specificity of DNA recognition described here is unique in requiring a specialized cofactor, which suggests an allosteric binding site for the SIN-dependent modulation of the restriction specificity.

Materials and methods

Bacterial strains, plasmids, media, and reagents

Native *Tsp*GWI protein and expression clone were obtained from Piotr Skowron's collection. Recombinant *Tsp*GWI protein was purified as described previously (14). *Escherichia coli* DH11S {*mcrA* Δ[*mrrhsdRMS*(rK^{-}, mK $^{+}$)-*mcrBC*] Δ(*lac-proAB*) Δ(*recA1398*) *deoR*, *rpsL*, *srl-thi*, *supE*/*F*′ *proAB*+ *lacI*QZΔ*M15*} (Life Technologies, Gaithersburg, MD, USA) was used for the transformation of ligation mixtures, DNA propagation, and *tspGWIRM* gene expression. Bacteria were grown in 2× yeast extract/tryptone (YT) medium (22). For protein expression, bacteria were cultivated in Terrific Broth (TB) medium (22). Media were supplemented with chloramphenicol (40 mg/mL) and 0.2% maltose. Difco media components were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA), phosphocellulose P11 resin was obtained from Whatman (Springfield Mill, UK), and hydroxyapatite HTP was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Other chromatographic resins were from GE Healthcare (Uppsala, Sweden).

The miniprep DNA purification kit, *Sma*I endonuclease, T4 DNA polymerase, Perfect Perfect 100-bp ladder, and Perfect Plus 1-kb ladder were from Eurx Molecular Biology Products (Gdansk, Poland). λ DNA and plasmids pBR322 and pUC19 were from Vivantis (Shah Alam, Malaysia). T4 DNA ligase was from Epicenter (Madison, WI, USA). The DNA sequencing and PCR primer synthesis were performed at Genomed (Warsaw, Poland). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

λ DNA cleavage assay

Cleavage was carried out in the presence or absence of 100 mM SIN in the optimal reaction buffer (50 mM Tris-HCl, pH 7.2, at 65°C, 10 mM $MgCl_2$,10 mM DTT) at 65°C. The reaction volume of 100 mL contained 12 pmol recognition sites and 120 pmol recombinant *Tsp*GWI protein. The protein to DNA recognition sites molar ratio was approximately 10:1. After 16 h, the digestion was quenched with phenol/chloroform, and DNA was ethanol-precipitated from the aqueous phase. DNA precipitate was collected by centrifugation and dissolved in 10 mM Tris-HCl, pH 8.0, at 25°C. To determine the recognition sequence and cleavage sites, the DNA samples were treated with T4 DNA polymerase in the presence of dNTP and subjected to shotgun cloning. All concentrations of *Tsp*GWI given here refer to the monomeric form of protein Mr 120,000.

PCR fragment DNA cleavage assay

The PCR(wt) fragment (390 bp) containing double convergent (→←) canonical sites for *Tsp*GWI was amplified from pBR322 plasmid DNA using *Taq* DNA polymerase and a pair of primers: FWT 5′-CTCGACCTGAATGGAAGCCG-3′ and RWT 5′-GGTGCAGGGCGCTGACTTCC-3′. The selected fragment contained a previously described refractory *Tsp*GWI recognition site, located within the tetracycline resistance gene. Cleavage of pBR322 supercoiled DNA showed a stable partial digestion pattern, where all sites except this one were efficiently cleaved (16). Complete digestion of PCR(wt) yielded 282 bp, 56 bp, and 48 bp (Figure 1A).

Figure 1. PCR fragment DNA substrates. (Click to enlarge)

For the PCR(mut) substrate (390 bp), the existing *Tsp*GWI sites of PCR(wt) were converted to *Taq*II sites using a pair of primers introducing specific point mutations within the native *Tsp*GWI sites (the changed nucleotides are written in small letters; *Taq*II sites are in bold and underlined): FMUT 5′-CTCGACCTGAATGGAAGCCGGCGGCACTCGCT**gACcGA**TTCACCACT-3′ and RMUT 5′-GGTGCAGGGCGCTGACTTCCGCGTTTCCAGACTTTACGAAA**CAccCA**AACCGAAGA-3′ The resulting PCR fragment contained two convergent (→←) sites for *Taq*II. The complete digestion of the PCR(mut) fragment with *Taq*II should yield 282 bp, 56 bp, and 48 bp (Figure 1B).

The PCR(single) fragment (390 bp) containing a single (→) canonical site for *Tsp*GWI was amplified from the PCR(wt) fragment using a combination of primers: FWT and RMUT. The second canonical *Tsp*GWI site was removed (Figure 1C).

The PCR(oligo) fragment (89 bp) containing double convergent (→←) canonical sites for *Tsp*GWI was amplified from PCR(wt) DNA using *Taq* DNA polymerase and a pair of primers: 5′-GGAAGCCGGCaGCACCTCGC-3′ and 5′-ccgccccGCGCATTC**ACgGa**TCTCgGCAAGA-3′. The second canonical *Tsp*GWI site was introduced. All the existing variants with a single degeneracy within the *Tsp*GWI canonical site had been eliminated using a pair of primers introducing specific point mutations (the changed or added nucleotides are written in small letters; the *Tsp*GWI site is in bold and underlined) (Figure 1D).

*Tsp*GWI or *Taq*II cleavage of PCR fragments was carried out in the presence or absence of 100 mM SIN in the appropriate reaction buffer at 65°C. The *Taq*II cleavage reaction of the PCR(mut) DNA fragment was performed in the following buffer: 10 mM Tris-HCl, pH 7.2, at 65°C, 10 mM MgCl2,1 mM DTT, BSA 100 μg/mL. All reaction mixtures contained 19 pmol recognition sites and 190 pmol recombinant *Tsp*GWI protein. The protein to DNA recognition sites molar ratio was approximately 10:1. The reaction volume was 100 mL. After 16 h, the reactions were quenched with phenol/chloroform, and DNA was ethanol-precipitated from the aqueous phase. The DNA precipitate was collected by centrifugation and dissolved in 10 mM Tris-HCl, pH 8.0, at 25°C. The products were analyzed by electrophoresis in either 1.5% agarose or 15% polyacrylamide gels.

Determination of *Tsp*GWI* recognition and cleavage sites in the presence of SIN

The *Tsp*GWI* recognition site and cleavage positions were established by shotgun cloning and sequencing of the digestion products of bacteriophage λ DNA and the 390-bp PCR(wt) fragment. The *Tsp*GWI*-generated restriction fragment ends were blunted with T4 DNA polymerase in the presence of dNTPs, cloned into the *Sma*I site of pUC19 vector, transformed into *E. coli* DH11S, and plated onto X-gal/IPTG plates (22). Plasmid DNA was isolated from white colonies, and the fragment/vector junctions were sequenced. The sequence data obtained were then analyzed using ABI Chromas 1.45 software (Perkin Elmer Applied Biosystems, Monza, Italy) and DNASIS 2.5 software (Hitachi Software, San Bruno, CA, USA).

Methyltransferase assay

The ability of *Tsp*GWI enzyme to cleave methylated canonical DNA recognition sequences in the presence of SIN was tested by the DNA protection assay, in which 0.5 µg (16.5 pmol) PCR(oligo) DNA fragment was used as a substrate in 50 µL *Tsp*GWI reaction buffer (50 mM Tris-HCl, pH 8.5, 10 mM $CaCl_2$, 10 mM DTT), supplemented with 200 µM AdoMet. In this buffer, devoid of Mg^{2+}, only the methylation activity of the *Tsp*GWI bifunctional enzyme becomes apparent. After addition of 33 pmol *Tsp*GWI protein, the reaction mixture was incubated for 3 h at 65 °C. Proteinase K was added to the solution, and the incubation was continued for a further 60 min at 55°C. Samples were purified to remove all traces of proteins and divalent cations from the methylation reaction mixture, and the resulting DNA was challenged with an excess of *Tsp*GWI (10 U) for 1 h in 50 µL *Tsp*GWI reaction buffer at 65°C in the presence and absence of SIN. One unit of the endonuclease *Tsp*GWI is defined as the amount of enzyme required to hydrolyze 1 µg pBR322 in 1 h at 65°C, resulting in a stable partial cleavage pattern. The reaction products were then resolved by PAGE.

Polyacrylamide gel electrophoresis

Polyacrylamide gels (15%) were prepared in 1× Tris-Borate-EDTA (TBE) buffer (22). The gels were visualized after staining with Sybr Green I using a 312-nm UV transilluminator and photographed with a SYBR Green gel stain photographic filter.

Results and discussion

In the absence of SIN, *Tsp*GWI REase recognizes its canonical 5′-ACGGA-3′ sequence and generates a defined partial fragmentation pattern (16). Surprisingly, the addition of SIN strongly stimulates *Tsp*GWI REase activity, as observed from the digestion of bacteriophage λ DNA (Figure 2B, lane 3). A concentration dependence experiment showed that the optimal SIN concentration for this stimulation of *Tsp*GWI activity seemed to be 50–100 µM (Supplementary Figure S1).

Further examination revealed that the presence of SIN in the reaction mixture induces a relaxation in the specificity of DNA recognition by *Tsp*GWI REase. This star activity is demonstrated by the appearance of multiple additional bands in the *Tsp*GWI/SIN digest of the PCR(wt) substrate containing two canonical *Tsp*GWI sites (Figure 1A; Figure 2A, lane 4; and Figure 3A, lane 3), the PCR(mut) substrate containing no canonical *Tsp*GWI site (Figure 1B and Figure 3B, lane 9), as well as of the PCR(single) substrate containing a single canonical *Tsp*GWI site (Figure 1C and Figure 4B). In the presence of SIN, methylated canonical *Tsp*GWI sites remain completely resistant to cleavage by *Tsp*GWI endonucleolytic activity (Supplementary Figure S2, lane 9).

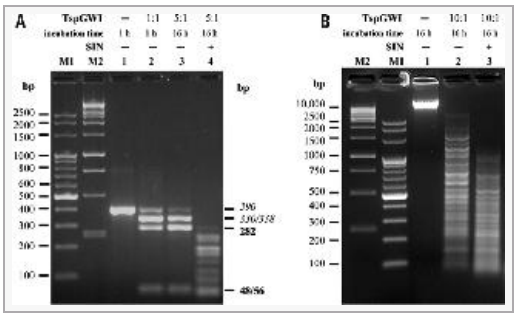

Figure 2. Cleavage patterns of *Tsp*GWI REase in the presence or absence of SIN. (Click to enlarge)

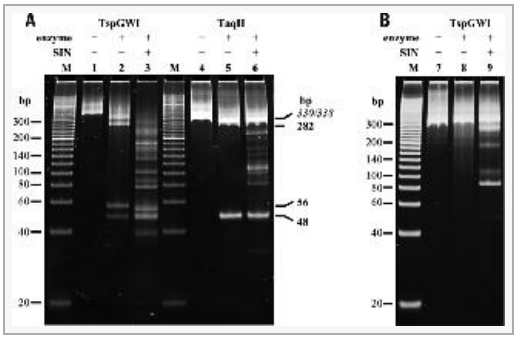

Figure 3. Comparison of SIN influence on *Tsp*GWI and TaqII cleavage patterns. (Click to enlarge)

***Tsp*GWI/SIN recognition sequence and cleavage site**

To establish the recognition site and cleavage positions of *Tsp*GWI star activity induced by SIN, shotgun cloning of the restriction fragments from bacteriophage λ DNA digested with *Tsp*GWI/SIN was performed (Figure 2B, lane 3). Restriction fragments were repaired with T4 DNA polymerase/dNTPs and cloned into the *Sma*I site of the pUC19 vector. The 218 resulting clones were sequenced to identify vector-insert junctions. Thirty-two identified junctions are shown in Supplementary Table S1. These initially determined *Tsp*GWI* recognition sequences were confirmed by sequencing an additional 50 clones generated by digesting the PCR(wt) fragment with *Tsp*GWI/SIN (Figure 2A, lane 4).

Analysis of the sequence data established that in the presence of SIN and with *Tsp*GWI in molar excess over recognition sites, the enzyme recognizes and cleaves at least 12 variants of the canonical 5′-ACGGA-3′ sequence, although a bias favoring the first five variants was observed (Table 1). Relaxed sites are distributed more or less uniformly along the target DNA, and changes in the canonical restriction site involve only single base pairs out of the original 5-bp DNA sequence (Table 1 and Supplementary Table S1). However, a higher level of degeneracy of the *Tsp*GWI recognition sequence cannot be excluded. Interestingly, no fixed “core”-invariable recognition sequence was identified; a single base departure per variant is allowed, regardless of location (Table 1 and Supplementary Table S1).

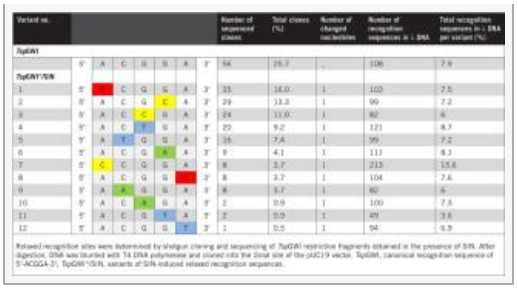

Table 1. Specificity change of *Tsp*GWI REase/MTase in the presence of SIN (Click to enlarge)

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Relationship between *Tsp*GWI and *Tsp*GWI* REase activity

To verify the proposed recognition sequences and test the relationship between the star activity and digestion at canonical sites, the PCR(oligo) substrate with two cognate sites and no star sites was generated (Figure 1D). Digestion of this fragment in the presence of SIN and increasing concentrations of *Tsp*GWI demonstrated that SIN stimulates cleavage of canonical *Tsp*GWI sites in the absence of star sites (Figure 5). However, even with SIN in the reaction buffer, *Tsp*GWI REase was not able to cleave the designed DNA substrate to completion (Figure 5B). Conversely, we observed slow cleavage of star sites in the PCR(mut) substrate with no *Tsp*GWI canonical sites (Figure 3B, lane 9) in comparison to the PCR(wt) substrate with two *Tsp*GWI canonical sites (Figure 3A, lane 3). Analysis of the sequencing data from the cloned λ DNA *Tsp*GWI/SIN restriction fragments showed a lack of uncleaved canonical *Tsp*GWI sites, while the star sites were not cleaved to completion. It should be noted that single *Tsp*GWI canonical sites cannot be cleaved in the absence of SIN. As shown in Figure 4A, the single canonical *Tsp*GWI site located near the 5′-end of the 390-bp PCR(single) fragment (Figure 1C) was not cleaved after 16 h of incubation. In contrast, the addition of the cofactor permitted the cleavage of this site (Figure 4B) with a 48-bp DNA fragment clearly visible after 30 min incubation. Several additional bands of different sizes gradually appeared after additional incubation of the reactions (Figure 4B) at 65°C, indicating that cleavage of this canonical site goes hand-in-hand with cleavage of the star sites. Remarkably, the same *Tsp*GWI site in the PCR(wt) DNA substrate could be cleaved in the absence of SIN when a second canonical site was located nearby (Figure 2A, lanes 2 and 3). In the light of these data, it would appear that in the presence of SIN, star sites can compensate for the second canonical recognition site required for the effective DNA cleavage. Additional experiments, however, should be performed in order to confirm this hypothesis.

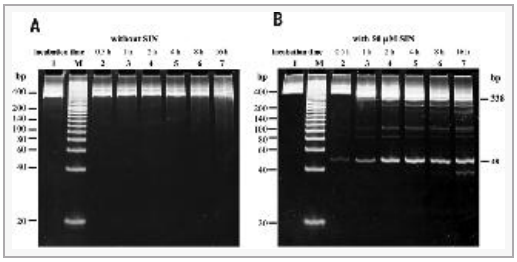

Figure 4. Time course of *Tsp*GWI digestion of the PCR(single) substrate in the absence and presence of SIN. (Click to enlarge)

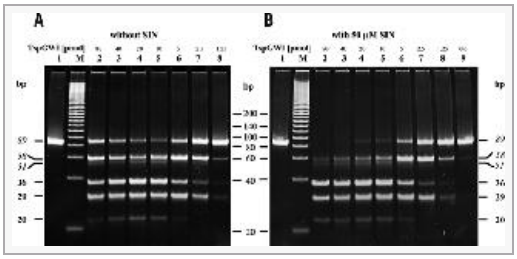

Figure 5. The stimulatory effect of SIN on *Tsp*GWI cleavage of canonical sites in the absence of star sites. (Click to enlarge)

A similar effect of SIN to compensate the second recognition site has been described for the type III restriction endonuclease *Eco*P15I (23). However, this observation has not been confirmed by other research groups, with several contradictory data sets concerning the influence of AdoMet and SIN on *Eco*P15I having been published over the past few years (23-26), suggesting that the observed effect of SIN may depend on how the enzyme is prepared and on the DNA substrates used.

Although the mechanism of SIN-induced star activity is currently unknown, this does represent a novel type of relaxation, at least according to the inducing factor criterion. Even the unusual *Cvi*JI REase, which recognizes the 5′-RGCY-3′ site following the addition of ATP, cleaving most 5′-GC-3′ central sites (12 out of 16 permutations of the neighborhood), retains the core sequence. Under standard conditions, *Tsp*GWI cleaves DNA on average every 1024 bp, however, SIN-induced *Tsp*GWI* results in frequent cleavage, approaching that of *Cvi*JI/*Cvi*JI*. Theoretically, complete digestion of DNA by *Tsp*GWI* would result in cleavage every 78.8 bp (1024/13) on average, corresponding to a putative 3-bp long recognition site. However, as is the case with the 2/3 bp cutter *Cvi*JI/*Cvi*JI*, complete digestion does not take place (1). Nevertheless, under partial digestion conditions, very frequent cutters can prove to be useful for genomic library preparation, yielding a quasi-random accumulation of DNA sequences (1), as well as for other specialized cloning applications, exemplified by ultrasensitive DNA labeling/amplification or high resolution restriction mapping and RFLP (1,27). Recently, we successfully took advantage of the combination of *Tsp*GWI/SIN to construct a representative genomic library from horse liver DNA (manuscript and patent in preparation).

In contrast to the recognition site relaxation induced by SIN, the site of actually DNA cleavage by *Tsp*GWI (11 and 9 nucleotides downstream of either the asymmetric star or canonical sites) remains unchanged, producing sticky ends with 3′ overhangs (Supplementary Table S1). Effective recognition and cleavage of the star sequences was found to be highly dependent on the presence of 5′-ACGGA-3′ or 5′-TCCGT-3′ sites within the same DNA molecule. As can be seen in Figure 3B, in the absence of the *Tsp*GWI canonical sequences in the PCR(mut) DNA substrate (Figure 1B), the star sites are cleaved at a very slow rate. In contrast, the presence of two convergently oriented canonical sequences in the

corresponding PCR(wt) fragment actually permitted effective cleavage of star sites (Figure 3A, lane 3). A similar effect was observed for the PCR(single) substrate (Figure 4B) and a substrate with a divergent orientation of *Tsp*GWI canonical sites (data not shown).

This phenomenon applies to the other member of the *Thermus* enzyme family, including the *Taq*II REase (Figure 3A, lane 6). Preliminary data also suggests that other members of the family—*Tsp*DTI and *Tth*111II REases—could behave in a similar way following the addition of SIN to the reaction buffer (data not shown). To our knowledge, such an unusual, SIN-induced change in the specificity of REases has not yet been described. Interestingly, the type IIS REase *Bpu*JI, which recognizes the asymmetric sequence 5′-CCCGT-3′ (a sequence that resembles the inversed recognition sequence of *Tsp*GWI: 5′-TCCGT-3′), cleaves DNA at multiple sites in the vicinity of the target sequence, on both DNA strands at distances varying from 2 to 23 nucleotides (28). This unusual enzyme has a 3′-end–directed endonucleolytic activity that can also cleave nonspecific DNA in *trans* once activated through binding to a recognition site, a situation that is reminiscent of the SIN-induced ability of *Tsp*GWI REase to cleave star *Tsp*GWI recognition sequences following activation by canonical sites. It is also worth mentioning that both *Tsp*GWI and *Bpu*JI need two restriction sites for their optimal activity. Moreover, the major product of the cleavage reaction matches the *Bpu*JI cut 2 and 11 nucleotides downstream (28), which resembles the reversed *Tsp*GWI cut 11 and 9 nucleotides.

*Tsp*GWI star activity resembles another unusual type II REase, *Sgr*AI, in its dependence on cognate sites being within the same DNA molecule (29). *Sgr*AI exhibits relaxation of sequence specificity under standard conditions and in the presence of DNA termini generated by cleavage of the canonical site 5′-CPu′CCGGPyG-3′. Moreover, both *Sgr*AI and *Tsp*GWI canonical recognition sequences contain a common core region—5′-CGG-3′. It is possible that *Tsp*GWI follows the same unconventional reaction pathway by which *Sgr*AI utilizes the self-generated DNA termini to cleave its DNA targets.

Potential regulatory role for AdoMet in *Tsp*GWI REase activity

Considering the fact that AdoMet is slightly inhibitory to *Tsp*GWI REase activity (14), we speculate that a second AdoMet binding site may exist in the *Thermus* family of REases/MTases. One such site would cause the allosteric stimulation of REase activity, the other would participate in the methylation reaction. The allosteric site of *Tsp*GWI could have lost the ability to be effectively stimulated by AdoMet, while retaining the altered binding properties. SIN—an analog of AdoMet—would compensate for the lost stimulatory function, but would alter the recognition of the canonical sequence. That would make the described affinity-star activity of *Tsp*GWI radically different from the other mechanisms known from prokaryotic REases; even so, it would be somewhat similar to the effect of ATP (or other adenine nucleotide) on *Cvi*JI eukaryotic REase (30). This SIN-type of relaxation may extend to other AdoMet-dependent subtype IIC REases.

The biological role of AdoMet in the *Tsp*GWI restriction reaction could be to prevent promiscuous DNA cleavage, resembling the role of this cofactor described for the *Eco*PI type III restriction enzyme (31). Irrespective of buffer conditions, in the presence of AdoMet, and irrespective of buffer conditions, only substrates with two *Eco*PI sites in inverted repeats were susceptible to cleavage. It is also interesting to note that AdoHcy and DNA ends can stimulate nuclease activities of *Eco*PI. Such extensive processing may even result in digestion of nonspecific DNA in *trans* (32). Through allosteric regulation, SIN could trigger an effect opposite to that of AdoMet by stimulating the promiscuous cleavage of degenerate sequences and the observed relaxation of *Tsp*GWI enzyme specificity. Hence, *Tsp*GWI, *Taq*II, and potentially two other members of the *Thermus* enzyme family would resemble type III REases with regards to this unique feature.

Acknowledgments

This work was supported by Gdansk University, Department of Chemistry grants: BW/8000-5-0143-8, BW 8000-5-0265-9, DS/8221-0-140-0, and by Ministry of Science and Higher Education (Poland) grant NR12-0070-06/2009. We thank Katarzyna Maczyszyn for the digital picture imaging and her valuable technical assistance.

Competing interests

The authors declare no competing interests.

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