The Two-step Cleavage Activity of PI-TfuI Intein Endonuclease Demonstrated by Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry*

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PI-TfuI, an intein spliced from the DNA polymerase of Thermococcus fumicolans, is a highly specific endonuclease, whose cleavage efficiency and specificity depend on both the substrate topology and the divalent cation used as cofactor. An open circular intermediate was observed during the cleavage of supercoiled DNA by PI-TfuI, suggesting a two-step cleavage of the DNA. We characterized this nicked intermediate and, through the development of a method of analysis of the cleavage reaction based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry, we demonstrated that the cleavage of DNA by PI-TfuI indeed results from two cleavage events. One step results in the cleavage of the bottom strand, which is independent of the DNA conformation or choice of the metal ion cofactor. A second step, which is slower, leads to the cleavage of the top strand and governs the specific requirements of PI-TfuI concerning the essential cofactor and the DNA topology. These two steps were shown to be independent in optimal conditions of cleavage. These data give support to the existence of two distinct and independent active sites in the endonuclease domain of the archaeal intein.

Among the 34 proteins known to harbor inteins, archaeal DNA polymerases are the preferred hosts (Inbase, the New England Biolabs intein data base at www.neb.com/inteins/intein_intro.html; Inteins-Protein introns web site at bioinfo.weizmann.ac.il/~pietro/inteins/). Indeed, 8 of the DNA polymerase genes from archaeabacteria sequenced to date encode 1 to 3 invading sequences. The intein coding sequences are inserted in-frame in the host genes, which are transcribed and translated into large precursor peptides. The subsequent protein splicing of these precursors is autocatalytic; it produces the mature DNA polymerases and liberates the inteins, which are also stable proteins. Among the 15 inteins recessed in archaeal DNA polymerase, 14 are potential endonucleases and only one is a mini-intein lacking the central endonuclease domain (1, 2). A specific activity of double-stranded DNA cleavage has been demonstrated for 8 of them (3–7). Besides, six other inteins are known to possess a specific endonuclease activity. Two are inserted in the Pyrococcus furiosus ribonucleotide reductase (8, 9), two in the Pps1 protein of Mycobacterium gastri (10) and Mycobacterium tuberculosis (11), one in RecA of M. tuberculosis (12), and one in the vacuolar ATPase of Saccharomyces cerevisiae (13). In this yeast, it was shown that the highly specific endonuclease activity of the PI-SceI intein confers to the intein coding sequence a specific mobility known as “homing” (13).

The endonuclease activity of these 14 inteins was individually studied (3–13) and common features are observed. They recognize and cleave long asymmetrical sequences: a 16 to 31-bp sequence spanning the intein insertion site in the inteinless allele of their host gene is the substrate of the intein, the cleavage leaving a 4-base long 3'-hydroxyl overhang. Whereas PI-SceI and mycobacterial inteins cleave DNA at 37 °C, all other inteins are thermophilic enzymes that are active at temperatures ranging from 50 to 100 °C. The endonuclease activity of inteins requires a divalent cation, usually Mg2+. This cation is an essential cofactor for the phosphodiester bonds hydrolysis but is usually not required for DNA binding with the exception of PI-PfuI binding to its target sequence. All these enzymes are active at pH about 8–8.5 and, in a few cases, the catalysis is enhanced in the presence of monovalent ions.

Although some inteins have been found to form only one specific complex with their target DNA, PI-SceI interacts with DNA, as a monomer, in a biphasic pathway aimed to settle the scissible bonds into the active site, and thus induces the double-stranded DNA cleavage without accumulation of a nicked intermediate (14, 15). These observations raised the question of whether the cleavage reaction of the double-stranded DNA is performed by only one catalytic center that simultaneously cleaves the two DNA strands following a single binding event or by two catalytic centers that act in a concerted reaction. The existence of two separate active sites is fairly suggested. First, the 2 LAGLIDADG motifs, which are spaced by ~100 amino acids in the primary structure of most inteins are involved in the endonuclease activity (9, 16, 17). Second, the structural homology between the endonuclease domain of the monomeric PI-SceI (18) and the homodimer of I-CreI (19, 20), a dedecapeptide homing endonuclease encoded by a self-splicing intron, is high. Whereas Christ and collaborators (21) clearly defined the catalytic residues of PI-SceI, which are implied in the two active centers that specifically cleave each DNA strand of the substrate, this hypothesis remains to be explored for DNA cleavage by other inteins.

To address this critical issue, we further studied the mechanism of DNA cleavage by PI-TfuI, an intein endonuclease from the DNA polymerase of Thermococcus fumicolans (GenBank™ accession number Z69882). A previous study (6) showed that PI-TfuI recognizes and cleaves a minimal se-

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The presence of Mn$^{2+}$ ions as a cofactor but the enzyme is 5–10-fold more active in the presence of Mn$^{2+}$ ions. Otherwise, it cleaves linear DNA only in the presence of Mn$^{2+}$ ions and requires a 19-bp minimal recognition sequence. Hence, the cleavage efficiency and/or specificity of PI-TfuI depend on both the substrate topology and the divalent cation cofactor. By contrast with PI-SceI, an open circular nicked DNA is observed over the cleavage of supercoiled DNA by PI-TfuI (6). That suggests a two-step cleavage of the DNA, giving support to the existence of two distinct active sites in the endonuclease domain of the archaeal intein.

In the first part of this study, we characterized the nicked DNA as an intermediate of the double-stranded DNA cleavage reaction. We then set up a new method of analysis of the DNA cleavage using MALDI-TOF$^1$ mass spectrometry. This technology had already been applied to the analysis of nucleic acids (22, 23) when searching for single nucleotide polymorphism in DNA sequences (24), when sequencing DNA (25) or RNA (26), and also when characterizing DNA fragments generated by a nuclease cleavage (27, 28). Here, the approach using MALDI-TOF mass spectrometry was optimized for the detection of small amounts of the DNA fragments typically generated by the intein cleavage in saline buffers, allowing the detection of less than 250 fmol of oligonucleotides produced by the enzymatic reaction. Finally, using this methodology, we demonstrated that the cleavage of DNA by PI-TfuI does indeed result from two independent reactions of cleavage.

EXPERIMENTAL PROCEDURES

Production and Purification of the PI-TfuI Intein—The recombinant intein was expressed in Escherichia coli, as previously described, after transformation of BL21(DE3)pLysS bacteria with the inducible expression vector pET26-TfuI. The purification procedure was unchanged from that previously described (6) and homogeneous fractions of PI-TfuI were obtained after a single step of anion exchange chromatography.

DNA Substrates and Cleavage Assays—Previously described plasmids S1 and S4 (6), containing, respectively, the 41-bp spanning the Pol-α homing site or the 16 bp constituting the minimal recognition and cleavage site of PI-TfuI, were used. The supercoiled form of these plasmids or the open circular intermediate of the cleavage were purified from a 1% agarose gel in TBE buffer (90 mM Tris borate, 2 mM EDTA) using the Qiaquick purification kit (Qiagen) and were diluted in water to a concentration of 100 ng/μl (58 fmol/μl). Endonuclease assays using these substrates were performed in a final volume of 10 μl, in 50 mM Tris acetate, pH 7.5, 50% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 200 μg/ml bovine serum albumin, and 50 mM NaCl, for storage.

DNA and beads were lyophilized and resuspended in 25 mM ammonium acetate at different concentrations ranging from 0.05 to 0.5 μM. The desalting procedure was adapted from the Langley’s procedure (22) to lower the MALDI-TOF detection limit. One μl of oligonucleotide sample was desalted by mixing with NH$_4$-loaded ion exchange Dowex beads for 15 min at room temperature and 0.5 μl of the desalted solutions (25–250 fmol of calibrating oligonucleotides) were directly loaded on the sample plate together with an equal volume of matrix using the dried droplet method. The matrix used was a saturated solution of 3HPA (Aldrich Chemical Co.) in H$_2$0/CH$_3$CN (1/1, v/v) with 0.1% trifluoroacetic acid (Sigma).

RESULTS

Open Circular DNA Is an Intermediate of the Cleavage of Supercoiled DNA by PI-TfuI—In our earlier examination of PI-TfuI endonuclease activity (6), an open circular form of the DNA substrate was observed during the cleavage of supercoiled DNA into linear DNA. A kinetic analysis of the cleavage of the supercoiled substrate S1 containing the 41 bp spanning the intein insertion site was undertaken to determine whether this open circular DNA is an unspacific product of the reaction or a long-lived intermediate of the specific cleavage.

Fig. 2A shows the electrophoretic separation of the different forms of plasmid S1 that was incubated with PI-TfuI in the presence of 25 mM MnSO$_4$ at 70°C. The amount of each DNA form after different incubation times was quantified (Fig. 2B). In the presence of Mn$^{2+}$ ions, the amount of open circular DNA increased during the first 8 min of the reaction and then progressively decreased, whereas the quantity of linear DNA (lin) was increased. The time courses of disappearance of super-

$^1$ The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; BM, bottom modifid; TM, top modified; WT, wild type.
The evolution of the amounts of each DNA forms during the reactions described in the Two-step Cleavage of DNA by the Intein PI-TfuI

were performed at 70°C, its rate of appearance being estimated around 0.01 to 0.02 min⁻¹. Rather stable and linear DNA appeared only very slowly (Fig. 4). As before, the supercoiled plasmid S1 was rapidly nicked into an open circular DNA (Fig. 2A), instead of MnSO₄. As before, the supercoiled plasmid S1 was used as substrate of the cleavage reaction (Fig. 3A) in the presence of Mg²⁺ ions. Considering this hypothesis, we purified the open circular DNA formed during the reaction and submitted it to cleavage assays by PI-TfuI in the buffer containing 25 mM MnSO₄ or 50 mM Mg(OAc)₂ (Fig 3B). As expected, this open circular DNA was a good substrate of the enzyme in the presence of Mn²⁺ ions because it was readily processed into DNA cleaved on both strands, but was very slowly converted into linear DNA in the presence of Mg²⁺ ions. Moreover, the amounts of linear DNA produced in both conditions were similar to those obtained when supercoiled DNA was used as substrate of the cleavage reaction (Fig. 3A). These observations reinforce the hypothesis of a two-step cleavage including a nonlimiting first step of DNA nicking.

Identical observations were made when the plasmid S4, containing the minimal 16 bp recognition and cleavage sequence, was used as substrate of PI-TfuI (not shown). Whatever the size of the target sequence, linear DNA was obtained after the incubation at 70°C of the supercoiled DNA with PI-TfuI in the presence of 10–100 mM MnSO₄, whereas accumulation of nicked plasmid was observed in the buffer containing 10–100 mM Mg(OAc)₂.

Optimization of the MALDI-TOF Analysis of DNA—To go further in the characterization of the open circular intermediate of the reaction and in particular to determine whether one of the DNA strands is preferentially nicked in the first stage of the reaction, we developed a strategy to analyze the process of DNA cleavage using MALDI-TOF mass spectrometry. One crit-

coiled DNA and of appearance of linear DNA were fit to two exponentials using Prism software (GraphPad Software, Inc. San Diego, CA) to estimate the half-life of reaction. The rate of disappearance of the supercoiled substrate was thus estimated to be around 0.2 min⁻¹ (half-life of 5.3 min) and the rate of appearance of the linear product was estimated around 0.06 min⁻¹ (half-life of 16.5 min).

A similar kinetic analysis of supercoiled DNA cleavage was performed in the same buffer containing 50 mM Mg(OAc)₂ instead of MnSO₄. As before, the supercoiled plasmid S1 was rapidly nicked into an open circular DNA (Fig. 2C), the rate of disappearance of the supercoiled DNA being at least equivalent to the rate in the presence of Mn²⁺ (0.2 min⁻¹). However, in the presence of Mg²⁺ ions, the open circular intermediate was rather stable and linear DNA appeared only very slowly (Fig. 2D), its rate of appearance being estimated around 0.01 to 0.02 min⁻¹ (half-life superior to 50 min). The stability of the open circular intermediate was observed independently of the Mg(OAc)₂ concentration in the reaction buffer (data not shown). Moreover, a shifted DNA form, probably consisting of a complex between open circular DNA and the intein, was observed.

To compare the efficiencies of supercoiled DNA nicking by PI-TfuI in the presence of Mg²⁺ or Mn²⁺ ions, additional cleavage assays were performed using a lower amount of enzyme in the reaction mixture. As shown in Fig. 3A, around 80% of the supercoiled substrate had disappeared after 20 min of reaction in both buffers, meaning that the nicking activity is not dependent on the metal ion cofactor. Consistently with data in Fig. 2, B and D, the amount of linear DNA produced was 3-fold lower in the presence of magnesium ions.

These preliminary results suggest that the open circular DNA is an intermediate of the cleavage reaction that is produced independently in the presence of Mn²⁺ or Mn²⁺ ions and is subsequently cleaved into linear DNA preferentially in the presence of Mn²⁺ ions. Considering this hypothesis, we purified the open circular DNA formed during the reaction and submitted it to cleavage assays by PI-TfuI in the buffer containing 25 mM MnSO₄ or 50 mM Mg(OAc)₂ (Fig 3B). As expected, this open circular DNA was a good substrate of the enzyme in the presence of Mn²⁺ ions because it was readily processed into DNA cleaved on both strands, but was very slowly converted into linear DNA in the presence of Mg²⁺ ions. Moreover, the amounts of linear DNA produced in both conditions were similar to those obtained when supercoiled DNA was used as substrate of the cleavage reaction (Fig. 3A). These observations reinforce the hypothesis of a two-step cleavage including a nonlimiting first step of DNA nicking.
tical step was the desalting of the oligonucleotides, the sensitivity of the analysis being mostly dependent on the efficiency of this particular process. Four synthetic oligonucleotides, F1 to F4, were used to optimize the DNA detection limits. Their sequences, shown in Table I, were chosen on the basis of the oligonucleotides are identical to the ones generated by the cleavage of the oligonucleotide duplex WT, a synthetic double-stranded substrate of PI-TfuI (Fig. 1). Among the various desalting procedures assayed, a variant of the procedure proposed by Langley and collaborators (22) resulted in the lowest detection limits. The oligonucleotides were incubated with NH$_4^+$-loaded Dowex beads, whereas Langley used H$^-$-loaded Dowex beads for the desalting process previous to the MALDI-TOF analyses. Analyses of various amounts of each of the four synthetic oligonucleotides showed that the total ion current decreases when the length of the oligonucleotide increases. Despite that, as little as 25 fmol of the 23-mer oligonucleotide F4 could be detected with a signal/noise of 3 (Fig. 4A), whereas Langley’s (22) detection limit was reached with 5 pmol of 20-mer oligonucleotides. The measured masses of these oligonucleotides, under the protonated form, were in good agreement with the masses expected from their nucleotide sequences, differences between measured and expected masses being less than 0.5 Da (Table I).

The analysis, in the same conditions, of an equimolar mixture of the four oligonucleotides showed that the detection limit of each oligonucleotide present in the mixture was higher than the detection limit of each oligonucleotide analyzed individually (Fig. 4B). Consequently, because the total ion current obtained for each oligonucleotide is dependent on oligonucleotide length, the detection limit of the smallest oligonucleotide F3 remains around 25 fmol, whereas that of the oligonucleotide F4 is 250 fmol in the presence of the other oligonucleotides.

The WT DNA substrate of PI-TfuI was obtained by annealing the complementary oligonucleotides P1 and P2 (Table I). This double-stranded DNA (Fig. 1) corresponds to the 40-bp sequence spanning the intein insertion site in T. fumicola$\text{ns}$ genome, which is the target of the specific endonuclease PI-TfuI. Each of the 40-mer oligonucleotides P1 and P2 were independently analyzed by MALDI-TOF mass spectrometry (Fig. 4C and D). It is noteworthy that the resolution of the two oligonucleotides peaks was rather low compared with that of oligonucleotides F1 to F4. This decrease in resolution may be explained by a poor desalting efficiency because of the size of these oligonucleotides because the analyses of P1 and P2 gave similar signals. Nevertheless, for both oligonucleotides, as little as 250 fmol were detected with a signal/noise of 4 and the measured masses corresponded to the calculated masses within 1 Da (Table I). By contrast, the analysis of the oligonucleotide duplex formed by the hybridization of P1 and P2 in the intein reaction buffer highlighted that the ion current observed for oligonucleotide P2 was 3 times higher than that of P1, suggesting that P2 may be more efficiently desalted than P1 in the buffered solution (Fig. 4E). One likely explanation, based on the observations of Gross and collaborators (29), is that the affinity of the oligonucleotides for NH$_4^+$ ions and, as a consequence, the desalting efficiencies of these oligonucleotides, when in competition, are dependent on the nucleotide sequence.

Further modifications of the desalting procedure were then pursued to improve the DNA detection limits in the reaction buffer. Hence, a precipitation step with acetone was added, DNA was then resuspended in water, incubated with NH$_4^+$-loaded ion exchange beads, and lyophilized in the presence of the beads. The matrix was directly added to the dried DNA mixture to be analyzed. This desalting procedure allowed us to detect less than 100 fmol of the cleavage fragments in the buffer containing 200 mM salts with a signal/noise superior to 3. The resolution of the two 40-bp parent oligonucleotides remained low so that it was not possible to follow the disappearance of each DNA strand during the reaction. The analysis of the duplex cleavage by the intein PI-TfuI was thus restricted to the mass range of the cleavage products, i.e. to $m/z$ from 4,000 to 8,000.

The Bottom Strand of DNA Is More Rapidly Cleaved Than the Top Strand—Fifty pmol of the WT duplex DNA were used as a substrate in digestion reactions and 1 pmol of digested DNA was analyzed by MALDI-TOF after desalting. The cleavage fragments generated by the incubation of the double-stranded DNA substrate with a large amount of PI-TfuI, at 70 °C in the optimal reaction buffer containing 25 mM MnSO$_4$, were analyzed at various time points from 0 to 60 min of reaction. As expected, no cleavage fragment was detected when the reaction had not yet begun (Fig. 5A). After 60 min of reaction, 4 peaks corresponding to the 4 generated fragments were detected (Fig. 5F), showing that, as expected, the site of cleavage of each strand is unique. Moreover, the measured masses for these fragments are in agreement with the previ-
ously proposed cleavage site of PI-TfuI indicated in Fig. 1.

MALDI-TOF analyses at intermediary times of reaction revealed that the four products of the reaction did not appear simultaneously in the reaction mixture. The two fragments generated by the cleavage of P2 and corresponding to the sequence of the controls F3 and F4 were clearly detected after 2 min of reaction (Fig. 5B), whereas the two fragments generated by the cleavage of P1 and corresponding to the sequence of oligonucleotides F1 and F2 were only weakly observed after 5 min of reaction (Fig. 5C). Analyses at longer reaction times showed that each fragment pair progressively appeared at different rates in the digestion mixture (Fig. 5, D–F), the cleavage of oligonucleotide P2 being faster than that of P1. These results demonstrated that the bottom strand of the DNA substrate is more rapidly cleaved by PI-TfuI than the top strand.

Based on the variation of the absolute peak intensities observed on the mass spectra, the amount of fragments generated by the cleavage of oligonucleotide P2 (Fig. 5) may be considered as constant after 15 min of reaction, indicating that the cleavage of the P2 oligonucleotide was then complete, whereas the cleavage of P1 appeared to be complete after 60 min of reaction. The rate of cleavage of the top strand can thus be considered to be roughly 4-fold lower than the rate of cleavage of the bottom strand.

The Cleavage of the Top Strand Is Mns²⁺-dependent—A similar kinetic analysis was performed in the same buffer but in the presence of 50 mM Mg(OAc)₂ instead of 25 mM MnSO₄. Here again, the fragments corresponding to the cleavage of P2 oligonucleotide were rapidly generated (Fig. 6A). The fragments corresponding to the cleavage of P1, however, were only weakly detected after 60 min of reaction (Fig. 6B). The observed peak intensities for these fragments are comparable with the intensities obtained after 5 min of reaction with PI-TfuI in the presence of Mn²⁺ ions (Fig. 5C). This observation suggested that the rate of cleavage of P1 is decreased by at least 10 times when Mn²⁺ ions are replaced by Mg²⁺ ions, whereas the bottom strand is cleaved at a comparable rate whatever the divalent cation used as a cofactor.

The Cleavage of the Top Strand Is Not Subsequent to the Cleavage of the Bottom Strand in the Presence of Mn²⁺ Ions—Two additional oligonucleotide duplexes were constructed to determine whether the cleavage of the top strand is necessarily consecutive to that of the bottom strand or if the cleavage reactions of the two strands are unrelated. These duplexes,
named BM and TM, possess the same sequence as the WT duplex but a phosphorothioate bond in place of each phosphodiester bond was cleavable by the intein either within the bottom or the top strand, respectively (Fig. 1). These modified bonds are aimed to prevent the cleavage of one strand while allowing the cleavage of the second strand.

The measured masses of P1-mod and P2-mod oligonucleotides, harboring a phosphorothioate bond and used to form TM and BM duplexes through the annealing with P2 and P1 parent oligonucleotides, respectively, were in good agreement with the calculated masses (Table I). As for the WT duplex, the cleavage of BM and TM duplexes was studied by following the appearance of the cleavage fragments by MALDI-TOF mass spectrometry.

The cleavage of the TM substrate generated, either in the presence of Mn$^{2+}$ (Fig. 7A) or Mg$^{2+}$ ions (Fig. 7B), the two expected fragments of 23- and 17-mers identical to the synthetic oligonucleotides F4 and F3, respectively. The fragment
peak intensities obtained after 60 min of reaction indicate that the digestion efficiency of P2, in both cases, is equivalent to the one obtained with the WT duplex. These results showed that the cleavage of the bottom strand does not depend on the cleavage of the top strand.

Similarly, the oligonucleotide P1 constituting the top strand of the BM substrate was cleaved in two fragments of 19- and 21-mer identical to the synthetic oligonucleotides P2 and P1, respectively, in the presence of Mn$^{2+}$ ions (Fig. 7C). The cleavage efficiency of the P1 oligonucleotide was roughly the same using BM and WT duplexes as substrates. By contrast, no cleavage fragment was detected when the BM duplex was incubated with PI-TfuI in the presence of Mg$^{2+}$ ions (Fig. 7D) after 60 min of reaction. The amount of the two cleavage fragments generated, if any, is thus smaller than the amount obtained using WT duplex. That could be explained either by a decreased rate of cleavage or by the absence of cleavage of the top strand when the bottom strand is uncleaved.

In conclusion, in the presence of Mn$^{2+}$ ions, the cleavage efficiency of each DNA strand does not depend on the cleavage of the other strand. The top strand, however, appears uncleaved in the presence of Mg$^{2+}$ ions when the bottom strand is uncleaved.

DISCUSSION

Among the inteins known to possess an endonuclease activity, PI-TfuI is singular because the most appropriate divalent metal ion cofactor of the DNA cleavage is Mn$^{2+}$, and not Mg$^{2+}$ as for all other inteins excepted the recently described mycobacterial RecA intein (12). As described for other dodecapeptide endonucleases such as PI-SceI and PI-PfuI inteins (8, 14), its specificity of cleavage depends on both the DNA substrate topology and the cation used as a cofactor.

An observation emerging from our previous analysis of the endonuclease activity of PI-TfuI was that open circular DNA was produced when supercoiled DNA was used as substrate of the cleavage reaction. Similarly, an open circular form of DNA was observed during the cleavage reaction by few other inteins (9, 12) and other endonucleases, such as EcoRV (30, 31) and I-CpaII (32), under nonoptimal conditions of reaction. Because independent studies suggested that the PI-SceI intein possesses two catalytic centers able to cleave each strand of the DNA substrate (14, 21, 33), we hypothesized that the observed open circular DNA could be an intermediate of the cleavage reaction, in which only one strand of the DNA was nicked. Thus, PI-TfuI appeared to be an interesting enzyme to gain further insights into the understanding of the DNA cleavage mechanism by inteins and more generally by dodecapeptide endonucleases.

In the present study, the kinetics of cleavage of supercoiled DNA by PI-TfuI in optimal conditions, involving the presence of Mn$^{2+}$ as a cofactor, confirmed that open circular DNA was formed as an intermediate of the cleavage because it appears transiently during the time course of the reaction (Fig. 2, A and B). Similar experiments performed in different biochemical conditions showed that this open circular intermediate accumulates when Mn$^{2+}$ ions are replaced by Mg$^{2+}$ ions (Fig. 2, C and D). Moreover, additional cleavage assays showed that the rate of disappearance of the supercoiled DNA substrate is independent of the divalent cation used (Fig. 3A), showing that the production of the nicked intermediate is not sensitive to the ion available as a cofactor. On the contrary, the rate of production of linear DNA is decreased by a factor 3–5 when Mg$^{2+}$ ions are used in place of Mn$^{2+}$ ions in cleavage assays using either the supercoiled plasmid or the purified open circular intermediate as substrate of PI-TfuI (Figs. 2 and 3). Identical results were obtained using either the plasmid harboring the 41-bp sequence spanning the intein insertion site or that harboring the 16-bp minimal cleavable sequence as supercoiled DNA substrates of PI-TfuI. This observation confirms that the 16-bp sequence is sufficient for the cleavage of DNA under torsional conditions.

FIG. 7. MALDI-TOF analysis of the cleavage of the phosphorothioate-modified duplexes by PI-TfuI. 50 pmol (5 μm) of TM and BM duplexes were incubated with 23 μg (55 μm) of PI-TfuI in a 50 mM Tris acetate, pH 8, buffer containing 100 mM NH$_4$OAc and 25 mM MnSO$_4$ (A and C, respectively) or 50 mM Mg(OAc)$_2$ (B and D, respectively) for 60 min at 70°C.
constraints, whereas this sequence remains uncleaved under the relaxed state (6), and demonstrates that the mechanism of supercoiled DNA cleavage is independent of the length of the cleavable sequence.

These previous data gave support to the idea of a two-step cleavage of DNA by PI-TfuI. The next critical point was thus to demonstrate that one strand of the DNA was preferentially cleaved before the other. With this goal in mind, we developed a method to follow the time course of the DNA cleavage reaction by MALDI-TOF mass spectrometry.

A 40-bp oligonucleotide duplex (WT), corresponding to the PI-TfuI target sequence (Fig. 1) and known to be efficiently cleaved by PI-TfuI in optimal conditions was used as substrate of the DNA cleavage. The products of the cleavage reaction were then analyzed by MALDI-TOF mass spectrometry, which allows us to follow the cleavage of each DNA strand by PI-TfuI. Because the thermophilic intein is active at high temperature of reaction (70 °C), it was not possible to reduce the size of the DNA duplex used as substrate in a way that would have facilitated the detection of the fragments by MALDI-TOF mass spectrometry. As a consequence, the optimization of the desalting process of these 17- to 23-mer DNA fragments from the reaction mixture was crucial. Indeed, the procedures previously used to analyze nucleic acids (22–28) were not directly applicable to our study because of the size and the low amount of DNA fragments to be detected.

The desalting procedure proposed by Langley and collaborators (22) is based on H⁺-loaded ion exchange beads to remove Na⁺ ions. This step was modified by using NH₄⁺-loaded ion exchange beads. Probably thanks to the volatility of ammonium ions and/or to its affinity for nucleic acids, the detection limit of MALDI-TOF analyses was improved, as little as 25 fmol of a 23-mer oligonucleotide being detected with a signal/noise around 3, whereas the detection limit by Langley et al. (22) of a 20-mer oligonucleotide is 5 pmol. Further modifications of the procedure, involving the addition of a DNA precipitation step and its lyophilization in the presence of the beads, were then necessary to analyze the mixture of DNA fragments from the intein reaction buffer containing high salt concentrations. It was thus possible to detect as little as 100 fmol of each of the cleavage fragments present in the reaction mixture.

Based on the measured mass of the fragments, MALDI-TOF analyses allowed us to confirm the location of the cleavage site on each DNA strand. Moreover, analyses of the formation of each DNA fragment in the reaction mixture containing manganese ions, at different stages of the digestion (Fig. 5), allowed us to demonstrate that only the bottom strand was cleaved during the first minutes of the reaction. The global rate of cleavage of the bottom strand was approximately four times higher than that of the top strand. These results are coherent with the kinetic analysis of the supercoiled DNA cleavage, because the consumption of supercoiled DNA is more rapid than the production of linear DNA. We then concluded that supercoiled and linear DNA substrates were submitted to the same two-step mechanism of cleavage consisting of one step of cleavage of the bottom strand and a slower step of cleavage of the top strand.

As we previously observed that the open circular form of DNA accumulated when supercoiled DNA was incubated with PI-TfuI in the presence of manganese ions, it was not surprising to observe, by MALDI-TOF, that only the bottom strand of the 40-bp DNA substrate was efficiently cleaved in these conditions (Fig. 6). Indeed, whereas the bottom strand was cleaved with the same efficiency whatever the cation used, the cleavage efficiency of the top strand was decreased by a factor greater than 10 in the presence of magnesium ions. These results definitively established that the nicking of the supercoiled DNA, which is not dependent on the metal ion, corresponds to the cleavage of the bottom strand. Thus, linear DNA, which is produced only in the presence of Mn²⁺ ions, results from the additional cleavage of the top strand. We then concluded that the cleavage of DNA by the intein PI-TfuI is promoted by two distinct active sites, each of these sites being specialized in the cleavage of one DNA strand with a specific cofactor requirement.

The catalytic residues involved in each catalytic site of PI- TfuI are still unknown. The acidic residues of each LAGLIDADG-type motifs are good candidates. The Glu-125 and Asp-225 of PI-TfuI certainly play similar roles than Asp-21S and Asp-326 of PI-SceI (34), which participate to the binding of one metal ion in each catalytic center as shown by the structural homology between PI-SceI endonuclease domain and I-CreI (18–20). The presence of a glutamic acid residue in place of an aspartic acid residue in one active center of PI-TfuI does not account for the different metal ions requirements between the two centers because the intein PI-PkoI, which is highly homologous to PI-TfuI, harbors the same Glu-125 residue and uses Mg²⁺ ions as an essential cofactor of the double-stranded DNA cleavage (5). Unfortunately, the low sequence homology between PI-TfuI and the dodecapeptide endonucleases, of which the tridimensional structure is known, do not allow us to locate the partners of the 2 acidic residues, which could be responsible for the specific metal requirement in one catalytic center of PI-TfuI.

Two additional duplexes, which possess the same 40-bp target sequence and a phosphorothioate bond in place of the scissible bond, either on the bottom or the top strand (Fig. 1), were used as substrates of PI-TfuI to study the cleavage of one DNA strand in the absence of cleavage of the other strand. The analysis by MALDI-TOF mass spectrometry of the digestion of these 2 duplexes by PI-TfuI showed that the unmodified strand can still be cleaved when the modified strand remains uncleaved. As expected, the bottom strand from the TM duplex was efficiently cleaved in two oligonucleotides either in the presence of Mn²⁺ or Mg²⁺ ions (Fig. 7). However, whereas a slow activity of cleavage of the top DNA strand of the WT duplex was observed in the presence of Mg²⁺ ions (Fig. 6), the cleavage of the top strand of the BM duplex strictly necessitated Mn²⁺ ions (Fig. 7). This suggests that even if the two cleavage activities are independent in optimal conditions, the top strand cleavage may depend on the bottom strand cleavage in the presence of magnesium ions. This is consistent with previous experiments, which suggested an effect of the DNA topology on the top strand cleavage activity. Hence, the activity of cleavage of the top strand is not solely varying with the metal cofactor but also with the topology of the DNA substrate.

In conclusion, through the development of a rapid and simple method of analysis of DNA cleavage fragments using MALDI-TOF mass spectrometry, the present study clearly shows that the intein PI-TfuI possesses two active sites, which are independent and specific of one DNA strand. Furthermore, we showed that the specific requirements of PI-TfuI concerning the essential cofactor and the DNA substrate topology are governed by the limiting step of cleavage of the top strand of the DNA substrate.

In this context, the biological relevance of the intein activity in the presence of manganese remains questionable because this metal cation is known to stimulate the activity of various endonucleases, including PI-SceI (13, 35), and clearly relaxes the topological specificity of PI-TfuI. Whereas the efficiency of the bottom strand cleavage is not affected by the divergent cation used as cofactor, the efficiency of the top strand cleavage...
is significantly enhanced in the presence of manganese, reflecting a dissimilar setting of the metal ion in the two distinct active sites of the intein. Hence, the higher activity level of the top strand cleavage site observed in the presence of manganese in vitro may be because of a decreased specificity of this cleavage site referable to the metal cation, whereas magnesium may constitute the in vivo cofactor of the endonuclease activity.

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