Mutational analysis of active-site residues in the *Mycobacterium leprae* RecA intein, a LAGLIDADG homing endonuclease: Asp^{122} and Asp^{193} are crucial to the double-stranded DNA cleavage activity whereas Asp^{218} is not

Pawan Singh, Pankaj Tripathi, and K. Muniyappa*

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, Karnataka, India

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Abstract: *Mycobacterium leprae recA* harbors an in-frame insertion sequence that encodes an intein homing endonuclease (PI-MleI). Most inteins (intein endonucleases) possess two conserved LAGLIDADG (DOD) motifs at their active center. A common feature of LAGLIDADG-type homing endonucleases is that they recognize and cleave the same or very similar DNA sequences. However, PI-MleI is distinctive from other members of the family of LAGLIDADG-type HEases for its modular structure with functionally separable domains *for* DNA-binding and cleavage, each with distinct sequence preferences. Sequence alignment analyses of PI-MleI revealed three putative LAGLIDADG motifs; however, there is conflicting bioinformatics data in regard to their identity and specific location within the intein polypeptide. To resolve this conflict and to determine the active-site residues essential for DNA target site recognition and double-stranded DNA cleavage, we performed site-directed mutagenesis of presumptive catalytic residues in the LAGLIDADG motifs. Analysis of target DNA recognition and kinetic parameters of the wild-type PI-MleI and its variants disclosed that the two amino acid residues, Asp^{122} (in Block C) and Asp^{193} (in functional Block E), are crucial to the double-stranded DNA endonuclease activity, whereas Asp^{218} (in pseudo-Block E) is not. However, despite the reduced catalytic activity, the PI-MleI variants, like the wild-type PI-MleI, generated a footprint of the same length around the insertion site. The D122T variant showed significantly reduced catalytic activity, and D122A and D193A mutations although failed to affect their DNA-binding affinities, but abolished the double-stranded DNA cleavage activity. On the other hand, D122C variant showed approximately twofold higher double-stranded DNA cleavage activity, compared with the wild-type PI-MleI. These results provide compelling evidence that Asp^{122} and

Additional Supporting Information may be found in the online version of this article.

Abbreviations: 2-AP, 2 aminopurine; bp, base pair; BPB, bromophenol blue; EDTA, ethylene diamine tetraacetic acid; form I DNA, negatively supercoiled DNA; form II DNA, nicked circular DNA; form III DNA, linear double-stranded DNA; ODN, oligonucleotide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PI-MleI, *M. leprae* RecA intein; SDS, sodium dodecyl sulfate.

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**Correspondence to:* K. Muniyappa, Department of Biochemistry, Indian Institute of Science, Bangalore 560012, Karnataka, India. E-mail: kmbc@biochem.iisc.ernet.in

Asp[193] in DOD motif I and II, respectively, are bona fide active-site residues essential for DNA cleavage activity. The implications of these results are discussed in this report.

Keywords: RecA intein; homing endonucleases; LAGLIDADG endonucleases; inteins

Introduction

Homing endonucleases (HEases) are a class of site-specific, rare-cutting DNA endonucleases encoded by self-splicing introns, inteins, or free standing genes found across all biological kingdoms.[1–6] These enzymes confer mobility to genetic determinants by a gene conversion event termed "homing."[1–6] During the homing process, the endonuclease inflicts a double-strand break at or near the homing site of the intein-/intron-less allele, which is subsequently repaired by the host DNA repair machinery resulting in the inheritance of intein/intron.[1–6] Consequently, efforts are being made to explore the possibility of using HEases as tools for genome mapping, cloning of megabase DNA fragments, and gene targeting.[1,7,8] One hallmark of HEases is their ability to recognize and cleave long DNA target sites (14–40 bp) spanning their insertion site in the intron-/intein-less allele.[1–6] The family of HEases is divided into four subfamilies based on the primary structure of their active site motifs: LAGLIDADG, GIY-YIG, H-N-H, and His-Cys box families.[1–6] Recently, the list of HEases is expanded with the addition of PD-(D/E)-XK nuclease family.[9] Among these, LAGLIDADG family is the largest, most prevalent, and well-studied class.[1–6] HEases that contain a single copy of LAGLIDADG motif per polypeptide chain, such as I-CreI, I-MsoI, and I-CeuI, function as homodimers and recognize and cleave palindromic and pseudo-palindromic DNA sequences.[3–5] On the other hand, HEases that harbor two copies of LAGLIDADG motifs, including I-AniI, PI-SceI, and I-SceI, act as monomers and recognize and cleave their DNA target sites with considerable asymmetry.[3–5]

Several lines of evidence suggest that a variety of divalent cations can support cleavage reactions by HEases, but they do not influence DNA-binding.[10–12] Most HEases prefer oxophilic Mg^{2+}, some use thiophilic Mn^{2+}, whereas others can use both for optimal catalysis.[10,11] Cocrystal structures of several LAGLIDADG endonucleases including I-CreI,[13] I-MsoI,[14] and I-SceI[15] indicated the presence of three divalent cations, coordinated by a pair of conserved aspartate residues at the active site. On the other hand, cocrystal structures of I-AniI[16] and PI-SceI[17] showed the presence of two divalent cations with shared metal being not visible. Based on metal mapping studies, in intron-encoded enzymes, I-DmoI and I-PorI, four metal ions at or near the catalytic centre have been proposed to participate in catalysis.[18] Sequence similarity searches indicate that a typical intein harbors both splicing and endonuclease domains, which further contain conserved amino acid sequence blocks.[19–21] The intein HEase-associated region encompasses canonical C and E or DOD1 and DOD2 blocks, called dodecapeptide motifs.[19,20] Among known LAGLIDADG-type enzymes, these motifs contain arrays of 9–10 residues, which are separated by a linker of 92–133 residues.[19]

Sequence analysis has revealed that there are strong similarities between *Mycobacterium tuberculosis* and *Mycobacterium leprae* gene products.[22] For instance, the *recA* genes are 92% identical.[23] In contrast, the sequences of *recA* intervening sequences are different in the two organisms.[23] Previously, we have shown that *M. tuberculosis* RecA intein (PI-MtuI) recognizes a unique 45-bp asymmetrical DNA sequence,[12] a feature similar to that of many LAGLIDADG-type HEases.[24] However, unlike other HEases, PI-MtuI possesses DNA-stimulated ATPase activity consistent with the presence of its ATP-binding motif.[25] On the other hand, characterization of PI-MleI has revealed that it possesses a modular structure with functionally separable domains for DNA-binding and cleavage, each with distinct sequence preferences. In previous work, analysis of an MACAW-generated multiple alignment of LAGLIDADG family members indicated the presence of C, D, and E motifs in several intein-encoded HEases, including PI-MtuI and *Mycobacterium leprae* RecA intein (PI-MleI).[20] However, sequence alignment analyses have also led to conflicting data in regard to the identity and the specific position of Blocks C and E within the PI-MleI polypeptide (Fig. 1). Using MACAW program, multiple sequence alignment of Blocks C and E of PI-MleI assigned ^{115}VLGSLMGDGP^{124} and ^{185}LQRAVYLGDG^{194} as the DOD motifs I and II, respectively.[20] On the other hand, HMM-based multiple sequence alignment provided an equally good match to consensus motifs ^{115}VLGSLMGDGP^{124} and ^{210}VLAIWYMDDG^{219} as the DOD motifs I and II in PI-MleI, respectively.[26] In addition, a lysine residue in Block D, which is known to be involved in catalysis in several LAGLIDADG-type enzymes,[13,27] is absent in PI-MleI (Fig. 1).[20]

To resolve this conflict and to determine the bona fide active-site residues of PI-MleI, we have performed site-directed mutagenesis of conserved amino acid residues presumed to be involved in catalysis. Furthermore, comparative analyses of DNA-binding and cleavage properties of PI-MleI, a novel LAGLIDADG-type enzyme with other well-studied members of this family, might offer unique insights into how

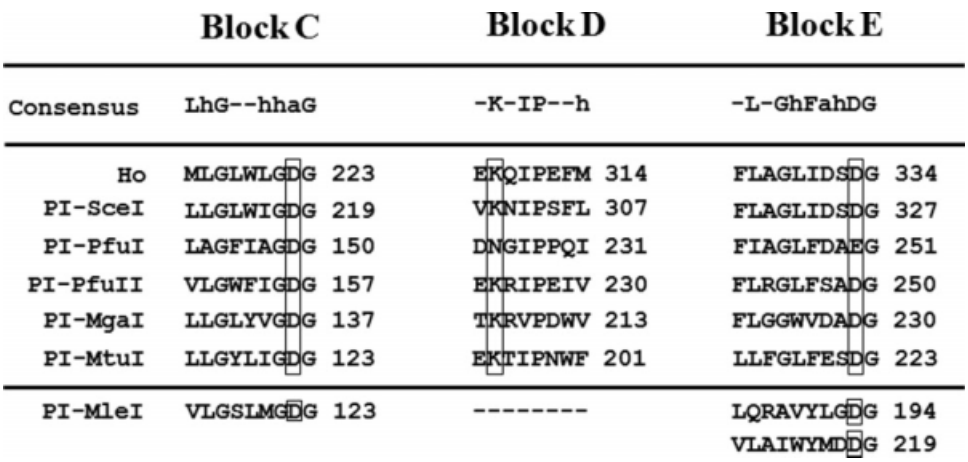

Figure 1. Alignment of endonuclease motifs of the LAGLIDADG-type homing endonucleases. Amino acid residues are represented by the single letter code. Blocks C and E represent the LAGLIDADG or dodecapeptide motifs involved in DNA cleavage, whereas Block D is another conserved motif involved in DNA catalysis. Conserved acidic (D or E) and basic (K) residues within blocks C, E, and D are boxed. The position of the last amino acid in each block is indicated to the right of the block. The consensus sequence signifies the conserved amino acid residues or groups from multiple sequence alignment. h, hydrophobic residues (G, V, L, I, A, M); a, acidic residues (D, E); (-), nonconserved residues; upper case letters, single letter amino acid code. The prefix "PI" indicates intein-encoded LAGLIDADG homing enzymes.

HEases bind and cleave their recognition sequences and also how they evolved different DNA-binding specificities. In addition, it would be interesting to identify the metal-binding catalytic residues in PI-MleI, and whether mutations in the catalytic domain would affect its DNA-binding properties. Here, we report the effects of these substitutions on DNA-binding affinities and kinetic parameters of PI-MleI. Together, these data provide compelling evidence to conclude that ^{115}VLGSLMGDGP^{124} and ^{185}LQRAVYLGDG^{194} motifs (Blocks C and E, respectively), but not ^{210}VLAIWYMDDG^{219} motif (Block E), and that residues Asp^{122} and Asp^{193} play a direct role with respect to the catalytic mechanism of PI-MleI.

Results

Rationale for the selection of mutants and purification of wild-type PI-MleI and its variants

Two different bioinformatics approaches to data analysis have led to the identification of three discrete dodecapeptide sequences in PI-MleI: one motif sequence in Block C and two in Block E (Fig. 1).[20,26] To determine the precise dodecapeptide motif(s) and active site residues responsible for mediating substrate specificity and catalytic mechanism of PI-MleI, we constructed a series of site-specific amino acid substitutions in the DOD motifs. A wealth of mutagenesis and structural data, which exist concerning HEases, suggest that catalytic centers carry essential aspartate residues, one in each of the LAGLIDADG motifs.[3,28] Accordingly, we chose to mutate conserved residues that have been previously implicated in catalysis. By site-directed mutagenesis, we constructed five mutant proteins, in which Asp^{122} was mutated to alanine, cysteine, and threonine, whereas Asp^{193} and Asp^{218} were mutated to alanine. The identity of each mutant was ascertained by determining the complete nucleotide sequence of the mutant gene. *E. coli* ER2566 strain was used as the host for overexpression of wild-type PI-MleI and its variants and were purified using IMPACT-T7 system, which allows protein purification under native conditions. PI-MleI mutants behaved similarly to the wild-type throughout the purification process and were purified to >95% homogeneity, as judged by silver staining of SDS-PAGE gel (Fig. 2). Purified proteins were devoid of both 5′ → 3′ and 3′ → 5′ exonuclease activities (data

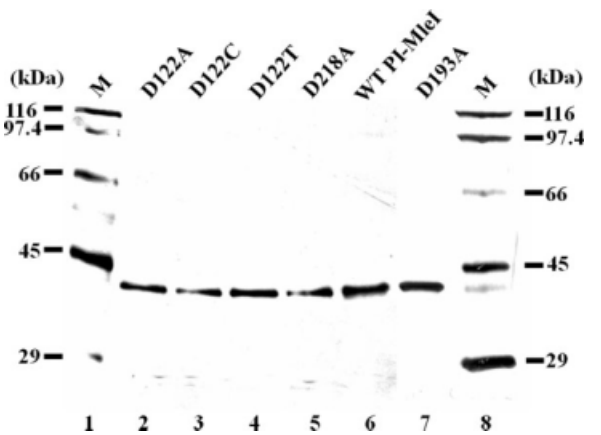

Figure 2. SDS-PAGE analysis of purified wild-type PI-MleI and its variants. Wild-type PI-MleI and its variants (0.5 μg) were analyzed by SDS-PAGE and visualized by silver staining. Lanes: 1 and 8, molecular weight standards; 2 to 7, purified wild-type PI-MleI or its variants as indicated on top of each lane. The sizes of the marker protein in kDa are indicated on either side of the gel.

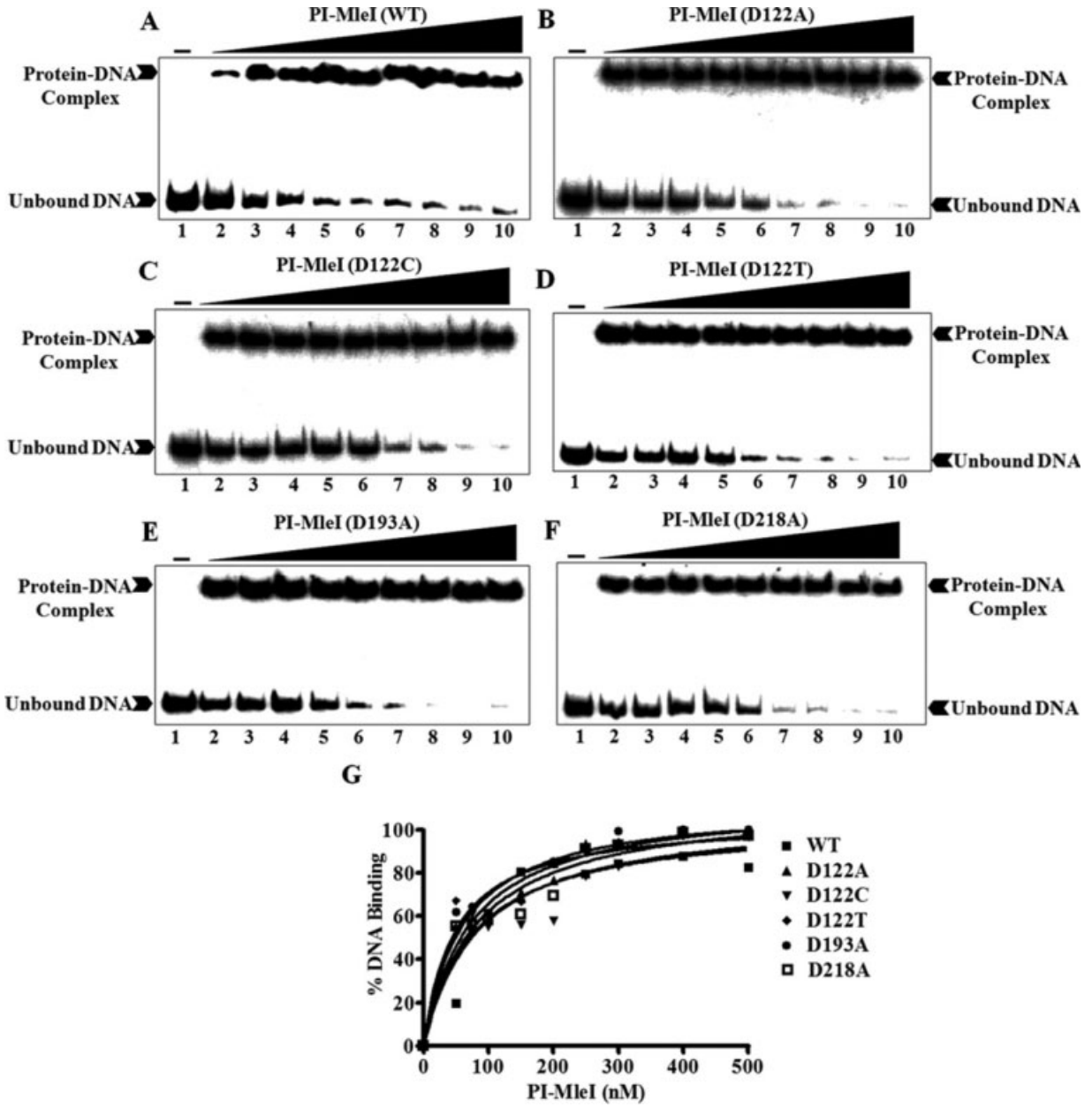

Figure 3. Binding of wild-type PI-MleI and its variants to cognate DNA. Binding reactions were performed with 1 nM ^{32}P-labeled cognate 88-bp duplex DNA in the absence (lane 1, A–F) or presence of 50, 75, 100, 150, 200, 250, 300, 400, or 500 nM wild-type PI-MleI or its mutant proteins (lanes 2–10, A–F), respectively. Samples were incubated at 37 °C, electrophoresed on native PAGE, and visualized by autoradiography as described under Materials and Methods. Panel A depicts the binding of wild-type PI-MleI to cognate duplex DNA. Panels B–F depict the binding of PI-MleI aspartate variants D122A, D122C, D122T, D193A, and D218A to cognate duplex DNA, respectively. Filled triangle on top of the gel images represents increasing concentrations of the specified protein. The positions of the unbound DNA and protein–DNA complexes are indicated. Panel G, Graph showing the extent of binding of wild-type PI-MleI or its variants. The extent of formation of protein–DNA complexes in panels A–F is plotted versus increasing concentrations of protein. (■), wild-type PI-MleI; (▲), D122A; (▼), D122C; (◆), D122T; (●), D193A; (□), D218A.

not shown) and, thus, were suitable for investigating their DNA-binding and cleavage activities.

DNA-binding analysis of PI-MleI and its variants by electrophoretic mobility shift assays

To evaluate the potential effects of PI-MleI mutations in active-site residues on their DNA-binding affinity, we used gel mobility shift assays,[29,30] which permit the acquisition of biochemical data on nucleic acid-binding proteins under relatively mild conditions. This assay has been previously applied to the wild-type PI-MleI to assess its DNA-binding properties.[31] Reactions were performed with a fixed amount of ^{32}P-labeled 88-bp DNA, containing a

Table I. *Apparent Dissociation Constants (K_d) of Wild-Type PI-MleI and Mutant Enzymes*

	K_d (n*M*)
PI-MleI (WT)	84.5
PI-MleI (D122A)	73.61
PI-MleI (D122C)	77.38
PI-MleI (D122T)	48.46
PI-MleI (D193A)	60.19
PI-MleI (D218A)	81.51

single centrally located PI-MleI target recognition sequence, hereafter referred to as the cognate DNA, and increasing concentrations of wild-type PI-MleI or its variants in the absence of divalent cations as described under Materials and Methods. Although the presence of divalent cations was not essential for DNA-binding, Mg^{2+} or Mn^{2+} was indispensable for the display of DNA-cleavage activity,[31] see later. Figure 3(A) shows that wild-type PI-MleI formed a discrete complex with cognate DNA in a concentration-dependent manner. Some complex formation was apparent at 50 nM PI-MleI, but with increasing amounts of the protein, >85% DNA were bound by the protein. Under similar conditions, PI-MleI variants were as proficient as the wild-type PI-MleI in the formation of protein–DNA complexes with cognate DNA and with comparable efficiency (Fig. 3, compare panel A with panels B–F). The amounts of free and bound DNA on the gels were analyzed using a phosphorimager, and all the proteins (wild type and mutants) displayed hyperbolic saturation curves [Fig. 3(G)].

The dissociation constants (K_d) were determined by titrating under conditions of relatively low concentrations of cognate duplex DNA and increasing concentrations of PI-MleI. The DNA substrates were incubated with increasing concentrations of PI-MleI or its variants, and the complexes were analyzed on native gels. Our results reveal the dissociation constants for PI-MleI and its variants with cognate duplex DNA in the low nanomolar range of the protein (Table I). These results suggest that PI-MleI variants have similar binding affinity as the wild-type PI-MleI to cognate duplex DNA.

To gain greater insight into the effect of aspartate substitutions on the interaction of PI-MleI and its variants with cognate DNA, we treated the protein–DNA complexes with increasing concentrations of NaCl in the standard assay buffer. As shown in Figure 4(A), the stability of wild-type PI-MleI-DNA complex and complexes formed by its variants with cognate DNA [Fig. 4(B–F)] progressively declined with increasing amounts of NaCl. The salt-titration midpoint for dissociation of protein–DNA complexes was about 0.3 M (Fig. 4, compare panels A with B–E). No difference in salt-titration midpoint was found between the complexes formed by the wild-type and some of its variants, indicating similar stability characteristics. However, although the binding affinity of D218A mutant to cognate DNA was analogous to the wild-type PI-MleI, the salt-titration midpoint for this mutant protein was in the range of $\sim$ 0.17 M [Fig. 4(G)]. It is likely that Asp^{218} may contribute towards the stability of PI-MleI-DNA complex.

Mutations in active-site aspartate residues fail to alter target DNA recognition by PI-MleI

Although the foregoing results indicate the occurrence of stable complexes between PI-MleI variants and target DNA, to further define the DNA-binding properties of each mutant protein, wild-type PI-MleI and its variants were assayed by DNase I footprinting, an approach which has been extensively used to characterize the interaction of DNA-binding proteins with their target sites.[32,33] Notably, DNase I footprinting is also an equilibrium binding assay and thus more sensitive than nonequilibrium assay such as EMSA. In addition, we believe that the analysis of different mutants in the DNase I footprinting assay has clear implications for target DNA recognition and cleavage activity.

DNase I footprinting assays were carried out with ^{32}P-labeled 88-bp DNA fragment, with the intein insertion site embedded at the centre, as described under Materials and Methods. The incubations were performed in the absence of divalent metal ions to prevent HEase activity and thus maintain integrity of the DNA substrate structure. The control reaction carried out in the absence of PI-MleI showed optimal cleavage of both the strands of the duplex DNA by DNase I (Supporting Information Fig. S1, lane 2 in panels A and B). In agreement with previous studies,[31] binding of wild-type PI-MleI to its target DNA resulted in the generation of an asymmetric footprint and protection of $\sim$ 16 nucleotide residues on the upper and 12 nucleotide residues on the lower strand, respectively. Importantly, the protected regions flank the intein-insertion site, indicating that the protein as whole binds to only one side of the DNA double helix (Supporting Information Fig. S1). A comparison of the pattern of footprints generated by wild-type PI-MleI and its variants (D122A, D122C, D122T, D193A, and D218A) on both the upper and lower strands (at all the concentrations tested) indicate that they are essentially identical (Supporting Information Figs. S1–S6). Figure 5 depicts linear projection of the relevant portion of the target DNA sequence and a summary of protection conferred by PI-MleI or its variants from DNase I digestion. These results are consistent with the notion that the aspartate substitutions in the catalytic motifs do not alter DNA recognition specificity of PI-MleI or its variants and may not play a direct role in protein–DNA

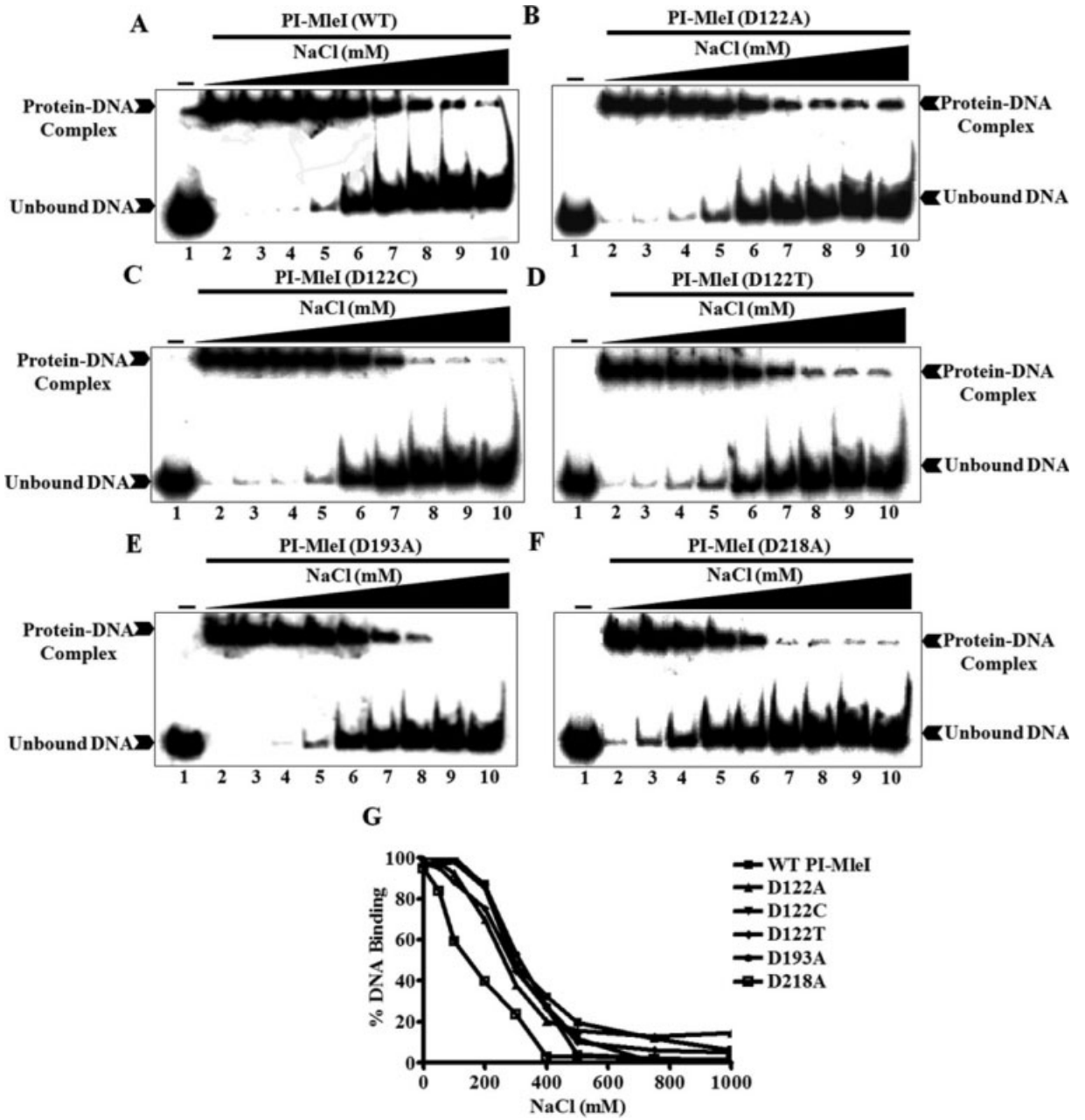

Figure 4. Effect of NaCl on the stability of PI-MleI-DNA complexes. Reaction mixtures contained 1 nM ^{32}P-labeled 88-bp cognate duplex DNA and 150 nM of wild-type PI-MleI (panel A) or its variants D122A (B); D122C (C); D122T (D); D193A (E); and D218A (F). After incubation at 37 °C for 30 min, NaCl was added to a final concentration of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1 M (lanes 3–10, panels A–F), respectively. Samples were further incubated at 37 °C for 30 min. Reactions were stopped by the addition of loading dye, electrophoresed on native PAGE, and visualized by autoradiography as described under the Materials and Methods. Lane 1 (A–F), buffer control; lane 2 (A–F), complete reaction without addition of NaCl. Panel G, Graph showing the extent of dissociation of protein–DNA complexes as a function of increasing concentrations of NaCl. (■), wild-type PI-MleI; (▲), D122A; (▼), D122C; (◆), D122T; (●), D193A; (□), D218A.

interactions. Furthermore, the data are similar to the wild-type PI-MleI with respect to the DNase I footprints near the intein-insertion site.[31]

Asp^{122} and Asp^{193} are essential for DNA distortion

Previous studies have shown that the wild-type PI-MleI although binds near the insertion site, but induces helical distortions only at the cleavage sites.[31] Here, we have extended these studies to gain quantitative information on the functional properties of PI-MleI variants. To this end, DNA-binding was performed with substrates in which adenine was substituted by a fluorescent analog, 2-aminopurine (2-AP), which is sensitive to the changes in base-pairing interactions and thereby serves as a probe to gauge helical distortions or opening.[34] We used six cognate duplex DNA substrates each with a 2-AP

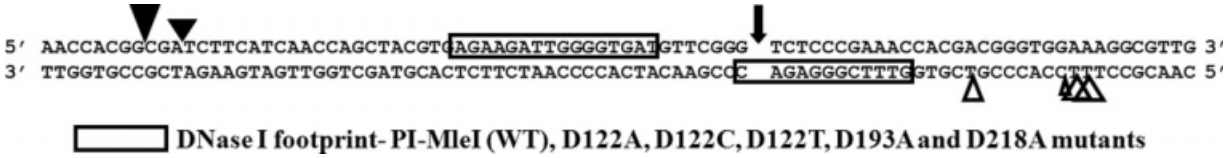

Figure 5. Schematic diagram showing protection from DNase I cleavage by wild-type PI-MleI and its variants. A relevant portion of *M. leprae* intein-less *recA* sequence showing the regions of protection (boxed regions) on both upper and lower strands, conferred by wild-type PI-MleI and its variants (D122A, D122C, D122T, D193A, and D218A). Inverted triangles on the upper and lower strands correspond to sites of PI-MleI cleavage. For details, see the Supporting Information figures and text.

embedded at the indicated position (Table II). Briefly, in one substrate 2-AP was positioned at the insertion site (AL +1); in the second, within the PI-MleI-binding site (AU −18), and in others either at the cleavage site (AU −45 and AU +16) or adjacent to it (AU −51 and AL +31) in upper or lower strands, respectively. 2-AP-containing duplexes were incubated with increasing concentrations of wild-type PI-MleI or its variants, and fluorescence intensity changes were measured in the spectral region between 330 and 450 nm. In agreement with previous studies,[31] a broad fluorescence enhancement was observed in the spectral region of 365–370 nm as a function of increasing concentrations of wild-type PI-MleI. Fluorescence enhancement was observed with target DNA containing 2-AP only at the cleavage sites, but not at its binding sites (Fig. S7, compare panels C and D with panels A, B, E, and F). Similar experiments were performed with the same set of 2-AP-containing DNA substrates with PI-MleI variants. The pattern and the extent of fluorescence enhancement induced by D122C, D122T, and D218A variants was indistinguishable from the wild-type PI-MleI; however, D122A and D193A disclosed obvious differences (compare spectral changes in Supporting Information Figs. S8–S10 with Figs. S11 and S12). To characterize the magnitude of spectral changes induced by PI-MleI and its variants, we computed the fluorescence intensities at 367 nm. Figure 6 shows the extent of fluorescence intensities (in arbitrary units) as a function of increasing concentrations of wild-type PI-MleI or its variants with DNA substrates containing 2-AP at the indicated positions. Interestingly, increase in fluorescence enhancement was observed with DNA substrates containing 2-AP at the cleavage positions (AU −45 and AU +16), and almost no change at the binding sites (AU −18 and AL +1). Although wild-type PI-MleI and D122C, D122T, and D218A variants caused six- to sevenfold increase in fluorescence intensity, D122A and D193A mutants were able to induce approximately threefold. The fluorescence enhancement increases linearly with increasing protein concentrations and does go to completion.

Asp^{122} and Asp^{193} are essential for DNA cleavage activity, whereas Asp^{218} is not

To validate the participation of individual Asp substitutions on the catalytic activity, we performed cleavage assays using form I pMLR DNA, which harbors a single copy of *M. leprae recA* intein-less allele, as the substrate. The assay was performed with increasing concentrations of wild-type PI-MleI or its variants, and the products were separated on an agarose gel and visualized as described under Materials and Methods. As shown in Figure 7(A), wild-type PI-MleI cleaves both the target DNA strands resulting in the generation of both form II and form

Table II. *Oligonucleotides Used for Synthesizing 2-Aminopurine (2-AP) Cognate DNA Substrates*

Oligonucleotides	Sequences
ODN 1	5′ AACCACGGCGATCTTCATCAACCAGCTACGTGAGAAGATTGGGGTGATGTTCGGGTCTCCC GAAACCACGACGGGTGGAAAGGCGTTG 3′
ODN 2	5′ CAACGCCTTTCCACCCGTCGTGGTTTCGGGAGACCCGAACATCACCCCAATCTTCTCACGT AGCTGGTTGATGAAGATCGCCGTGGTT 3′
ODN 3	5′ AACCACGGCGATCTTCATCAACCAGCTACGTGAGAAGATTGGGGTGATGTTCGGGTCTCCCG AAACCACGACGGGTGGAAAGGCGTTG 3′
ODN 4	5′ AACCACGGCGATCTTCATCAACCAGCTACGTGAGAAGATTGGGGTGATGTTCGGGTCTCCC GAAACCACGACGGGTGGAAAGGCGTTG 3′
ODN 5	5′ AACCACGGCGATCTTCATCAACCAGCTACGTGAGAAGATTGGGGTGATGTTCGGGTCTCCCG AAACCACGACGGGTGGAAAGGCGTTG 3′
ODN 6	5′ AACCACGGCGATCTTCATCAACCAGCTACGTGAGAAGATTGGGGTGATGTTCGGGTCTCCCG AAACCACGACGGGTGGAAAGGCGTTG 3′
ODN 7	5′ CAACGCCTTTCCACCCGTCGTGGTTTCGGGAGACCCGAACATCACCCCAATCTTCTCACGTA GCTGGTTGATGAAGATCGCCGTGGTT 3′
ODN 8	5′ CAACGCCTTTCCACCCGTCGTGGTTTCGGGAGACCCGAACATCACCCCAATCTTCTCACGTA GCTGGTTGATGAAGATCGCCGTGGTT 3′

The underlined A residues indicate the position of 2-AP substitution in the oligonucleotide sequence.

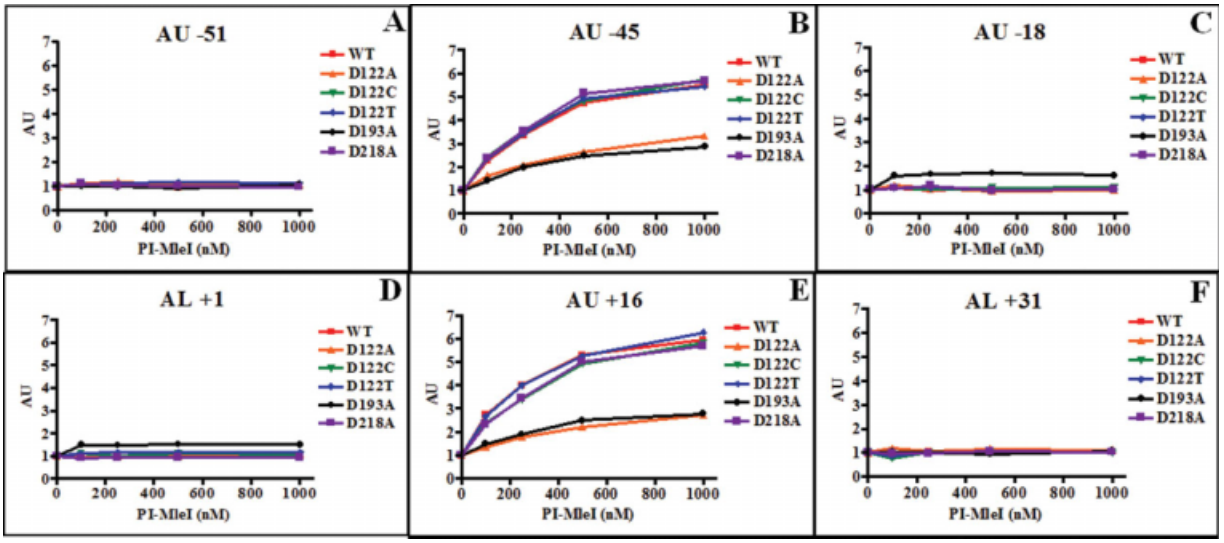

Figure 6. Graphs showing the enhancement of 2-aminopurine fluorescence by wild-type PI-MleI and its variants. The reactions were carried out as described under Materials and Methods. The fluorescence intensity induced by wild-type PI-MleI and its variants (D122A, D122C, D122T, D193A, or D218A) at 367 nm was plotted against increasing concentrations of the indicated protein on the *x*-axis. The *y*-axis represents relative fluorescence intensity changes (in arbitrary units). Each datum point represents mean of two experiments. Panels A–F, target DNA substrates with 2-AP embedded at different positions. AU or AL denote 2-AP placed on the upper or lower strand of cognate DNA sequence, whereas numbers designate the position of the base upstream (−1) or downstream (+1) from the intein insertion site. For details, see the Supporting Information figures and text.

III DNA. At lower concentrations, these enzymes target single-strand cleavage to either the upper or lower strands, but when their concentrations were raised double-strand cleavage occurred in accordance with the two-step reaction [Figs. 7(A,C,F, lanes 2–6)]. For direct comparison, we have considered the double-stranded DNA cleavage activity of wild-type PI-MleI and its variants, which results in the generation of linear duplex or form III DNA. Figure 7(G) displays a graph showing the percentage of form III DNA as a function of increasing concentration of the indicated wild-type or mutant PI-MleI. Although the D218A variant displayed wild-type levels of cleavage activity [compare Fig. 7(F) with Fig. 7(A)], D122T mutant showed substantially reduced activity [Fig. 7(D)]. The residual activity observed with D122T mutant implicates a possible role for nucleophilic oxygen atom in the threonine side chain in catalysis. We also generated D > T and D > C mutants for residues at position 193. Unfortunately, we were unable to purify the variant enzymes to homogeneity because they were found in the insoluble inclusion body fractions. On the other hand, mutant enzymes containing the D122A [Fig. 7(B)] and D193A [Fig. 7(E)] substitution individually displayed low single-strand nicking activity, but were totally inactive in this assay [Fig. 7(B,E,G)]. Not surprisingly, a double mutant (D122A and D193A), similar to the D193A single mutant, displayed no detectable double-stranded DNA cleavage activity (data not shown). It is noteworthy that D122A and D193A mutants, whose ability to distort DNA was weakened (Fig. 6), were also defective in DNA cleavage. Strikingly, D122C variant showed approximately twofold enhanced double-stranded DNA cleavage activity, compared with the wild-type PI-MleI [Fig. 7(C,G)]. At present, we have no mechanistic insight into the increased catalytic activity displayed by the D122C variant. However, these findings indicate that Asp^{122} in Block C and Asp^{193} in Block E are crucial to the double-stranded DNA HEase activity of PI-MleI, whereas Asp^{218} in Block E is not.

Discussion

In this study, we have identified the bona fide LAGLIDADG motifs of PI-MleI and the active site residues responsible for its HEase catalytic mechanism. Site-directed mutagenesis and cleavage assays disclosed that Asp^{122} and Asp^{193} are crucial to the double-stranded DNA HEase activity, whereas Asp^{218} is not. The EMSA and DNase I footprinting experiments show that PI-MleI and its variants bind specifically to the target DNA recognition sequence spanning about 16 and 12 bases on upper and lower strands, respectively, flanking the intein-insertion site in intein-less substrate. Intriguingly, binding of PI-MleI or its variants was not centered on the cleavage sites. The fact that PI-MleI binding was not centered on the cleavage sites was unexpected for a HEase, but it is not without precedent. Supporting this notion, I-TevI, a two-domain protein, generates DNase I footprints centered over the

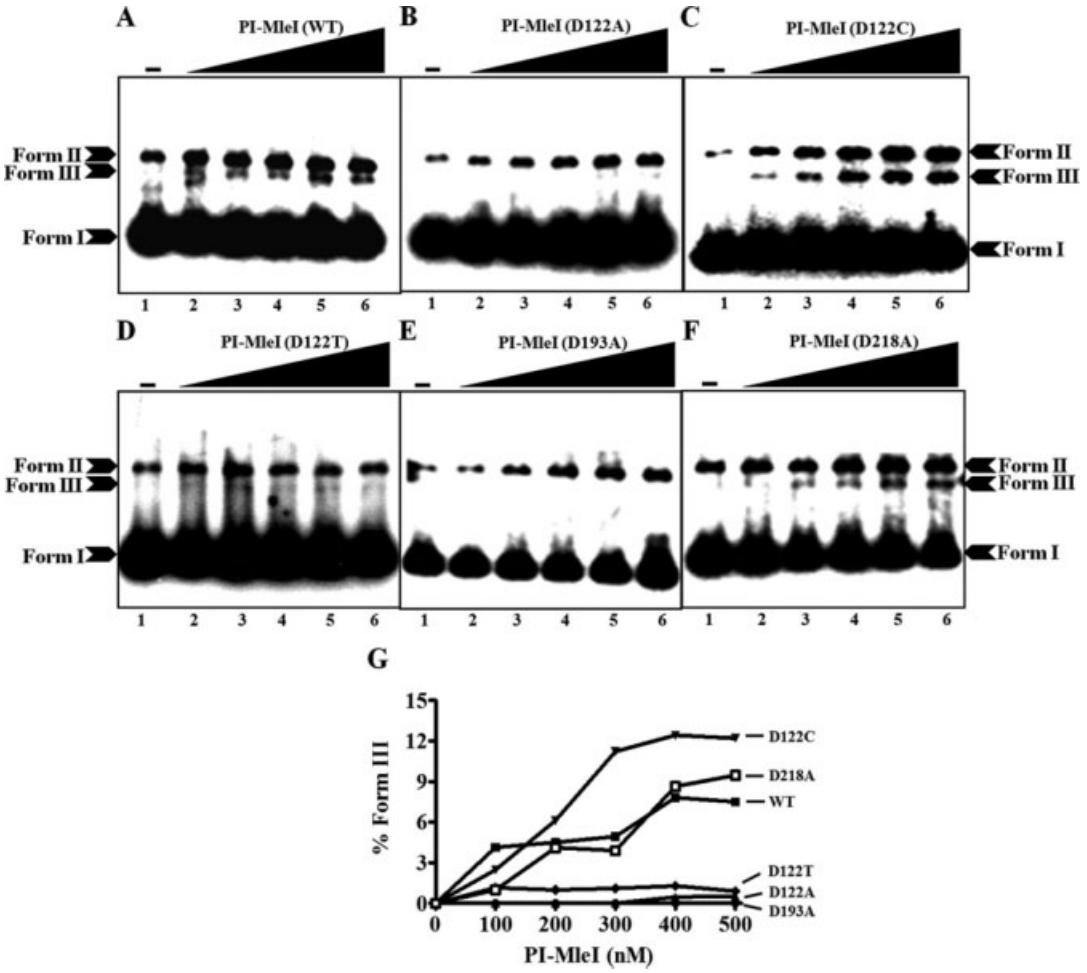

Figure 7. Comparison of DNA cleavage activity between wild-type PI-MleI and its variants. Reaction mixtures (20 μL) contained 25 mM Tris-HCl (pH7.5), 0.4 mM DTT, 5 mM $MnCl_2$, 16 μM pMLR DNA [a mixture of form I (>85%) and form II (<15%)], and increasing concentrations of wild-type PI-MleI (panel A) or its variants (panels B–F). After incubation at 37 °C for 30 min, samples were deproteinized, analyzed by agarose gel electrophoresis, and visualized by Southern hybridization as described under Materials and Methods. Lane 1 (A–F), no enzyme; lanes 2–6 (A–F), 100, 200, 300, 400, or 500 nM of wild-type PI-MleI or its variant as indicated at the top of each panel. G, Graph depicting the generation of form III DNA by wild-type PI-MleI and its variants as a function of protein concentration. (■), wild-type PI-MleI; (▲), D122A; (▼), D122C; (◆), D122T; (●), D193A; (□), D218A.

intron insertion-site of intron-less substrate, but cleavage occurs 23–25 bases further upstream.[35]

The equilibrium binding constant (K_d) for binding of PI-MleI and its variants to the target DNA sequence is of the same order, suggesting that mutations of active site Asp residues have no effect on their affinity to the target DNA substrate. D122T and D193A variants displayed slightly higher binding affinity for target *DNA* as indicated by lower K_d values. The K_d values for PI-MleI and its variants are comparable to the K_d values determined for the binding of other LAGLIDADG-type enzymes to their cognate DNA sequences.[12,36,37] The specificity of interaction of PI-MleI variants with target DNA sequence was determined using a salt-titration assay. The higher salt-titration midpoint value (~ 0.3 M), except in the case of D128A variant (Fig. 4), suggests that in addition to electrostatic and ionic interactions other forces contribute to the stability of protein–DNA complexes. The salt-induced dissociation of D218A-DNA complex at slightly lower NaCl concentration is in contrast to its higher binding affinity for the target DNA. It is possible that the low-salt standard assay conditions may stabilize the binding of D218A variant with the target DNA resulting in lower K_d. Nonetheless, the salt-induced dissociation isotherms and the K_d values, which allow a direct comparison of binding of PI-MleI and its variants to the target DNA sequence, indicate no gross structural changes in the mutant proteins. In addition, these observations suggest that the Asp residues in LAGLIDADG motifs of PI-MleI might not play a direct role in its binding to DNA, implicating the existence of a modular structure with distinct DNA-binding and catalytic domains. This inference is in agreement with some of the members of

LAGLIDADG-type HEases such as PI-SceI[28,38,39] and PI-PfuI.[37,40]

Several lines of evidence suggest that the conformational changes in the protein and/or DNA often accompany protein–DNA interaction.[41] Fluorescent base analogs, such as 2-AP, are often used to monitor such conformational changes involving protein–nucleic acid interaction.[42–45] To gain insights into the functional differences in the target DNA selectivity of wild-type PI-MleI and its variants, we used 2-AP fluorescence to monitor changes induced in double-helical DNA by these enzymes. We observed that the fluorescence intensities induced by PI-MleI and its variants with the target DNA substrate at the cleavage site followed a hierarchical manner in the following order: wild-type PI-MleI ≡ D122C ≡ D122T ≡ D218A > D122A > D193A (Fig. 6). The enhancement of 2-AP fluorescence induced by DNA-binding proteins has been interpreted previously as evidence for DNA strand separation and/or base flipped out of its helical conformation.[42–45] Although the precise mechanism remains obscure, helical distortions may account for the fluorescence enhancement induced by PI-MleI. We conclude, however, that changes in DNA conformation are apparent at the cleavage sites upon binding of PI-MleI and its variants; the absence of such distortion at the binding DNA sequence (Fig. 6) suggests that binding and cleavage processes are unlikely to be mechanistically linked.

A large amount of data obtained from site-directed mutagenesis and 3-dimensional crystal structure of PI-SceI have shown the spatial arrangement of Asp218, Lys301, and Asp326 and their role in catalysis.[17,27,28] These three residues, which form a triad of the catalytic center, are embedded in Blocks C, D, and E, respectively, of the eight conserved intein motifs.[20] Other studies have provided compelling evidence that the last Asp residues, Asp218 and Asp326, in the PI-SceI LAGLIDADG motifs independently coordinate a single divalent cation, one per catalytic center.[46] In strand cleavage reactions catalyzed by LAGLIDADG-type HEases, the Asp nucleophile comes from one each of DOD motif. Likewise, Asp residues in motifs C or E are actually important for the cleavage activities of PI-PfuI and PI-PfuII.[37] These results are also in agreement with the studies from other enzymes in this family.[47] Alignment of amino acid sequence of PI-MleI with other HEases in the LAGLIDADG family revealed the presence of single copy of DOD motif (sequence) in Block C, two copies of the same motif in Block E, and absence of Block D (Fig. 1). According to the alignment, Asp^{122}, Asp^{193}, and Asp^{218} of PI-MleI were predicted to be the corresponding putative catalytic ligands; therefore, we made different substituted proteins of PI-MleI (D122A, D122C, D122T, D193A, and D218A) by site-specific mutagenesis. This approach to identify acidic amino acid ligands involved in positioning the divalent cation at the catalytic center was successfully used previously for RAG1 and RAG2[48] as well as for the Tn7 and Tn10 transposases.[49] Our results show that that two Asp residues, Asp^{122} and Asp^{193}, each of which are in the two LAGLIDADG motifs (Block C and E), respectively, are actually crucial for the double-stranded DNA cleavage activities of PI-MleI. In addition, substitution of Asp^{122} and Asp^{193} to alanine was found to affect the DNA helix distortion suggesting the essential role of these residues in PI-MleI-mediated DNA catalysis. The PI-MleI variant D122C was catalytically hyperactive as it exhibits enhanced catalytic activity. These results indicate that the cysteine residue in D122C provides a functional group, which can help coordinate Mn^{2+} in a more or less isosteric manner comparable to the aspartic acid residue in the wild-type enzyme, thereby supporting higher activity. This was not unexpected, because upon substitution of aspartic acid by cysteine the postulated residue for the metal ion is changed from oxygen to sulfur.[50] PI-MleI lacks Block D and, consequently, the lysine residue which has been suggested as a general base in several LAGLIDADG HEases such as I-CreI[13] and PI-SceI.[27] The absence of catalytic lysine in PI-MleI as judged from sequence alignments has also been seen earlier in other LAGLIDADG enzymes such as PI-PfuI, wherein its absence in one of the catalytic centre has been linked with the differential cutting activity of the enzyme on two strands of the substrate DNA.[40]

The LAGLIDADG-type HEases have been the subject of intense study over the past 2 decades. A combination of genetic, biochemical, structural, and bioinformatics approaches have provided insights into the conserved DOD motifs and the importance of the acidic amino acid residues in HEase activity. The challenging goals that remain include the plasticity of their DNA target site recognition, use of different cofactors, and the different mechanisms of cleavage. Finally, since inteins/introns are being discovered in increasing numbers of organisms across all biological kingdoms and host proteins, in-depth understanding of HEases is essential for gaining insights into the horizontal transfer of these genetic determinants between different organisms.

Materials and Methods

DNA, reagents, and enzymes

All the chemicals and reagents used in this study are of analytical grade. DNA-modifying enzymes, restriction endonucleases, IMPACT-T7 cloning system, and chitin resin were procured from New England Biolabs (UK). DNA gel extraction kit was purchased from QIAGEN. Mutagenesis primers were obtained from Sigma-Genosys. Bacterial strains

were grown in liquid or solid agar Luria Broth (LB) media supplemented with appropriate antibiotics. The recombinant plasmids were maintained in *E. coli* DH5α or DH10B strains (Invitrogen). Protein expression was carried out in *E. coli* ER2566 (NEB) encoding T7 RNA polymerase. Plasmid pMLR bearing *M. leprae* intein-less *recA* allele in pUC19 vector was prepared as described.[31] The concentration of plasmid DNA substrates was expressed in moles of nucleotide residues per liter.

Table II displays the sequences of oligonucleotides used in this study. Duplexes were prepared by mixing equimolar amounts of the single strands. Briefly, oligonucleotide (ODN1) labeled at the 5′ end[51] was annealed to the complementary oligonucleotide (ODN2) to make radiolabeled duplex DNA. Annealing was done by incubating the two ODNs at 95 °C for 5 min followed by gradual cooling to 24 °C. Similarly, 2-aminopurine-containing cognate duplexes were prepared by annealing ODN2 or ODN1 with the indicated complementary oligonucleotide, ODN3 to ODN8. The annealed duplex DNAs were separated from the single-stranded oligonucleotides by electrophoresis on a 6% (w/v) native PAGE. The bands corresponding to the ^{32}P-labeled or 2-AP-modified cognate duplexes were excised from the gel, and the DNA was eluted into a buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The concentration of oligonucleotide substrates was expressed in moles of DNA ends/liter.

Site-directed mutagenesis

The expression plasmid pTMLRI encoding wild-type PI-MleI[31] served as a template for the introduction of single amino acid substitutions by double PCR protocol as described.[52] The complementary forward and reverse primer pairs used to construct each PI-MleI variant discussed are listed in Table III. Amplified mutant genes were directionally cloned into pTXB1 expression vector using NheI and XhoI restriction sites. The mutational changes were confirmed by DNA sequencing (data not shown).

Expression and purification of PI-MleI variants

Expression and purification of wild-type PI-MleI and its variants were performed as described previously.[31] The purity of PI-MleI and its variants was assessed by SDS-PAGE and visualized by silver staining (Fig. 2). The concentrations of the wild-type PI-MleI and its variants were determined by dye-binding method using bovine serum albumin as standard[53] and expressed in moles per liter.

Electrophoretic mobility shift assays

Reaction mixtures (20 μL) contained 25 mM Tris-HCl (pH 7.5), 0.4 mM DTT, 1 nM (5′-^{32}P)-labeled 88-bp cognate duplex DNA, and increasing concentrations of wild-type PI-MleI or its variants. Reaction mixtures were incubated at 37 °C for 30 min and terminated by the addition of 2.2 μL of 10× gel-loading dye [50% glycerol containing 0.42% (w/v) each of bromophenol blue and xylene cyanol]. The samples were subjected to electrophoresis using a 6% native PAGE in 0.5× TBE (45 mM Tris-borate buffer, pH 8.3, containing 1 mM EDTA) buffer at 150 V for 3 h and analyzed using a Fuji phosphorimager 5000 screen followed by autoradiography. The bands were quantified using UVI-BAND MAP, and the resulting data were plotted in Graph pad prism version 4.0.

Table III. *List of Oligonucleotide Primers Used for Generating PI-MleI Variants by Double Polymerase Chain Reaction*[52]

Oligonucleotides Sequences (5′→ 3′)	Variants
CGAGAGGTTCCC**TGC**ACCCATCAGTGA	D122A
CGAGAGGTTCCC**GCA**ACCCATCAGTGA	D122C
CGAGAGGTTCCC**GGT**ACCCATCAGTGA	D122T
TAAGAACTTCTTACC**TGC**TCCGAGGTAGAC	D193A
TGTGAATGAACC**TGC**GTCCATATACCA	D218A
ATTGGGGTGATGGCTAGCTGCATGAAT	Forward primer
CGTGGTTTCCTCGAGATTGTGTACCAT	Reverse primer

Nucleotide sequences that are bold indicate the mutated residues, whereas underlined sequences indicate the restriction sites.

DNase I footprinting

DNase I footprinting was done as described previously.[32,54] The footprinting reactions (20 μL) contained 10 nM ^{32}P-labeled 88-bp cognate duplex DNA, 25 mM Tris-HCl (pH 7.5), 0.4 mM DTT, and increasing concentrations of wild-type PI-MleI or its variants. Reaction mixtures were incubated at 37 °C for 30 min. Footprinting reactions were initiated by the addition of 10 μL cofactor solution containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$, and DNase I to a final concentration of 0.005 U. After incubation at 24 °C for 1 min, the reactions were then stopped by the addition of 100 μL stop solution (20 mM EDTA, 5% SDS, 200 mM NaCl, and 25 μg/μL calf thymus DNA). DNA was precipitated with ethanol and resuspended in formamide loading dye (80% formamide, 0.1% BPB, and 0.1% xylene cyanol). Samples were heated to 95 °C for 5 min, cooled quickly on ice, and analyzed on a 12% polyacrylamide sequencing gel in the presence of 7 M urea along with G + A ladder.[55] The gel was dried, and the bands were visualized by Fuji FLA-5000 phosphorimager.

Steady-state fluorescence measurements

All steady-state fluorescence experiments were performed in a SPEX Fluromax-3 spectrofluorometer (Jobin Yvon Horiba, USA). Reaction mixtures (300 μL) contained 10 nM of 2-AP-labeled 88-bp cognate duplex, and wild-type PI-MleI or its variants at the

indicated concentrations. The samples were excited at 315 nm wavelength, and the emission profile was monitored in the wavelength range of 330–450 nm. Band passes [Band pass (nm) = slit width (mm) × dispersion (nm/mm)] were set at 5 and 6 nm on excitation and emission monochromators, respectively. Spectral corrections for lamp fluctuation and instrumental variation were done. The spectra were also corrected for background emission and tryptophan fluorescence. Spectral measurements were taken in 5 mm × 5 mm cuvette at 30 °C. Three independent scans were taken to derive integrated spectra for acquiring the binding curves. These binding curves were normalized to the values obtained for the 2-AP-labeled duplexes in the absence of protein.

DNA cleavage assays

Cleavage reactions (20 μL) contained 25 mM Tris-HCL (pH 7.5), 0.4 mM DTT, 5 mM $MnCl_2$, 16 μM form I plasmid pMLR DNA, and wild-type PI-MleI or its variants at the indicated concentrations. Reaction mixtures were incubated at 37 °C for 30 min, and the reactions were stopped by the addition of 0.1% SDS. Samples were deproteinized by adding 0.5 mg/mL proteinase K followed by incubation at 37 °C for 15–20 min. The reaction was stopped by the addition of 2.5 μL of gel-loading dye [0.42% (w/v) of bromophenol blue and xylene cyanol in 50% glycerol]. Samples were electrophoresed on a 0.8% agarose gel in 89 mM Tris borate (pH 8.3) buffer containing 2 mM EDTA. Reaction products were visualized under UV light after staining with ethidium bromide (0.5 μg/mL ethidium bromide). *DNA* from the gel was then transferred to Nylon N+ membrane and visualized by Southern hybridization.[51,56] The reaction products corresponding to substrate cleavage were quantified using UVI-Band MAP software version 97.4 and plotted using Graph pad Prism.

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